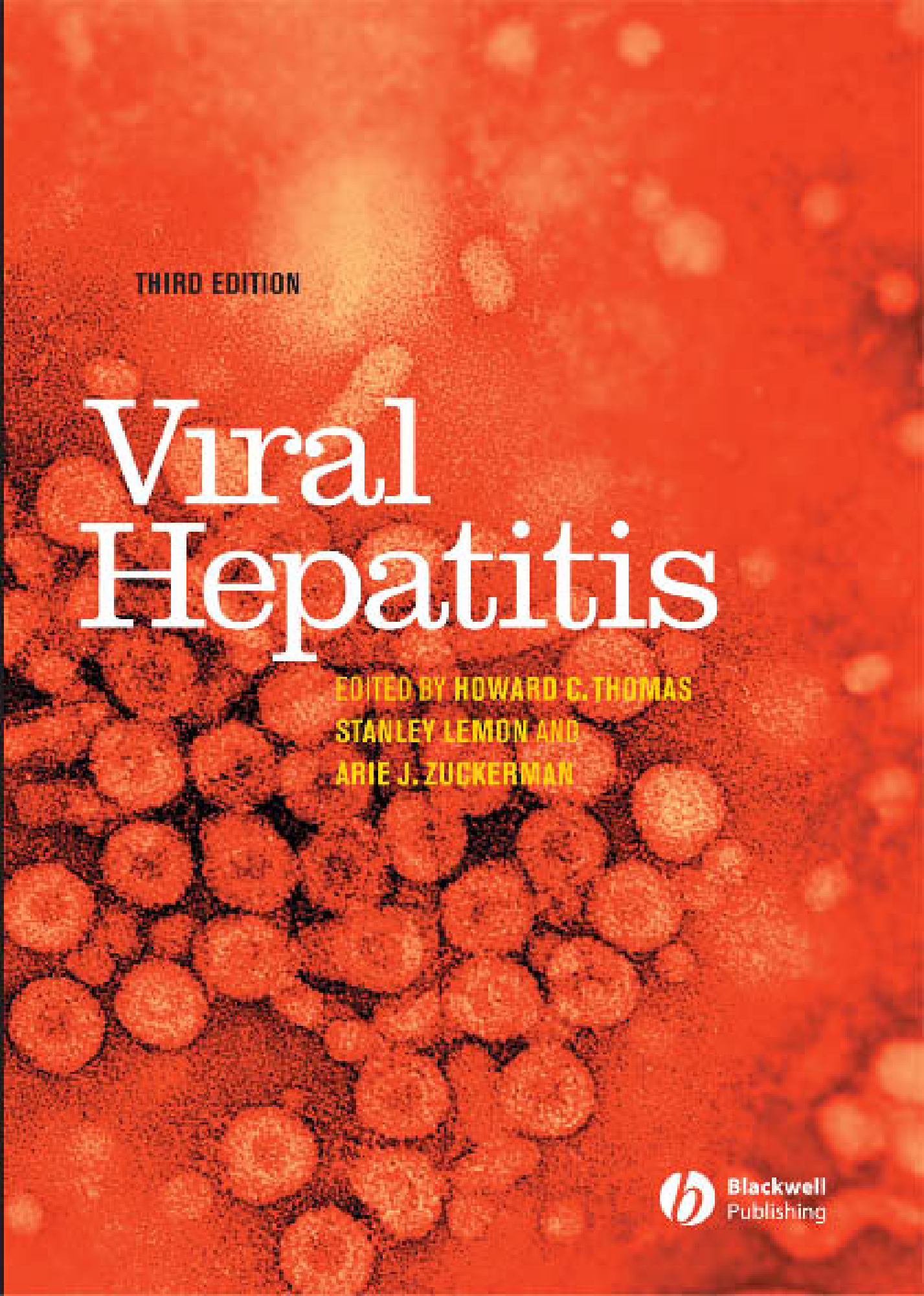
THIRD EDITION

# Viral Hepatitis

EDITED BY HOWARD C. THOMAS
STANLEY LEMON AND
ARIE J. ZUCKERMAN



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# Contents

Colour plate section appears facing p. 786

# Preface to the Third Edition

I am pleased to welcome Professor Stan Lemon to the editorial group for the third edition of *Viral Hepatitis*: he brings considerable expertise in molecular virology to the editing process. Since the second edition was published in 1998, an additional 14,000 peer-reviewed papers have been published in this field, and many important advances have been made. The book includes over 50 chapters, ranging from new contributions on hepatic stem cell biology and the liver's immune system, through to updated reviews of the basic virology of the five hepatitis viruses and the increasing importance of viral variants, insights into the pathogenesis of persistent infection and the role of hepatitis B and C in tumorogenesis.

To appeal to the clinician, we have included more specialized chapters on the treatment of fulminant hepatitis, liver cancer, persistent hepatitis in children and in haemophiliacs, treatment of the extra-hepatic manifestations of hepatitis B and C, and of the specific problems occurring in HIV-infected patients with viral liver disease. Two of the more important chapters for the clinician will be those dealing with the treatment of chronic hepatitis B and C written by clinicians practising in Europe and North America. Finally, we have included a new chapter on occupational health issues, and reviewed the subjects of sterilisation and prevention by vaccination.

We hope the book will appeal to virologists, immunologists, clinicians in infectious diseases, hepatology and gastroenterology and, of course, to public health and occupational health physicians. It is a book for healthcare workers addressing today's problems and researchers projecting us forward to solve the remaining issues in a disease area affecting over half a billion of the world's population!

Recognising the rapidity with which this field is evolving, we would welcome your suggestions on how the Journal and Textbooks of *Viral Hepatitis*, both now published by Blackwell's, might interact to better serve your needs in this electronic publishing era.

*Howard Thomas*
*March 2005*

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# Section I
# Introduction to Liver Biology

# Chapter 1
# Liver stem cells in persistent viral infection, liver regeneration and cancer

Stuart Forbes, Malcolm Alison

## Stem cells: definitions and properties

It is widely believed that the most versatile stem cells are embryonic stem (ES) cells, which are isolated from the inner cell mass (ICM) of the early blastocyst or fetal gonadal tissue. These blastocysts are usually 'spares' from IVF programmes, although some have been deliberately created. Tissues generated from such ES cells have still to overcome the problem of histocompatibility, but somatic cell nuclear transfer techniques (SCNT – also called therapeutic cloning) offer the possibility of using the patient's own genome to generate ES cells and so overcome this obstacle.[1]

### A hierarchy of potential

Stem cells have varying degrees of potential. The most versatile is of course the fertilized oocyte (the zygote) and the descendants of the first two divisions. These cells are *totipotent*, able to form the embryo and the trophoblasts of the placenta. After about 4 days these totipotent cells begin to specialize, forming a hollow ball of cells, the blastocyst, and a cluster of cells called the ICM from which the embryo develops. The ICM cells are considered to be *pluripotent*, able to differentiate into almost all cells that arise from the three germ layers, but not the embryo because they are unable to give rise to the placenta and supporting tissues. A large number of studies point to the fact that these ES cells are able to differentiate down the hepatocyte lineage.[2–5] Another cell with pluripotency is the so-called 'multipotent adult progenitor cell' (MAPC) that has been isolated by Catherine Verfaillie and colleagues from mesenchymal cell cultures obtained from human and rodent bone marrow.[6,7] These MAPCs are capable of in excess of a hundred population doublings, and can be induced to differentiate not only into mesenchymal lineages, but also into endothelial, neuroectodermal cells (neurones, astrocytes and oligodendrocytes) and endodermal cells (hepatocytes).

Most adult tissues have *multipotential* stem cells, which are capable of producing a limited range of differentiated cell lineages appropriate to their location, e.g. small intestinal stem cells can produce all four indigenous lineages (Paneth, goblet, absorptive columnar and entero-endocrine). Central nervous system (CNS) stem cells have trilineage potential generating neurones, oligodendrocytes and astrocytes,[8] while the recently discovered stem cells of the heart can give rise to cardiomyocytes, endothelial cells and smooth muscle.[9] In the liver, such multipotential stem cells are found in the canals of Hering, giving rise to a transit amplifying population of cells called either oval cells or hepatic progenitor cells (HPCs). Ordinarily, these cells are essentially bipotent, giving rise to hepatocytes and biliary epithelial cells, but under some circumstances they can give rise to other cell types, notably cells of an intestinal phenotype. The least versatile of stem cells are *unipotential* stem cells, which are capable of generating one specific cell type. Into this category we could place epidermal stem cells in the basal layer that produce only keratinized squamous cells and certain adult hepatocytes that have long-term repopulating ability. Some would argue that there is no such thing as a unipotential stem cell, and really these cells should be called *committed progenitors*. While there is no doubt that in some tissues, e.g. the gastrointestinal tract and haematopoietic renewal systems, there are committed stem cells (progenitors) with more limited division potential than their multipotent predecessors, in the liver there do appear to be some unipotent cells with a very large clonogenic potential.[10]

### Self-maintenance

In many renewing tissues the ability of stem cells to self-renew is one of the most defining characteristics. Stem cells are normally located in a protected environment (niche; *Fr*. recess), and in a tissue such as the small intestine where the cell flux is in one direction, they are

found at the origin of the flux. In the heart, they are located in areas of least haemodynamic stress. Although only a small percentage of a tissue's total cellularity, stem cells maintain their numbers if, on average, each stem cell division gives rise to one replacement stem cell and one transit amplifying cell (an *asymmetric* cell division). The interactions with the stem cell niche are likely to be crucial to this process. We are still largely ignorant about the identity of both multipotential and unipotential stem cells in the liver and so any discussion of a stem cell niche is premature.

### Proliferation, clonogenicity and genomic integrity

In renewing tissues, stem cells are slowly cycling but highly clonogenic. Teleologically, it would seem prudent to restrict stem cell division because DNA synthesis can be error-prone. Thus, in many tissues stem cells divide less frequently than transit amplifying cells. In the intestine, stem cells cycle less frequently than the more luminally located transit amplifying cells, and in the human epidermis basal cells highly expressive of the β1-integrin (receptor for type IV collagen) have a lower level of proliferation than the other basal cells. In hair follicles, the hair shaft and its surrounding sheaths are produced by the hair matrix that is itself replenished by the bulge stem cells. As befits true stem cells, the bulge cells divide less frequently but are more clonogenic than the transit amplifying cells of the hair matrix. Combined with an infrequently dividing nature, stem cells would also appear to have devised a strategy for maintaining genome integrity. Termed the immortal strand hypothesis or Cairns hypothesis, stem cells can apparently designate one of the two strands of DNA in each chromosome as a template strand, such that in each round of DNA synthesis while both strands of DNA are copied, only the template strand and its copy are allocated to the daughter cell that remains a stem cell.[11] Thus, any errors in replication are readily transferred (within one generation) to transit amplifying cells that are soon lost from the population. Such a mechanism probably accounts for the ability of stem cells to be 'label-retaining cells' (LRCs) after injection of DNA labels when stem cells are being formed. As the healthy adult liver is very largely proliferatively quiescent, the foregoing stem cell attributes (apart from clonogenicity) are not readily identified within the liver.

### Molecular control of stem cell behaviour

It appears likely that the local microenvironment, through a combination of cells and extracellular matrix components, will govern all aspects of stem cell behaviour. This led to the concept of the stem cell niche (not yet identified in the liver) that supports and controls stem cell activity. Wnt signalling, in particular, seems critical to stem cell self-renewal in many tissues. The active Wnt pathway maintains the pluripotency of ES cells,[12] myofibroblast-secreted Wnts preserve the intestinal stem cell phenotype,[13] while haematopoietic stem cell (HSC) self-renewal also requires Wnts, in particular Wnt-3A.[14] Interestingly, Wnt-3A has been implicated in the maintenance of a hepatobiliary (reservoir) cell with bipotential capabilities in cultures derived from fetal liver.[15]

### Adult stem cell plasticity

There is some evidence to support the idea that certain adult stem cells, particularly those of bone marrow origin, can engraft alternative locations (e.g. non-haematopoietic organs), particularly when the recipient organ is damaged, and transdifferentiate into phenotypes appropriate to their new location. However, the field is not without its critics.[16] The reason for this is twofold: 1) certain instances of so-called plasticity have now been attributed to cell fusion between bone marrow cells and cells of the recipient organ (see below), and 2) several remarkable claims have not been able to be confirmed, including conflicting reports regarding the ability of HSCs to contribute to hepatocyte replacement in the damaged liver (see below).

### Stem cell diseases – metaplasia, fibrosis and cancer

Under normal circumstances tissue-specific stem cells generate the range of cell types appropriate to their location. However, chronic inflammation and damage are often accompanied by metaplastic change – a major switch in tissue differentiation, and it is reasonable to suppose that this switch occurs at the level of stem cells rather than between terminally differentiated cells. Adult stem cells, and particularly HSCs, may also contribute to organ fibrosis including liver cirrhosis (see below). It is also likely that many cancers, including primary liver tumours are of stem cell origin (see below).

## Liver stem cells: hepatocytes

Hepatocytes are highly differentiated cells with multiple synthetic and metabolic functions. They are also the functional stem cell in the liver under most circumstances. In health, individual hepatocytes have a life expectancy of over a year. Therefore, in the normal adult liver there is little cell proliferation detectable with only 0.01% of hepatocytes in the cell cycle at any one time. However, in response to parenchymal cell loss, the hepatocytes restore the liver mass by self-replication. This is a very efficient system, and in rodents when two-thirds of

the liver is resected the remaining remnant can re-grow to the original liver size in approximately 10 days. This model has been intensively studied and has provided many data on the mechanisms controlling liver regeneration.[17] In response to this stimulus the hepatocytes initially hypertrophy, accumulate triglycerides and amino acids and activate enzymes that are associated with cell proliferation. The normally quiescent hepatocytes leave G0 to enter the cell cycle under the influence of growth factors and cell cycle-dependent kinases. The hepatocyte proliferation begins in the periportal region of the liver and spreads to the central region of the lobule. This situation requires the hepatocytes to undergo on average less than two rounds of replication to restore the liver to its original size. However, this does not mean that the hepatocytes have a replication potential limited to this degree. Hepatocyte transplantation models in mice have shown that the transplanted cells are capable of significant clonal expansion within the diseased livers of experimental animals. In the FAH-deficient mouse, a model of hereditary type 1 tyrosinaemia, there is strong positive selection pressure on the transplanted wild-type cells as the host hepatocytes are metabolically deficient. This phenotype is lethal unless the mice are administered the compound 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), which prevents the accumulation of toxic metabolites in the tyrosine catabolic pathway. When $10^4$ normal hepatocytes from congenic male wild-type mice are intrasplenically injected into mutant female mice and the NTBC treatment is withdrawn, these cells colonize the mutant liver efficiently.[10] Moreover, serial transplantations from these colonized livers to other mutant livers indicated that at least 69 hepatocyte doublings can occur, thereby confirming the clonogenic potential of hepatocytes and fulfilling a crucial property required of stem cells.

The clonogenic ability of human hepatocytes in chronic hepatitis can be indirectly estimated. Using mathematical modelling of viral kinetics it has been estimated that in chronic hepatitis B between 0.3% and 3% of all hepatocytes are killed and therefore replaced each day to maintain a stable liver cell mass (this approximates to $10^9$ of the liver's $2 \times 10^{11}$ hepatocytes).[18] This estimation accords with proliferation indices seen in chronic hepatitis B and C with proliferating cell nuclear antigen indices of 0.1–3.6% of hepatocytes and Ki-67 labelling indices in hepatitis C of 1–14%.[19,20] In chronic hepatitis the parenchymal mass can therefore be maintained through hepatocyte self-replication.

The hepatocyte proliferation rate increases in hepatitis C with increasing histological damage until cirrhosis is reached when the proliferation rates fall.[21] The reason for this fall in hepatocyte proliferation rate is not fully resolved. It may represent the hepatocytes coming to the end of their replicative potential and indeed Wiemann *et al*. have found that in cirrhosis the hepatocyte telomerase shortening correlates with cell cycle senescence and the degree of fibrosis.[22] However, other factors contributing to the inhibition of hepatocyte proliferation seen in cirrhosis may be the accumulation of hepatic collagen and activated stellate cells acting to inhibit hepatocyte proliferation,[23] or simply the distortion of the lobular anatomy and blood flow seen in cirrhosis. Whatever the reason, the reductions in hepatocyte proliferation indices in cirrhosis are coincident with the production of cells from a second potential stem cell compartment, located within the smallest branches of the intrahepatic biliary tree. This so-called 'oval cell' compartment identified in rodent models has historically been termed the 'ductular reaction' when seen in human liver disease. The development of the oval cell reaction in response to hepatocyte replicative senescence has recently been demonstrated in a transgenic mouse model of fatty liver and DNA damage. In this model the mice developed fatty livers and a large number of senescent hepatocytes. A striking oval cell response developed in these mice which related to the number of senescent mature hepatocytes.[24]

## Liver stem cells: oval cells

### Rodent models

Following very extensive liver damage or in situations where hepatocyte regeneration after damage is compromised, a potential stem cell compartment located within the smallest branches of the intrahepatic biliary tree is activated. This 'oval cell' or 'ductular reaction' amplifies a biliary population that has a bipotential capacity capable of differentiating into either hepatocytes or cholangiocytes. Most rodent models of oval cell activation have employed potential carcinogens to inhibit hepatocyte replication in the face of a regenerative stimulus. For example in the rat, protocols have included administering 2-acetylaminofluorene (2-AAF) to inhibit hepatocyte proliferation before creating a demand for hepatocyte proliferation by partial hepatectomy or a necrogenic dose of carbon tetrachloride. The need to maintain parenchymal cell mass results in the development of an oval cell response in the mouse liver that spreads from the canals of Hering into the parenchyma. The oval cells initially express cell surface markers of both hepatocytes and biliary epithelia, but over time the oval cells differentiate into a hepatocyte phenotype.[25] Oval cells are small cells with a large nucleus to cytoplasm ratio, in which the nucleus also has a distinctive ovoid shape. A wide range of markers has been used to identify oval cells including gamma-glutathione transferase and glutathione-S-transferase activity, along with a host of monoclonal antibodies raised against cy-

**Table 1.1** Markers that have aided in the identification of oval cells

| Marker/antibody | Nature |
|---|---|
| OV-6 | Monoclonal antibody generated by immunizing mice with cell preparations isolated from carcinogen-treated rat livers |
| OC.2, OC.3 | Biliary/oval cell antigens |
| BDS7 | Monoclonal antibody generated against surface-exposed components of bile ductular cells |
| Thy-1 | Haematopoietic stem cell marker |
| c-kit | Haematopoietic stem cell marker/receptor for stem cell factor |
| CD34 | Haematopoietic stem cell marker |
| ABCG2/BRCP1 | ATP-binding cassette transporter |
| Connexin 43 | Gap-junction proteins |
| CK7, CK19, CK14 | Intermediate filament proteins that impart mechanical strength to cells |
| AFP (alpha-fetoprotein) | The predominant serum protein produced during early liver development, replaced later in development by albumin |
| Gamma-glutamyl transpeptidase (GGT) | A major enzyme of glutathione homeostasis present in normal adult liver in bile duct epithelium and membranes of bile canaliculi |
| Placental form of glutathione-S-transferase (GST-P) | Dimeric proteins involved in the intracellular transport of hydrophobic molecules and the metabolism of toxic compounds |
| Flt-3 | Receptor with tyrosine kinase activity present on haematopoietic stem cells |
| DMBT1 | Deleted in malignant brain tumour 1: proteins involved in the mucosal defence system and epithelial differentiation |
| (M2-PK) | Antibody against the fetal M2 isoform of pyruvate-kinase |

toskeletal proteins and surface antigens (see Table 1.1). Of great interest was the finding that oval cells express antigens traditionally associated with haematopoietic cells such as c-kit, Flt-3, Thy-1 and CD34.[26–28] This led to speculation that some hepatic oval cells may be directly derived from a precursor of bone marrow origin, particularly when the biliary tree is damaged[29] and this will be reviewed in the following section. Most researchers believe that the location of a stem cell niche for oval cells is in the canals of Hering, which is a transitional zone between the periportal hepatocytes and the biliary cells lining the smallest terminal bile ducts. In rats and mice, the canals of Hering barely extend beyond the limiting plate, but the resultant oval cell proliferation can result in branching ducts that express alpha-fetoprotein (AFP) and stretch to the midzonal areas before these cells differentiate into hepatocytes in the hepatic parenchyma (see Plate 1.1, facing p.786). Using oval cell transplant experiments into the FAH-deficient mouse Wang *et al*. found that the oval cells from metabolically competent mice repopulated the mutant livers at least as efficiently as mature hepatocytes, demonstrating their hepatocyte differentiation potential.[30]

## Human liver

The human counterparts to the oval cells described in rodent models are often referred to as hepatic progenitor cells. These have been studied after severe hepatocellular necrosis, chronic viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease. It is thought that activation of hepatic progenitor cells and differentiation towards the biliary lineage leads to formation of reactive ductules, which are anastomosing strands of immature biliary cells with an oval nucleus and a small rim of cytoplasm.

Differentiation towards the hepatocyte lineage occurs via intermediate hepatocytes. These are polygonal cells with a size and phenotype intermediate between progenitor cells and hepatocytes.[31] After submassive liver cell necrosis, reactive ductules, in continuity with intermediate hepatocytes, are seen at the periphery of the necrotic areas. In patients studied with sequential liver biopsies, intermediate hepatocytes become more numerous with time and extend further into the liver lobule. This sequence of changes suggests gradual differentiation of human putative progenitor cells into intermediate hepatocytes, analogous with what is seen in rat models of chemical injury associated with impaired hepatocyte replication. In chronic viral hepatitis, progenitor cells are activated, even in mild degrees of inflammation. The number of progenitor cells (the degree of activation) correlates with the degree of inflammatory activity.[32] Intermediate hepatocytes are only seen when a certain level of inflammation is reached, suggesting that progenitor cells only differentiate towards hepatocytes when a certain threshold of damage is reached.

Elegant three-dimensional reconstructions of serial sections of human liver immunostained for cytokeratin-19 have shown that the smallest biliary ducts, the canals

of Hering, normally extend into the proximal third of the lobule (unlike those in rodents), and it is envisaged that these canals react to massive liver damage (akin to a tripwire), proliferating and then differentiating into hepatocytes[33] (see Plate 1.2, facing p.786). Oval cell numbers in human liver rise with increasing severity of liver disease[34] and this ductular reaction is thought to be a stem cell response rather than a ductular metaplasia of 'damaged' hepatocytes. However, others have argued that this cellular gradient does not necessarily represent differentiation of a progenitor cell, but rather a de-differentiation of mature hepatocytes towards a biliary phenotype. Falkowski *et al*.[21] have clarified this issue in human liver. Using serial sections and three-dimensional reconstructions they found in human liver cirrhosis that in most cases (94%) the intraseptal hepatocyte buds (small nodules of hepatocytes) were connected to areas of ductular reaction. Furthermore, they found that 'cholestatic' hepatocytes were very largely not associated with a ductular reaction, clearly supporting the notion that ductular reactions represent a stem cell response.

Recently, human bipotent cell lines have been identified from human liver; the HepaRG cell line has been established from a liver tumour associated with chronic hepatitis C. These cells display both hepatocyte-like and biliary-like epithelial phenotypes, but hepatocyte differentiation can be produced by culture with epidermal growth factor (EGF).[35] Hopefully, the study of cell lines such as this will aid the understanding of the molecular mechanisms of stem cell differentiation

## Liver stem cells: bone marrow cells

### Rodent data

Within an adult tissue, the locally resident stem cells were formerly considered to be capable of only giving rise to the cell lineage(s) normally present. However, adult haematopoietic stem cells (HSCs) in particular may be more flexible: removed from their usual niche, they are capable of differentiating into non-haematopoietic lineages. Oval cells/hepatocytes were first thought to be derived from circulating bone marrow cells in the rat. Petersen *et al*.[29] followed the fate of syngeneic male bone marrow cells transplanted into lethally irradiated female recipient animals whose livers were subsequently injured by carbon tetrachloride (to cause hepatocyte necrosis) in the presence of 2-acetylaminofluorene (to block hepatocyte regeneration) in order to induce an oval cell activation. Y chromosome-positive-containing cells were tracked by *in situ* hybridization. Oval cells and hepatocytes that were Y chromosome-positive were identified at 9 and 13 days, respectively. Additional evidence for hepatic engraftment of bone marrow cells was sought from a rat whole liver transplant model. Lewis rats expressing the major histocompatibility complex (MHC) class II antigen L21-6 received liver transplants from Brown Norway rats that were negative for L21-6. Subsequently, ductular structures in the transplants contained both L21-6-negative and L21-6-positive cells, indicating that the biliary epithelium was of both donor and recipient origin. These cells of host origin were thought to originate from circulating bone marrow cells. Using a similar gender mismatch bone marrow transplantation model (female mice transplanted with male bone marrow), Theise *et al*.[36] reported that, over a 6-month period, 1–2% of hepatocytes in the murine liver may be derived from bone marrow in the absence of any obvious liver damage, suggesting that bone marrow contributes to normal 'wear and tear' renewal of the liver. In the most striking demonstration of the potential therapeutic utility of bone marrow, FAH-deficient mice (see above) were rescued biochemically by a million unfractionated bone marrow cells that were wild-type for FAH. Moreover, only purified HSCs (c-kit$^{high}$Thy$^{low}$Lin$^{-}$Sca-1$^{+}$) were capable of this functional repopulation, with as few as 50 of these cells being capable of hepatic engraftment when haematopoiesis was supported by $2 \times 10^5$ fah–/– congenic adult female bone marrow cells.[37] Importantly, although the initial engraftment was low, approximately one bone marrow cell for every million indigenous hepatocytes, the strong selection pressure exerted (induced liver failure) thereafter on the engrafted bone marrow cells resulted in their expansion to eventually occupy almost half the liver. However, it was later found that the healthy liver cells in the fah–/– mouse contained chromosomes from both the recipient and donor cells, with presumably the donor haematopoietic cell nuclei being reprogrammed when they fused with the unhealthy fah–/– hepatocyte nuclei to become functional.[38,39] The key to this discovery was to perform the gender mismatch bone marrow transplantation experiments between female donors and male recipients. In one experiment, a million donor bone marrow cells (fah+/+) from Fanconi anaemia group C (fancc–/–) homozygous mutant mice were serially transplanted into lethally irradiated fah–/– recipients.[39] The usual repopulation (approximately 50%) of the mutant liver by Fah-positive hepatocytes was noted, but Southern blot analysis of the purified repopulating cells revealed that they were heterozygous for alleles (fah+/+; fancc–/–) that were unique to the donor marrow – fusion with host liver cells must have occurred. To confirm this conclusion, in a second experiment, fah+/+ bone marrow from ROSA26 female mice was transplanted into male fah knockout mice. Cytogenetic analysis of the LacZ-positive, sorted bone marrow-derived hepatocytes revealed that most if not all had a Y chromosome, thus confirming fusion. Before bone marrow transplant, most host hepatocytes had a karyotype of either 40,XY or 80,XXYY, but after transplantation

with fah+/+ bone marrow, the commonest karyotype of Fah-positive hepatocytes was either 80,XXXY, suggesting fusion between a diploid female donor cell and a diploid male host cell, or 120,XXXXYY, suggesting fusion between a female donor diploid blood cell and a tetraploid male host hepatocyte. However, a substantial proportion of bone marrow-derived hepatocytes were aneuploid, suggesting that fusion had created some sort of genetic instability with the hybrid cells randomly shedding chromosomes. In the companion paper (Vassilopoulos *et al*.[38]) to that of Grompe and colleagues,[39] lineage-depleted wild-type bone marrow was transplanted into lethally irradiated female fah–/– mice and, following withdrawal of NTBC, the usual Fah-positive nodules emerged 4–5 months later. When restriction enzyme-digested genomic DNA from these nodules was probed for fah sequences, the mean level of donor DNA was found to be only 26%, again leading to the conclusion that the donor haematopoietic cells had fused with the host fah–/–, generating polyploid hepatocytes.

Fusion of bone marrow cells has also been found to occur in the normal mouse, not only with hepatocytes, but also with Purkinje cells and cardiomyocytes.[40] These were very elegant studies *in vivo* and *in vitro* in which a reporter gene was activated only when cells fused. However, unlike the Fah null mouse, no selection pressure (liver damage) was operative, and even after 10 months only 9–59 fused cells/$5.5 \times 10^5$ hepatocytes were found. Importantly, they also found evidence that with time donor genes had been either inactivated or eliminated, again suggestive of genetic instability in heterokaryons.

Mouse bone 'marrow-derived' hepatocytes can also be expanded selectively if they are engineered to overexpress Bcl-2, and then the indigenous cells are targeted for destruction by an anti-Fas antibody. It is as yet unknown whether cell fusion operates in this model.[41]

Not all murine studies are consistent. Terai *et al*.[42] reported an impressive 25% contribution of bone marrow to the parenchyma after CCL4-induced injury, but Kanazawa and Verma[43] failed to find any evidence for bone marrow engraftment in three models of chronic liver injury, including CCL4 (see Table 1.2).[29,37–39,41–53] However, many studies have testified to the ability of human cord blood cells to transdifferentiate into hepatocytes in the liver of the immunodeficient mouse (Table 1.2), albeit at a low level.[48,49,51–53] While it seems logical to believe that parenchymal damage is a stimulus to hepatic engraftment by HSCs, the molecules that mediate this homing reaction to the liver are not well understood. Petrenko *et al*.[54] speculated that in mice the molecule AA4 (murine homologue of the C1q receptor protein) may be involved in the homing of haematopoietic progenitors to the fetal liver – it may be that this receptor protein is expressed on HSCs that engraft to the damaged liver. An alternative explanation is that cells in the liver express the stem cell chemoattractant 'stromal derived factor-1' (SDF-1) for which HSCs have the appropriate receptor known as CXCR4.[55] Hatch *et al*.[56] have provided persuasive evidence that SDF-1 is involved in oval cell activation, furthermore speculating that this chemokine may secondarily recruit bone marrow to the injured liver. More definitive proof was provided by Kollet *et al*.[53] who observed increased SDF-1 expression (particularly in biliary epithelia) after parenchymal damage, and concomitant with such damage was increased HGF production, a cytokine that was very effective in promoting protrusion formation and CXCR4 up-regulation in human CD34+ haematopoietic progenitors. Intriguingly, Ratajczak *et al*.[57] have identified a population of cells in the bone marrow of normal mice that have features of hepatocyte differentiation (alpha-fetoprotein and cytokeratin-19 production). They speculated that the bone marrow could be a location for already differentiated CXCR4-positive tissue-committed stem/progenitor cells to reside. Furthermore, following organ specific damage, SDF-1 is unregulated, the cells follow the SDF-1 gradient, mobilize into peripheral blood and subsequently take part in organ regeneration.

Other cells found to have an origin, at least in part, from the bone marrow are the hepatic stellate cells. Baba *et al*.[58] used bone marrow transplants from mice transgenic for green fluorescent protein (GFP) into non-transgenic recipients prior to injections with carbon tetrachloride to induce liver fibrosis. Surprisingly, they found that a third of the stellate cells in recipients were GFP-positive and therefore likely to be from the bone marrow. This is in accord with recent data in mice where myofibroblasts in various organs including gut, lung and skin were identified to be of bone marrow origin.[59,60]

## Human data

In two contemporaneous papers, Alison *et al*.[61] and Theise *et al*.[62] demonstrated that hepatocytes can also be derived from bone marrow cell populations in humans (see Table 1.3).[61–67] Two approaches were adopted – firstly, the livers of female patients who had previously received a bone marrow transplant from a male donor were examined for cells of donor origin using a DNA probe specific for the Y chromosome, localized using *in situ* hybridization. Secondly, Y chromosome-positive cells were sought in female livers engrafted into male patients but which were later removed for recurrent disease. In both sets of patients, Y chromosome-positive hepatocytes were readily identified, but the degree of hepatic engraftment of HSCs into human liver was highly variable. Most probably related to the severity of parenchymal damage, up to 40% of hepatocytes and cholangiocytes appeared to be derived from bone marrow in a liver transplant recipient with recurrent hepati-

**Table 1.2** Rodent models of bone marrow-liver 'plasticity'

| Authors | Model | Technique | Evidence of marrow origin | Proportion of marrow-derived hepatocytes | Comments |
|---|---|---|---|---|---|
| Petersen *et al.* 1999[29] | Rat | Male BMTx to females<br>Male wild-type to DPPIV-null<br>2- AAF/CCL4 liver injury | Y+ cells in female DPPIV+ hepatocytes in DPPIV- liver | 0.16% | Also noted Y+ oval cells |
| Avital *et al.* 2001[44] | Rat | Strain mismatch (c3- into c3-positive rats) liver transplant with organ rejection | c3-negative cells integrated into hepatic plates | NS | No definite evidence that the cells were hepatocytes |
| Dahlke *et al.* 2003[45] | Rat | CD45 mismatch BMTx<br>retrorsine/CCL4 liver injury | Donor MHC antigens | None | Liver mass restored by hepatocyte hypertrophy |
| Theise *et al.* 2000[46] | Mouse | Male BMTx into female mice<br>No liver injury | Y chromosome-positive/albumin mRNA-positive cells | Up to 2.2% | ... |
| Lagasse *et al.* 2000[37] | Mouse | Male BMTx into female Fah–/– mice, liver failure induced by NTBC withdrawal | Y+/Fah+ hepatocytes | 30–50% | Strong selection pressure for healthy, wild-type hepatocytes |
| Mallet *et al.* 2002[41] | Mouse | BMTx from male Bcl-2 transgenic mice into female recipients | Y chromosome +/Bcl-2+/CK 8/18+ | 0.8% | FAS-mediated necrosis of 50% of recipient hepatocytes |
| Fujii *et al.* 2002[47] | Mouse | GFP-positive BMTx into negative recipients | GFP+ hepatocytes | Nil | BM contributed to endothelium and Kupffer cells |
| Wang *et al.* 2003[39] | Mouse | Female BMTx into male Fah–/– mice, liver failure induced by NTBC withdrawal | Fah+/Y+ cells, genotype of Fah+ cells | Approximately 50% | Fah+ cells had both the recipient and donor genotype, i.e. due to fusion |
| Vassilopoulos *et al.* 2003[38] | Mouse | Male BMTx into female Fah–/– mice | Genotype of Fah+ cells | NS | Mixed genotype, i.e. cell fusion |
| Terai *et al.* 2002[42] | Mouse | GFP-positive BMTx into negative recipients and CCL4-induced cirrhosis | Cords of GFP+ cells<br>Most Liv2+ | Approximately 25% | Recipients had a rise in serum albumin |
| Kanazawa and Verma 2003[43] | Mouse | EGFP+ or LacZ + BMTx<br>Three models of liver injury | Y+/EGFP+/LacZ+ | Nil | ... |
| Danet *et al.* 2002[48] | hUCB into NOD/SCID mice | CD34+/–, C1qRp+ BM fraction used, no liver injury | Human albumin (RT-PCR)<br>c-Met (immuno-detection) | 0.05–0.1% | ... |
| Ishikawa *et al.* 2003[49] | hUCB into NOD/SCID mice | CD34+ or CD45+ fraction used<br>No liver injury | Human albumin (RT-PCR)<br>HepPar1 (immuno-detection) | 1–2% | No evidence of fusion |
| Newsome *et al.* 2003[50] | hUCB into NOD/SCID mice | No liver injury | HepPar1 (IHC) h.DNA sequences by FISH | NS | No evidence of fusion |
| Wang *et al.* 2003[51] | hUCB into NOD/SCID and NOD/SCID/BMG- mice | CD34+ fraction<br>CCL4-induced liver injury | Human albumin mRNA | 1–10%, but only 1 in 20 expressed albumin | ... |
| Kakinuma *et al.* 2003[52] | hUCB into NOD/SCID mice | Liver injury induced by partial hepatectomy and 2-AAF | Human X chromosome<br>Human albumin (immuno-detection) | 0.1–1% | ... |
| Kollet *et al.* 2003[53] | hUCB into NOD/SCID mice | CD34+ cells<br>CCL4-induced liver injury | Human albumin | 50–175 per $1.5 \times 10^6$ | Occasional clusters of human cells next to SDF-1+ bile ducts |

2-AAF/PH, 2-acetylaminofluorene followed by partial hepatectomy; 2-AAF/CCL4, 2-acetylaminofluorene followed by carbon tetrachloride; BMTx, bone marrow transplant; DPPIV, dipeptidyl peptidase IV; EGFP, enhanced green fluorescent protein; Fah, fumarylacetoacetate hydrolase; FISH, fluorescence *in situ* hybridization; GFP, green fluorescent protein; hUCB, human umbilical cord blood; IHC, immunohistochemistry; NOD/SCID/BMG-, non-obese/severe combined immunodeficient/β2 microglobulin negative; NS, not stated; NTBC, 2-(2-nitro-4-trifluromethylbenzoyl)-1, 3-cylohexanedione; PBSC, peripheral blood stem cell; RT-PCR, reverse transcription-polymerase chain reaction.

**Table 1.3** Analysis of human tissue – the contribution to the hepatocyte population from circulating extrahepatic cells

| Authors | Procedures | Evidence of marrow origin | Marrow-derived hepatocytes | Comments |
|---|---|---|---|---|
| Alison *et al*. 2000[61] | Male BMTx into female Sex mismatched liver transplant | Y+/CK 8+ cells | 1–2% | ... |
| Theise *et al*. 2000[62] | Male BMTx into female Sex mismatched liver transplant | Y+ CAM 5.2 positive cells | 4–43% | High percentage in a case of fibrosing cholestatic hepatitis in recurrent hepatitis C |
| Korbling *et al*. 2002[63] | Male BMTx into female | Y+ CAM 5.2 positive cells | 4.7% | ... |
| Kleeberger *et al*. 2002[64] | Liver allograft | Genotype chimerism | NS | Chimerism of cholangiocytes also |
| Fogt *et al*. 2002[65] | Sex mismatched liver transplant | Sex chromosome detection | Nil | Long-term follow-up |
| Ng *et al*. 2003[66] | Sex mismatched liver transplant | Genotype chimerism | 0.62% | Most male cells were macrophages |
| Wu *et al*. 2003[67] | Sex mismatched liver transplant | Y+ cells | Very rare or nil | Long-term follow-up |

tis.[62] Subsequent human investigations with granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood CD34+ stem cells have shown that these cells are also apparently able to transdifferentiate into hepatocytes, with 4–7% of hepatocytes in female livers being Y chromosome-positive after the cell transplant from male donors.[63] However, it is worth noting that some other studies, examining the contribution of recipient cells to liver allografts in humans, have failed to register any real engraftment in the allografted liver (see Table 1.3).[65–67] Given the rodent data suggesting that the mechanism of these observations is likely to be cell fusion (except the NOD/SCID experiments), it is possible that cell fusion explains these observations. Technical difficulties make it hard to demonstrate clearly whether all these 'marrow-derived hepatocytes' are the result of fusion, transdifferentiation or a mixture of both.

While there is a limited degree of differentiation of bone marrow cells into hepatocytes, there is a more robust axis of regeneration of non-parenchymal cells from the bone marrow. It has been well described that, within the liver, the Kupffer cells and inflammatory cells traffic between the host's bone marrow and the liver graft. Gao *et al*.[68] demonstrated that in human liver grafts the hepatic endothelium became replaced by cells of recipient origin. This was studied further in female mice that had received a male bone marrow transplant, and it was demonstrated that it was the bone marrow-derived cells that were responsible for repopulating the liver endothelium, in contrast to the endothelium of the heart, kidney or musculature. The contribution of the bone marrow to hepatic myofibroblasts in humans has been demonstrated by Forbes *et al*.[69] who analysed male patients who received female liver transplants and then developed recurrent hepatitis and fibrosis. Here, up to 40% of the liver's myofibroblasts were of male origin. In order to identify the origin of these circulating extrahepatic cells, liver tissue was analysed from a female patient who had undergone a male bone marrow transplant before developing hepatitis C-induced cirrhosis. In this case a quarter of the liver's myofibroblasts were of bone marrow origin. The exact relationship between the bone marrow and the Ito cells/quiescent stellate cells remains to be determined, but it may be that both contribute to the collagen-forming cells (myofibroblasts) in cirrhosis.

## Stem cells and cancer

There is no doubt that the integration of hepatitis B virus (HBV)-DNA into the human genome is a significant event in hepatocarcinogenesis.[70,71] Moreover, inspection of viral integration sites among tumour cells clearly indicates that each tumour is monoclonal, i.e. is derived from a single cell.[72,73] The important question is, which cell? Stem cell biology and cancer are inextricably linked. In continually renewing tissues such as the intestinal mucosa and epidermis, where a steady flux of cells occurs from the stem cell zone to the terminally differentiated cells that are imminently to be lost, it is widely accepted that cancer is a disease of stem cells because these are the only cells that persist in the tissue for a sufficient length of time to acquire the requisite number of genetic changes for neoplastic development. In the liver, the identity of the founder cells for the two major primary tumours, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC), is more problematic. The reason for this is that no such unidirectional flux occurs in the liver. Moreover, the existence of bipotential hepatic progenitor cells (HPCs), often called oval cells, along with hepatocytes endowed with longevity and long-term repopulating potential, suggests that there may be more than one type of carcinogen target cell. Irrespective of

which target cell is involved, what is clear is that cell proliferation at the time of carcinogen exposure is pivotal for 'fixation' of the genotoxic injury into a heritable form. Taking this view, Sell has opined that in models of experimental hepatocarcinogenesis as a whole, there may be at least four distinct cell lineages susceptible to neoplastic transformation.[74,75] This supposition is based on the fact that there is considerable heterogeneity in the proliferative responses that ensue after injury in the many different models of hepatocarcinogenesis. Thus, hepatocytes are implicated in some models of HCC, direct injury to the biliary epithelium implicates unipotent cholangiocytes in some models of cholangiocarcinoma, while HPC/oval cell activation accompanies very many instances of liver damage irrespective of aetiology, making such cells very likely carcinogen targets. A fourth cell type that might be susceptible to neoplastic transformation is the so-called nondescript periductular cell that responds to periportal injury. The suggestion that such a cell may be of bone marrow origin would be experimentally verifiable in the context of a sex-mismatch bone marrow transplantation (see above) and the appropriate carcinogenic regimen.

The direct involvement of hepatocytes in hepatocarcinogenesis has been clearly established in rats. Gournay *et al*.[76] found that some preneoplastic foci (expressing gamma glutamyl-transpeptidase and the placental form of glutathione-S-transferase) were directly descended from hepatocytes. This was achieved by stably labelling hepatocytes at 1 day after a two-thirds partial hepatectomy, with β-galactosidase, using a recombinant retroviral vector containing the β-galactosidase gene. Subsequent feeding with 2-acetylaminofluorene led to foci, some of which were composed of β-galactosidase-expressing cells. Using the same labelling protocol, Bralet *et al*.[77] observed that 18% of hepatocytes expressed β-galactosidase at the completion of regeneration after a two-thirds partial hepatectomy. Subsequent chronic treatment with diethylnitrosamine (DEN) resulted in many HCCs of which 17.7% of the tumours expressed β-galactosidase, leading to the conclusion that a random clonal origin of HCC from mature hepatocytes was operative in the model.

As discussed above, there is now compelling evidence that oval cells/HPCs are at the very least bipotent, capable of giving rise to both hepatocytes and cholangiocytes. The fact that oval cell activation (ductular cell reaction) precedes the development of HCC in almost all models of hepatocarcinogenesis and invariably accompanies chronic liver damage in humans,[21,31,34,78] makes it almost certain that the mature hepatocyte is not the cell of origin of all HCCs, indeed perhaps only a small minority of HCCs are derived from the mature hepatocyte. The fact that oval cells/HPCs can be infected with HBV is also consistent with a possible histogenesis of HCC from such cells.[79] An origin of HCC from HPCs is often inferred from the fact that many tumours contain an admixture of mature cells and cells phenotypically similar to HPCs. This would include small oval-shaped cells expressing OV-6, CK7 and 19, and chromogranin-A, along with cells with a phenotype intermediate between HPCs and the more mature malignant hepatocytes.[80] Likewise, foci of small cell dysplasia (possible preneoplastic lesions) have an HPC phenotype while foci of large-cell dysplasia, whose relationship to HCC is unclear, do not have an HPC phenotype.[81] Cells resembling HPCs (OV-1+ or OV-6+) have also been noted in hepatoblastoma.[78] This tumour, the most common liver tumour in childhood, is widely believed to be derived from a multipotent progenitor cell, and can even have structures mimicking intrahepatic bile duct formation with the formation of a ductal plate-like structure.[82] Cells with an HPC phenotype have also been noted in a relatively rare subset of hepatic malignancies where there are clearly two major components, an HCC component and a cholangiocarcinoma component, again suggestive of an origin from a bipotential progenitor.[83] Direct evidence of a role for oval cells in the histogenesis of HCC can also be obtained experimentally. Dumble *et al*.[84] isolated oval cells from p53 null mice and when the cells were transplanted into athymic nude mice they produced HCCs. Thus, HCCs in particular, like many tumours, are heterogeneous populations in which many cells are probably terminally differentiated (reproductively sterile) or transit amplifying cells with limited division potential, while at the same time some cells may have stem cell-like properties. In many human tumours, it is becoming apparent that only tumour stem cells are capable of 'transferring the disease'. For example, in human acute myeloid leukaemia, only the CD34+CD38– cells are capable of transferring the disease to NOD/SCID mice, while in human breast cancer, the CD44+ESA+CD24–/low fraction has a similar potential.[85] It would be of enormous interest to know if HPCs in HCCs are the only cells capable of propagating the tumour in immunodeficient mice.

## Conclusions

In this chapter we have tried to summarize the role of the several cell lineages that might be involved in liver regeneration in the virally infected and chronically damaged liver (see Plate 1.3, facing p.786). The ability of hepatocytes, the unipotent cholangiocytes and the bipotent oval/HPCs to contribute to liver regeneration is not in doubt, although the identity of the cells within the parenchymal mass and biliary tree deserving of the appellation of 'stem cells,' is still unclear. The role of extrahepatic cells in liver regeneration, in particular HSCs, is also uncertain with claims for and against a participation in the regenerative process. However, HSCs do

contribute to the scarring which is an inevitable feature of the chronically damaged liver and the evidence proffered for an origin of many HCCs from oval/HPCs is becoming increasingly persuasive.

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# Chapter 2
# Hepatic immunology

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## Overview

The liver represents a unique immunological environment, and this has major implications for our understanding of liver pathology. Until recently, the immune system has been viewed as a single circulating system, with cellular and molecular components designed to patrol the body, constantly on the alert to detect potential harm. However, a more sophisticated view of immunology is emerging, in which the unique features of specific regional immune systems function, sometimes to preempt and sometimes to complement systemic immune mechanisms. Several organs in the body, including the gut, lungs, uterus and of course the liver, are now recognized to have specialized local immune systems that respond to specific immunological challenges offered by distinctive sets of locally presented antigens, both harmful and harmless. The large volume of blood from the gastrointestinal tract bearing dietary antigens and those derived from commensal microbes, and potentially laced with pathogens and toxins, provides specific challenges for the hepatic immune system. The fact that the intestine is a frequent site of carcinogenesis and the large circulating blood supply constantly exposes the liver to encounter with any possible metastatic cells, makes it a potentially important site of tumour surveillance. The liver's role in detoxification of ingested toxins may also contribute to increased risk of local malignancy, due to exposure to and metabolic activation of carcinogens. In addition, neo-antigens may result from active hepatic xenobiotic metabolism (particularly by the P450 oxidase system), running the risk of inadvertent haptenation of self proteins, thus promoting autoimmune-type reactivity.

Cellular and molecular components of innate and adaptive immunity that function locally, some of which may originate locally, combine with elements of the circulating immune system to provide the liver with its own, distinctive regional immune system. This regional immune system is not quietly dozing, waiting for some signal to become active, but is continuously in action, constantly surveying and sampling, distinguishing harmful from harmless stimuli, thus providing persistent protection against infection and malignancy, but tolerating dietary, commensal-derived and self-antigens.

## Relevance of hepatic structure to immunological function

The location, structure and physiological role of the liver are all critical to its immunological function. The liver is the largest solid organ in the body, comprising approximately 2% of the adult body weight, and is responsible for a remarkable variety of functions, including anabolic and catabolic metabolism, exocrine, endocrine, reticuloendothelial and immunologic functions.[1] The liver represents a unique tissue microenvironment, reflecting its anatomic location as well as its physiologic and metabolic roles, many of which are hepatocytic, although important liver functions also reside in other cell types. All of these activities require a high-capacitance and low-velocity vascular bed to facilitate exchange between the cellular components of the liver and blood.

### Gross anatomy and blood flow

The gross and microscopic anatomy represent the morphologic solution to multiple physiological demands. The roughly wedge-shaped liver is covered by a fibrous tissue capsule, containing type I collagen, which invaginates into the portal hilus and continues to invest the portal tracts with supportive connective tissue as the portal tracts sequentially arborize, ultimately ending in the portal triad of the hepatic lobule, the functional anatomic subunit of the liver. Within the connective tissue of the portal tracts run similarly arborizing branches of the hepatic artery, portal vein, bile duct and lymphatics.[2] This dual blood supply (portal venous and arterial blood) to the liver is significant for many aspects of hepatic biology and is of particular importance in understanding the liver as a unique immunologic microenvironment. In humans, the liver receives more than a quarter of the total cardiovascular output. Approximately 25–30% of hepatic blood is well oxygenated arterial

blood from the hepatic artery branch of the abdominal aorta.[3] The remaining blood supply comes from the portal vein, which drains the intestines and spleen. This venous blood is low in $O_2$ tension, low pressure and high in dietary products as well as products derived from the commensal gut flora, such as lipopolysaccharide (LPS). In addition, portal venous blood brings bile-derived material reabsorbed from the gut back to the liver, the so-called enterohepatic circulation.[3] As such, the liver is the portal through which foreign molecules from the outside world of the gut must pass on their way to the general circulation.

## The functional subunit: the hepatic lobule

The functional anatomic unit of the liver has been variably described as the 'lobule' or 'acinus', made up of hepatocyte plates and multiple portal triads surrounding a single central vein.[4] The portal structures (the portal venules, hepatic arterioles, bile duct and associated ductules as well as lymphatic channels) are embedded within supportive connective tissue within the portal tract. This connective tissue contains abundant structural fibres of type I collagen (Plate 2.1, found between p.786–7). Under normal circumstances, type 1 collagen is not present to any significant degree in the parenchyma of the lobule.[4] The portal tract vasculature is of the usual, non-fenestrated type and is invested with a basement membrane, which includes collagens type IV and V. Bile ducts and lymphatic channels also have associated basement membrane.[5] The portal tracts must allow for transport of blood into the sinusoidal spaces of the lobule as well as allow bile and lymph to move in the opposite direction, from the lobule to the portal tract. Although there is some evidence suggesting that a small minority (5–10%) of sinusoids receive only arterial blood, it is clear that the majority of the sinusoids contain mixed portal venous and hepatic arterial blood.[6]

The portal tract is delimited by a single ring of adjacent hepatocytes, termed the limiting plate. In addition to hepatocytes, the cells of the hepatic lobule include a specialized type of endothelial cell (liver sinusoidal endothelial cells, LSECs), resident liver macrophages called Kupffer cells, myofibroblast-like hepatic stellate cells (also known as the Ito cell, or the hepatic lipocyte) and resident natural killer (NK) cells (formerly known as 'pit cells'). The LSECs and Kupffer cells are interspersed in a mosaic fashion to make up the sinusoidal lining, although Kupffer cells can also be highly motile and travel within the intravascular sinusoidal space and in the subendothelial space of Disse. Liver resident NK cells and stellate cells reside within the space of Disse. Each of these cell types will be described below, with particular emphasis on their role in modulating immunobiological parameters in the liver.

## The unique vascular architecture of the lobule and its functional consequences

Blood in the lobule slowly percolates along the sinusoidal space within an anastomosing network of hepatic cords or plates, moving towards the central vein. The flow is dynamic and can be affected locally by a number of factors, including contraction of actin-filament-containing stellate cells.[7] Not only do the high-capacitance and low-flow haemodynamics of the lobule facilitate diffusion of molecules between the blood and cells of the lobule, but exchange is further augmented by a unique ultrastructural feature of the LSEC, the fenestrae, or pores (~ 0.1 nm in diameter) which often cluster into sieve-plates.[8] Unlike most other vascular beds, the LSECs lining the sinusoids do not rest on a continuous basement membrane, but rather on a fine, discontinuous scaffolding of collagen type IV and fibronectin.[9] This fine meshwork of collagen IV is dramatically increased during cirrhosis (Plate 2.2, found between p.786–7). Although the cellular components of blood are excluded from passage through the fenestrae, plasma components can freely diffuse into the subendothelial space of Disse and gain access to the apical hepatocyte cell membrane, which is rich in microvilli, increasing the surface area of hepatocyte/plasma interactions. As can be readily appreciated in scanning electron micrographs, the space of Disse in the normal liver is a potential space (expanded pathologically at times by oedema, fibrosis or cellular infiltrates). Scanning electron micrographs show that Kupffer cell pseudopodia often extend into the vascular space to explore passing erythrocytes and other intravascular cells, while other pseudopodia penetrate into the subendothelial space of Disse, where they may make contact with hepatocytes or other cells.[10]

## Hepatic lymph flow and drainage

One consequence of the extraordinary permeability of the normal sinusoidal vasculature is the production of large quantities of protein-rich lymph (~6 g/dL or 80–90% of plasma levels). Under resting conditions, approximately 50% of lymph is formed in the liver and drains from the space of Disse into the portal tract lymphatic channels. Recently, collagen-lined channels have been described which penetrate the hepatocytes of the limiting plate and act as a conduit for the passage of interstitial fluid and, presumably, dendritic cells, into the portal lymphatic space.[11] Large-calibre, efferent lymphatics are also found within the large portal tracts as well as in the subcapsular region.[12]

### Plasma proteins, complement, coagulation factors and acute phase reactants: innate immunity in the liver

Innate immunity is the initial rapid response to potentially dangerous stimuli, including pathogens, tissue injury, stress and malignancy. The liver is the primary site of systemic innate immune responses and hepatocytes and their products are major players. Expression of receptors for inflammatory cytokines and bacterial constituents by hepatocytes mediates the response to systemic inflammatory stimuli by stimulating synthesis and secretion of inflammatory cytokines, acute phase proteins and antimicrobial peptides. Hepatocytes are also the primary producers of complement proteins, key molecular mediators of the innate immune response. The complement system consists of a tightly regulated group of serum zymogens. Activation of the complement cascade can lead to the production of activated protein fragments with a variety of functions, including chemotaxis, opsonization, activation of immune cells as well as target cell lysis. They are also important for liver repair after toxic injury: the complement components C3 and C5 have been shown to be essential for liver regeneration, and to mediate their effect by interacting with key signalling networks that promote hepatocyte proliferation. Mice lacking C3 or C5 exhibited high mortality, parenchymal damage and impaired liver regeneration after partial hepatectomy.[13] In response to pro-inflammatory cytokines – particularly tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-6 – hepatocytes increase the synthesis of plasma acute phase reactants such as C-reactive protein, amyloid and fibrinogen, which facilitate tissue regeneration as part of the systemic immune activation. These cytokines and granulocyte-macrophage colony-stimulating factor (GM-CSF) are also known to have an important role in liver regeneration.[14] Hepatocytes also produce serum mannose-binding lectin, which recognizes microbial-specific sugar motifs, leading to activation of the immune system and microbial clearance through opsonization.[15]

## Hepatic reticuloendothelial cells

Kupffer cells represent the largest population of tissue-resident macrophages in the body. They are interspersed with LSECs in a mosaic fashion to make up the cellular aspect of the sinusoidal lining (Plate 2.3, found between p.786–7). They can be highly motile and are active phagocytes, playing an important role in surveillance and uptake of intravascular debris, including effete cells (particularly erythrocytes), bacteria and other blood-borne particulates.[16,17] Taken together, the intra-sinusoidal location of the Kupffer cells, the high-capacitance/low-flow haemodynamics of the liver, the location of the liver 'downstream' of the portal circulation and the diverse sets of cell surface receptors present on the Kupffer cells, all significantly contribute to making the liver an efficient 'filtration system' for the blood. The hepatic reticuloendothelial system is not simply a passive filter, but also functions as a sentinel to identify microbial threats and activate both innate and adaptive immune responses.

The consensus is that that Kupffer cells are derived from circulating bone marrow-derived monocytes. However, myeloid stem cells have been demonstrated in adult liver,[18] suggesting local development of perhaps some populations and Kupffer cells may also be capable of limited self-renewal.[19] It is likely therefore that there are several populations of Kupffer cells and it is not surprising that there is considerable overlap in phenotypic markers between Kupffer cells and 'newly recruited' monocytes/macrophages, or for that matter, other closely related myeloid-derived cell types such as liver dendritic cells.[20] In mice and rats, the presence of F4/80 antigen (which becomes expressed as monocytes mature into tissue resident macrophages) on sinusoidal liver cells has been used to 'define' Kupffer cells, but low levels of F4/80 have also been reported to be expressed on dendritic cells as well. Using electron microscopy, Kupffer cells can be identified by endogenous peroxidase localized to the rough endoplasmic reticulum and perinuclear envelope.[21]

### Kupffer cells as reticuloendothelial phagocytes

To facilitate this reticuloendothelial function, Kupffer cells are endowed with a number of cell surface receptors and receptor complexes, including a variety of complement receptors (CR), Fc-receptors and receptors for lectin-containing opsonins such as plasma mannose-binding lectin. Human Kupffer cells also express CR1 (CD35), CR3 (Mac-1; CD11b/CD18) and CR4 (CD11c/CD18, p150/95).[22] These receptors appear to have multiple ligands apart from complement-derived proteins, including intercellular adhesion molecule-1 (ICAM-1) and polysaccharides of microbial and host origin.[23] Murine Kupffer cells appear to have some significant differences in receptor expression patterns. For instance, it has been reported that Kupffer cells in mice do not express significant amounts of CD11c.[24] Kupffer cells express both high-affinity Fcγ receptors, which facilitate phagocytosis of IgG-coated particles, and receptors for IgA.[25,26] This ability to bind IgA-coated particles is thought to represent an important 'second line of defence' in the case of a breach of lower gastrointestinal mucosal immune barriers. In addition to these opsonin receptors, Kupffer cells express galactose and mannose receptors[27] and scavenger receptors, which are capable of directly binding microbial surface compo-

nents such as sugars and polyanionic moieties,[28] as well as receptors for bacterial N-formylmethione-containing peptides.[29] The capacity of Kupffer cells to phagocytose intravenously-injected latex microspheres is illustrated in Plate 2.4 (found between p.786–7).

## Toll-like receptors: sentinels of innate immunity

Toll-like receptors (TLRs) represent an evolutionarily conserved group of molecular pattern recognition receptors, which bind different microbe-derived molecules and activate phagocytes including Kupffer cells.[30] At the time of writing, 10 mammalian TLRs have been identified, all of which function through receptor-associated kinases.[31] The TLR may associate with other non-TLR cell surface receptors (such as CD14 in TLR4-binding of enodotoxin [lipopolysaccaride, LPS]) or may form heterodimers with other TLRs to achieve unique binding specificities. In the prototypic LPS-driven activation of TLR4 signalling in macrophages, NF-κB is activated, leading to production of pro-inflammatory cytokines (TNF-α, IL-10, IL-12, interferon-gamma – IFN-γ), as well as upregulation of microbicidal mechanisms such as the production of reactive oxygen and nitrogen species. Kupffer cells may represent a unique population of tissue-resident macrophages in that they are subjected to unusually high basal levels of gut-derived TLR ligands such as LPS.[32,33] As a consequence of this chronic LPS stimulation, Kupffer cells are thought to constitutively produce IL-10, leading to the establishment of the predominant anti-inflammatory cytokine milieu in the liver.[34]

## Linking to adaptive immunity: cytokines, chemokines and antigen presentation

Like other macrophage populations, Kupffer cells play an important role in activating adaptive immune responses and integrating adaptive and innate immune functions. Depending on the context, Kupffer cells can produce pro- and anti-inflammatory cytokines as well as other immune-regulatory molecules such as prostaglandins, reactive oxygen and nitrogen species and chemokines.[35] In fact, Kupffer cell-derived chemokines have been shown to play an important role in the recruitment of NK cells, dendritic cells, macrophages, neutrophils and lymphocytes in various experimental settings.[36–40] Kupffer cells have also been shown to play a role in the determination as to whether inflammation in the liver is biased towards a Th1- or Th2-type response.[41,42] In addition to the production of immunemodulatory molecules, Kupffer cells can play a direct role in adaptive immunity by functioning as antigen-presenting cells. Numerous studies have shown that Kupffer cells can cross-present antigen, and present antigen both in the context of major histocompatibility complex (MHC) class I[43] and class II molecules.[44] Although in certain experimental conditions, Kupffer cells are capable of expressing positive co-stimulatory molecules and stimulating naïve CD4 and CD8 T cells,[45] antigen presentation by Kupffer cells is generally found to be tolerogenic, and this may involve expression of negative co-stimulatory molecules such as PD-L1.[46]

## Dendritic cells

Dendritic cells (DCs) are bone marrow-derived cells, which are thought to represent the critical antigen-presenting cell required for the stimulation of naïve T cells. Morphologically, DCs show thin membranous projections and are currently believed to be derived from both myeloid and lymphoid lineages, although the latter is controversial. Immature DCs can be found residing within the epithelial compartment of organs such as the gut, skin and lungs, well positioned to intercept microbial antigens. After capturing antigen, DCs begin to mature, and transport the antigens to draining lymph nodes to initiate an adaptive immune response.[47] The regional lymph nodes draining the liver to which the DCs presumably migrate include the hilar, hepatoduodenal ligament lymph nodes and the caval lymph nodes, which include the hepatic artery and portal vein nodes.

The maturation of DCs depends on signals from the environment, including cytokines and the engagement of pattern-recognition receptors that respond to conserved structural motifs of viruses and bacteria.[48] Thus, microbial endotoxin engages TLR4 and TLR2, while structural features of microbial DNA (CpG) engage TLR9. In the presence of these signals, full maturation of DCs occurs. In contrast, if such signals are absent, DCs may differentiate to a semi-mature state, in which they will interact with T cells to promote abortive T-cell differentiation, or suppressor rather than effector T-cell function.[49]

Only recently have investigators begun to focus on liver-specific DC populations, and the evidence suggests that distinct subpopulations of liver dendritic cells bias the immunological microenvironment of the liver towards tolerance. Hepatic DCs are present in very low numbers in fresh tissue but they can be expanded upon stimulation with Flt3-ligand. They are then found to have the phenotype of immature DCs, expressing low MHC class II as well as low levels of the co-stimulatory molecules CD80 and CD86. These cells are likely to induce tolerance rather than activation of CD4+ cells.[50–52] Also, a recent study comparing human DCs obtained from surgical explants of skin and liver demonstrated that liver-derived DCs lack CD1a, produce IL-10 and are less efficient at stimulating naïve T cells.[53] There is evidence to suggest that hepatic DCs may differentiate locally in the liver from stem cells. Normal liver is rich in

differentiating haematopoietic stem cells[54–56] and some express the myeloid differentiation marker CD33.[18] Cytokines required for haematopoietic lineage cell proliferation are also found in adult liver including TGF-β, IL-7, IL-10, IL-15 and GM-CSF.[57,58] The normal liver therefore has a cytokine milieu conducive to DC differentiation. Interestingly, transforming growth factor-beta (TGF-β), possibly expressed by hepatocytes, inhibits the maturation of DCs, thus preventing them from becoming activating antigen-presenting cells, while promoting their expansion in GM-CSF.[51]

### Antigen presentation by liver sinusoidal endothelial cells

The immunological role of liver sinusoidal endothelial cells (LSECs) is a subject of debate. Presentation of antigen and activation of T cells has been shown to be a property of LSECs.[59] Physiologically, LSECs play an important role in filtration, endocytosis and regulation of sinusoidal blood flow. Although they possess fenestrations with a mean diameter of approximately 100 nm, particles are excluded. Thus, LSECs function as a barrier between the macromolecules in the sinusoidal lumen and hepatocytes. Receptor-mediated uptake of macromolecules occurs quickly and efficiently via various pattern-recognizing receptors such as mannose receptors. LSECs have a unique immune phenotype, expressing markers typical of cells of myeloid lineage (CD1, CD4, CD11c), even though the evidence suggests that these cells differentiate from hepatocyte progenitors. Thus, LSECs resemble immature DCs more than typical microvascular endothelial cells from other organs, and seem to be a new type of organ-specific antigen-presenting cell.[60] LSECs constitutively express MHC class II molecules together with CD80, CD86 and CD40, indicating an antigen-presenting function that will result in activation of CD4+ T cells.[61] However, CD4+ T cells primed by antigen-presenting LSECs fail to differentiate towards effector Th1 cells but express high levels of immunosuppressive cytokines, including IL-10 and IL-4. This may be due to the prostenoids and TGF-β secreted by LSECs, both of which are known to limit T-cell activation. Interestingly, antigen presentation to CD8+ T cells by LSECs *in vivo* leads to CD8+ cell proliferation followed by the development of antigen-specific tolerance. These cells also lose their ability to respond to specific antigen upon restimulation.[62,63]

## Hepatic lymphocytes

### Circulating T cells with α/β antigen receptors

T lymphocytes expressing clonotypic receptors coded for by rearranged genes normally circulate through the blood, lymphatic vessels and lymphoid organs in a resting state. Activation depends on encounter with a cell that expresses an HLA (human leucocyte antigen) molecule with which the T cell can interact, carrying a peptide for which the T-cell receptor (TCR) is specific. Thus, CD4+ T cells may only be activated by cells bearing HLA class II molecules, while CD8+ T cells may be activated by cells expressing HLA class I molecules. In addition to the signal through the TCR, the outcome of T-cell activation is determined by a series of other intercellular signals, collectively termed co-stimulation. T cells subjected to TCR signals in the presence of strong co-stimulation typically proliferate, mature and become able to deliver defensive immune functions, and may further differentiate into memory cells that provide enhanced protection against a second encounter with the same antigen.

When fully activated, CD8+ T cells display two known functions: cytotoxic killing of target cells, and the synthesis of cytokines including IL-2, IFN-γ and TNF-α. The cytotoxic function is mediated by two distinct mechanisms: the cell surface expression of a cytotoxic ligand, FasL, and the targeted delivery by exocytosis of perforin and granzymes. All of these mechanisms cause apoptosis of the target cell.[64] The defensive function of CD8+ T-cell cytotoxicity is manifest as the elimination of virus-infected cells early in the infection cycle, before the assembly of new infectious virions, but this mechanism may also cause immunopathology. Thus, in a transgenic mouse model of hepatitis B, the capacity of CD8+ T cells to synthesize IFN-γ was important in suppressing viral RNA transcription, while the cytotoxic function was more closely linked to hepatocyte damage.[65]

Activated CD4+ T cells are potentially cytotoxic through their expression of FasL, but they do not secrete granules containing perforin and granzymes. They do secrete cytokines and chemokines. Individual CD4+ T cells may secrete a wide variety of cytokines, or they may become polarized to secrete pro-inflammatory cytokines including IFN-γ and TNF-α (in Th1 cells). Alternatively, they produce a set of cytokines that promote B-cell differentiation and parasite expulsion, but suppress inflammation (in Th2 cells). The signature cytokines of Th2 cells are IL-4, IL-5 and IL-10. A population of activated cells frequently becomes polarized such that either Th1 or Th2 cells predominate. Mixtures containing both kinds of T cells are unstable, as Th1 cells suppress Th2 function, and vice versa.[66] Activated CD4+ T cells may also acquire regulatory function, in which they suppress the activation of other T cells. A subset of regulatory CD4+ T cells differentiates as a result of encounter with self-antigens in the medulla of the thymus, where many self-proteins from diverse tissues are expressed as a mosaic, each in a small proportion of medullary epithelial cells.[67] It is controversial whether peripheral encounter

with antigens on tissue cells or semi-mature DCs simply activates pre-existing regulatory CD4+ T cells, or instead drives undifferentiated CD4+ T-cell precursors to acquire regulatory function.[68]

## α/β T cells in the liver

The normal, healthy liver contains a resident population of α/β T cells, which are found in the sinusoids, in the space of Disse, and in the portal tracts. However, this population differs from the circulating T-cell pool in several respects. First, the normal ratio of CD4+ to CD8+ T cells is reversed (Plate 2.5, found between p.786–7) and CD8+ T cells outnumber the CD4+ T cells in the liver.[69] Second, liver T cells include a high frequency of cells expressing activation markers, and of cells secreting cytokines.[70] Third, liver T cells contain an unusually high frequency of cells undergoing apoptosis.[71]

Many hepatic T cells are cytotoxic effector cells, and kill target cells upon TCR ligation. The majority rapidly secrete cytokines of the inflammatory (Th1/Tc1) type upon pharmacological stimulation or CD3/TCR cross-linking.[72] These include IFN-γ, TNF-α and/or IL-2. Production of pro-inflammatory cytokines by hepatocytes and Kupffer cells is clearly critical for activation of local T-cell populations in the liver, and promotes the inflammatory Th1 context characteristic of the healthy liver.[73] About 5% of human hepatic T cells produce IL-4, a cytokine of the Th2/Tc2 (helper) type, but do not produce IL-5.[72,74] Interestingly, while cytokine production is generally polarized at other sites to the mutually inhibitory Th1/Tc1 or Th2/Tc2 profiles, the majority of hepatic T cells that produce IL-4 also produce IFN-γ, indicating that they are of the Th0 type, rather than conventional Th2 cells. Th0 cells are thought to either be undifferentiated or regulatory cells and there is evidence that both of these T-cell types are present in the liver.

The excess of CD8+ T cells over CD4+ T cells among the resident liver lymphocytes is likely to reflect activation of specific recruiting mechanisms from the peripheral blood. The cytokine milieu and expression of adhesion molecules particularly by liver sinusoidal endothelium will affect this process.[75] Liver sinusoidal endothelium expresses a high level of ICAM-1 as well as vascular cell adhesion molecule-1 (VCAM-1) and vascular adhesion protein-1 (VAP-1).[76–78] Activated T cells express lymphocyte function antigen-1 (LFA-1), the counter-receptor for ICAM-1, and CD8+ T cells express more than CD4+ T cells.[79] The VAP-1 molecule also preferentially promotes CD8+ T cell adhesion. On this basis, the liver will preferentially trap activated CD8+ T cells from a mixture that passes through the sinusoids.[80]

The presence of activated T cells in the normal liver is mysterious. A large proportion of lymphocytes in the healthy gastrointestinal tract – another important site of regional immunity – express high levels of activation markers, suggesting ongoing immunological activity.[81] Another possibility is that liver T cells share some features with resident T cells in other non-lymphoid tissues. It has recently become clear that many tissues, including skeletal muscle, body fat, kidney, lungs and brain, contain T cells, and that many of these have an activated and/or memory phenotype. Some of the tissue T cells result from the 'diaspora', a redistribution of memory T cells from lymphoid to non-lymphoid tissue at the end of a primary immune response.[82,83] Tissue memory T cells differ from lymphoid (central) memory cells, in that they have more functional activity *ex vivo*, and a lower rate of apoptosis.[84] The activation status of liver T cells places them in this category, although they also contain a high frequency of cells undergoing apoptosis.

The activation and apoptosis of liver T cells could be linked, as part of the induction of liver tolerance. The liver is involved in T-cell tolerance on several levels. First, the process of oral tolerance maintains T-cell unresponsiveness to harmless antigenic components in food. Oral tolerance depends on the portal circulation, which carries food antigens and antigen-presenting cells from the gut to the liver. Thus, a surgical porta-caval anastomosis prevents the induction of oral tolerance, while injection of antigen-presenting cells into the portal vein promotes systemic tolerance.[85] The co-existence of activated and apoptotic T cells in the liver could be explained partly by the induction of oral tolerance. The activation and apoptosis of liver T cells also co-exist during liver transplantation tolerance. In many mammalian species, including mice, rats and humans, a liver allograft may be accepted without immunosuppression, and the tolerance induced by the liver graft is systemic, because it promotes the survival of normally immunogenic allografts from the same donor, such as kidney grafts. In a recently allografted liver, there is a large T-cell influx, but these T cells undergo apoptosis, leaving the liver graft unharmed.[86] This has been interpreted as the induction of tolerance through activation-induced cell death in the T cells.

Apoptotic CD8+ T cells are also found in the liver as a consequence of the activation of T cells at other sites. Thus, in experimental influenza infection in mice, the use of a peptide-MHC tetramer to identify CD8+ T cells specific for a particular flu-encoded epitope found these cells in the lungs at the time of peak virus infection, but subsequently in the liver.[87] Similarly, the activation of mouse CD8+ T cells using an antigenic peptide causes massive pooling of dying T cells in the liver.[88] The extent to which these phenomena are liver-specific is controversial, but the accumulation of activated CD8+ T cells during systemic T-cell activation is sufficient to cause hepatocyte damage, evident both histologically

and in terms of elevated serum amino-transaminase enzymes.[89]

## Non-classical lymphoid populations in the liver

While conventional single-positive CD4+CD8– and CD8+CD4– αβ T cells account for many hepatic CD3+ T cells, the liver also contains significant numbers of T cells that express neither CD4 nor CD8 (double-negative) and T cells that are double-positive for both CD4 and CD8, as well as T cells that express the CD8α chain in the absence of the β chain (CD8 $\alpha^{+}\beta^{-}$ cells[90]). A significant proportion of T cells with unconventional CD4/CD8 expression express the γ/δ TCR. Indeed, healthy human liver has a surprisingly high population of γ/δ TCR+ T cells.[91,92]

## T cells with γ/δ antigen receptors.

The identification of the genes encoding the TCR α and β chains resulted in the accidental discovery of a second pair of TCR genes, γ and δ. These genes encode a distinct category of antigen receptors that are expressed on early fetal thymocytes, in γ/δ T lymphocytes resident in many epithelia, and on a small subset of T cells in the circulation, the lymphoid organs, and many tissues including the liver. The specificity and function of γ/δ T cells are much less well understood than is the case for α/β T cells. Many γ/δ T cells arise early in ontogeny, and express TCRs of limited diversity. They are found in significant numbers in the intestinal epithelium, skin, lungs and the pregnant uterus where they respond to the stress-inducible proteins MICA and MICB and CD1c and contribute to tissue remodelling as well as immune surveillance.[93,94] It appears that different γ/δ T-cell subsets have distinct cytokine secretion patterns and thus may have several functions as well as targets.[95] In the mouse, dendritic epidermal T cells of the skin express invariant γ/δ receptors, while cells that populate the female genital tract and tongue also show limited γ/δ receptor diversity. A large population of γ/δ cells is found among the intestinal intra-epithelial lymphocytes, and these cells express more diverse receptors.[92] The availability of gene-targeted mice that specifically lack γ/δ T cells has not revealed a clear and uniform role in the resistance to infection. A full discussion of this issue is outside the scope of the present brief summary, but several contradictory examples are given in the following section on liver γ/δ cells. One aspect of γ/δ T cell function seems to be the maintenance of tissue integrity. The dendritic epidermal T cells of mice respond to stressed keratinocytes in tissue culture, and secrete keratinocyte growth factor. The lack of these cells in gene-targeted mice impairs the healing of skin wounds.[95] In the intestine, infection with the parasitic protozoan *Eimeria vermiformis* usually results in minimal damage to the intestinal mucosa, but in the absence of γ/δ T cells this damage is more severe.[96] The failure to contain *Klebsiella pneumoniae* in the lungs of infected mice that lacked γ/δ cells could also be seen as a failure of tissue integrity,[98] while the correlation of γ/δ cell frequencies with the severity of liver damage in several studies (see below) is also consistent with this view of γ/δ cell function.

## γ/δ T cells in the liver

Up to 35% of hepatic T cells express γ/δ TCRs, making the liver one of the richest sources of γ/δ T cells in the body. There is a striking appearance of γ/δ T lymphocytes in the liver of mice after birth. The majority of hepatic γ/δ T cells express CD8+ or double-negative (DN) phenotypes and NK cell markers (CD56, CD161, KIRs, CD94) and they account for up to 60% of the population of hepatic T cells that co-express NK receptors. Despite their unusually large percentage in normal liver, the role of hepatic γ/δ T cells is unclear, although their heterogeneity suggests several functions. Unlike conventional α/β T cells, γ/δ T cells express oligoclonal or invariant TCRs that recognize soluble non-peptide antigens and stress-inducible proteins, sometimes without the need for MHC restriction. This suggests that T-cell responses to non-peptide antigens through non-classical mechanisms may be of greater importance in the liver than in other organs. Antigenic ligands recognized by γ/δ T cells include phosphorylated thymidine metabolites derived from mycobacteria,[99] bacterial and plant alkylamines[100] and viral glycoproteins.[101] The antigenic targets of hepatic γ/δ T cells are not known, but their high frequency in the liver suggests that they may have an important role in the rapid elimination of microbes and virally infected and tumour cells from the liver, an interface where a large number of external antigens are constantly sampled. The presence of CD1c in the liver has not been definitively documented. Therefore, it is unclear if this molecule plays a role in hepatic γ/δ T-cell activation or if these cells recognize alternative ligands in the liver.[102]

Like intrahepatic α/β T cells, liver γ/δ T cells are activated and appear as large blast cells that proliferate *ex vivo*. In the mouse, liver γ/δ cells are enriched for a subset of receptors encoded by Vγ1 and Vδ6,[103] while in the human, the majority of hepatic γ/δ T cells express Vγ9 and/or Vδ2 chains.[92] Unlike γ/δ cells of the skin, these receptors are neither exclusive to this site nor predominant in it. However, over 20% of hepatic γ/δ T cells do express Vδ3, a receptor rarely found in the blood, suggesting a possible antigen-specific expansion of this cell population within the liver. In tumour-bearing hepatic tissue, there was an expansion of Vδ1+ γ/δ cells which recognize the stress-inducible MICA and MICB molecules that are upregulated as a result of tumour transformation and virus infection.[105]

Bacterial infection of mice with two very different pathogens, *Listeria monocytogenes* and *Leishmania major*, resulted in an increase in the frequency of activated liver γ/δ T cells,[105,106] but it is not clear whether this was a specific response to the infection, or an effect of the accompanying tissue destruction. In the specific setting of very young (14-day-old) mice, the lack of γ/δ T cells caused increased susceptibility to *Listeria* infection.[107] However, in adult mice, specific depletion of the Vγ1 subset of γ/δ cells improved resistance to *Listeria* infection. Thus, different subsets of γ/δ cells might augment or suppress antibacterial immunity.

While γ/δ T cells are thought to have a role in antiviral immunity in the liver, their involvement in hepatitis C is particularly difficult to decipher. In humans infected with hepatitis C virus (HCV), T cells grown from the liver in a cytokine cocktail were enriched in γ/δ cells.[109] In another study, there was an increased frequency of γ/δ T cells using the Vδ1 chain, and this frequency correlated with hepatocellular damage.[110] In contrast, in liver biopsies of humans infected with HCV, quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) indicated a reduction in TCRδ chain RNA compared with markers of α/β T cells.[111] Another study showed selective expansion of classical CD4+ α/β T cells in HCV-infected livers.[112] These data, and many others, are contradictory even on the simple question as to whether HCV causes an increase, or a decrease, in liver γ/δ T cells. It is therefore very difficult to accommodate the data within a unified model.

## Natural killer cells

Natural killer (NK) cells, key players in non-adaptive immune responses, are the predominant innate immunocyte population in the livers of mice and humans, accounting for up to 50% of the total lymphoid pool in the non-diseased liver. The first description of hepatic NK cells used immunohistological analyses of liver tissue from rats to describe large granular cells present in the liver sinusoids. These cells, originally termed pit cells, are capable of spontaneously lysing tumour cell lines and participate in innate immune responses against viruses, intracellular bacteria, parasites and transformed cells.[113–115] The higher numbers of NK cells in liver compared with blood is reflected by higher levels of NK cytotoxic activity that are associated with hepatic mononuclear cells. NK cells are even more elevated in patients with hepatic malignancy.[116–119] NK cells do not have antigen-specific receptors but detect changes in membrane glycoprotein expression on target cells. Their activity is controlled by receptors that mediate activation or inhibition upon ligation of surface molecules on target cells, and by cytokines in the environment such as IFN-γ, IL-2, IL-12 and IL-15. In mice, the NKR-P1 family of molecules, which includes NK1.1, mediates NK cell activation. The only known human NKR-P1 molecule, CD161, does not, although it is present on most NK cells. Human NK receptors that mediate activation include CD16, the Fc receptor for IgG, responsible for antibody-dependent cellular cytotoxicity (ADCC) and cytokine secretion upon ligation by antibody-coated target cells; and the NKG2D molecule which binds to the stress-inducible MICA molecule on target cells. In addition, several other co-stimulatory and adhesion molecules including CD2, CD11a/CD18 (LFA-1), CD69 and CD49d/CD29 (VLA-4) have been implicated in NK cell activation.[120] MHC class I ligation of NK surface molecules (p58.1/p50.1, p58.2/p50.2 and CD94) can result in either activation or inactivation of NK cells with the inhibitory signal exerting a dominant effect over the activating signal. The complex interactions between NK receptors and classical human leucocyte antigen (HLA) class I molecules which present intracytoplasmic peptides (markers of self), between CD94 and the non-classical HLA-E molecule which presents peptides derived from classical class I molecules (a marker of class I turnover), and between NKG2D and the MHC class I-like MICA molecule (a marker of stress) allow NK cells to respond to subtle changes in MHC class I expression and antigen presentation which may occur in tumour cells and virus-infected cells.

### *Natural T cells*

A large proportion of γ/δ T cells in the liver co-express NK markers, as do a significant proportion of α/β T cells. T lymphocytes that co-express NK receptors include phenotypically and functionally diverse populations of cells that can account for more than half of all hepatic CD3+ cells. These T cells have restriction patterns, antigen recognition mechanisms, cytokine profiles and responses to stimulation which differ significantly from conventional T lymphocyte populations and are shared with NK cells. Thus, these cells are called natural T (NT) or NKR+T cells and are thought to represent an evolutionary and functional bridge between innate and adaptive immunity.[90]

### *NKT cells*

In the mouse, a significant population of hepatic NKR+ T cells expresses an invariant TCR consisting of the Vα14Jα281 TCRα chain with little or no N region additions, and a limited number of β chains (Vβ2, Vβ7 and Vβ8). These cells are restricted by CD1.[121,122] They comprise CD4+ or CD4–CD8–DN T cells and express TCR levels intermediate between those on immature and mature thymocytes (and have thus been termed $TCR^{int}$ cells) as well as the NK receptors, NK1.1, Ly49

and IL-2Rα (therefore, they are also known as NK1.1T cells). These cells are characterized by their potent effector function, revealed by their ability to kill tumour cells in the absence of prior antigenic stimulation, and to rapidly produce high levels of both Th1 and Th2 cytokines upon activation through their TCR or NK1.1 molecules. This can mobilize NK cells, other lymphocytes and additional components of the innate and adaptive immune response. Murine NKT cells rapidly produce IL-4 and were thought to have a role in the initiation and regulation of Th2 cell differentiation and antibody isotype switching,[123] although they are not Th2-like as they can also produce IFN-γ, as well as IL-5 and IL-10. NKT cells respond to IL-12 and IL-18, and the liver has been shown to be a rich source of these cytokines. NKT cells cross-reactively recognize α-galactosylceramide (α-Gal-Cer), a glycolipid antigen found in marine sponges. Such recognition is restricted by the MHC-like molecule, CD1d. However, the natural hepatic ligand for CD1d-restricted T cells remains a mystery. There is evidence that NKT cells accumulate in the liver, and it has also been shown that the liver and marginal zones appear to be preferential sites of NKT activation and early NKT cell cytokine production.[124] Preferential residence in the liver raises questions about the reasons and consequences for the accumulation of NKT cells at this site.

There is now significant evidence that NKT cells have a major role in tumour surveillance. Stimulation of Vα14Jα281+ T cells in mice by α-Gal-Cer or IL-12 can result in the prevention of tumour metastasis by a mechanism that is dependent upon IFN-γ production and/or anti-tumour cytotoxicity by NKT cells.[125–128] These findings have encouraged the use of both IL-12 and α-Gal-Cer in patients with malignancy, and phase I trials using both compounds in human patients are ongoing. In contrast to mice, human NKT cells are found in small numbers in healthy liver (0.5% of CD3+ cells) and are not expanded in tumour-bearing tissue.[129] Most human hepatic Vα24+ NKT cells express the Vβ11 chain. They include CD4+, CD8+, and CD4–, CD8– cells, and many express the NK cell markers CD56, CD161 and/or CD69. Importantly, human hepatic Vα24+ T cells are potent producers of IFN-γ and TNF-α, but not IL-2 or IL-4, when stimulated pharmacologically or with α-Gal-Cer.[129]

## Hepatic immunoregulation

The liver appears to represent a tolerogenic local immune environment, based both on transplantation and portal vein tolerance models, in which initial exposure to antigens delivered to the gut and/or directly into the portal circulation preferentially lead to the induction of tolerance rather than immunity.[33] It is well known that liver transplant patients require less immunosuppression than the recipients of other organs. Moreover, additional organs are more likely to be tolerated if transplanted at the same time as the liver, indicating generation of systemic tolerance to antigens expressed on the liver.[130] Several mechanisms are likely to contribute to local tolerogenic effects, all of which will influence hepatic immune responses to local pathological challenge.[33,45,49,61] Recent work has focused on the mechanisms of activation of T cells in the hepatic environment and the subsequent generation of systemic tolerance.[131] There is growing appreciation for the contribution of various lymphoid and antigen-presenting cell populations to local regulation of hepatic immunity and its influence on systemic responses.

### Immune regulation and tolerance: contribution of hepatic NKR+T cells

It is becoming apparent that NKR+T cells are extremely heterogeneous and highly versatile and have important immunoregulatory activities.[132] The high levels of inflammatory cytokines and high proportions of hepatic T cells that display activation/memory phenotypes and exhibit potent lymphocyte-activated killer (LAK) cytotoxic activity suggest that rigorous immunoregulatory control is required in the healthy liver. One such mechanism is via signals through the MHC class I molecules (KIRs and CD94) on NKR+T cells. These receptors (p58.1/p50.1, p58.2/p50.2, p70 and CD94) are expressed by similar frequencies of hepatic NKT cells and NK cells.[119] As outlined above, identical signals can induce activating or inhibitory signals to the cell depending on the nature of the cytoplasmic domains of these receptors. However, KIR cross-linking has been shown to result in an overall net inhibition of TCR-mediated cytotoxicity by hepatic NKT cells, rather than activation.[119] This would suggest either that hepatic NKT cells express more inhibitory than activating KIR receptors, or that the inhibitory signals exert a dominant effect over the activating signals. The contradictory cytokine elaboration patterns of these cells reflect the complexity of their immunoregulatory role.

### The immunoregulatory role of Kupffer cells and hepatic DCs

Kupffer cells play a key role in establishing the tolerogenic milieu of the liver[133–135] and both co-stimulation,[136] and direct killing of activated antigen/alloreactive T cells by Kupffer cells have been shown to be involved.[135] Kupffer cells make pro- and anti-inflammatory cytokines and present antigen. In addition to phagocytosis (Plate 2.4, found between p.786–7), an important role for Kupffer cells is the production of inflammatory cytokines. In the IFN-γ rich hepatic environment, high levels of class II

molecules are constitutively expressed, making Kupffer cells potentially important in the presentation of antigen to CD4+ as well as CD8+ T cells of the adaptive immune system. Kupffer cells and T cells form structured inflammatory foci in the liver in various conditions (Plates 2.6 and 2.7, found between p.786–7). The relative proportions of IL-10, IL-12 and IFN-γ in the cytokine environment of the liver, together with the phenotype of the responding T-cell population, determines whether the outcome of activation will be the generation of an effector T-cell response, or tolerance.

IL-12[136] produced constitutively by hepatic myeloid cells, including monocytes, Kupffer cells and DCs, contributes to the local inflammatory environment. High levels of IL-12 have been demonstrated in healthy liver and it is upregulated in tumour-bearing tissue.[137] IL-12 influences the maturation of NK cells, CD8+ T cells and NKT cells, all of which have potent cytotoxic activities. Murine studies have shown that liver DCs induce IL-10 and IL-4 secretion by mononuclear cells.[138] By contrast, comparatively high numbers of IFN-γ+ cells are induced by bone marrow DCs. Modulation of Th2 cytokine production by donor-derived DCs is likely to contribute to the comparative immune privilege of hepatic allografts.

### Non-classical antigen presentation and its consequences

Expression of the classical restriction elements class I and class II MHC molecules that present small peptides to conventional CD4+ and CD8+ T cells is well described in human liver. The presentation of antigen to other non-classical populations of hepatic T cells has not been explored in any great detail. This issue is particularly complicated as the targets for these T cells may not even be exclusively protein in nature. NKT cells respond to the glycolipid α-Gal-Cer presented by CD1d, but other human non-invariant NT cell clones from the liver, peripheral blood and bone marrow are also restricted by CD1.[139,140] Human CD1 molecules, CD1a, CD1b, CD1c and CD1d, are structurally related to MHC molecules and can bind lipid molecules and present them to T cells leading to their activation. CD1a, CD1b and CD1c can present various lipid and glycolipid components of bacterial cell walls, including mycolic acids, inositol-containing phospholipids, and mycolyl glycolipids, to CD8+ and double-negative CD4–CD8– T cells while the significantly more divergent CD1d isoform presents glycosylceramide and glycosyl-phosphatidylinositols antigens to Vα24JαQ+ T cells. Recently, CD1a has been shown to present lipopeptides derived from *Mycobacterium tuberculosis*.[141] While the human liver contains CD1d-reactive Vα24JαQ+ T cells and γ/δ T cells of unknown restriction, additional populations of CD1a, CD1b, CD1c and CD1d-reactive T cells may also be present. The high numbers of multiple populations of DN cells, NT cells and γ/δ T cells, all of which have been shown in various systems to recognize CD1 isotypes, suggest that the liver is a specialized site of CD1-restricted T cells that respond to lipid and glycolipid antigens. Populations of γ/δ T cells of limited TCR variability are restricted by MICA and MICB. It is unknown whether these restriction elements are expressed in the liver, although it is likely that these and additional recognition molecules will be described, leading to the identification of further populations of lymphocytes of limited TCR variability. Although liver DCs have lower CD1d surface expression than hepatocytes, they have been shown to be potent stimulators of IFN-γ and IL-4 release by liver NKT cells when pulsed with α-Gal-Cer *in vitro* or *in vivo*.[142] Liver DCs are thus potent stimulators of pro-inflammatory cytokine release by NKT cells, are activated themselves in the process of NKT cell activation, and express an activated phenotype after the NKT cell population is eliminated following α-Gal-Cer stimulation.

### Leucocyte circulation and hepatic infiltration

Hepatic-specific accumulation of lymphocyte populations will inevitably influence subsequent immune responses. Molecular and physiological features determine which subpopulations are selected.[75] The sinusoids allow oxygenated blood arriving from the portal tract to percolate slowly past the hepatocyte cell plates, allowing maximal exchange of cells and materials before the blood leaves the liver through the central vein branches that run to the hepatic vein (described above). Thus, cells entering the liver can arrive through vascular endothelial cells lining the portal vein in the portal tract, through the microvascular endothelial cells that line the sinusoids or through the terminal hepatic veins. Expression of adhesion molecules including chemokines and their receptors, by LSECs and circulating lymphocytes plays a determining role in controlling leucocyte infiltration of hepatic tissue. For example, expression of CCL21 in association with mucosal addressin cell adhesion molecule-1 (MADCAM-1) in the portal tracts of patients with primary sclerosing cholangitis promotes recruitment and retention of CCR7+ mucosal lymphocytes, leading to the establishment of chronic portal inflammation and expanded portal-associated lymphoid tissue typical of this hepatic autoimmune disease.[143]

## Hepatic influence on the systemic immune system

### Generation of tolerance

The transplantation of an allogeneic liver appears to induce systemic immune tolerance, which is specific for antigens expressed on the transplanted liver.[144] This may be manifest as acceptance of a kidney allograft or a skin

graft expressing the same antigens. Such tolerance is antigen-specific, as third-party allografts expressing distinct antigens are rejected.[145] This tolerance may result from multiple mechanisms, including T-cell inactivation, or the activation of anti-inflammatory T cells following antigen presentation by liver DCs, Kupffer cells or LSECs (see above). An alternative hypothesis is that liver transplantation tolerance is due to the dispersal of small numbers of liver-derived tolerance-promoting antigen-presenting cells throughout the host ('microchimerism').[146] This hypothesis requires that such liver-derived cells should have distinctive properties, as the 'passenger leucocytes' of other solid organ allografts, such as kidney grafts, promote immunity and graft rejection rather than tolerance.[147]

The induction of liver transplantation tolerance raises the possibility that other antigens expressed in the liver, including exogenous antigens encoded by pathogens, could induce systemic tolerance. One remarkable example where this may occur is in HCV infection. In experiments in which peptide-MHC tetramers were used to identify circulating HCV-specific CD8+ T cells, these cells were unable to synthesize IFN-γ in response to stimulation, while other CD8+ T cells specific for different pathogens responded well.[148] As a strong CD8+ T-cell response is associated with virus elimination in both human and chimpanzee HCV infections,[149,150] this selective CD8+ T-cell inactivation may promote virus persistence. The possible involvement of liver-specific tolerance mechanisms in the persistence of other infections of the liver, such as malaria, has not been extensively investigated.

### Deletion of activated T cells

An important biological property of the liver linked to systemic immune regulation is the accumulation and apoptosis of CD8+ T cells that occurs during a systemic immune response. This effect has been observed in diverse immune responses: during the lymphopenia associated with diabetes in BB rats;[151] in the immune response of TCR transgenic T cells to a systemically administered peptide;[152,153] and during the response to influenza infection that is localized to the respiratory system.[154] This trapping phenomenon is augmented when the liver vasculature is able to present the antigen, but is not dependent on it.[155] Activated CD8+ T cells bind in part to ICAM-1 and in part to VCAM-1, both of which are expressed in the liver.[156] Once trapped in the liver, the T cells die by unknown mechanisms. Interestingly, the liver in the diabetes-prone BB rat is deficient in NKT cells, suggesting a role for these cells in the regulation of this process.[157] The significance for the overall kinetics and magnitude of systemic immune responses is not understood. One possibility is that the liver acts as a 'sink' for activated T cells, limiting the numbers that are able to participate in a defensive response, or competing against the formation of memory cells that migrate into other tissues and take up long-term residence.[158]

## Concluding remarks

In this chapter, we have summarized the distinctive features of innate immunity, natural lymphocytes and adaptive immune cells that occur in the liver. The haemodynamics and micro-architecture of the liver promote interactions with circulating immune cells, while the passage of material from the intestine presents problems in balancing immune tolerance with host defence, and reactive environmental toxins from the same source may create new antigens and pose a risk of oncogenesis. As a result of these multiple conflicting demands, the liver has evolved a baseline state of mixed tolerance and immunity. The immunity is manifest in the adhesion and antigen-presenting properties of the LSECs, the high frequency of activated T cells, and the abundance of inflammatory cytokines including IFN-γ and TNF-α. The tolerance is manifest during CD8+ T cell apoptosis, in liver transplantation tolerance, by high levels of inhibitory cytokines including IL-10 and TGF-β and possibly in the persistence of some liver pathogens including HCV. The task for the future is to determine how these contradictory states can co-exist in the liver, and what this means for the understanding and amelioration of liver disease.

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# Section II
# Clinical Aspects of Viral Hepatitis

# Chapter 3
# Clinical features of hepatitis

Arie Regev, Eugene R Schiff

Viral hepatitis A through E may be associated with a wide spectrum of hepatic and extrahepatic clinical manifestations. Hepatitis A and E are both self-limited, faecally spread diseases, whereas hepatitis B, C and D are transmitted parenterally and may occasionally lead to chronic hepatitis. In contrast to hepatitis B, C and D, hepatitis A and E have never been shown to evolve into a chronic liver disease. Hepatitis B has a low chronicity rate (<5%) in healthy adults and a high chronicity rate (>90%) in infants, whereas hepatitis C has a high chronicity rate (80–85%) in all age groups. Hepatitis D affects only individuals with hepatitis B infection. Despite distinct differences in many clinical aspects, general clinical features are common to all the viral hepatitides.[1–3]

## General features

### Acute viral hepatitis

Acute hepatitis A to E run similar clinical courses, and there are no features that unequivocally distinguish the individual types. Typically, most patients experience a mild disease, usually without symptoms, that is marked only by a rise in serum aminotransferase levels. Alternatively, the patient may be anicteric but suffer gastrointestinal and influenza-like symptoms. Such patients are likely to remain undiagnosed unless there is a clear history of exposure. Increasing grades of severity are then encountered, ranging from a mild icteric disease, which is associated with almost universal recovery, to fulminant, fatal viral hepatitis.

#### *Prodromal phase*

The usual icteric disease in adults is marked by a prodromal period of low-grade fever, malaise, myalgia and headache. This presentation may follow an incubation period that may range from 2 weeks to 6 months. Although the incubation periods of the various hepatitis viruses differ, they can overlap sufficiently to prevent an accurate diagnosis. Anorexia, nausea and vomiting commonly occur early in the illness. Right upper abdominal discomfort and ache may develop but severe abdominal pain is unusual. There may be changes in taste and smell with resultant aversion to food and often to cigarettes and alcohol. Less commonly, pharyngitis and cough may also occur. Low-grade fever is common, although rigors are unusual. The prodromal phase usually lasts from 3 to 7 days, but may continue for up to 3 weeks. Occasionally, headache may be severe and, in children, may be associated with photophobia and neck rigidity. Protein and lymphocytes in the cerebrospinal fluid (CSF) may be raised. In approximately a quarter of the patients, predominantly but not exclusively those with hepatitis B, the early prodromal phase is characterized by a serum sickness-like syndrome, consisting of fever, arthralgia, arthritis, rash and angioneurotic oedema. These symptoms usually begin 2–3 weeks before jaundice develops and resolve before jaundice becomes apparent. Less frequently, they may persist for variable periods during the course of the acute illness.

Laboratory findings during the prodromal phase commonly include leukopenia and relative lymphocytosis, frequently with atypical lymphocytes. The most sensitive tests to identify the development of hepatitis are serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Serum ALT and AST are the first biochemical tests to become abnormal, typically preceding the development of jaundice. They may reach levels greater than 100 times the upper limit of normal, ALT levels usually being higher than AST. The serum level of alkaline phosphatase (AlkP) is abnormal in most cases however, it rarely exceeds four times the upper limit of normal. The exception is the uncommon variant of cholestatic viral hepatitis, which has been described in hepatitis A, B and C. Serum levels of lactate dehydrogenase (LDH) are usually elevated, but the increase is mild compared with the elevation of the aminotransferases.

### *Icteric phase*

The prodromal period is followed by darkening of the urine and lightening of the faeces. Symptoms subside slowly and jaundice develops. The temperature returns to normal within a few days and bradycardia may develop. Appetite returns, and abdominal discomfort and vomiting cease. At this time, pruritus may appear transiently for a few days. In some patients the prodromal symptoms may subside without a subsequent jaundice. This anicteric form appears to be more common among children with hepatitis A and individuals with hepatitis C. The duration of the icteric period ranges from a few days to 6 weeks. During the active illness physical examination may be unrevealing or may identify mild lymphadenopathy (involving particularly the posterior cervical region), mild hepatomegaly and occasionally splenomegaly. Generalized lymphadenopathy is not a feature of acute viral hepatitis. The liver is palpable in 70% of patients however, marked enlargement is uncommon. The liver edge is usually smooth, soft and slightly tender to palpation. The spleen is palpable in 10–20% of patients. A significant weight loss may occur during the acute illness and a few vascular spiders may appear transiently on rare occasions. The appearance of jaundice in the icteric phase usually indicates serum bilirubin level in excess of 2.5 mg/dL. Both conjugated and unconjugated bilirubin levels are elevated, but total serum bilirubin level usually does not exceed 20 mg/dL. A level in excess of 30 mg/dL suggests an additional bilirubin load due to overproduction (haemolysis) or impaired secretion (renal failure). Serum levels of alpha-fetoprotein (AFP) may increase, possibly due to hepatocellular regeneration.[4] AFP levels may be higher in patients with fulminant hepatitis.

### *Convalescent phase*

After the icteric period most patients make an uninterrupted recovery. In children, improvement is particularly rapid. The stools regain their colour, appetite becomes normal and pruritus abates. After the resolution of jaundice, lassitude and fatigue may persist for several weeks. The enlarged liver and spleen begin to shrink gradually. Clinical and biochemical recovery is usual within 6 months of the onset in almost all patients with acute hepatitis A, in the majority of adult patients with hepatitis B (>95%) and in a minority of patients with hepatitis C (15–20%).

### *Acute (fulminant) liver failure*

All types of viral hepatitis may lead to acute (fulminant) liver failure, although it is an uncommon complication in healthy individuals. Acute liver failure occurs more frequently in individuals who become infected past the age of 40 years and in those with a pre-existing chronic liver disease.[5–8] Hepatitis C is considered a very rare cause of acute liver failure in the Western world. Fulminant hepatitis usually develops within 10 days in patients with viral hepatitis. The patients may become hyperkinetic and combative. This may develop so rapidly that jaundice is inconspicuous, and the diagnosis is confused with an acute psychosis or meningoencephalitis. Alternatively, the patient, after a typical acute onset, becomes deeply jaundiced. Ominous signs are repeated vomiting, fetor hepaticus, confusion and drowsiness. The flapping tremor may be only transient, but rigidity is usual. Coma supervenes rapidly, and the picture becomes that of acute liver failure. Temperature rises, jaundice deepens and the liver shrinks. Widespread haemorrhage may develop.

## Chronic hepatitis

The symptoms of chronic viral hepatitis are generally vague, non-specific and in many cases intermittent. They do not distinguish between chronic hepatitis B, C or D. Many patients are completely asymptomatic, and in some patients symptoms do not develop until cirrhosis is present.[1–3] Consequently, many patients with chronic viral hepatitis are identified incidentally through routine blood tests or evaluation for an unrelated problem. The most common symptom of chronic viral hepatitis is fatigue, which can be variously described as malaise, weakness or lack of stamina.

Other less common symptoms include: right upper quadrant pain or discomfort, decreased appetite, nausea, muscle pain and joint pain. The severity of symptoms does not correlate with the severity of liver disease, with the level of serum aminotransferases or with the histologic activity.

Symptoms of extrahepatic involvement occur in a small percentage of the patients.

Most patients have few, if any, physical signs. The examination is usually normal or may show mild hepatomegaly or hepatic tenderness. Splenomegaly is an early sign suggestive of cirrhosis.

The characteristic laboratory finding is elevation in serum levels of both ALT and AST, usually to a similar degree. Typically, the ALT is mildly higher than the AST, the ratio ranging from 1:1 to 2:1. In some patients with cirrhosis this ratio may decrease to slightly less than 1:1.[9] The serum levels of AlkP and gamma-glutamyl transpeptidase (GGT) are usually within normal limits, but may be moderately elevated in patients with cirrhosis, usually to less than twice the upper limit of normal. Jaundice is rare in chronic hepatitis except for severe exacerbation or when liver failure occurs. Serum albumin levels and prothrombin time remain normal until late in

the course of the disease, and become abnormal in cirrhotic patients with the development of decompensated liver disease. Mild hyperglobulinaemia is common in chronic viral hepatitis, with immunoglobulin G (IgG) being the most elevated, usually in the range of 2.0–3.0 g/dL.[10] These levels are lower than those found in autoimmune hepatitis. Anti-nuclear antibodies and anti-smooth muscle antibodies may be detected in 10–20% and 20–40% of the patients, respectively, typically in low titres.[11] Anti-liver kidney microsomal antibody type 1 (anti-LKM-1) has also been detected in viral hepatitis. Platelet and white blood cell count may be mildly decreased in patients with chronic hepatitis, although markedly decreased counts suggest cirrhosis and hypersplenism.

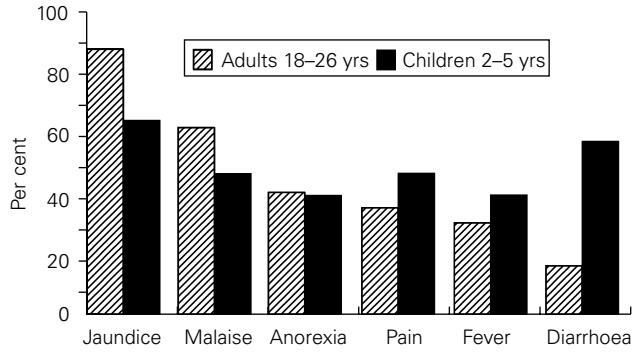


**Figure 3.1** Symptoms and signs associated with acute hepatitis A. (From: Lemon SM. Type A viral hepatitis: new developments in an old disease. *N Engl J Med* 1985;**313**:1059–67, with permission.)

## Hepatitis A

The incidence of hepatitis A virus (HAV) infection has decreased substantially worldwide over the last decades, mostly due to improved standards of living. However, due to the decreasing incidence of natural immunity, hepatitis A is more often encountered as a severe disease in adults. In urban areas, about 30% of adults show IgG anti-HAV, whereas in underdeveloped countries, 90% of children have the antibody by the age of 10. Hepatitis A occurs sporadically or in epidemic form. It is usually spread by the faecal–oral route. Parenteral transmission is extremely rare, but can follow transfusion of blood from a donor who is in the incubation stage of the disease.[12] Young people, not previously exposed, visiting endemic areas, are increasingly becoming affected. In addition, there are multiple reports of outbreaks among men who have sex with men.[13–15] Age 5–14 years is the group most affected, and adults are often infected by spread from children as a result of overcrowding, poor hygiene and poor sanitation. The incubation time ranges from 15 to 50 days. Clinically evident hepatitis develops in fewer than 1 in 10 infected children under the age of 3 years, whereas the vast majority of infected adults are symptomatic, many of them (40–70%) with overt jaundice.[16] The typical prodromal period is manifested with low-grade fever, malaise anorexia and nausea. Headache and myalgia may occur, and diarrhoea is frequent, especially among children[17] (Fig. 3.1). Rash, arthralgia and, rarely, arthritis may be a component of the prodromal phase of HAV infection.[18] The serum ALT level varies but may be increased to 100-fold above normal in more severe cases (Fig. 3.2). The increase in ALT level is followed by an increase in the serum levels of bilirubin

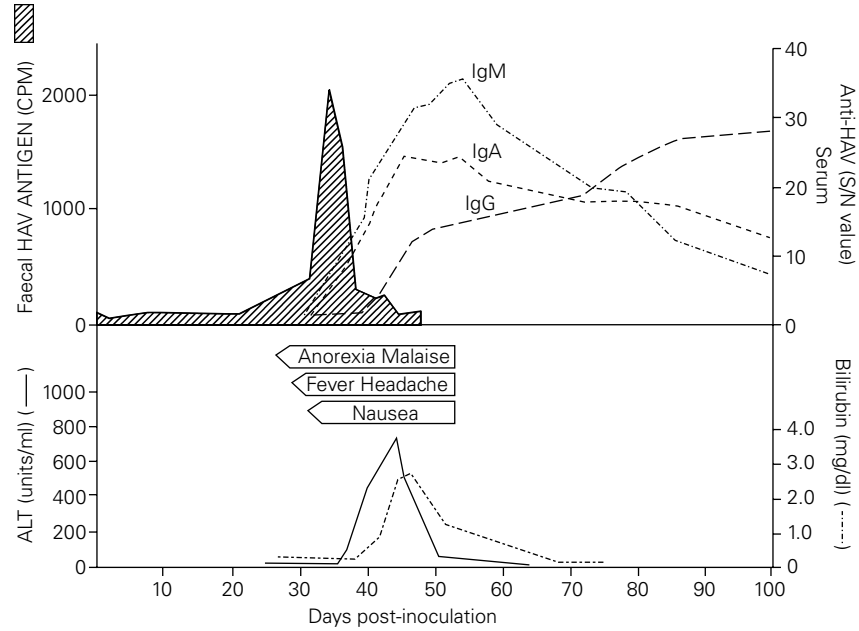


**Figure 3.2** Clinical course of HAV infection. (From: Lemon SM. Type A viral hepatitis: new developments in an old disease. *N Engl J Med* 1985;**313**:1059–67, with permission.

and AlkP. These biochemical abnormalities usually resolve within weeks, although it is not uncommon for the ALT level to remain mildly elevated for 3 months and occasionally longer. In the vast majority of patients the serum ALT level returns to normal within 6 months.

## Relapsing hepatitis

Relapses have been described in 1.8–15% of patients with acute hepatitis A, usually within several months after a typical acute infection (Fig. 3.3).[19] The relapse may occur 30–90 days after the initial presentation. In some, it is clinically similar to the original attack, usually in a milder form. More often, relapse presents mainly as an increase in serum aminotransferases and occasionally bilirubin. Serum IgM anti-HAV is positive, and there is continuous viraemia with shedding of the virus in the stools during the relapse phase.[19,20] Multiple episodes may occur, but recovery after relapse is almost invariably complete. Rarely, the relapse episode may be associated with arthritis, vasculitis and cryoglobulinaemia.[21,22]

## Cholestatic hepatitis

Occasionally, jaundice may be prolonged and may be associated with cholestatic features persisting for several weeks or more. The onset of disease is similar to that of the usual hepatitis patient however, the jaundice deepens and within 3 weeks the patient starts to itch. After the first few weeks, the patient feels better and gains weight. However, they continue to suffer from pruritus, and jaundice may persist for 8–29 weeks. This pattern is not indicative of severe hepatocellular disease. Subsequently, the jaundice subsides and the patients invariably make a complete recovery without sequelae.[23] This type of viral hepatitis must be differentiated from obstructive jaundice, and from drug-induced liver disease.

## HAV and autoimmune hepatitis

Several authors have reported rare cases of chronic autoimmune hepatitis possibly triggered by acute hepatitis A in susceptible individuals.[24,25] Autoimmune hepatitis type I developed within 5 months of the occurrence of acute hepatitis A. Individuals experiencing this rare sequence of events are likely to have an inherited defect of immune regulation.

## Extrahepatic manifestations

Extrahepatic manifestations of HAV infection include renal, neurological, pancreatic and haematological complications.

**Renal involvement** has been reported infrequently in association with HAV infection, and when present it is characterized by mild proteinuria, microscopic haematuria and slight urinary sediment abnormalities. Acute renal failure is a rare but well-described complication of non-fulminant HAV infection.[26–30] The nephrotic syndrome has been reported with immune complex-mesangial, proliferative glomerulonephritis.[30]

**Neurological complications** are uncommon. There are rare reports of aseptic meningitis, encephalitis, myelitis and seizures during the course of acute hepatitis A, in the absence of fulminant liver failure.[31,32] Several authors reported Guillain-Barré syndrome following acute HAV infection.[32]

**Pure red cell aplasia** is encountered rarely in patients with acute hepatitis A.[33,34] In most reported cases it is as-

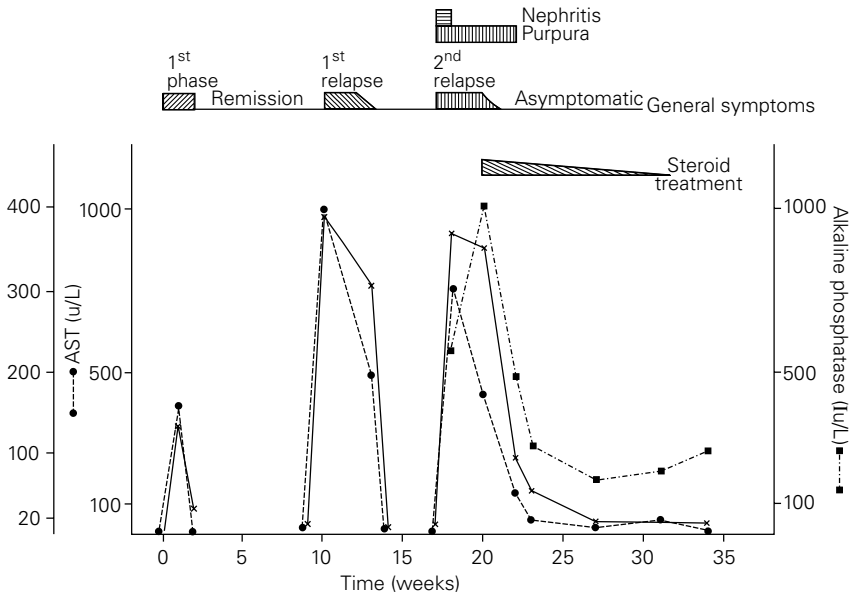


**Figure 3.3** Clinical and biochemical course of relapsing hepatitis A. (From: Glikson M *et al*. Relapsing hepatitis A. Review of 14 cases and literature survey. *Medicine (Baltimore)* 2002;**71**:14–23, with permission.)

sociated with a severe or fulminant hepatitis. Response to corticosteroids and immunosuppressant drugs is variable.[33,34]

### Fulminant hepatitis

HAV infection accounts for fewer than 10% of cases of acute liver failure (ALF) in most series. Although ALF due to severe hepatitis A is well documented in young children, it is significantly more frequent in middle-aged and older individuals and in those with underlying chronic liver disease.[5–8,35] The survival rate is probably better than other viral hepatitides, reported to be as high as 60% by some authors.[36,37]

## Hepatitis B

It is estimated that over 350 million people are chronically infected with hepatitis B virus (HBV) throughout the world. The carrier rate of hepatitis B surface antigen (HBsAg) varies worldwide from 0.1 to 0.2% in Britain, the United States and Scandinavia to more than 3% in Greece and Southern Italy, and up to 10–15% in Africa and the Far East. Carriage of HBsAg is even higher in some isolated communities such as Alaskan Eskimos and Australian Aborigines. HBV is typically transmitted parenterally, perinatally or by sexual contact. In low carriage rate areas the major risk factors for transmission are intravenous drug use and sexual contact. Historically, transfusion of blood products was an important risk factor. However, screening of donated blood for HBsAg has almost eliminated post-transfusion hepatitis B.[38] According to recent studies, the rare cases of transfusion-transmitted hepatitis B are mostly the results of donations collected from HBsAg-negative donors with acute HBV infection.[39] Blood transfusion continues to be an important cause of hepatitis B in countries where donor blood is not screened for HBsAg. Other modes of parenteral transmission include the use of unsterile instruments for dental treatment, ear piercing and manicures; prophylactic inoculations; subcutaneous injections; acupuncture; and tattooing. In high carriage rate areas, infection is acquired by vertical transmission at the time of birth and during close contact afterwards. The risk of transmission increases as term approaches and is greater in acute than in chronic carriers, and in replicating state than in inactive carriers state. HBs antigenaemia develops in the newborn within 2 months of birth and tends to persist.[40] There is an inverse relationship between the risk of chronicity and the age of infection. The chronicity rate for infections before the age of 1 year is 80–90%, and for infections in early childhood it is 20–50%, whereas HBV infection in healthy adults gives rise to a chronic infection or a carrier state in only 1–2%.[41,42]

### Acute hepatitis B

The incubation period ranges from 1 to 5 months, but it may be shorter in patients who were exposed to a large viral load.[43] During the prodromal phase serum sickness-like syndrome tends to occur more commonly than with the other hepatitis viruses. Polyarthralgia and polyarthritis may be seen involving mostly small joints of the upper extremities. Urticaria is occasionally present, and is associated with vascular deposition of circulating immune complexes containing HBeAg.[44] The clinical course of acute HBV infection is anicteric in the majority of infected individuals, which explains the high positivity rate of serum markers in those who give no history of acute HBV infection. It is well established that the anicteric patients are more liable to become chronically infected than the icteric ones. In patients who recover, the serum ALT levels usually return to normal values within 1–4 months. Persistent elevation of serum ALT for more than 6 months suggests chronic hepatitis and persistent infection.[42]

### Chronic hepatitis B

Chronic HBV infection accounts for 5–10% of cases of chronic liver disease and cirrhosis in the USA.[45] In most patients with chronic hepatitis B the acute phase had been mild or asymptomatic. In areas of low or intermittent prevalence, about 70% of patients with chronic hepatitis B do not have a history of acute hepatitis. The patients who have an explosive onset and deep jaundice usually recover completely. Similarly, survivors of fulminant hepatitis seldom, if ever, develop a chronic disease. The course of chronic hepatitis B generally consists of two phases: an earlier replicative phase with active liver disease and a later, non-replicative phase with remission of liver disease.[2] In patients with chronic HBV infection acquired during adulthood (Fig. 3.4A), the initial phase is characterized by active viral replication (presence of HBeAg and HBV DNA in serum), elevated ALT levels and chronic hepatitis in liver biopsy. During this phase the patient may be symptom-free, with only biochemical evidence of continued activity, or may have non-specific symptoms such as fatigue, or occasional right upper quandrant discomfort. Symptoms do not correlate with the severity of liver damage. The diagnosis is usually made on a routine blood test detecting mild elevation of serum aminotransferases. During the replicative phase patients may experience biochemical exacerbations (an abrupt increase in ALT levels), which may be asymptomatic or may mimic acute hepatitis, causing symptoms of fatigue, anorexia, nausea and jaundice. These exacerbations are often, but not always, associated with HBeAg seroconversion.[46,47] They may occasionally be misdiag-

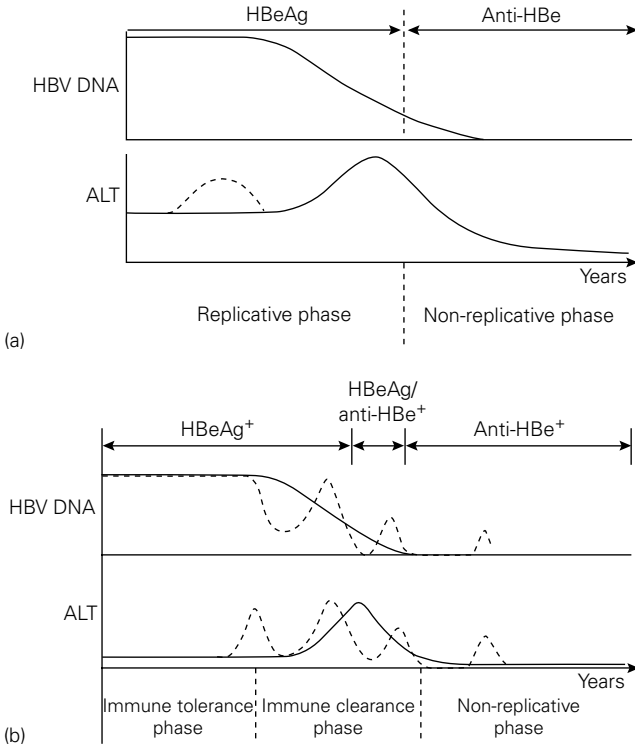


**Figure 3.4** Natural history of chronic HBV infection. (a) HBV infection acquired in adulthood. (b) Perinatally acquired HBV infection.

nosed as acute hepatitis B, due to elevated serum levels of IgM anti-HBc.[40,48–50] Exacerbations are occasionally associated with increased serum levels of AFP to >400 ng/mL,[50] and may rarely lead to hepatic decompensation.[51] In any severe exacerbation, superinfection with hepatitis D virus (HDV), HAV, or hepatitis C virus (HCV) must be considered. The second phase of chronic HBV infection is characterized by a marked decrease in serum HBV DNA levels and a spontaneous HBeAg seroconversion. This usually represents transition from chronic hepatitis B to an 'inactive carrier' state. In this phase the patients are almost invariably asymptomatic, and usually have inactive liver disease on liver biopsy. However, spontaneous reactivation from HBeAg-negative to HBeAg- and HBV DNA-positive has been described. The clinical picture ranges from absence of manifestations to fulminant liver failure.[46] Reactivation is particularly severe in immunosuppressed patients and patients who are human immunodeficiency virus (HIV)-positive. Many patients, usually with established stable cirrhosis, may present with hepatocellular carcinoma.

In perinatally acquired HBV infection (Fig. 3.4B) the initial replicative phase is characterized by virological features of active replication (high serum levels of HBV DNA and presence of HBeAg). However, these findings are not accompanied by signs or symptoms of liver disease, and are usually associated with normal levels of serum aminotransferases. This phase may last 10–30 years, and is most probably related to an early development of immune tolerance to HBV antigens.

The transition from the first to the second phase most commonly occurs between the ages of 15 and 35 years and is characterized by a high rate of spontaneous clearance of HBeAg (10–20% yearly). This phase is frequently accompanied by biochemical exacerbations, and occasionally by symptoms of acute hepatitis.

## Extrahepatic manifestations

### *Renal manifestations*

HBV infection has been associated with several forms of glomerulonephritis, including membranous glomerulonephritis, mesangial proliferative glomerulonephritis, minimal change nephropathy, membranoproliferative glomerulonephritis and IgA nephropathy. The most frequently reported kidney pathology is membranous glomerulonephritis, which has been described mostly in children. Proteinuria and nephritic syndrome are common manifestations.[52,53] In most of the patients the liver disease is mild, but HBeAg is usually positive. Immune complexes of HBsAg, HBeAg, anti-HBc or anti-HBe are found in glomerular and papillary basement membranes.[54,55] In children, the glomerulonephritis resolves spontaneously in most of the patients in 6 months to 2 years. However, in adults, the disease is usually more severe, gradually progressing to renal failure in up to 30% of the patients. Treatment with corticosteroids is not effective and may increase viral replication. However, in some patients alpha-interferon (IFN-α) treatment may lead to a remission.[56,57] In Chinese patients, treatment with IFN has been disappointing, spontaneous remission is uncommon, and the disease usually progresses relentlessly to renal failure.[53] Recent evidence suggests that the incidence of HBV-associated membranous nephropathy in children has declined significantly since the introduction of the HBV vaccine.[58]

### *Neurological manifestations*

The Guillain-Barré syndrome has been well described in patients with HBV infection.[32] In some patients, examination of the cerebrospinal fluid revealed the presence of HBsAg or immune complexes containing HBsAg.[59] The majority of the reported patients recovered with mild neurological residua.[32]

### *Polyarteritis nodosa (PAN)*

PAN is a rare complication of hepatitis B. However, approximately a third of patients with established PAN are HBsAg-positive. HBV-related PAN usually presents as

an acute disease shortly after the HBV infection, and has similar characteristics to classic PAN.[60] HBsAg, immunoglobulins (IgG and IgM), and complement have been demonstrated by immunofluorescence in exudative and fibrinoid vascular lesions.[60,61] Immune complexes may be present in the serum, and CH50 and C3 titres are typically low.

### *Aplastic anaemia*

Aplastic anaemia has been associated with HBV infection and is usually fatal.[62] Suppression of haematopoietic stem cells has been shown *in vitro* in HBV infection.[63]

### *Other extrahepatic manifestations*

There are rare reports of myocarditis[64] and pancreatitis[65] in association with HBV infection. It has been suggested that mixed cryoglobulinaemia may be initiated by HBV infection.[66] However, this association has not been unequivocally established.[67] Conversely, the association with HCV infection is well documented.[68–72]

## Fulminant hepatitis

Fulminant hepatic failure (FHF) may result from an acute HBV infection or from a reactivation of a chronic infection or carrier state. Reactivation has been described during or after withdrawal of immunosuppressive or cytotoxic chemotherapy, or with no apparent precipitating factor.[73–77] A fulminant course of hepatitis B is related to an enhanced immune response with more rapid clearing of the virus. In most cases replication of the virus ceases rapidly, and the majority of the patients lose the HBV DNA and HBeAg within a few days of clinical presentation.[78] Antibodies to surface and e antigen may be detected in high titres and the HBsAg may be in low titre or undetectable. The diagnosis in these cases may be made only by finding serum IgM anti-HBc. However, a Japanese study in patients with FHF have found that anti-HBc IgM was not detected in the serum in nearly 50% of patients in whom HBV DNA was detected by polymerase chain reaction (PCR) technique.[79] Several studies reported an association between the precore mutant HBV and fulminant liver failure,[80,81] although both the wild-type and mutant HBV strains may cause fulminant hepatitis.[82] Another viral hepatitis, superimposed on a symptomless hepatitis B carrier, may precipitate a fulminant course. The superimposed agent may be HAV, HDV or HCV.

## Clinical significance of HBV genotypes

HBV is classified into eight genotypes (A–H) based on inter-group divergence of 8% or more in the complete nucleotide sequence.[83,84] The predominant genotypes among patients born in the USA, Europe, the Far East, and Southeast Asia are A, D, C and B, respectively. In the USA genotype A is more common among whites and African Americans, whereas genotypes B and C are predominantly found in Asian Americans.[85,86] Unlike HCV genotyping, HBV genotyping is still used predominantly as a research tool. However, there is growing evidence that HBV genotype may correlate with the natural history of HBV infection and the severity of liver disease. Several authors have shown a strong relationship between HBV genotype and mutations in the precore and core promoter region that abolish or diminish the production of HBeAg.[35,87,88] For example, it has been suggested that precore mutations are found more commonly in patients with genotypes B, C and D but not in genotype A. This may account for the higher prevalence of HBeAg-negative chronic hepatitis B in Southern Europe and Asia. Recent studies suggest that HBV genotype B is associated with less active liver disease than genotype C, and that spontaneous HBeAg seroconversion occurs earlier and at a higher rate among patients with genotype B compared with patients with genotype C.[88–90] HBV genotype has also been related to response to IFN treatment, which was found to be higher in patients with genotype A or B than in those with genotype D or C, respectively.[91,92]

# Hepatitis C

It is estimated that about 5 million people have chronic HCV infection in Western Europe, which account for 40% of end-stage liver disease and 30% of liver transplant candidates.[93] In the USA, approximately 3.5 million people (1.8% of the population) have chronic HCV infections, but prospective surveillance studies indicate a marked decline in the incidence of new cases in the last two decades.[94–96] The most common risk factor for new infections is intravenous drug use. Other risk factors include injections with reusable non-sterilized needles, transfusion of blood products and transplantation of organs from infected donors before the introduction of screening in the early 1990s. Contaminated anti-rhesus D immunoglobulin (anti-D) has caused large outbreaks of HCV in Ireland[97] and Germany.[98] Current transmission methods may also include needle-stick accidents, tattoos, body piercing and intranasal cocaine use.[99] Haemodialysis patients have been at increased risk for HCV infection and the incidence of new cases of hepatitis C in the USA ranges from 1% to 3% yearly despite increased adherence to preventive measures.[100–102] The risk of HCV transmission after needle-stick injury varies in most studies from 2% to 5%.[103] The risk of vertical transmission is 2–5% and is related to the maternal viral titre. It is significantly increased when the mother is co-

infected with HIV.[104] HCV is not found in semen, and sexual transmission is rare. Sexual transmission among homosexual men appears to be infrequent[105] and infection of sexual partners in long-term relationships occurs rarely.[106,107]

## Acute hepatitis C

Acute hepatitis C represents less than 15% of all acute hepatitis cases in the USA.[108] The acute attack is usually mild and often unrecognized. Consequently, most patients with chronic hepatitis C infection lack a clinical history of acute hepatitis. When present, symptoms are often vague, such as anorexia, nausea or malaise. Often they are attributed to a gastrointestinal upset. Alternatively, there may be an abrupt onset with fever, arthralgia and abdominal discomfort. Less than 1% of HCV-infected patients report an acute illness associated with jaundice in relation to HCV infection. As there is no accepted serological definition of acute hepatitis C, and because many individuals will not have had documentation of a previously negative anti-HCV test, the diagnosis of acute hepatitis C is often difficult to establish. The incubation period of HCV ranges from 5 to 26 weeks. Serum hepatitis C RNA can be detected 1–2 weeks after infection.[109] Anti-HIV-positive patients and patients on immunosuppression therapy may have a rapidly progressive course.[110] A fulminant course, however, is rare, and co-infection with another virus must be considered. It is currently estimated that acute HCV infection clears in about 15–20% of cases. The chronicity rate in most patient populations is, therefore, 80–85%.[111,112] However, lower rates have been reported in specific cohorts. In children infected from blood transfusions, and in young healthy women infected from contaminated anti-D immune globulin chronicity rates have been only 50–60%.[113–115]

In addition, recent data have suggested that patients with symptomatic onset (especially flu-like symptoms and jaundice) are more likely to have self-limited disease.[116–118] Conversely, several studies have shown a chronicity rate greater than 90% in African American men.[119,120]

## Chronic hepatitis C

Most patients with chronic hepatitis C have asymptomatic elevation of serum aminotransferase levels, which is often discovered on a routine blood screen, and only about 6% have symptomatic liver disease.[121] Moreover, up to one-third of patients may have normal serum aminotransferases.[3,122,123] In most patients diagnosed with chronic hepatitis C the HCV infection may have been acquired 15–25 years previously. Long-term follow-up studies have shown that it takes 15–18 years to develop significant chronic hepatitis, 20 years to develop cirrhosis and 28 years to develop hepatocellular carcinoma.[123] Most infected patients will have mild to moderate liver disease, and only 20–35% will develop significant liver complications.[124] It is estimated that approximately 20% of the patients develop cirrhosis after 20 years' duration of infection. The patient may present insidiously with fatigue as the major symptom and right upper quadrant discomfort or pain as the second most common complaint. Other less common symptoms include anorexia, nausea, arthralgia, myalgia and pruritus. The course is slow, marked by fluctuating serum aminotransferase levels over many years. Serum bilirubin and albumin concentrations are usually normal until the late stages of liver failure. Serum gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase levels show slight increases. Serum gamma-globulin levels are increased modestly but rarely exceed 2 g/L. Routine haematology is usually normal and prothrombin time remains within normal limits until the late stages of cirrhosis. Association with non-alcoholic fatty liver disease is common in the USA, and probably leads to accelerated progression of liver damage and increased frequency of cirrhosis in HCV patients.[125–127] Genotype 3 is an independent predictor of steatosis in patients with chronic hepatitis C.[126,128]

## Hepatitis C and HIV

Because the routes of transmission are similar, co-infection with HCV and HIV is frequent. In the USA and Europe 14–33% of HIV-infected patients are also infected with HCV.[129–131] The prevalence of HCV co-infection is highest in HIV-positive drug users, in whom it may reach 90%. Before the introduction of highly active antiretroviral therapy (HAART), HIV-infected patients died within 10 years from the consequences of AIDS. Currently, the morbidity and mortality in HIV seropositive patients are more frequently related to liver disease, and HCV-related liver disease is becoming the main cause of death in HIV patients.[132,133] Serum levels of HCV-RNA in HIV/HCV co-infected patient are about 10 times higher than in HIV-negative patients.[134,135] In addition, the progression of HCV-related liver disease in HIV/HCV co-infected patients is faster, with a consequent higher risk of liver cirrhosis, hepatic failure and hepatocellular carcinoma.[135–137] Several studies have suggested that the rate of liver fibrosis is related to CD4 cell depletion in HIV/HCV co-infected patients.[138] The presence of HIV decreases the likelihood of spontaneous clearance of HCV after acute infection, and the chronicity rate may be as high as 100%.[139] On the other hand, there is evidence to suggest that HCV infection is associated with more rapid progression to AIDS, reduced effect of HAART and increased hepatotoxicity during HAART therapy.[140]

It has been suggested that HIV co-infection may increase the risk of sexual transmission of HCV.[141,142] Co-infection with HIV has also been reported to increase the vertical transmission of HCV from 2–5% to 14–22%.[104,143,144] This may be related in part to the elevated HCV viraemia in HIV infection.

## Extrahepatic manifestations

HCV-related chronic liver disease may be associated with numerous extrahepatic features. For some of these the association with HCV infection has been well established, whereas for others it remains speculative (Tables 3.1 and 3.2).[145] A strong association has been recognized with mixed cryoglobulinaemia and various cryoglobulinaemic syndromes. The association is less strong, although well documented for disorders such as lymphoma, porphyria cutanea tarda, diabetes mellitus, thyroid disease, salivary gland disease corneal ulcers, lichen planus and pulmonary fibrosis.

### *Cryoglobulinaemia*

Mixed cryoglobulinaemia occurs in 36–59% of patients with HCV infection.[71,146,147] Although it was initially thought to be associated with HBV infection,[66] more recent studies have established that HCV accounts for 73–90% of all forms of mixed cryoglobulinaemia.[68–72] The IgM component of the cryoprecipitate causes high levels of rheumatoid factor activity, because it binds avidly to IgG to form immune complexes. In some laboratories the rheumatoid factor test may be more sensitive than the cryoglobulin test for detection of mixed cryoglobulinaemia. Serological evidence for activation of the complement system, such as low serum levels of C3, C4 and CH50, is commonly seen in HCV patients with mixed cryoglobulinaemia. C4 level is often lower than that of C3. It has been suggested that cryoglobulinaemia is more prevalent in patients with genotype 2 and 3, compared with genotype 1. However, this has not been established in large numbers of patients.[148] The clinical manifestations of mixed cryoglobulinaemia are caused by vascular deposition of the cryoprecipitate, which contains HCV RNA, low-density lipoprotein, polyclonal IgG and monoclonal IgM.[68] The cryoglobulinaemic syndromes may include various disorders, the most common of which are kidney disease, skin disease (Fig. 3.5), joint manifestations and neuropathy (Table 3.1). Nevertheless, many patients with detectable cryoglobulinaemia are asymptomatic.

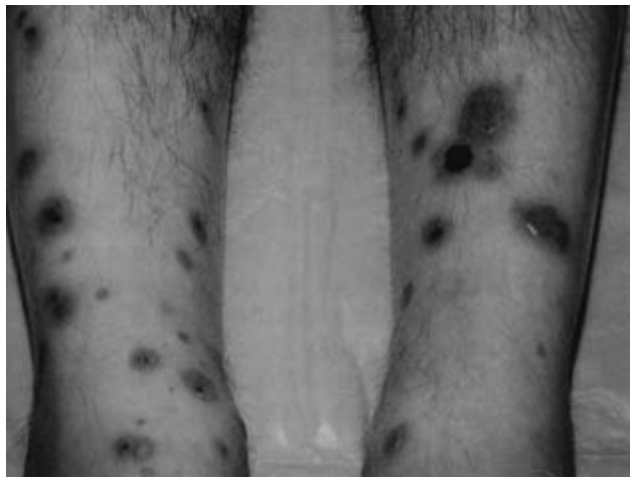

**Figure 3.5** Cutaneous vasculitis in a patient with chronic HCV infection and mixed cryoglobulinaemia. (Courtesy of E.G. McKay, MD.)

**Table 3.1** Extrahepatic manifestations of chronic HCV infection

| Manifestation | Prevalence |
|---|---|
| Cryoglobulinaemia | 49–59% |
| Kidney disease | 25–30% |
| Membranoproliferative glomerulonephritis* | |
| Proliferative glomerulonephritis | |
| Membranous glomerulonephritis | |
| Skin manifestations | 22% |
| Palpable purpura* | |
| Urticaria | |
| Livedo reticularis | |
| Necrotizing skin lesions* | |
| Lichen planus | |
| Porphyria cutanea tarda | |
| Neuropathy | 8–25% |
| Peripheral neuropathy* | |
| Optic neuropathy | |
| Encephalopathy | |
| Joint manifestations | 16% |
| Arthralgia | |
| Arthritis* | |
| Raynaud phenomenon | 3–35% |
| Sicca syndrome | 20% |
| Lymphadenopathy* | 3% |
| Lymphoproliferative disorders | 1.5–10% |
| Idiopathic thrombocytopenic purpura | Uncommon |
| Fever | 3% |
| Weakness | 65% |

*Strongly associated with cryoglobulinaemia.

### *Kidney disease*

The most common kidney manifestation of HCV is membranoproliferative glomerulonephritis (MPGN):[101,149,150] 10–20% of the cases of MPGN in the USA and up to 60% in Japan are associated with HCV infection.[150] Over 85% of the HCV patients with MPGN have cryoglobulinaemia, and about 90% have abnormal liver histology. These patients present with proteinuria, often in the nephrotic range, and decreased kidney function. Complement levels are depressed in the majority of the patients.[70,149] Kid-

**Table 3.2.** Extrahepatic disorders associated with HCV in a sample of hospitalized veterans (1992–1999): results of multivariable logistic regression analysis

| Disorder | Parameter estimate | Adjusted odds ratio | 95% CI | *p* value |
|---|---|---|---|---|
| Membranous glomerulonephritis | 0.02 | 1.03 | 0.78–1.35 | 0.86 |
| Membranoproliferative glomerulonephritis | 1.51 | 4.53 | 3.11–6.60 | <0.0001 |
| Adult-onset diabetes | –0.01 | 0.99 | 0.96–1.02 | <0.0001 |
| Lichen planus | 0.84 | 2.31 | 1.76–3.04 | <0.0001 |
| Vitiligo | 0.39 | 1.49 | 1.05–2.10 | 0.03 |
| Porphyria cutanea tarda | 2.23 | 9.31 | 7.16–12.11 | <0.0001 |
| Cryoglobulin | 2.68 | 14.68 | 10.60–20.33 | <0.0001 |
| Thyroiditis | –0.0669 | 0.94 | 0.67–1.30 | 0.69 |
| Hodgkin's lymphoma | –0.0004 | 1.00 | 0.77–1.30 | 1.00 |
| Non-Hodgkin's lymphoma | 0.20 | 1.22 | 1.01–1.39 | 0.04 |

CI, confidence intervals. From: El-Serag HB *et al*. Extrahepatic manifestations of hepatitis C among United States male veterans. *Hepatology* 2002;**36**:1439–45, with permission.[145]

ney biopsy is consistent with immune complex disease, and glomerular deposits are mainly of IgG, IgM and C3. IFN treatment was reported to reduce proteinuria, usually with no significant improvement in kidney function. Less common kidney pathologies encountered in HCV patients are proliferative glomerulonephritis and membranous glomerulonephritis.[151]

### *Skin manifestations*

The most frequent cutaneous manifestation of mixed cryoglobulinaemia in HCV patients is palpable purpura of the lower extremities. Other skin areas such as upper extremities, abdomen and buttocks may also be involved. Histological examination reveals leucocytoclastic vasculitis in the majority of cases and fibrinoid necrotizing arteritis in a distinct minority. Urticaria and livedo reticularis are less common features sharing the same underlying histological abnormalities.[152] Rarely, the vasculitic process can progress to necrotizing skin lesions leading to painful ulcers, mostly in the lower extremities (Fig. 3.6).

Lichen planus has also been reported in association with chronic HCV infection.[153]

### *Neuropathy*

Peripheral neuropathy is by far the most common neurological manifestation of HCV. Most cases are related to cryoglobulinaemia, although some have been associated with polyarteritis nodosa-like lesions.[154,155] In most cases it is an axonal sensory polyneuropathy, often causing pain for months or years before motor deficits develop. Pure motor polyneuropathies have never been described. Rare cases of optic neuropathy and encephalopathy related to cryoglobulinaemia have been reported. The response to treatment has been disappointing.[156]

### *Joint manifestations*

Arthralgia is a relatively common symptom in HCV infection, although frank arthritis unrelated to cryoglobulinaemia is rare. The most common joint manifestation of mixed cryoglobulinaemia is mono- or oligo-articular non-destructive arthropathy, most often affecting the proximal interphalangeal joints, metacarpophalangeal joints and the knees. These patients are occasionally misdiagnosed as having rheumatoid arthritis, as many of them have elevated serum levels of rheumatoid factor.[72]

### *Lymphoma*

Many studies suggest a possible association between chronic HCV infection, mixed cryoglobulinaemia and non-Hodgkin's lymphoma. The reported incidence varies. According to some articles, approximately 25% of HCV patients who have active mixed cryoglobulinae-

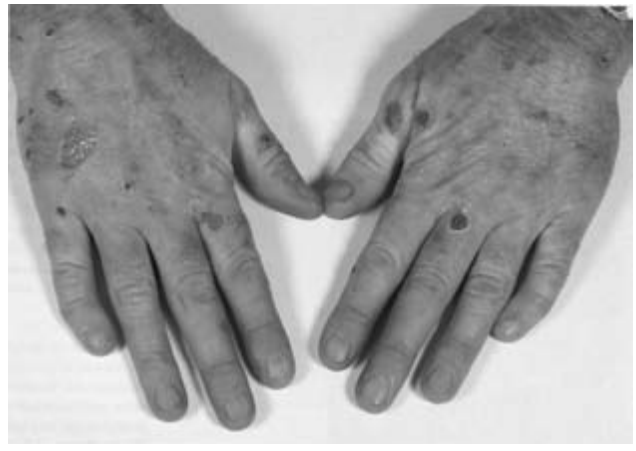

**Figure 3.6** Skin lesions due to porphyria cutanea tarda (PCT) in a patient with chronic HCV infection. (Courtesy of E.G. McKay, MD.)

mia have a lymphoproliferative disorder evident on random bone marrow biopsies.[157,158] HCV infection usually precedes the lymphoma by many years. Many are low grade, although high-grade lymphoma may occur. Extranodal involvement is common.[159]

### *Porphyria cutanea tarda (PCT)*

The association of PCT with HCV infection has been found in some but not all countries. In several studies from the USA and Western Europe, HCV infection was found in 56–79% of PCT patients.[160–162] The patients present with vesicles, bullae and skin ulcers in sun-exposed areas. Skin pigmentation and hirsutism are also common features. Many of these patients (up to 73%) are also found to have mutations in the HFE gene. Conversely, PCT is uncommon in HCV patients, and elevated urinary uroporphyrin levels are detected in 0.5–22% of patients with chronic HCV infection.[160,163]

### *Diabetes mellitus*

Large retrospective studies from the USA and Europe found the prevalence of type 2 diabetes to be 21–24% in patients with HCV infection compared with 9–12% in patients with HBV infection. These differences were most prominent in cirrhotic patients.[164,165]

### *Other disorders*

Thyroid disease,[166,167] sicca syndrome, lymphocytic sialadenitis resembling Sjögren's syndrome,[71,168] Moorentype corneal ulcers,[169] idiopathic thrombocytopathic purpura[170] and fibromyalgia[171] have all been reported in patients with chronic HCV infection. However, these associations have yet to be confirmed in larger populations (Table 3.2).

## Hepatitis D (delta)

The hepatitis delta virus (HDV) is a small RNA particle that replicates effectively only in the presence of HBsAg. It is capable of infection only when activated by the presence of hepatitis B infection.[172] The interaction between the two viruses is complex. Synthesis of HDV may depress the appearance of hepatitis B viral markers in infected cells and even lead to elimination of active viral replication.[173] HBV and HDV infection may be simultaneous (co-infection) or HDV may infect a chronic HBsAg carrier (superinfection). HDV infection is acquired parenterally and via close personal contact in endemic areas. Infection is strongly associated with intravenous drug use, but can affect all risk groups for hepatitis B infection. It can spread sexually, and within families.[174]

HDV infection is found worldwide, but is particularly prevalent in Southern Europe, the Balkans, the Middle East, South India and parts of Africa. In general, it is rare in the Far East (including Japan) and South America. However, epidemics of HDV infection have been reported from the Amazon Basin, Brazil (Labrea fever), Colombia (Santa Marta hepatitis), Venezuela and equatorial Africa. In these areas, children of the indigent population are affected and mortality is high.

With co-infection, acute hepatitis D is usually self-limited, as HDV cannot outlive the transient HBV antigenaemia. Only 2% of cases of co-infection have been reported to evolve to chronicity. The clinical picture is usually indistinguishable from hepatitis resulting from HBV alone. However, a biphasic rise in ALT may be noted, the second rise being due to the acute effects of HDV.

Clinically, overt co-infection is usually severe and icteric, with full but transient expression of both viruses. Fulminant hepatitis is rare. After recovery, the patient acquires immunity to HDV.

With superinfection, the acute attack may be severe and may rapidly lead to liver failure in HBV carriers with pre-existing chronic liver disease. Alternatively, superinfection may be marked only by a rise in serum transaminase levels. Delta infection should always be considered in any HBV carrier, usually clinically stable, who has a relapse. Superinfected HBV carriers are at high risk of becoming chronic carriers of HDV. Superinfection is followed by progression to chronicity in more than 90% of the patients. In the remaining cases, the superinfection resolves, with persistence of the original HBV infection, or clearance of HBsAg. In the early stages of chronic HDV infection, HDV has a marked inhibitory effect on HBV, and HBV DNA may not be detectable even by sensitive PCR assays.

The natural history of chronic HDV infection has been a matter of controversy. Although early studies linked HDV to severe and progressive liver disease, more recent surveys in a large population have shown that HDV infection may be present without causing liver damage. A large study from the Greek island of Rhodes showed that only a minority of delta-infected persons had liver disease, and the majority were healthy carriers.[175] In contrast, chronic hepatitis D may run a rapidly progressive course, leading to liver failure within several months to 2 years, in drug addicts with HBV and HDV co-infection.[176]

The HBV-infected patient who acquires HDV has more active necroinflammation and develops cirrhosis more rapidly than chronic hepatitis of other viral aetiologies. In addition, the risk of mortality in patients with HDV cirrhosis was twice, and the risk of hepatocellular carcinoma was three times that of patients with ordinary

HBV cirrhosis.[177] Fortunately, the incidence of hepatitis D is decreasing worldwide.

## Hepatitis E

Infection with hepatitis E virus (HEV) accounts for sporadic and major epidemics of viral hepatitis in developing countries. It is endemic in the Indian subcontinent and in south-eastern and central Asia. The incidence in other parts of the world is much lower, and the disease appears to be restricted predominantly to individuals who have travelled to areas where it is endemic.[178] HEV infection is enterically transmitted, usually by sewage-contaminated water, and the viral genome has been found in the stools of infected patients.[179] Person-to-person transmission is uncommon and vertical transmission is rare. Lower socio-economic status and drinking well water are both significant risk factors for infection.[180] In contrast to HAV, HEV affects young adults and is rare in children.[181] The incubation period ranges from 2 to 10 weeks. The illness is usually insidious in onset. The prodromal phase lasts about 1–4 days and is characterized by influenza-like symptoms, fever, anorexia and abdominal discomfort, with occasional vomiting and diarrhoea. Arthralgia and macular skin rash may occur. These symptoms last for a few days and are followed by the appearance of jaundice, with a subsequent symptomatic improvement.[182] HEV infection is associated with fulminant hepatitis and acute liver failure in endemic regions.[183] The course may be cholestatic. Chronicity does not develop. The mortality rate is very high (about 25%) in women in the last trimester of pregnancy. The severe picture in pregnancy is more common in epidemics than in sporadic cases. Fulminant HEV infection may be confused with acute fatty liver of pregnancy.[184] Antibodies to HEV (IgG anti-HEV) are protective and probably provide a long-term immunity.[185] The diagnosis is made by antibody tests using recombinant proteins expressed from cloned HEV. These detect both IgG and IgM antibodies.[186] Antibody tests are not completely satisfactory.[187] HEV RNA can be determined by PCR.

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# Chapter 4
# Diagnostic approach to viral hepatitis

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Viral hepatitis enters into the differential diagnosis of both acute and chronic liver disease. Infection caused by all five currently identified hepatitis viruses (A–E) can present as acute viral hepatitis, whereas only hepatitis B (HBV), C (HCV) and D (HDV, delta) viruses are important causes of chronic liver disease. For most cases of viral hepatitis, diagnosis of the specific viral cause is based on routine serological tests (Table 4.1). In selected cases, the application of molecular biological techniques, including polymerase chain reaction (PCR) amplification, can enhance the sensitivity and sophistication of diagnostic testing in viral hepatitis. Specific serological and virological identification of each of the five types of viral hepatitis must be guided by both an appreciation of the clinical setting and a recognition of the limitations of currently available diagnostic tests.

## Hepatitis A

Hepatitis A virus (HAV), which causes acute but not chronic hepatitis, is spread almost exclusively by the faecal-oral route. In clinical practice, a diagnosis of acute hepatitis A is made by serological detection of specific viral antibodies. Although HAV antigen and HAV RNA can be detected with research techniques,[1] by the time a patient presents with symptoms of acute hepatitis, virus levels in stool and blood are markedly diminished or undetectable by conventional serological/virological detection methods.

Antibody to HAV of the IgM class (IgM anti-HAV) is usually detectable in serum with the onset of symptoms and is a reliable marker of acute or recent HAV infection (Fig. 4.1). Typically, IgM anti-HAV persists for about 3–6 months after acute illness, very rarely up to 18 months.[2] False-negative IgM anti-HAV results are uncommon, but occasionally the duration of IgM anti-HAV positivity is as short as several days.[3] False-positive IgM anti-HAV results have been described and are attributable to confounding binding in the immunoassay, as may occur in the presence in serum of rheumatoid factor or hyperglobulinaemia.[4] In a small proportion of cases, hepatitis A relapses several weeks to several months after apparent recovery. Such relapses may be associated with recurrent faecal excretion of HAV[5] and, theoretically, infectivity. In patients with relapsing hepatitis A, IgM anti-HAV may reappear in serum after its initial disappearance. However, in most cases, relapses occur during the period of IgM anti-HAV positivity associated with the initial expression of hepatitis.[6]

The primary immune response to HAV is of the IgM class. However, antibody to HAV of the IgG class (IgG anti-HAV) also appears in serum following HAV infection, but its concentration rises more gradually than does that of IgM anti-HAV. After HAV infection, IgG anti-HAV persists indefinitely and confers permanent protection against reinfection.

Both radioimmunoassays (RIAs) and enzyme-linked immunoassays (EIAs) are available for detecting total and IgM anti-HAV antibodies,[7] but EIAs have supplanted RIAs in clinical practice. Microparticle-based, automated fluorescence immunoassays are available as well. The presence of IgG anti-HAV, which signifies HAV infection in the recent or remote past, is reflected by the

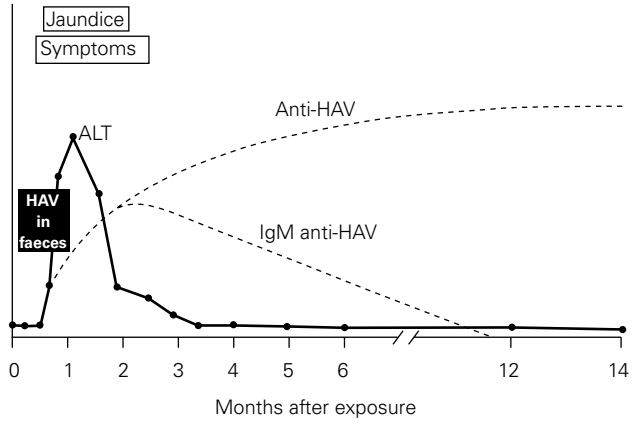


**Figure 4.1** Typical course of acute hepatitis A infection. HAV, hepatitis A virus; ALT, alanine aminotransferase; anti-HAV, antibody to HAV; IgM anti-HAV, antibody to HAV of the IgM class. (From Hoofnagle JH, DiBisceglie AM. Serologic diagnosis of acute and chronic viral hepatitis. *Semin Liver Dis* 1991;**11**:73–83, with permission.)

**Table 4.1** Routine serological diagnosis of viral hepatitis

| Interpretation | IgM anti-HAV | IgG anti-HAV | | | | |
|---|---|---|---|---|---|---|
| Acute HAV infection | + | – | | | | |
| Remote HAV infection | – | + | | | | |
| **Interpretation** | **HBsAg** | **HBeAg** | **Anti-HBs** | **Anti-HBe** | **Anti-HBc** | **HBV DNA** |
| Acute HBV infection | | | | | | |
| Early | + | + | – | – | IgM | + |
| Window period | – | – | – | – | IgM | +/– |
| Recovery | – | – | + | + | IgG | +/–* |
| Chronic HBV infection | | | | | | |
| Replicative | + | + | – | – | IgG, IgM† | >$10^5$copies/mL |
| Non-replicative/inactive carrier state | + | – | – | + | IgG | <$10^5$copies/mL |
| Reactivation HBV | + | +/– | – | – | IgM or IgG | + |
| HBeAg(–) chronic HBV (precore or core mutant) | + | – | – | + | IgG | >$10^5$copies/mL |
| **Interpretation** | **Anti-HDV** | **HBsAg** | **IgM anti-HBc** | | | |
| HDV infection | + | + | – | | | |
| HDV co-infection | + | + | + | | | |
| HDV superinfection | + | + | – | | | |
| **Interpretation** | **Anti-HCV** | **HCV RNA** | | | | |
| Acute or chronic HCV infection | + | + | | | | |
| Resolved HCV infection | + | – | | | | |

IgM anti-HAV, antibody to hepatitis A virus of the IgM class; IgG anti-HAV, antibody to hepatitis A virus of the IgG class; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBs, antibody to hepatitis B surface antigen; anti-HBe, antibody to hepatitis B e antigen; anti-HBc, antibody to hepatitis B core antigen; HBV DNA, hepatitis B virus DNA; anti-HDV, antibody to hepatitis delta virus; anti-HCV, antibody to hepatitis C virus; HCV RNA, hepatitis C virus RNA.
*Low levels ($10^1$–$10^2$ genome equivalents/mL) may be detected in serum up to 21 years after recovery from acute HBV infection.
†Low levels may also be detected.

detection of total anti-HAV in the absence of IgM anti-HAV, whereas the presence of IgM anti-HAV, characterized by the presence of both total and IgM anti-HAV, is indicative of acute or recent hepatitis A.

Generally, the diagnosis of acute hepatitis A is not particularly difficult or challenging, except in the rare patient with rheumatoid factor activity and a false-positive IgM anti-HAV result. Persons who have received prophylactic immune globulin (passive immunization) may have low levels of IgG anti-HAV in serum for several weeks, although protection against infection tends to last for several months and outlasts detectable anti-HAV in serum. Active immunization with HAV vaccines induces an antibody response only to structural proteins (initially IgM anti-HAV and later IgG anti-HAV). By contrast, during natural infection, antibodies are produced against both structural and non-structural proteins. Although not available routinely, an EIA specific for antibody to the non-structural 3C proteinase of HAV can be used to distinguish natural infection from a vaccine response.[8] Patients immunized against HAV will not have detectable antibody against the 3C proteinase.

As noted above, HAV antigen can be detected in stool by RIA during the incubation period and early symptomatic phase of the illness. In addition, HAV RNA can be detected in stool, serum and body fluids by PCR techniques.[9] However, these sophisticated techniques are not needed in routine clinical practice.

## Hepatitis B

The complexity of the genomic organization and replicative cycle of HBV,[10] a DNA virus, is reflected in the complexity of the antigenic expression of, and associated antibody responses to, the virus. Hepatitis B virus accounts for a sizable proportion of all cases of both acute and chronic viral hepatitis. Currently, routine serological diagnosis relies on the detection of three pairs of antigens and antibodies: hepatitis B surface antigen (HBsAg) and its corresponding antibody (anti-HBs); hepatitis B e antigen (HBeAg) and its corresponding antibody (anti-HBe); and antibody to hepatitis B core antigen (anti-HBc) (hepatitis B core antigen [HBcAg], against which anti-HBc is directed, does not circulate freely in the serum). Assays to detect HBV DNA by molecular hybridization were introduced in the 1980s as commercially available kits, none of which was approved for routine use (Table 4.2). Such hybridization

**Table 4.2** Commercially available HBV DNA quantification assays

| Assay | Manufacturer | Method | Lower detection cut-off | Dynamic range of quantification |
|---|---|---|---|---|
| Signal amplification | | | | |
| Genostics Assay | Abbott Labs, Chicago, IL, USA | Liquid hybridization | 1–2 pg/mL (~600 000 copies/mL) | 1–2 pg/mL to ~800 pg/mL (600 000–300 000 000 copies/mL) |
| Versant HBV DNA 1.0 Assay | Bayer Corp., Diagnostics Division, Tarrytown, NY, USA | Manual branched DNA (bDNA) signal amplification | 700 000 genome equivalents/mL | 700 000–5 000 000 000 genome equivalents/mL |
| Versant HBV DNA 3.0 Assay | Bayer Corp., Diagnostics Division, Tarrytown, NY, USA | Semi-automated bDNA signal amplification | 2000 copies/mL | 2000–100 000 000 copies/mL |
| HBV Hybrid-Capture I | Digene Corp., Gaithersburg, MD, USA | Hybrid capture signal amplification in tubes | 700 000 copies/mL | 700 000–560 000 000 copies/mL |
| HBV Hybrid-Capture II | Digene Corp., Gaithersburg, MD, USA | Hybrid capture signal amplification in microplates | 142 000 copies/mL | 142 000–1 700 000 000 copies/mL |
| Ultra-sensitive HBV Hybrid-Capture II | Digene Corp., Gaithersburg, MD, USA | Hybrid capture signal amplification in microplates after centrifugation | 4700 copies/mL | 4700–57 000 000 copies/mL |
| Target amplification | | | | |
| Amplicor HBV Monitor Test v2.0 | Roche Molecular Systems, Pleasanton, CA, USA | Manual quantitative RT-PCR | 1000 copies/mL | 1000–40 000 000 copies/mL |
| Cobas Amplicor HBV Monitor Test v2.0 | Roche Molecular Systems, Pleasanton, CA, USA | Semi-automated quantitative RT-PCR | 200 copies/mL | 200–200 000 copies/mL |

RT-PCR, reverse transcription polymerase chain reaction. Adapted from Pawlotsky J. Hepatitis B virus (HBV) DNA assays (methods and practical use) and viral kinetics. *J Hepatol* 2003;**39**:S31–S35, with permission.

assays, with sensitivity thresholds of $10^5$–$10^6$ HBV DNA copies/mL, have now been supplanted by PCR methodology, with sensitivities generally as low as $10^2$–$10^3$ viral copies/mL, some with a sensitivity of $10^1$ copies/mL (see below). Molecular amplification assays for HBV DNA can identify evidence of HBV replication that cannot be detected by molecular hybridization (slot, dot-blot, liquid-capture, etc.) assays, and, technically PCR assays can demonstrate HBV infection in the absence of serological markers of infection, such as HBsAg.[11] Practically, however, current immunoassays for HBV antigens are so sensitive that reliance on PCR amplification of HBV DNA to establish a diagnosis of acute or chronic hepatitis B is almost never required in routine clinical practice. Additional viral markers that circulate in serum during HBV infection, e.g. HBV DNA polymerase, as well as pre-S1 and pre-S2 viral envelope antigens and their respective antibodies (anti-pre-S1 and anti-pre-S2), can be detected by experimental techniques that have no routine clinical applications.

## Serological tests

During HBV infection, HBsAg, the product of the S gene of HBV, circulates at high serum concentrations (up to $10^{13}$ particles per mL).[12] This viral protein comprises the outer coat, or envelope, of the virion. In addition, HBsAg is produced in excess as non-virion 22-nm spheres and tubules, which outnumber intact virions by several orders of magnitude. Intact virion-associated HBsAg differs from smaller, non-virion-associated spherical forms in containing more pre-S gene product. Circulating HBsAg can be detected readily in serum by commercial immunoassays (formerly RIA, now EIA) 1–10 weeks after infection with HBV and 2–8 weeks before the onset of clinical hepatitis (Fig 4.2). In very rare cases of acute hepatitis B, HBsAg may be undetectable at the time of presentation, either because levels of HBsAg never reach, or have already declined below, the detection threshold of the assay. Typically, false-positive HBsAg results are borderline or only weakly positive and rarely encountered.

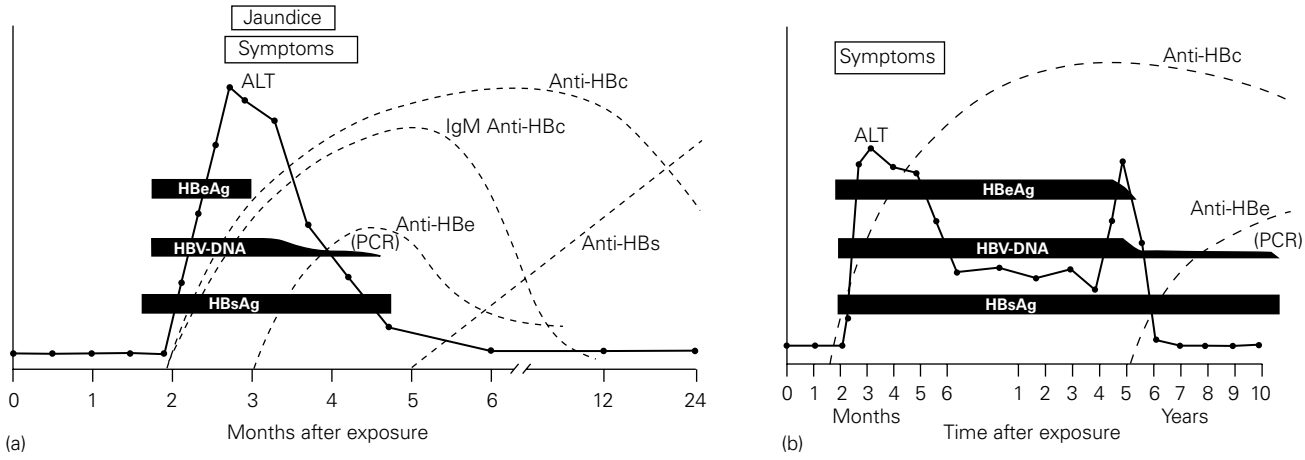


**Figure 4.2** Sequence of events after acute hepatitis B infection. (a) Acute HBV infection with resolution. (b) Acute HBV infection progressing to chronic HBV infection. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; anti-HBe, antibody to hepatitis B e antigen; anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to hepatitis B surface antigen; IgM anti-HBc, antibody to hepatitis B core antigen of the IgM class. (From Hoofnagle JH, DiBisceglie AM. Serologic diagnosis of acute and chronic viral hepatitis. *Semin Liver Dis* 1991;11:73–83, with permission.)

Although anti-HBs is produced early during acute HBV infection, marked HBsAg excess prevents routine detection of anti-HBs until HBsAg levels decline. Typically, with the resolution of acute hepatitis B, HBsAg becomes undetectable as anti-HBs becomes detectable.[13] Because of the sensitivity of current assays, the previously described 'window period' between the disappearance of HBsAg and the appearance of anti-HBs in serum is rarely, if ever, observed nowadays. Antibody to HBsAg is a neutralizing antibody, which, when present, is associated with life-long immunity. In addition, anti-HBs is the antibody produced in response to hepatitis B vaccination with current vaccines composed entirely of HBsAg.[14] Anti-HBs levels of at least 10 milli-international units are regarded as protective. Failure of acute HBV infection to resolve, with evolution to chronic HBV infection, is suggested by persistence of circulating HBsAg and absence of detectable anti-HBs.

Hepatitis B core antigen (HBcAg), a component of the viral nucleocapsid, is associated with the intact virion and does not circulate freely in the serum. Therefore, HBcAg is not detectable routinely in serum. During acute HBV infection, IgM anti-HBc appears shortly after HBsAg and persists for approximately 6 months (rarely longer but almost never beyond 12 months) after the onset of acute HBV infection. During the course of acute HBV infection, levels of IgG anti-HBc rise more slowly, and eventually IgG anti-HBc replaces IgM anti-HBc. The presence of IgG anti-HBc signifies either resolved HBV infection (when detected with anti-HBs after clearance of HBsAg) or chronic HBV infection (when detected in patients with persistent HBsAg). Commercially available EIAs for total anti-HBc and IgM anti-HBc are available. The presence of IgG anti-HBc is inferred when total anti-HBc is present but IgM anti-HBc is undetectable. Antibody to HBcAg has not been felt to be a neutralizing antibody. However, chimpanzees immunized with HBcAg have been protected against HBV challenge, and the T-cell immune response to HBcAg has been shown to provide help in promoting the humoral immune response to HBsAg. Therefore, testing for anti-HBc is an alternative screening test for immunity to HBV that can be used to determine the need for hepatitis B vaccination. The presence of IgM anti-HBc is a helpful diagnostic adjunct when the time of onset of HBV infection is not known (for example, in a blood donor found to harbour HBsAg).

Occasional persons are found to have an isolated anti-HBc in the absence of HBsAg or anti-HBs. For example, before the introduction of contemporary blood donor screening approaches, up to 5% of healthy blood donors had isolated anti-HBc in the serum. Among human immunodeficiency virus (HIV)-infected persons, the rate of isolated anti-HBc in serum has been reported to be as high as 42%.[15] Isolated anti-HBc can occur in four settings: 1) during the now rare window period of acute HBV infection (see earlier); 2) during chronic HBV infection as levels of HBsAg become undetectable; 3) after resolved HBV infection in the remote past as anti-HBs levels fall below the limits of detection; and 4) as a false-positive result. Among populations with a high prior probability of HBV infection, i.e. in HBV-endemic areas, up to 20% of persons with isolated anti-HBc in serum have circulating HBV DNA, signifying chronic HBV infection.[16–18] In contrast, among persons with chronic hepatitis C, isolated anti-HBc is common but is almost never associated with detectable HBV DNA. In the absence of HBV DNA, isolated anti-HBc usually represents a false-positive result or HBV infection in the remote past. Therefore, in persons with isolated anti-HBc who are at

high risk of HBV infection (e.g. have high-risk behaviour or are members of an HBV-endemic population), testing for HBV DNA by a molecular assay is helpful to exclude or establish occult chronic HBV infection.

Hepatitis B e antigen (HBeAg) is a product of the nucleocapsid (core) gene of HBV but, unlike HBcAg, is a soluble, non-particulate protein that is secreted in serum.[19] For HBeAg to be expressed, the precore region of the C gene must be intact and translated. Detectable by commercial immunoassay (formerly RIA, now EIA), HBeAg is a convenient marker of high virus replicative activity, which in turn is associated with infectivity and liver injury. HBeAg is invariably detectable early in the course of acute hepatitis B, coincident with or immediately after the appearance of HBsAg, and generally disappears within several weeks in acute, resolving cases. Persistence of HBeAg in serum beyond the first 3–4 months predicts protracted (chronic) HBV infection. In patients with chronic hepatitis B, HBeAg tends to persist for months to years and signifies the active replicative phase of chronic HBV infection. HBeAg disappears at a rate of approximately 10% (range 9–19%) per year in patients with chronic hepatitis B, coincident with a dramatic decline in viral replication to undetectable or nearly undetectable levels.[20] Testing for HBeAg is not necessary in most cases of acute hepatitis B but plays an important role in the assessment of patients with chronic hepatitis B.

Chronic HBV infection can be divided into HBeAg-positive and HBeAg-negative categories. Early in the course of chronic HBV infection, both HBsAg and HBeAg are typically detected in serum, reflecting active viral replication, infectivity and hepatic inflammation. Most patients will ultimately undergo seroconversion with loss of HBeAg and appearance of anti-HBe, coincident with a sustained decline in viral replication, return of serum aminotransferase levels to normal, and decline in serum HBV DNA to low ($<10^3$–$10^4$copies/mL) levels that are generally undetectable with standard molecular assays (see later) (although HBsAg persists in serum). These persons are known as *inactive carriers*.

Some persons who are HBeAg-negative and anti-HBe-positive do have active chronic HBV infection with detectable HBV DNA (usually $>10^5$ copies/mL but at levels one to several $\log_{10}$ lower than in HBeAg-reactive chronic hepatitis B) and elevated or fluctuating serum aminotransferase levels.[21] Absence of HBeAg in serum in these patients is the result of a mutation in the precore or core promoter region of HBV DNA that halts production of HBeAg. The most commonly seen precore mutation is a substitution of adenosine (A) for guanine (G) at codon 1896 (G1896A) that results in a premature stop codon, which prevents production of HBeAg.[22] This HBV mutant is found in up to 27% of persons with chronic HBV infection in the USA but 50% and 92% of persons with chronic HBV infection in Asia and the Mediterranean, respectively.[23,24] When compared with the clinical course of persons with HBeAg-positive chronic HBV infection, the course of HBeAg-negative chronic HBV infection is typically characterized by lower serum HBV DNA levels, as mentioned above, and intermittent, rather than sustained, periods of necroinflammatory activity in the liver. In addition, long-term (rather than short-term) antiviral therapy is generally required to maintain suppression of HBV replication.

## Molecular tests

Qualitative (PCR-based) and quantitative molecular assays for HBV DNA in serum are commercially available. Suppression of HBV DNA in serum by antiviral therapy is generally monitored with quantitative HBV DNA assays.

Quantification of HBV DNA in serum is performed with either signal or target amplification techniques (Table 4.2). Signal amplification techniques require the use of a specific 'capture' oligonucleotide probe that hybridizes denatured DNA.[25] The signal (radioisotope, chemiluminescence, etc.) from the probe-DNA hybrid is then amplified for detection and quantitation.[25] Target amplification requires amplification of the viral genome (amplicons). The amplicons are then detected and quantified.

Assays that rely on signal amplification include the Hybrid Capture System (Digene Hybrid-Capture II HBV DNA Test, Digene Corp., Gaithersburg, MD, USA) and a branched DNA (bDNA) assay (Bayer, Emeryville, CA, USA). In the Hybrid-Capture System,[25–27] specific RNA probes are hybridized to the target HBV DNA to create RNA-DNA hybrids. Then, multiple RNA-DNA hybrids are captured onto microplate wells by universal capture antibodies specific for the hybrids. The captured RNA-DNA hybrids are then detected with the use of multiple antibodies (creating signal amplification) conjugated to alkaline phosphatase. The bound alkaline phosphatase is detected with a chemiluminescent dioxetane substrate that produces light, which is then measured spectrophotometrically. The signal can be amplified 3000-fold. The lower limit of detection of the Hybrid-Capture System is 4700 copies/mL, i.e. approximately $5 \times 10^3$ IU/mL.[25]

The bDNA technique[25,28] involves the use of specific oligonucleotide probes to hybridize HBV DNA to plastic microwells. Signal amplification occurs when bDNA amplifier molecules are hybridized to the target HBV DNA hybrids in the microwell. Multiple repeat sequences within the bDNA amplifier molecule are then conjugated with an alkaline phosphatase-catalyzed chemiluminescence probe similar to that used in the Hybrid-Capture System. The lower limit of detection of the bDNA assay is $7 \times 10^5$ DNA equivalents/mL.[25]

Although signal amplification techniques offer highly specific assays to detect HBV DNA, these assays are too insensitive to detect low levels of HBV DNA ($<5 \times 10^3$ copies/mL). Target amplification techniques such as PCR-based assays are highly sensitive and are capable of detecting as few as 10 copies/mL of HBV DNA (TaqMan-based PCR).[29] PCR assays rely on the use of specific primers that attach to each strand of target DNA. Then, new DNA strands are synthesized and amplified behind the primer. This cycle of DNA denaturing, primer annealing and strand synthesis is repeated multiple times, thereby resulting in amplification of the target HBV DNA. The most common primers used in HBV DNA PCR assays are complementary to the precore or core region.[9] Commercially available assays include the Amplicor HBV Monitor Test, v2.0 (Roche Molecular Systems, Pleasanton, CA, USA) and the Cobas Amplicor HBV Monitor Test, v2.0 (Roche Molecular Systems). The ranges of HBV DNA detection with these assays are 1000–40 000 000 copies/mL and 200–200 000 copies/mL, respectively. The Cobas Amplicor system is the more sensitive of the two assays and uses an Amplicor analyser that automates the amplification and detection process.

Recent advances in PCR technology include the development of 'real-time' PCR techniques to increase the sensitivity of the assay. Real-time PCR refers to the simultaneous amplification and quantification of viral genomes, thereby obviating the need for post-PCR manipulations.[30,31] Real-time PCR can detect a wide range of HBV DNA levels and offers a more rapid assay than conventional PCR techniques. In a recent study, LightCycler (LC)-PCR (Roche Diagnostics), a real-time PCR technique, was compared to the Hybrid-Capture II HBV DNA test (Digene Corp., Gaithersburg, MD, USA),[31] and LC-PCR was found to be rapid (~2.5 h) and 500 times more sensitive than Hybrid-Capture II, with an HBV DNA detection range of 250 to $2.5 \times 10^9$ copies/mL.

Currently, real-time PCR using the TaqMan probe is the most sensitive quantitative HBV DNA assay, capable of detecting as few as 10 copies/mL.[29] TaqMan technology relies on a fluorescent probe annealed to target DNA sequences for quantitation of DNA.[32,33]

Until recently, the HBV DNA quantitative units reported by the various assays listed in Table 4.2 did not represent the same actual amount of DNA in a given patient sample. Therefore, the World Health Organization (WHO) established an international standard for HBV DNA in international units (IU).[34] Ultimately, all commercial HBV DNA quantitative assays will adopt this new calibration standard and express HBV DNA levels in IU/mL. One IU/mL is equivalent to approximately 5 copies/mL.

## Serological and molecular testing in practice

Quantitative tests for HBV DNA in serum are useful to distinguish replicative from non-replicative chronic HBV infection and to monitor a patient's response to antiviral therapy. With the use of conventional serological markers plus molecular assays for HBV DNA, we can distinguish between two phases of chronic HBV infection.[35] The early 'replicative' phase is characterized by the presence of circulating HBeAg and high levels of serum HBV DNA (usually $>10^5$–$10^6$ copies/mL) and infectious virions. Clinically, patients are often symptomatic, have elevated serum aminotransferase levels, demonstrate histological evidence of liver necroinflammatory injury, and are highly infectious for their contacts. Eventually, patients may experience transition to the relatively 'non-replicative' phase, characterized by loss of HBeAg, appearance of anti-HBe in serum, and a marked decrease in serum levels of HBV DNA, which may remain detectable only by PCR (generally $<10^3$ copies/mL). An improvement in symptoms and in biochemical and histological indicators of necroinflammatory activity as well as a marked reduction in infectivity follow. Often the transition from the replicative to the non-replicative phase is accompanied by a clinical exacerbation of chronic hepatitis (postulated to represent cytolytic T-cell clearance of HBV-infected hepatocytes), with a transient, marked elevation in serum aminotransferase levels and perhaps an exacerbation of symptoms. Rarely, primarily in patients with marginally compensated chronic liver disease, this clinical exacerbation can resemble fulminant hepatitis and result in hepatic failure.

Once the infection has entered a non-replicative phase, liver injury is unlikely. However, subsequent spontaneous or immunosuppression-induced reactivation of HBV replication remains possible. Reactivation of replicative HBV is accompanied by high levels of HBV DNA in serum and may occur with or without the reappearance of HBeAg.[36]

The threshold that distinguishes the replicative from the non-replicative state has been defined as an HBV DNA level of $10^5$ copies/mL, although $10^3$–$10^4$ may be more accurate. Patients are classified as having replicative, non-replicative or reactivation HBV infection. Because the reappearance of IgM anti-HBc during reactivations is variable, occurring in some cases but not others, the absence of IgM anti-HBc does not distinguish reliably between reactivation of chronic hepatitis B and acute hepatitis B.[37]

During the course of chronic HBV infection, transition to the non-replicative phase is associated with a shift from primarily episomal HBV DNA to HBV DNA that is primarily integrated into the host genome of infected

hepatocytes, a process that may set the stage (i.e. is necessary but not sufficient) for the later development of primary hepatocellular carcinoma.[38] However, viral integration and development of hepatocellular carcinoma are probably not causally linked. Instead, both events probably result independently from HBV infection of long duration. In fact, development of hepatocellular carcinoma is also associated with the ongoing hepatic necroinflammatory activity that characterizes the replicative phase of chronic HBV infection.[39]

In addition to characterizing the status of viral replication in patients with chronic HBV infection, quantitative HBV DNA assays are useful in monitoring response to antiviral treatment. Recently, the National Institute of Diabetes and Digestive and Kidney Diseases and the American Gastroenterological Association proposed criteria to classify responses to antiviral therapy based on biochemical, histological and virological criteria.[21] A biochemical response is signified by a decrease in the serum ALT level to the normal range, and a histological response is signified by a decrease from baseline in histological activity index of at least two points. A critical component of the virological response is a reduction in serum HBV DNA to $<10^5$ copies/mL and loss of HBeAg in patients who were initially HBeAg-positive. For patients with HBeAg-negative chronic HBV infection, however, the only measure of virological response is loss of detectable serum HBV DNA. Assays to detect precore and core-promoter HBV mutations are also available. However, for routine clinical purposes, the presence of HBV DNA and elevated aminotransferase activity is usually sufficient for the detection of HBeAg-negative chronic hepatitis B.

For patients with HBV infection who are treated with the antiviral nucleoside analogue lamivudine, the emergence of a lamivudine-resistant mutant is characterized by the reappearance of HBV DNA in serum after an initial decline in level or disappearance. Although assays to detect specific YMDD (tyrosine-methionine-aspartate-aspartate) lamivudine-resistant mutations are available, reversal of virological and biochemical responses is usually sufficient to infer emergence of this mutation.

## Hepatitis D (delta agent)

Hepatitis D virus (HDV), or the delta agent, is an RNA virus that requires the helper function of HBV to complete its replicative cycle. Therefore, HDV infection is not encountered in the absence of HBV infection. Infection caused by HDV can occur either simultaneously with acute HBV infection (co-infection), superimposed on chronic HBV infection (superinfection),[40] or prior to overt reinfection of a liver allograft with HBV (subclinical or latent HDV).[41] HDV infection, especially superinfection, may result in more severe liver disease than does HBV infection alone. Whereas patients co-infected with HDV and HBV often have acute resolving hepatitis no more severe than, and indistinguishable from, that caused by HBV alone (with occasional exceptions),[42] persons with HDV superinfection are more likely to have severe hepatitis with more rapid progression to cirrhosis, and even more likely to have fulminant hepatitis, than are persons with chronic hepatitis B alone.[43] Persons with long-standing chronic hepatitis D and B often have inactive cirrhosis.

The diagnosis of HDV infection in clinical practice rests on the detection of antibody to HDV antigen (anti-HDV) by commercial immunoassay (formerly RIA, now primarily EIA). The titre of total anti-HDV is generally low (<1:100) and undetectable in over 90% of cases in acute HDV infection.[44] However, the time to first appearance of anti-HDV is variable, and repeated testing may be necessary to confirm the diagnosis. Anti-HDV tends to persist for only a short time beyond the resolution of acute hepatitis D and may disappear subsequently, leaving no marker of previous infection. Anti-HDV of the IgM class appears during acute HDV infection, but the presence of IgM anti-HDV does not distinguish acute from chronic HDV infection. IgM anti-HDV persists in chronic infection, and high titres are often found in patients with severe liver inflammation.[4] In persons with chronic hepatitis B who become superinfected with HDV, anti-HDV appears early, reaches high titres, and persists indefinitely. Serodiagnosis of HDV infection in this setting is quite reliable.

Routine diagnosis of acute HDV-HBV co-infection is based on the detection of anti-HDV in serum in association with IgM anti-HBc (Fig. 4.3). Because HDV suppresses HBV replication, loss of HBsAg from serum may be accelerated in HDV-HBV co-infection and, occasionally, IgM anti-HBc may be the only marker of acute HBV infection in this setting.[4] A diagnosis of HDV superinfection in a patient with chronic hepatitis B is supported serologically by the presence of anti-HDV in a patient who harbours HBsAg and IgG anti-HBc. Persistence of high titre anti-HDV for more than 6 months also supports a diagnosis of chronic HDV infection.

Immunoassay methods (RIA and EIA) have been developed but are not widely available to detect HDV antigen (HDAg) in serum. These immunoassays permit detection of HDAg in many patients with early acute HDV infection but rarely in patients with chronic HDV infection.[4] In contrast, HDAg can be detected by Western blot in over 70% of cases of chronic HDV infection.[45] Western blotting for HDAg, however, is technically difficult, cumbersome, not readily adaptable for routine diagnostic purposes, and remains a research tool confined to a small number of specialized laboratories.

Other research tools have been applied to the evaluation of HDV infection. Hepatitis D RNA (HDV RNA)

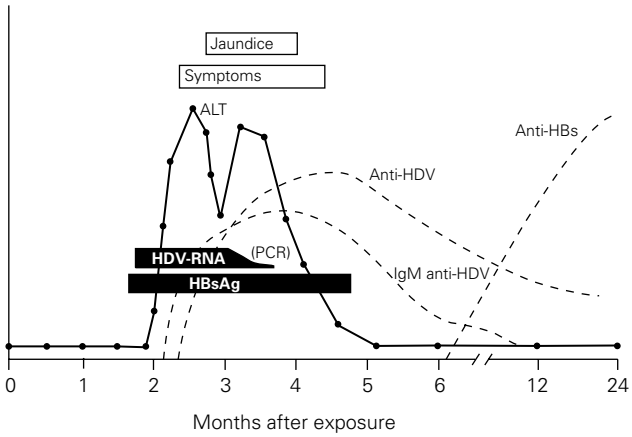


**Figure 4.3** Typical course of hepatitis B and hepatitis D (delta) virus co-infection. ALT, alanine aminotransferase; anti-HBs, antibody to hepatitis B surface antigen; anti-HDV, antibody to hepatitis D virus; HBsAg, hepatitis B surface antigen; HDV, hepatitis D virus. (From Berenguer M, Wright TL. Viral hepatitis. In: Feldman M, Friedman LS, Sleisenger MH, eds. *Sleisenger and Fordtran's Gastrointestinal and Liver Disease: Pathophysiology Diagnosis Management*, 7th edn. Philadelphia: WB Saunders, 2002: 1278–341, with permission.)

can be detected in serum by molecular hybridization transiently during acute HDV infection and persistently in chronic HDV infection.[46] Although testing for HDV RNA is now a research tool with limited availability, in the future, measurement of serum HDV RNA levels is likely to be used to monitor response to antiviral therapy in patients with chronic hepatitis D.[47] Research techniques for detection of intrahepatic HDV include demonstration of HDV RNA by *in situ* hybridization[48] and of HDAg by immunofluorescence and immunoperoxidase staining in frozen sections and formalin-fixed sections, respectively.[4] These techniques have limited utility because staining for HDAg becomes less accurate as specimens age. Only 50% of biopsy specimens obtained more than 10 years previously from patients with chronic HDV infection will stain positively.[9]

## Hepatitis C

Hepatitis C is characterized by an 85% rate of progression to chronicity following acute infection, a propensity for progression to cirrhosis, and an associated risk after protracted infection of primary hepatocellular carcinoma. Although HCV transmission has been most commonly the result of injection drug use and has been characterized most thoroughly following blood transfusion, non-percutaneous spread of HCV is thought to account for some sporadic cases of hepatitis C. Perinatal and sexual transmission of HCV are uncommon, however, and covert spread remains unexplained in many cases.[49,50] Even among persons who cannot recall a specific percutaneous exposure, however, blood-borne acquisition, often in the remote past, is the most likely source of infection.

### Serological tests

The identification of HCV,[51,52] an RNA virus responsible for most, if not all, cases of post-transfusion and sporadic (non-epidemic) 'non-A, non-B hepatitis', was followed by the rapid development of serological tests. Currently, anti-HCV is detected with a third-generation EIA. The first-generation RIA and then EIA (EIA-1) detected antibody directed against c100-3, a recombinant polypeptide derived from non-structural region NS4 of the viral genome. The value of this assay was limited, because anti-HCV was detectable no earlier than several weeks, and usually several months, after the onset of acute hepatitis and was not detectable at all in 30–40% of patients with acute, self-limited hepatitis C.[53] Even in patients with chronic transfusion-related non-A, non-B hepatitis shown by other techniques (see below) to be caused by HCV, anti-HCV was undetectable by first-generation EIA in approximately 20% of cases.

Because of the relative insensitivity of the first-generation anti-HCV assays, second-generation assays (EIA-2) were developed to detect antibodies to additional recombinant antigens: c22-3 derived from the core region and c33c from non-structural region NS4 of the HCV genome.[9] Second-generation assays detected anti-HCV earlier in the course of acute HCV infection and were much more sensitive, detectable in 95% of patients with chronic hepatitis C. Third-generation EIAs (EIA-3), designed to detect antibodies to an additional recombinant antigen from the NS5 region, have even higher sensitivities than those of EIA-1 or EIA-2 and can detect anti-HCV as early as 7–8 weeks after acquisition of HCV infection.[9]

Despite the improved sensitivity of second- and third-generation EIAs for anti-HCV, the specificities of the assays vary widely depending on the risk profile of the population being tested. For example, EIA-3 has a sensitivity of 95–98% when compared to HCV RNA testing, but the positive predictive value of EIA-3 in low-risk populations is only 25% (Table 4.3), confounded by many false-positive results.[9] False-positive EIA results have also been described in patients with autoimmune disorders such as autoimmune hepatitis.[54] The finding of an apparently biological false-positive anti-HCV result under these circumstances has been shown to correlate with hypergammaglobulinemia. In such cases, anti-HCV may become undetectable following therapy with corticosteroids, presumably because of a decrease in gamma globulin levels.[49,54,55] For persons from low-risk populations (i.e. with a low prior probability of in-

**Table 4.3** Enzyme immunoassays (EIAs) for the diagnosis of HCV infection

| Assay | Sensitivity (%)* | PPV in low-risk groups (%)† | PPV in high-risk groups (%)† | Window period (weeks)‡ |
|---|---|---|---|---|
| EIA-1 | 70–80 | 30–50 | 70–85 | 15 |
| EIA-2 | 90–95 | 50–60 | 90–95 | 9–10 |
| EIA-3 | 95–98 | 25 | – | 7–8 |

PPV, positive predictive value. (From Berenguer M, Wright TL. Viral hepatitis. In: Feldman M, Friedman LS, Sleisenger MH, eds. *Sleisenger and Fordtran's Gastrointestinal and Liver Disease: Pathophysiology Diagnosis Management*, 7th edn. Philadelphia: WB Saunders, 2002: 1278–341, with permission.)

*Compared with HCV RNA testing.

†High-risk patients are considered those with a definitive risk factor for HCV acquisition and elevated serum aminotransferase levels. Low-risk patients include blood donors, patients with normal aminotransferase levels, and those with no risk factor for HCV acquisition.

‡Window period: time between acquisition of infection and first positive result.

fection) or with autoimmune disorders and a positive EIA for anti-HCV, confirmatory testing for HCV infection should be performed (see below).

The specificity of EIA testing for anti-HCV can be improved by supplemental testing with a recombinant immunoblot assay (RIBA). The major use of RIBA testing has been to confirm (technically speaking to 'supplement') EIA reactivity in low-risk populations, such as asymptomatic blood donors with normal serum aminotransferase levels, of whom one-half to three-quarters may have false-positive EIA anti-HCV reactivity.[56] A positive RIBA result confirms that the positive EIA result is a true positive. Currently RIBA testing is rarely required but, for historical reasons, the application and evolution of this assay are reviewed. Three generations of RIBA assays, RIBA-1, -2, and -3 were developed successively. RIBA-2 has replaced RIBA-1 as the most commonly used assay in the clinical setting, while RIBA-3 has only been approved for use by blood banks.[9] The first-generation RIBA assay consisted of a nitrocellulose strip with separate bands containing the polypeptides c100-3 (recombinant yeast-derived) and 5-1-1 (recombinant bacteria-derived), which are the products of overlapping non-structural segments of the HCV genome, as well as superoxide dismutase, a fusion protein with which c100-3 is expressed in its yeast vector and which may account for some false-positive EIA reactivity.[57] RIBA-2 (RIBA HCV 2.0 Strip Immunoassay, Chiron Corporation, Emeryville, CA, USA) contains a larger panel of HCV antigens, incorporating both first- and second-generation EIA proteins (c100-3, 5-1-1, c33c from the non-structural region, c22-3 from the HCV core-associated region, and superoxide dismutase). A specimen is considered positive if ≥2 detectable bands derived from at least two different HCV gene products are detected. Seventy-two to 100% of persons with a positive RIBA-2 result will have detectable HCV RNA by PCR techniques.[58] Specimens are considered indeterminate if detectable bands from only one HCV gene region appear or if the specimen reacts with the superoxide dismutase band and two or more HCV gene products.[9] For persons with an indeterminate RIBA-2 result or a positive EIA-3 and undetectable HCV RNA, additional testing with RIBA-3 can be considered. RIBA-3 includes two recombinant antigens, c33c and NS5, and two synthetic peptides, one from the nucleocapsid (c22) and c100-3 from the NS4 region.[9] In practice, however, testing for HCV RNA has supplanted RIBA testing in most instances.

## Molecular tests

The advent of molecular assays for HCV RNA, such as PCR amplification, has enhanced the sensitivity of testing for HCV infection. The ability to perform molecular assays for HCV RNA is useful in several settings: 1) diagnosis of acute hepatitis C; 2) confirmation of chronic hepatitis C; 3) demonstration of perinatal transmission of HCV; 4) detection of occupational exposure to HCV; and 5) monitoring the response to antiviral therapy.[25] In a small proportion of persons with acute, symptomatic HCV infection, anti-HCV is undetectable during the early weeks of infection (Fig. 4.4).[25] In addition, HCV RNA becomes detectable before anti-HCV in all cases and may be the only marker of infection during early infection, before the infection becomes apparent clinically. In these instances, if clinical suspicion of acute HCV infection is high, assays for HCV RNA can be used to confirm or exclude the presence of viraemia. HCV RNA assays are also used routinely to confirm a diagnosis of chronic hepatitis C. In almost all patients with chronic hepatitis and anti-HCV in serum, HCV RNA is present in serum continuously, but occasionally HCV RNA levels decline transiently and become undetectable.[59] Thus, a single negative HCV RNA result does not exclude viraemia, and a follow-up HCV RNA assay should be performed.

Assays for HCV RNA may also be used to determine the infection status of infants born to mothers with HCV infection. Because anti-HCV can be transferred passively

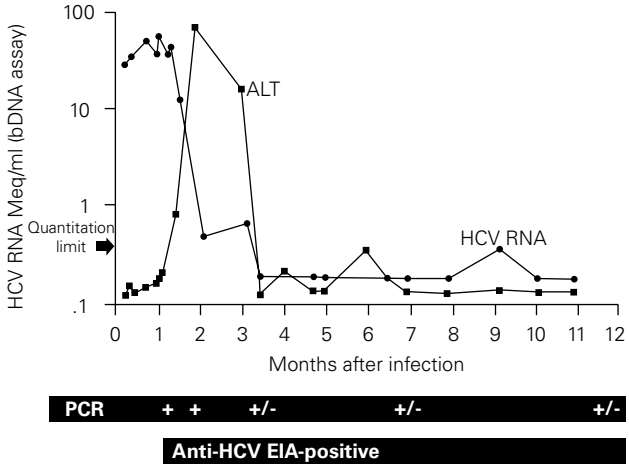


**Figure 4.4** Typical course of acute hepatitis C infection. Changes in hepatitis C virus (HCV) RNA and serum alanine aminotransferase levels (ALT) after acute post-transfusion hepatitis C. Note that viral RNA is detectable for several weeks before anti-HCV seroconversion. EIA, enzyme immunoassay; PCR, polymerase chain reaction. (From Chiron Corporation, Emeryville, CA, USA, with permission.)

from mother to infant and persist in the infant's serum for several months to 1 year, the diagnosis of HCV infection in the infant requires detection of HCV RNA.[60–64]

For blood-borne, occupational exposures to hepatitis C, HCV RNA can be detected before anti-HCV seroconversion, elevation of serum aminotransferase levels and symptoms of acute hepatitis. Thus, molecular assays are used frequently to determine whether transmission has occurred.

Finally, HCV RNA assays are also used to follow a patient's response to antiviral therapy. All treated patients are tested routinely for quantitative HCV RNA by PCR; an early virologic response (EVR) is defined as a ≥2-$\log_{10}$ reduction in HCV RNA at week 12; an end-treatment virologic response (ETR) is defined as undetectable HCV RNA at the completion of therapy; and a sustained virologic response (SVR) is defined as undetectable HCV RNA 6 months after completion of therapy. Absence of an EVR or a decline in serum HCV RNA levels (as determined by a quantitative assay) by <2-$\log_{10}$ at week 12 is predictive of a non-sustained virologic response (negative predictive value 98%).[65] Because qualitative assays are more sensitive than quantitative assays, the former are better indicators of ETR and SVR.

Both qualitative and quantitative HCV RNA assays are available (Table 4.4). The qualitative assays consist of transcription-mediated amplification (TMA) or PCR techniques and have lower limits of detection (50 IU/mL, or approximately 100 viral genomes/mL) than do the quantitative assays ($10^2$–$10^3$ IU/mL).[66] The TMA-based HCV RNA assay uses two enzymes, a reverse transcriptase and a T7 RNA polymerase. First, the reverse transcriptase synthesizes a double-stranded (dsDNA) template from single-stranded HCV RNA. Target amplification of the HCV RNA strand occurs when the T7 RNA polymerase transcribes new HCV RNA from the dsDNA template. Then, the new HCV RNA serves as a template for the reverse transcriptase inhibitor, and the cycle continues.[25] Recently, a TMA-based HCV RNA assay (VERSANT HCV RNA, Bayer Corp, Berkeley, CA, USA), yet to be approved by the US Food and Drug Administration (FDA), has been shown to have a lower limit of detection of 2.4 IU/mL.[67] PCR techniques can also be applied to HCV RNA. In contrast to PCR testing for HBV DNA, a reverse transcription step of HCV RNA is necessary to synthesize dsDNA, which then acts as a template for the PCR reactions. Once the HCV dsDNA is synthesized, specific primers attach to each strand of DNA. Then, new DNA strands are synthesized and amplified behind the primer. This cycle of DNA denaturing, primer annealing and strand synthesis is repeated multiple times, resulting in amplification of target DNA. This process is called reverse transcriptase-PCR (RT-PCR). Currently, Roche Molecular Systems has three RT-PCR assays with lower limits of detection ≤50 IU/mL (Table 4.4).

Quantitative HCV RNA assays have also been developed. Quantitative assays use target or signal amplification techniques such as RT-PCR or branched DNA (bDNA), respectively. The HCV quantitative assays are less sensitive than the qualitative assays, and their clinical use is limited to monitoring response to antiviral therapy. Two commercial RT-PCR quantitative assays are also available for HCV RNA (Roche Molecular Systems) with dynamic ranges of quantification of 600–850 000 IU/mL (Table 4.4). The bDNA technique[25] involves use of specific 'capture probes' to hybridize HCV RNA to microtitre plate wells. Signal amplification occurs when bDNA preamplifier molecules, which hybridize bDNA molecules, are in turn hybridized to the target HCV RNA in the well. Multiple repeat sequences within the bDNA amplifier molecule are then conjugated with an alkaline phosphatase-catalyzed chemiluminescence probe, and the chemiluminescence emission is measured. The newest generation of bDNA assays is the VERSANT HCV RNA 3.0 assay with a range of quantification of 615–7 690 000 IU/mL (1 IU = 5.2 copies) (Table 4.4).

Until recently, the HCV RNA quantitative units reported by the various assays did not represent the same actual amount of RNA in a given patient sample. Therefore, WHO established an international standard for HCV RNA quantification in international units (IU).[68] All commercial HCV RNA quantitative assays rely on this new standard and are calibrated to express the level of HCV RNA in IU/mL. For most assays, one IU/mL is equivalent to approximately 2.7 copies/mL.

**Table 4.4** Commercially available HCV RNA quantification assays

| Assay | Manufacturer | Method | Lower detection cut-off | Dynamic range of quantification |
|---|---|---|---|---|
| Qualitative assay | | | | |
| Versant HCV RNA | Bayer Corporation, Berkeley, CA, USA | Manual transcription-mediated amplification | 2.4 IU/mL | None |
| Amplicor HCV v2.0 | Roche Molecular Systems, Pleasanton, CA, USA | Manual RT-PCR | 50 IU/mL | None |
| Cobas Amplicor v2.0 | Roche Molecular Systems, Pleasanton, CA | Semi-automated RT-PCR | 50 IU/mL | None |
| Ampliscreen HCV | Roche Molecular Systems, Pleasanton, CA, USA | Semi-automated RT-PCR | <50 IU/mL | None |
| Quantitative assay | | | | |
| Signal amplification | | | | |
| Versant HCV RNA 2.0 Assay (bDNA) | Bayer Corporation Diagnostics, Tarrytown, NY, USA | Manual branched DNA (bDNA) | 200 000 genome equivalents/mL | 200 000–120 000 000 genome equivalents/mL |
| Versant HCV RNA 3.0 Assay (bDNA) | Bayer Corporation Diagnostics, Tarrytown, NY, USA | Semi-automated bDNA | 615 IU/mL | 615–7 690 000 IU/mL (1 IU = 5.2 copies) |
| Target amplification | | | | |
| Amplicor HCV Monitor v2.0 | Roche Molecular Systems, Pleasanton, CA, USA | Manual RT-PCR | 600 IU/mL | 600–850 000 IU/mL |
| Cobas Amplicor HCV Monitor v2.0 | Roche Molecular Systems, Pleasanton, CA, USA | Semi-automated RT-PCR | 600 IU/mL | 600–850 000 IU/mL |

Adapted from Pawlotsky J. Molecular diagnosis of viral hepatitis. *Gastroenterology* 2002;**122**:1554–68, with permission from the American Gastroenterological Association.

In addition to the detection and quantification of HCV RNA, molecular techniques are available to determine the HCV genotype. The HCV genotype is now determined routinely in candidates for therapy of HCV infection. For patients with HCV genotype 2 or 3, 6 months of therapy with weekly pegylated interferon and 800 mg of daily ribavirin suffices to yield an SVR in approximately 80%.[25,69] For patients with other genotypes, a full year of combination therapy involving high-dose ribavirin (1000 to 1200 mg/day based on weight < or ≥75 kg) is required. Achievement of SVR occurs in 42–51% of patients with these more refractory genotypes.

Two methods of HCV genotyping are commercially available, including a reverse hybridization assay and direct nucleic acid sequence analysis of the 5′ non-coding region of the HCV genome. The reverse hybridization line probe assay (VERSANT HCV Genotyping Assay (LiPA), Bayer Diagnostics, Tarrytown, NY, USA)[70,71] relies on 19 type-specific oligonucleotide probes attached to nitrocellulose strips to detect sequence variation in the 5′ non-coding region of the HCV genome. Initially, biotin-labelled HCV DNA PCR fragments are hybridized to the oligonucleotide probes on the strip. Next, the biotin group of the hybridization complex is revealed by incubation with a streptavidin-alkaline phosphatase complex and a chromagen resulting in a purple-brown precipitate when a match occurs between the probe and the biotinylated PCR product. Alternatively, direct nucleic acid sequencing of the 5′ non-coding region of the HCV genome can be performed with a commercial kit (Trugene HCV 5′ NC Genotyping Kit, Visible Genetics Inc., Toronto, ON, Canada), and both methods are reliable. A recent comparison of the Versant HCV Genotyping Assay (LiPA) (previously marketed as Inno-LiPA HCV II) and the Trugene HCV 5′ NC Genotyping Kit demonstrated 100% concordance between the two assays in determining HCV genotype.[72]

## Hepatitis E

Hepatitis E virus (HEV) is an RNA virus classified as a calicivirus. Hepatitis E virus causes acute, but not chronic, hepatitis. Similar in some ways to HAV,[4] HEV is spread by the faecal-oral route and is an important cause of sporadic, endemic and epidemic hepatitis in certain developing countries.[73,74] Hepatitis E virus is not indigenous to the USA, but 'imported' cases have been reported in travellers returning from endemic areas, including Pakistan and Mexico. For unknown reasons, acute hepatitis E has a particularly high mortality rate (up to 20%) in pregnant women during the third trimester of pregnancy.

Routine serological testing for HEV is not available at present. However, immunoassays (Western blot and EIAs) with reported sensitivity rates of 80–90% have been developed in research laboratories for the detection

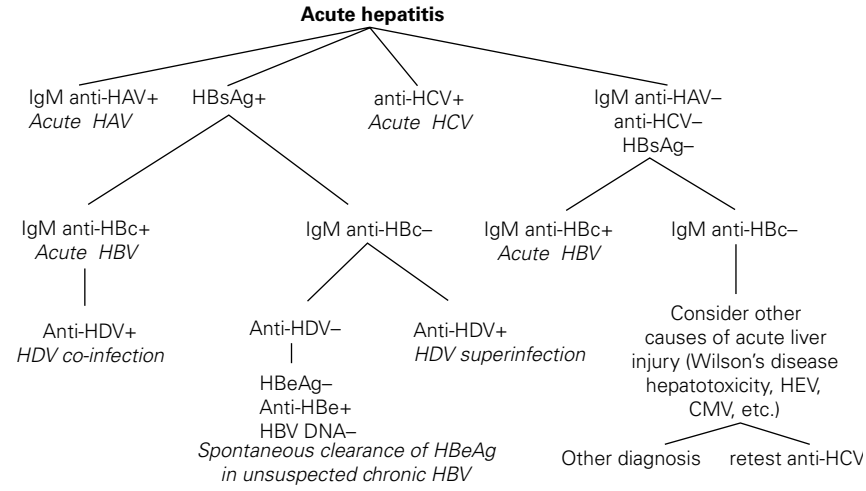


**Figure 4.5** Diagnostic algorithm for acute hepatitis.

of anti-HEV in serum based on recombinant proteins derived from the structural regions of the HEV genome (open reading frames 2 and 3).[9] Immunofluorescence assays can be used as research tools to detect HEV antigen in the liver and sensitive amplification assays (RT-PCR assays) available in research laboratories have been developed to detect HEV RNA. HEV antigen can be identified in stool, bile and liver during the incubation and symptomatic phases of acute HEV infection.[75] Antibody to HEV (anti-HEV) can also be detected in serum during acute hepatitis E.[73]

In practice, the diagnosis of HEV infection is currently made on the basis of compatible travel history and exclusion of other specific causes of acute viral hepatitis.

## Diagnostic application of serological tests

All five major hepatitis viruses can cause acute hepatitis, but only three – HBV, HDV and HCV – also cause chronic hepatitis. Therefore, serological diagnosis of viral hepatitis should be tailored to the particular clinical circumstances. As noted above, diagnostic testing for HEV is available on a research basis only. Infection with HDV does not occur in the absence of HBV infection, and testing for HDV should generally not be part of a first-line panel of clinical assays.

In suspected cases of acute viral hepatitis, appropriate initial tests should be directed towards detection of acute hepatitis A, B, or C (Fig. 4.5), with an initial diagnostic panel consisting of IgM anti-HAV, HBsAg, IgM anti-HBc and anti-HCV. The diagnosis of acute hepatitis A is established by detection of IgM anti-HAV. The diagnosis of acute hepatitis B is based on detection of HBsAg and IgM anti-HBc. The presence of IgM anti-HBc suggests that, if HBsAg is present, HBV infection is acute or that, if HBsAg is absent, the patient has acute resolving hepatitis B or acute hepatitis B with a level of HBsAg beneath the detection threshold of the immunoassay. Routine testing for HBeAg or HBV DNA during acute hepatitis B is not indicated, although both markers are present. If epidemiological or clinical features suggest HDV infection, such as an unusually severe episode of acute hepatitis or a risk factor such as injection drug use, testing for anti-HDV should be done. Finally, testing for anti-HCV should be done, especially in a patient with a history of injection drug use or with (now rare) post-transfusion hepatitis. Because of the low sensitivity of anti-HCV testing during acute hepatitis C, repeated testing for anti-HCV and testing for HCV RNA are appropriate if no other cause for acute hepatitis has been identified. If this initial battery of serological tests is negative, the diagnosis of acute viral hepatitis should be reconsidered. Other possible causes of acute liver injury, such as drug hepatotoxicity, biliary tract disease, metabolic disorders, autoimmune liver disease, cardiac failure, or involvement of the liver in a multisystem disease, such as infectious mononucleosis or metastatic malignancy, should be considered.

In cases of chronic liver disease, neither HAV nor HEV is included in the differential diagnosis. Testing for HBV

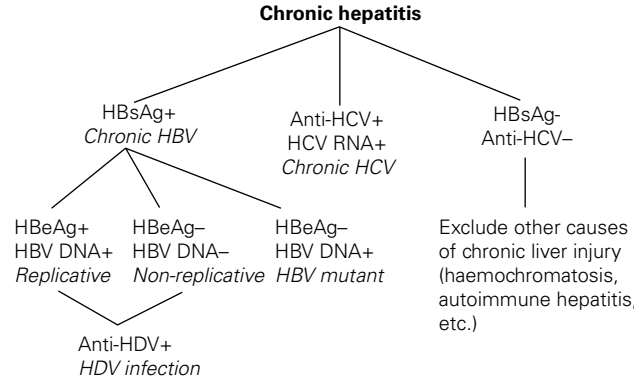


**Figure 4.6** Diagnostic algorithm for chronic hepatitis.

and HCV infection should be accomplished initially by immunoassays for HBsAg and anti-HCV, respectively (Fig. 4.6). If HBsAg is present, detection of IgG anti-HBc in the absence of IgM anti-HBc excludes acute hepatitis B and confirms the chronicity of hepatitis B. Additional information about HBV infection may be obtained by testing for HBeAg and HBV DNA in serum to document the presence of active viral replication. In addition, in patients with hepatitis B, anti-HDV testing is indicated to establish the presence of associated HDV infection, particularly if the clinical features of liver disease are severe or risk factors such as injection drug use exist. In a patient with a high risk of percutaneous exposure to HCV, the detection of anti-HCV by EIA usually confirms a diagnosis of chronic HCV infection. If doubt exists about the interpretation of the anti-HCV result (either falsely negative or falsely positive), testing for serum HCV RNA should be performed. Although liver biopsy is generally not indicated purely for diagnosis in chronic viral hepatitis, histological assessment is a routine component of pretreatment staging. Demonstration of viral antigens in liver tissue provides additional confirmation of a specific viral infection. However, apart from immunohistochemical detection of intrahepatic HBV antigens, techniques to detect intrahepatic viral markers are not widely available.

Common serological patterns and their interpretations are illustrated in Table 4.1 and diagnostic algorithms for acute and chronic hepatitis are illustrated in Figs 4.5 and 4.6.

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# Chapter 5
# Evolution of hepatitis viruses

Peter Simmonds

The origins and evolution of the major viral pathogens affecting humans remain poorly understood, even though such information can provide important perspectives on the spread of viruses and mechanisms underlying the diseases they cause.

For some of the viruses causing hepatitis in humans there is extensive and wide-ranging information on their transmission, geographical ranges and genetic variability that combined provides some clues about their origins and tempo of evolutionary change. This chapter attempts to synthesize some of the available information for hepatitis C virus (HCV) and related viruses, and for hepatitis B virus (HBV), now known to be widely distributed in a range of ape species as well as humans. Although not in any way conclusive at present, these attempts to reconstruct the evolution of the two virus groups provide an interesting and useful alternative paradigm for ongoing studies of pathogenicity, future transmission and prevention.

## General principles of virus evolution

The evolution of viruses resembles that of all organisms, and is a process that is ultimately dependent on mutations in their genetic material. In many ways, however, viruses differ from commonly studied organisms such as the geneticist's mouse or fruit fly, particularly in their speed of sequence change, large population sizes and in the nature of the selection pressures they encounter. For example, of major importance for the persistence of HCV and HBV infections in humans is the ability of these viruses to mutate rapidly to evade host immune defences.

To understand the evolution of viruses and other organisms, a little background information on natural selection is needed. In popular use, the word 'evolution' describes the process of adaptive change, whereby organisms change in their phenotype (such as body shape or behaviour) in response to external, sometimes changing, selection pressures, and by competition with other organisms for limited resources in a shared environment. In this process, a random mutation in the genome associated with copying errors or chromosomal damage occasionally (and entirely by chance) might improve the fitness of an organism, allowing the mutated gene to spread and eventually predominate in the population where the advantage it provides to the organism, in terms of reproductive success, is significant. In this model of evolution, the appearance of distinct species of animals, plants and bacteria is the net result of large numbers of incremental changes in phenotype associated with adaptation to the wide range of separate contemporary and previous environments.

Surprisingly, this 'Darwinian' type of evolution makes very little contribution to the genetic diversity of organisms, measured at the level of DNA or RNA sequences. As one of the most hotly contested hypotheses of evolution change when originally proposed,[1,2] neutral theory demonstrates that the majority of sequence change that occurs over time in and between species is 'neutral'.[3] In this revised evolutionary model, nucleotide changes in gene and non-coding sequences that have little or no effect on organism fitness are tolerated and frequently become fixed in the population by chance. Thus, geographically isolated members of species can become genetically very different, even though morphologically and behaviourally they remain similar over long periods in stable environments. The frequency of fixation of neutral changes can be predicted to be relatively constant over time, and indeed there is a remarkably close correlation between sequence divergence of certain genes such as haemoglobin with known chronological times of splitting of different mammalian species and orders over the past 150 million years.

Evidence for both 'Darwinian' and neutral evolution can be found in human hepatitis viruses. Examples of adaptive changes include a specific amino acid substitution in the polymerase protein of HBV in patients receiving lamivudine therapy that confers antiviral resistance (see Chapters 20 and 21), and changes to a major B cell epitope in the HBV surface antigen (HBsAg) in individuals infected with HBV after immunization (Chapter 14). In these cases, single nucleotide mutations emerge that cause dramatic changes to the phenotype of the virus,

conferring major fitness advantages to the virus under new selection pressures (such as antiviral treatment and immunoglobulin immunotherapy).

In contrast to these adaptive changes, 'neutral' sequence drift undoubtedly accounts for much of the genetic diversity observed between geographically or epidemiologically separated populations of hepatitis viruses, such as the genotypes of HCV and HBV infecting humans and other ape species. In such cases, the process of divergence associated with the fixation of neutral changes does not greatly alter the phenotype of the viruses. For example, despite the greater than 30% sequence differences observed between genotypes of HCV, each retains a similar tendency for persistence in humans, to cause similar inflammatory liver damage and a slow progression to cirrhosis and hepatocellular carcinoma (HCC). Putting this viral diversity into context, coding sequences of (morphologically and behaviourally very different) humans and ostriches are also approximately 30% divergent, a comparison that highlights the contrasting highly conservative nature of HCV evolution over the period in which the major genotypes emerged. Indeed, this lack of phenotypic innovation demonstrates, perhaps, how the evolution of HCV (and HBV) is entirely shaped and constrained by their close adaptation to the particular ecological niche they inhabit, the human liver, and their requirement for efficient transmission between individuals.

## Hepatitis C virus

The genetic diversity of HCV exists at several different levels. Most obvious is the great genetic divergence of the currently classified six major genotypes of HCV. These frequently show specific geographical ranges in the human population and associations with particular risk groups for infection (see next section). At a lower level is the variability observed between different HCV strains within a genotype. Much of the sequence diversity observed between such HCV strains (such as the 5–8% divergence observed between variants in epidemiologically unlinked HCV genotype 1a, 1b and 3a infections) reflects processes of neutral sequence drift over time after the introduction of HCV into new risk groups in the 20th Century. It is also possible that some of the sequence divergence may be phenotypically selected changes associated with adaptation for replication in different individuals with different immune responses to infection. Finally, HCV diversifies measurably within an infected individual over time, forming what has been described as a 'quasispecies'. This pre-existing genetic variability, combined with an extremely large replicating population size of HCV in a chronically infected individual, provides a large pool of genetic variants that can readily adapt to new selection pressures such as antiviral treatment.

### HCV genotypes

HCV shows quite extensive genetic diversity, a feature shared with many other RNA viruses that infect humans and other mammals. Comparison of the nucleotide sequences of HCV variants recovered from infected individuals in different risk groups for infection, and from different geographical regions has revealed the existence of at least six major genetic groups (Fig. 5.1). As an average over the complete genome, these differ in sequence by 30–35% of nucleotide sites, with more variability concentrated in regions such as the E1 and E2 glycoproteins, while sequences of the core gene and some of the non-structural protein genes such as NS5 are more conserved. Least sequence variability between genotypes is found in the 5′ and the terminal region of the 3′ untranslated regions, where sequence conservation is thought to be essential for their replication and translation functions (see Chapter 24). In broad terms, the sequence differences between HCV genotypes are comparable to those observed between serotypes of other viruses, such as dengue virus 1–4 in the *Flaviviridae*, and between those of poliovirus serotypes (member of the *Picornaviridae*). It is therefore likely that the genotypes of HCV are antigenically distinct, representing a potential problem with the future development of broadly effective vaccines against HCV (Chapter 35).

Each of the six major genetic groups of HCV contains a series of more closely related subtypes, typically different from each other by 20% in nucleotide sequences compared with the >30% between genotypes (Fig. 5.1).[4] Some, such as genotypes 1a, 1b and 3a, have become very widely distributed as a result of transmission through blood transfusion and needle sharing between injecting drug users (IDUs) over the past 30–70 years (Chapter 25) and now represent the vast majority of HCV infections in western countries. These are the genotypes most commonly encountered clinically, and for which most information has been collected on response to interferon and other antiviral treatments (see Chapter 33).

A different pattern of HCV sequence diversity is observed in parts of Africa and south-east (SE) Asia. Here, there are close associations between HCV genotypes and specific geographical regions (Fig. 5.2). For example, HCV infections in Western Africa are almost invariably by genotype 2,[5–9] while those in Central Africa, such as the Democratic Republic of Congo (DRC) and Gabon are by genotype 1 and 4.[7,10–15] In both regions, there is a remarkable diversity of subtypes. For example, 20 of 23 HCV-seropositive blood donors in Ghana (West Africa) were infected by genotype 2, but each corresponded to

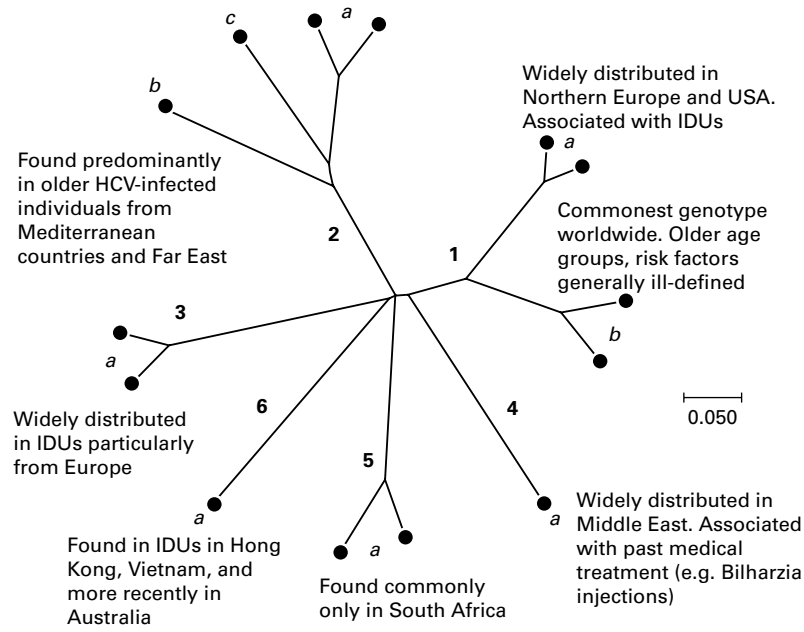


**Figure 5.1** Evolutionary tree of the principal genotypes of HCV found in industrialized countries, and their main epidemiological associations with specific risk groups. These genotypes of HCV are believed to have become prevalent over the course of the 20th century. The tree was constructed using the phylogeny programme Neighbour-Joining implemented in the MEGA package, using Jukes-Cantor corrected distances between complete genome sequences. IDU, injecting drug user.

different and previously undescribed subtypes.[5] This diversity is reproduced in Guinea-Bissau (West Central Africa), where 18 different subtypes of HCV genotypes 1 and 2 were found in samples from 41 HCV-infected individuals.[6] These field observations reflect both the huge genetic diversity of HCV genotypes 1, 2 and 4, and also its likely long-term presence in human populations in these parts of Africa. Taking this geographical mapping further, genotypes 3 and 6 show similar genetic diversity in south and eastern Asia.[7,16–18]

The model suggested by these genotype distributions is that HCV has been endemic in sub-Saharan Africa and SE Asia for a considerable time, and the occurrence of HCV infection in western and other non-tropical countries represents a relatively recent emergence of infection in new risk groups for infection.[13,19] In the 20th Century, parenteral exposure to blood-borne viruses became frequent through the widespread adoption of blood transfusion since the 1940s, the medical use of often unsterilized needles for injections and vaccinations (a practice that continues in many developing counties) and most specifically to industrialized countries, injecting drug use and the sharing of injection equipment. These new routes for transmission plausibly account for the epidemiological and genetic evidence for recent epidemic spread of HCV over the past 50 years in Europe, Egypt and elsewhere.[13,20–22]

However, one of the puzzles about the origins of HCV is the absence of obvious transmission routes for HCV in human populations of Africa and SE Asia, where the greatest genetic diversity is observed. Transmission by either sexual contact or from mother to child is uncommon (see Chapter 25), and there is little evidence historically for the type of widespread parenteral exposure that fuelled the HCV epidemic in western countries. However, recent field investigations in southern Burkino Faso (Central/Western Africa), where HCV genotypes 1 and 2 are prevalent and highly diverse in sequence, have shown associations between HCV infection with previous sexually transmitted diseases (STDs), circumcision

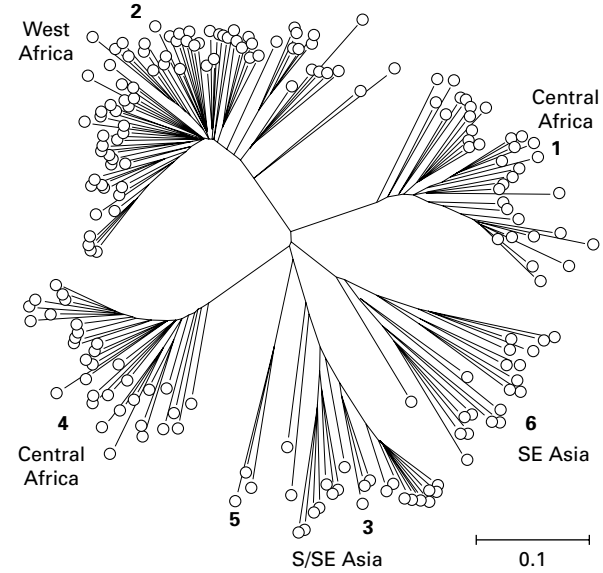


**Figure 5.2** Evolutionary tree of all available NS5B sequences of subtypes and genotypes of HCV, including those found in high diversity areas of of genotypes 1, 2 and 4 (sub-Saharan Africa), and 3 and 6 (south-east Asia). HCV variants still fall into six distinct clades, but with far greater numbers of genetic variants corresponding to subtypes in industrialized countries. The tree was constructed using the phylogeny programme Neighbour-Joining implemented in the MEGA package, using Jukes-Cantor corrected distances between partial NS5B sequences (320 bases).

and scarification practices.[23] Although the association with STDs has not been documented in studies carried out in western countries (see Chapter 25), it is possible that lack of mucosal integrity associated with long-term untreated infections may facilitate the entry of HCV into the genital tract. Determining how long HCV has been in these 'endemically' infected populations would clearly be of value in understanding the epidemiology of HCV in these regions (see below).

Even less is known about the earlier divergence of the six major genotypes of HCV, the origins of HCV infection in humans, and the underlying basis for the current geographic distribution of HCV genotypes. Strangely, those areas where HCV is endemic and highly diverse are precisely those where HBV is prevalent (see below), and also represent regions where human and ape population ranges overlap. However, in contrast to HBV, there is currently no evidence that HCV or HCV-like variants infect Old World ape or monkey species.[24] Therefore, despite the tempting analogies with the introduction and spread of human immunodeficiency virus (HIV)-1 and -2 infections in humans through cross-species transmission of simian viruses from chimpanzees and mangabeys,[25,26] it would be highly speculative and currently unjustified to imagine that HCV originated in these human populations as a result of similar cross-species transmissions. On the other hand, it has been discovered that a very distantly related HCV-like virus, GB virus B,[27] infects tamarins and/or other New World primates. The existence of this homologue in such a distantly related primate species certainly allows the possibility that HCV or HCV-like viruses may indeed be more widely distributed in primates than previously thought. Larger-scale serological and PCR-based surveys of a much larger range of primates in Africa, Asia and South America are required to resolve this issue.

## Sequence variability within genotypes

Several studies have described the rapid sequence drift of HCV over time, a process of diversification that leads to the existence of identifiably separate HCV strains or isolates within human populations. By comparing HCV sequences from sequential samples from chronically infected individuals, or from those infected from a common source, rates of sequence change of HCV were measured to be $1.44 \times 10^{-3}$ nucleotide changes per site per year over the whole genome, or 4 4.1 and $7.1 \times 10^{-4}$ changes per site per year in the NS5 and E1 regions.[28,29] In coding regions of the genome, these changes occur predominantly at synonymous sites (sites that do not alter the encoded amino acid), and are therefore likely to represent the accumulation of phenotypically neutral changes. The expectation from neutral theory that such diversification should occur at a constant rate over time is implicit in attempts to use this rate to estimate times of spread of HCV in specific transmission networks such as IDUs,[20–22] and indeed extrapolation to calculating times of introduction of specific HCV genotypes such as 1a, 1b, 3a and 4a into new risk groups for infection in western countries.[29] For example, the current sequence diversity and phylogenetic tree structure of genotype 4a in Egypt is compatible with the introduction of HCV into that population through parenteral treatment for schistosomiasis (tragically with non-disposable and poorly sterilized needles) in the 1950s and 1960s.[21,30,31] The increasing sequence diversity within genotypes 3a, 1a, 1b, 2a and 2b, respectively, suggests times of introduction of these viruses at increasingly earlier times in the 20th Century associated with other parenteral risk factors for infection such as injecting drug use, blood transfusion, large-scale immunizations and syphilis treatment.[32]

While regions of the genome such as NS5B have frequently been used for epidemiological reconstruction, other parts of the genome such as the 'hypervariable' regions (HVRs) of E2 and NS5A show much greater variability and much more rapid amino acid sequence change over time. This variability may arise through specific selection (Darwinian) mechanisms operating on the virus associated with immune escape. For example, the HVR in E2 may be a target for neutralizing antibody, and HCV persistence may therefore require continuous virus sequence change to evade B-cell responses.[33–36] For HIV-1, it is known that initial infection is accompanied by a number of amino acid changes in class I binding motifs in potential T-cell epitopes, such as the Arg → Lys or Gly change in the immunodominant epitope in $p24^{gag}$ recognized by the B27 allele.[37] These mutations occur with some fitness cost and revert to wild type on the development of severe immunodeficiency, and probably on further transmission to other individuals with different HLA types. It is therefore possible that many of the amino acid polymorphisms observed in HCV are also driven sequentially by selection from different major histocompatibility complex (MHC) class I or II alleles encountered during a virus's passage through human populations, comparable to the observed immune-selected changes in cytotoxic T-lymphocyte (CTL) epitopes in experimentally infected chimpanzees.[38] Supporting this hypothesis is the observation that HCV sequence change was slower in individuals with defects in T- or B-cell immunity,[39] interpreted as indicating reduced immune selection on CTL or B-cell epitopes. Indeed, recovery from HCV infection is associated with strong and sustained CTL responses around the time of primary infection,[40–42] a time where there was evidence for specific changes in CTL epitopes, and accelerated sequence change in the HCV coding sequence was observed in those who became chronic carriers.[43,44]

The high degree of sequence variability observed within genotypes in the NS5A region is puzzling, because it has been demonstrated that, at least in some human populations, certain sequences in the 'interferon sensitivity determining region' (ISDR) of genotypes 1b, 2a and 2b (described as 'prototype') are associated with resistance to interferon (IFN) treatment[45] (see Chapter 33). NS5A is a multifunctional protein with a number of activities, such as binding to protein kinase (PKR), that contribute to the evasion of host cell antiviral mechanisms[46,47] (Fig. 5.3). As the 'prototype' ISDR sequence of NS5A is specifically found in individuals who resist IFN therapy, it is difficult to imagine why there should not be selection and the acquisition of resistance to treatment in those with non-'prototype' sequences. However, NS5A is known to contain a high concentration of T- and B-cell epitopes,[48–51] and it is possible that immune selection drives the ISDR or neighbouring sequence away from the optimal functioning protein (corresponding to the prototype sequence) in individuals with certain human leukocyte antigen (HLA) types, analogously to the changes in p24$^{gag}$ of HIV-1 selected by the B27 class I allele. A poorly functioning NS5A protein may make the infecting virus more sensitive to intracellular antiviral responses and thus to a greater likelihood of spontaneous viral clearance, as well as increased susceptibility to IFN therapy in those who remain viraemic.

The balance in this 'trade-off' between NS5A function and immunological recognition may differ between genotypes of HCV. For example, the reason why genotypes 2 and 3 infections are generally much more responsive to IFN treatment (see Chapter 33) may be because a far greater proportion of individuals immunologically recognize the prototype NS5A protein. Subsequent evolution of the infecting virus with a functionally impaired NS5A protein makes it less able to resist the further assault of exogenously administered IFN used for therapy. Human population-specific differences in the frequencies of HLA types in different study groups may also go some way to explaining why the association of 'prototype' ISDR sequences with treatment resistance varies so much between studies in Japan and Europe (Chapter 33).

## HCV 'quasi-species'

The processes of neutral and adaptive evolution of HCV operate during the course of chronic HCV infection within an individual, leading to both continued fixation of nucleotide changes over time, and the development of variable degrees of sequence diversity within the HCV replicating population at a given time point. Sequence diversity is generated continuously during virus replication because RNA copying by the virally encoded RNA polymerase (NS5B) is error-prone, and because the replicating population of HCV is so large. For example, ongoing error rates ranging from 1 in 10 000 to 100 000 bases copied typically found in RNA polymerases,[52,53] combined with a rate of virus production of up to $10^{12}$ virions per day,[54] would produce a genetically diverse population of variants. It could be predicted statistically that the 'quasi-species' would include mutants differing at every nucleotide position and every combination of paired nucleotide substitutions from the population average or consensus.

Even though the consensus sequence may be optimal in fitness at any one time, the existence of a large and diverse population would allow very rapid adaptive (Darwinian) changes associated with changes in the replication environment. This might take the form of evolving immune responses that select against viruses with specific T- or B-cell epitopes. It might also confer resistance to antiviral agents. The rapid and reproducible independent appearance of specific amino acid changes associated with the acquisition of HIV-1 resistance to reverse transcriptase and protease inhibitors is a dramatic demonstration of 'quasi-species' evolution. This may be

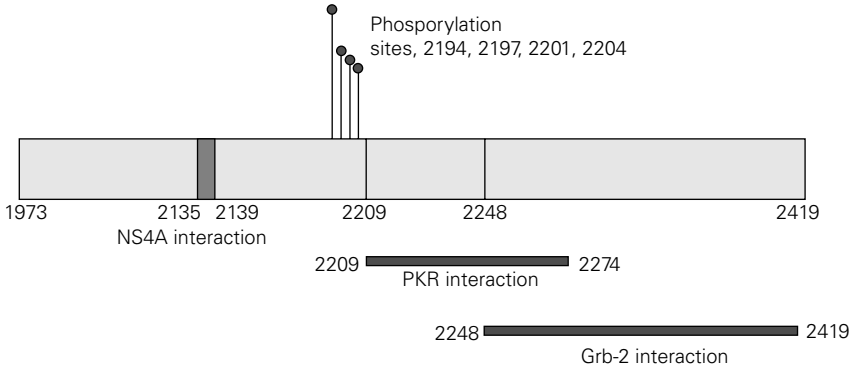


**Figure 5.3** Diagram of the NS5A gene, showing the interferon-sensitivity determining region (ISDR), and regions of the protein known to interact with protein kinase (PKR) and other cellular pathways associated with innate cell defences against viruses. Sequence changes in the ISDR correlate with sensitivity of the virus to interferon therapy, potentially representing a trade-off between functional activity and immune selection.

reproduced in HCV infections treated with the new generation of antiviral agents, such as BILN2061 and other protease inhibitors (Chapter 34).[55–57]

### Origin of HCV genotypes

How long HCV has been present in human populations is difficult to estimate. As described above, the diversity of HCV variants within genotypes 1, 2 and 4 in sub-Saharan Africa and of genotypes 3 and 6 in SE Asia suggests that HCV may have been endemic in these populations for considerably longer than in western countries. As the evolutionary process of HCV sequence divergence that led to the diversity of subtypes in these regions is likely to have been predominantly neutral in mechanism, it may therefore be possible to calculate the times of splitting of HCV subtypes, and possibly also the time of divergence of the six main clades of HCV through use of published rates of HCV sequence change over time.[28,29]

Extrapolation of these rates to time the 20% and 30% sequence divergence observed between HCV subtypes and genotypes produces relatively recent times of origin that in many ways are difficult to reconcile with the epidemiology of HCV and its global distribution. For example, the diversity of HCV variants observed in West African genotype 2 sequences predicts a time of origin for this endemic pattern of infection approximately 200–250 years ago, while the HCV genotypes 1–6 would have diverged about 100 years earlier. Even using complex methods for correction for multiple substitutions and allowing rate variation between sites, the current diversity of HCV genotypes predicts an origin no earlier than 1000 years ago.[29] This seems recent for such a widely distributed virus infecting often relatively isolated human populations in equatorial Africa and SE Asia.

We have argued that hidden constraints of HCV sequence change, such as the extensive RNA secondary structure in coding regions of the genome,[58,59] may severely limit the number of 'neutral' sites in the genome. In the case of HCV, such constraints would operate on base positions where sequence changes would disrupt internal base-pairing of RNA structured elements.[19,58] Given the complexity and large scale of these HCV RNA secondary structures, truly 'neutral' sites not involved in base-pairing may be rare indeed. Although neutral drift evidently still occurs in HCV, reconstructing the timescale of diversification is hampered by not knowing how many and at which sites sequence changes can be fixed without fitness cost. Until these issues are better understood, there remain major problems in making adequate correction for multiple substitutions in the calculation of evolutionary distances. In making the estimates above for 350–1000 years for the time of divergence of HCV genotypes, we are in danger of telescoping a much longer period of virus evolution into an unrealistically short time-frame.

## Hepatitis B virus

The tempo and mode of HBV evolution show many similarities with those of HCV. Several genotypes of HBV exist, many with specific geographical locations overlapping those of HCV. Similarly, much of the sequence change observed on chronic infection shows evidence for adaptive selection, with evidence for amino acid changes in response to immune pressure and antiviral treatment. There are, however, important differences, most strikingly the existence of widespread infection of non-human apes with HBV variants that show a complex evolutionary relationship with human genotypes. Furthermore, there is frequent recombination between HBV genotypes infecting humans, whereas this is extremely uncommon in HCV. Persistence of HBV infection *in vivo* is also characterized by modulation of gene expression, including the appearance of mutations in the precore region that prevent translation of the HBV e antigen (HBeAg), probably originating through some kind of immunological selection.

### HBV genotypes

Variants of HBV can be currently classified into a total of approximately seven or eight genotypes.[60,61] The genotypes designated A–E and G differ from each other by around 9% in nucleotide sequence (Fig. 5.4). However, genotype F and the related genotype H, are outliers and more divergent in sequence (approximately 13%). Certain genotypes show specific geographical distributions: B and C in East and SE Asia; E in West, sub-Saharan Africa; A predominantly in Central Africa; and genotypes H and F largely confined to aboriginal Indian populations in Central and South America. There is currently insufficient information for genotype G to allow its geographical distribution to be defined. Genotype D appears to be globally distributed (Europe, Africa, Asia) without evidence for a specific geographical origin as found for other genotypes. Regions where certain genotypes can be localized correspond generally to where HBV in human populations is endemic and highly prevalent (sub-Saharan Africa [A, E], east/SE Asia [B, C] and South America [F]). As described above, the first two areas are also those characterized by HCV endemicity and genetic variability.

Recombination between different genotypes frequently leads to the appearance of viable hybrid viruses with highly variable and frequently complex combinations of genetic elements derived from the parental viruses.[62] Although many recombinant viruses have been observed only sporadically, others may be widely distributed,

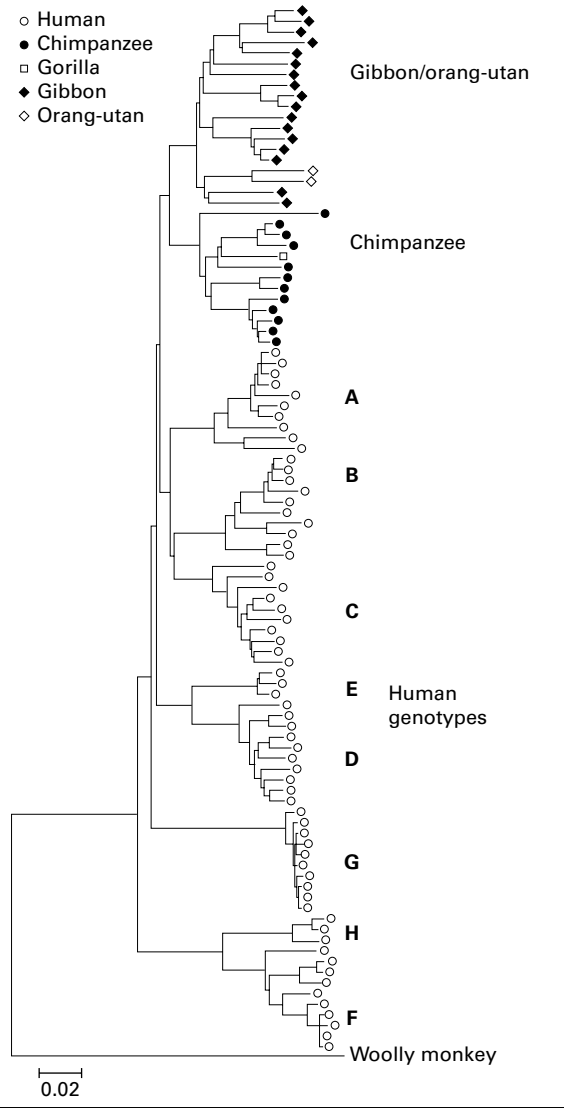


**Figure 5.4** Genotypes of HBV infecting humans (A–H), and relationship with those infecting chimpanzees, gibbons, other ape species and the woolly monkey. Human genotypes A–E and G are approximately equally divergent from each other and from the two clades of HBV variants infecting predominantly chimpanzees and gibbons (9% sequence divergence). These latter sequences are more similar to these human genotypes than those found in human populations in Central and South America (F and H), and the highly divergent HBV variant recovered from a captive woolly monkey (of uncertain origin). The tree was constructed using the phylogeny programme Neighbour-Joining implemented in the MEGA package, using Jukes-Cantor corrected distances between complete genome sequences.

such as a C/D recombinant virus in Tibet,[63] A/C in Vietnam[64] and B/C recombinant frequently found in eastern Asia.[65] Infection with the latter is more frequently associated with HBeAg expression than the non-recombinant form of genotype B, and it may therefore differ in long-term pathogenicity, transmissibility and treatment response from non-recombinant forms.[66]

HBV infection is also highly prevalent in chimpanzees and gorillas in sub-Saharan Africa, and in gibbons and orang-utans in SE Asia, each species showing frequencies of HBsAg carriage ranging from 10% to 30%.[24,67] In general, these different ape species are infected with genotypes of HBV distinct from each other, with suggestions that ape-derived HBV variants can be classified into chimpanzee, gibbon and orang-utan-specific genotypes.[67–74] This may be an oversimplification, as further analysis of HBV variants in SE Asian primates showed that phylogenetic grouping correlated better with geographical range than host species.[67] For example, variants from orangutans more closely resembled those from gibbon species at the southern end of their geographical range, a result of an overlap in their natural habitat, rather than forming a separate species-specific phylogenetic group (Fig. 5.4). Similarly, from the minimal data available at present, HBV variants infecting gorillas appear most similar to HBV variants recovered from central African chimpanzees,[68] rather than forming a separate species-associated lineage.

What are the origins of HBV infection in humans? Despite the existence of widespread infection of ape species in areas where human HBV infection is endemic and highly prevalent, there is no evidence for direct cross-species transmission, as human- and primate-derived HBV genotypes are invariably distinct and non-overlapping. Thus, despite large surveys, there is no evidence for infection in human populations in Central or West Africa with HBV variants resembling those found in chimpanzees, nor have the human genotypes from this region (A and E) been detected in chimpanzees (with the exception of one genotype E-infected chimpanzee, although in this case, iatrogenic transmission of HBV in captivity cannot be ruled out[72]). There is similarly no overlap between human genotypes B and C with gibbon- or orang-utan-derived HBV variants. There is therefore no support for the type of cross-species transmission model proposed for the origins of HIV-1 or -2 infection in humans.[25,26]

The inter-relationships of human- and non-human ape-derived HBV genotypes are indeed complex, and difficult to reconcile with any straightforward evolutionary hypothesis for human HBV origins.[19] Ape-derived genotypes of HBV are interspersed with human genotypes A–E and G, differing from each other and from these human genotypes by the same 9% level of divergence found between most human genotypes. Although the chimpanzee and gibbon/orang-utan groups are internally relatively diverse, this does not reproduce the multiple genotype diversity observed in human populations, and the outlier genotype F/H from South America. It could be argued that humans acquired HBV

infection through cross-species transmission of HBV variants infecting a different set of non-human primate species in Africa and Asia. However, several serological and PCR-based surveys concur that HBV infection in the wild is confined to the four species of ape described above.[24,67]

## HBV sequence variability within genotypes

The phylogenetic tree of human and ape HBV variants (Fig. 5.4) includes a selection of published sequences from each genotype. From this it is apparent that variable degrees of sequence divergence exist within each genotype, with genotype E sequences being perhaps the least variable, and the gibbon/orang-utan clade being the most diverse. Just as the human and other ape genotypes show different geographic distributions, sequence variability within genotypes may also correlate with geographical origin. For example, while both genotype C and D variants are widely distributed in SE Asia, those from mainland Asia are genetically distinct from HBV variants found in the aboriginal population in Papua New Guinea and from those in Australia.[75,76] Variants of genotype A (but not E) are similarly segregated between Central and West Africa, while Central American variants designated as genotype H[77] could perhaps be more consistently classified as geographical variants of genotype F found in South America. These observations are consistent with long-standing endemic infections in what are frequently aboriginal and highly isolated human populations, implying extremely slow long-term sequence change over time. How this can be reconciled with observation for rapid sequence change over short periods is discussed in the next section.

The previous evolutionary history of HBV in humans and non-human ape populations therefore remains difficult to reconstruct, and the current distribution of HBV infection and association of certain genotypes with specific geographical areas and/or host species cannot be easily explained by any of the currently discussed theories for HBV origins. As for HCV, attempting this type of reconstruction would be assisted by knowledge of the rate of sequence change of HBV, providing some kind of estimate of the times of divergence of the human and ape genotypes. Although short-term rates of sequence change of HBV have been measured (see next section), extrapolating these to longer periods leads to the same intractable problems encountered for HCV (see above).

## Mechanisms of HBV sequence change

For HCV, neutral sequence drift undoubtedly accounts for much of the genetic variability observed between different genotypes, as well as between isolates of the same genotype found in different infected individuals. For HBV the situation is much less certain. The rate of fixation of sequence changes of HBV has been calculated by sequence comparisons of HBV variants recovered from sequential samples from chronically infected individuals,[78] averaged at $2.1 \times 10^{-5}$ substitutions per site per year for those who were HBeAg-positive. Remarkably, however, sequence change was more than 10 times faster in those who were HBeAg-negative. The continued production of HBeAg during chronic infections reflects partial immune tolerance to HBV, and is typically the outcome of perinatal infection (see Chapter 11). The much higher rate observed in those who have cleared HBeAg therefore suggests that the majority of sequence changes in HBV are immunologically driven. This might take the form of changes to class I or II epitopes presented to T cells (as described for HIV-1), or alterations to linear or conformational epitopes recognized by immunoglobulins.

Understanding the relative contributions of neutral and adaptive mechanisms of sequence change of HBV is complicated by a number of factors peculiar to HBV. Most of the genome of HBV is configured to contain not one but two or in some regions three coding sequences in different reading frames (Chapter 10). Given that the remaining single coding and non-coding regions of the genome are packed with RNA structural elements and transcriptional and translational regulatory sequences (Chapter 10), it is doubtful whether there are any sites where sequence change has no significant effect on phenotype as demanded by neutral theory.[3] Combined with the evidence for intense immune system-mediated selection for sequence change, a large part of the sequence diversification over time observed in chronically infected individuals and on transmission must represent the evolution of HBV mutants changing under intense Darwinian selection for immune evasion. As already described for HIV-1 epitopes, such sequence changes incur fitness costs and may revert on transmission of infection to individuals with different HLA types. Thus, the apparent relatively rapid sequence change observed in chronic infection, rather than being accumulative, may simply involve flip-flopping of certain key amino acid residues that are most immunogenic. There may therefore be no net sequence evolution over longer periods, each genotype gravitating towards a particular phenotypically optimized master sequence constrained in a relatively rugged fitness landscape. As described earlier for HCV and related viruses, this restriction of neutral sites suggests that while only 10–13% divergent, times of divergence of genotypes of HBV infecting human and primates are not only incalculable but also potentially ancient in the absence of sustained neutral drift. Indeed, the apparently simple and largely unstructured branching of human and ape genotypes may well conceal a complex evolutionary history that is now obscured by

large numbers of selected, convergent sequence changes. These may defeat attempts to construct a true phylogeny for HBV by nucleotide sequence comparisons alone.

Further evidence for the genetic plasticity of HBV under selection pressure is provided by observation of the rapid and stereotyped amino acid changes in the *pol* gene that confer resistance to lamivudine[79] and other antiviral agents targeting the HBV reverse transcriptase (see Chapter 20). As for HIV-1 and HCV, this is the likely outcome of a large 'quasi-species' population present in infected individuals, in which mutants with the required resistance change may be present by chance at the start of antiviral therapy, and expand under drug-induced selection pressure to become the dominant variant over the initial weeks of therapy. Comparable is the observation that treatment of immunosuppressed transplant recipients with anti-HBsAg antibodies frequently generates HBV mutants with mutations in the immunodominant 'a' determinant that escape from antibody binding.[80,81] Less certain are the selection pressures that drive sequence change in the precore region, and which inhibit the expression of HBeAg.[82] These nucleotide changes include a stop codon in the coding sequence, and substitutions in the core promoter region that decrease transcription of the HBeAg/core mRNA. The regularity with which pre-core mutants emerge during the course of chronic infection, particularly in HBV genotypes B, C, D and E suggests that such changes have a selective advantage over wild-type HBV variants. At present, however, the functional significance of this change, and indeed of HBeAg expression itself, remain unclear.

## Summary

The evolution of HCV and HBV comprises highly dynamic processes, occurring both through multiple processes of adaptive selection that drive sequence change (such as those resulting from the host immune response and from antiviral treatment) and through drift in which phenotypically neutral sequence changes accumulate over time without altering the phenotype or behaviour of the virus. However, despite their potential to evolve rapidly, the longer-term evolution of both viruses appears remarkably conservative. Little fundamental change in the relationship with their human hosts in terms of persistence and transmission has developed between genetic variants of HBV and HCV, despite the probably extremely long periods over which this viral diversity has developed. HCV and HBV thus appear to have successfully filled very specific, different ecological niches in human populations. The intimate host/parasite relationships in which these viruses thrive are therefore likely to have a degree of complexity that we are only just beginning to understand in current studies of their pathogenesis.

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# Section III
# Hepatitis A Virus

# Chapter 6
# Structure and molecular virology

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## Introduction

Hepatitis A virus (HAV), the causative agent of acute, infectious type A hepatitis, is a positive-strand RNA virus classified within the family *Picornaviridae*. While both its structure and the organization of its genome share features in common with other picornaviruses, such as poliovirus or encephalomyocarditis virus, several important features of HAV distinguish it from other human and animal members of this large virus family. These unique features of HAV include its relatively high level of resistance to thermal denaturation, its remarkable stability under low pH conditions, and the composition and details of the assembly of the viral capsid structure (in as much as it is understood). Coupled with the fact that HAV shares only very limited nucleotide homology with other picornaviruses,[1] these features have contributed to the classification of HAV within a novel genus of the family *Picornaviridae*, the genus *Hepatovirus*.[2] One other distinct species of picornavirus, avian encephalomyelitis virus, has been placed within this genus on the basis of a remarkably close phylogenetic relationship to HAV.[3]

## Overview of HAV structure and the viral life-cycle

As with other picornaviruses, the infectious HAV particle is small, non-enveloped, and approximately 27 nm in diameter.[4] It comprises a highly stable, icosahedral protein capsid containing an RNA genome consisting of a single, messenger-sense molecule approximately 7.5 kb in length.[1] The HAV genome generally resembles that of other picornaviruses in terms of its overall organization and, quite likely, the mechanisms by which it replicates (Figs 6.1 and 6.2). There is a small viral protein (VPg) linked to the 5′ terminus of the genomic RNA,[5] and a lengthy, templated poly-A tract at the 3′ end. Like the genomic RNAs of other positive-strand viruses, this positive-sense RNA acts as messenger RNA, directing the translation of viral proteins upon its release into the cytoplasm following the entry of virus particles into the hepatocyte, the major cell type infected by the virus. Viral RNA replication takes place exclusively within the cytoplasm of the infected cell. A single open reading frame (ORF) extends through much of the length of the genomic RNA and encodes a large viral polyprotein that is co-translationally processed into individual structural and non-structural proteins, in large part by a single, virally encoded proteinase. This results in the production of three to four mature structural proteins (encoded by the 5′ third of the ORF) that assemble into the capsids required for production of progeny virus, as well as at least seven non-structural proteins (encoded by the remainder of the ORF) that function to support replication of the genome. The organization of the viral genome and the proteins it encodes is depicted in Fig. 6.1.

A recent detailed review of the viral replication cycle is available elsewhere,[6] but key steps in the life-cycle of HAV include (1) the attachment of the virus to a cellular receptor and its penetration into the hepatocyte, (2) uncoating of the viral RNA with its release into the cytosol, (3) translation of the RNA genome by a process involving internal ribosome entry and resulting in the production of the viral polyprotein, (4) co- and post-translational proteolytic processing of the polyprotein into both structural and non-structural proteins, (5) assembly of an RNA replicase complex at the 3′ end of the viral RNA and initiation of viral negative-strand (intermediate) RNA synthesis, (6) non-conservative synthesis of multiple copies of positive-strand RNA molecules produced from single negative-strand RNA intermediates, which are then directed to further polyprotein translation and RNA replication cycles, or (7) packaged into immature viral particles, followed by (8) maturation of the viral particles and their transport out of the cell. These events are depicted in Fig. 6.2.

Although many of these steps in the viral life-cycle have been studied in detail with other picornaviruses, relatively few particulars are known of these events for

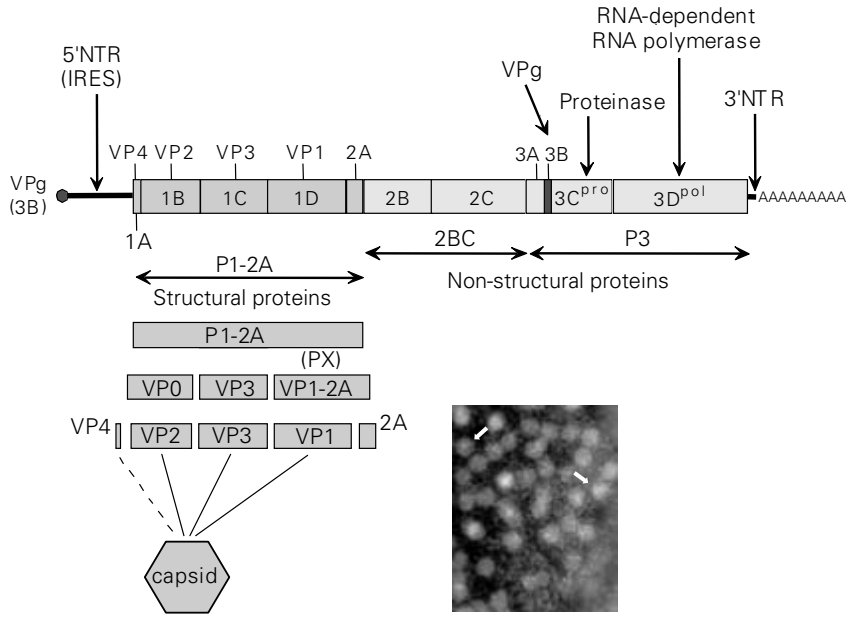


**Figure 6.1** Organization of the HAV genome and processing of the structural protein precursors. The virion RNA is 7.5 kb in length, positive-sense, and has 5′ and 3′ non-translated regions (5′NTR and 3′NTR) flanking a single large open reading frame encoding the viral polyprotein (shown as the large rectangular box). The 5′ terminal uridine residue is covalently linked to a small viral protein (3B, otherwise known as VPg). The 5′NTR contains substantial RNA secondary structures which comprise both replicase-recognition signals and the viral internal ribosomal entry site (IRES) that directs the cap-independent translation of the viral polyprotein (see also Fig. 6.3). The 3′NTR terminates in a poly(A) tract. The polyprotein is composed of three functionally distinct domains (P1–2A, 2BC and P3): each of these domains is proteolytically processed to yield smaller precursors as well as the mature viral proteins (1A, 1B, 1C, etc.). The structural proteins (shown in mid-grey), VP1 (1D), VP2 (1B), VP3 (1C), and possibly VP4 (1A), are encoded by the P1 region. 2A can also be considered a structural protein, as it functions in particle assembly and is not required for RNA replication. However, it is not a component of the mature virus particle. The non-structural proteins (shown in pale grey, except for 3B, shown in dark grey) have functions related to RNA replication, and are encoded by the 2BC and P3 regions. These proteins include an NTPase (2C) with possible helicase activity, VPg (3B) which functions in protein priming of RNA synthesis, the viral specific cysteine proteinase ($3C^{pro}$), and an RNA-dependent RNA polymerase ($3D^{pol}$). Primary proteolytic cleavage occurs between the 2A and 2B proteins and is directed by $3C^{pro}$, which also has RNA-binding activity and has functions related to RNA replication. Other processing events are also mediated by this protease, except for two cleavages within the structural protein precursors (the 1A/1B and 1D/2A cleavages) that occur during particle morphogenesis. The inset shows an electron microscopic image of 27 nm diameter virus particles with shape suggesting icosahedral symmetry (arrows).

HAV. The viral RNA replicase is an assembled structure comprising several different non-structural proteins that are associated with membranous vesicles that appear to be formed by redirection of membranes from the endoplasmic reticulum.[7,8] The association of the viral replicase with intracellular membranes is typical of positive-strand viruses, and is also observed with hepatitis C virus (HCV).[9]

## Growth of HAV in cultured cells

Unlike other human hepatitis viruses, HAV can be propagated in a variety of different types of mammalian cells, although most often cells of primate origin are used. Interestingly, and in contrast to the invariably transient nature of HAV infections in infected humans, infection of cultured cells is typically not associated with any dramatic cellular injury and commonly leads to long-term persistence of the virus. These observations are consistent with the fact that HAV replication does not interfere specifically with cellular macromolecular synthetic processes, and does not induce the shutdown of cellular protein or nucleic acid synthesis as observed with poliovirus in cultured cells.[10] Variants of HAV that are cytopathic for cultured cells have been described, however (see below).

HAV was first isolated in marmoset liver explant cultures and was subsequently propagated in continuous fetal rhesus monkey kidney cells.[11,12] However, the virus may be directly isolated from clinical materials in primary African green monkey kidney (AGMK) cells, or, with somewhat greater difficulty, in continuous cell lines including BS-C-1 cells, MRC-5, FRhK-4, PLC/PRF-5 (human hepatoma cells), and others.[13–15] HAV is also

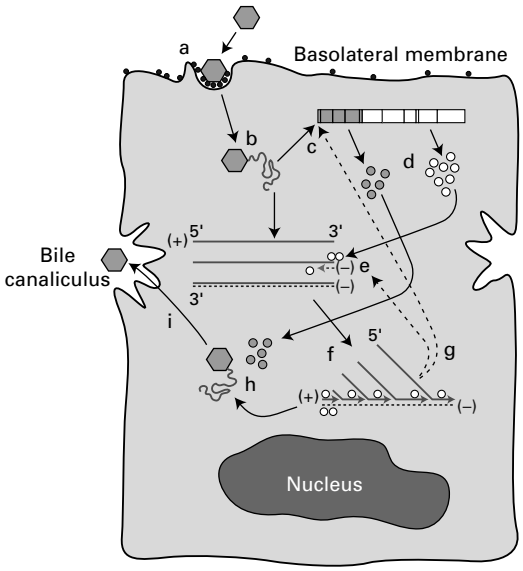


**Figure 6.2** Schematic showing major features of the replication cycle of HAV. (a) Virus attachment occurs at a specific cellular receptor on the basolateral surface of the hepatoctye, within the space of Disse, and is followed by (b) penetration and uncoating of the virus particle. Subsequent events take place exclusively within the cytoplasm of the cell, and do not disrupt cellular homeostasis. These include (c) translation of the polyprotein by a 5′ cap-independent mechanism under control of the viral IRES, (d) co-translational processing of the polyprotein by the $3C^{pro}$ proteinase into structural and non-structural proteins, (e) assembly of a membrane-bound viral replicase complex at the 3′ end of the positive-strand genomic RNA (dark grey) resulting in the synthesis of a negative-strand intermediate RNA molecule (dotted line), (f) synthesis of new positive-strand progeny RNA (dark grey), which can either (g) reinitiate the cycle by undergoing translation or replication (dashed lines) or (h) be packaged into new virus particles. Finally, (i) newly formed virus particles are transported to the apical surface of the cell and exported into the biliary canaliculus.

capable of replicating in a broader diversity of mammalian-derived cell types, including cells of guinea pig or dolphin origin.[16]

Wild-type virus usually replicates very slowly and to relatively low titres in cultured cells. Thus, there is no utility to virus isolation as a diagnostic modality. Low passage virus typically requires several days to weeks to reach maximal titres in cell cultures, and usually requires immunological or nucleic acid hybridization techniques to be detected because of the lack of virus-induced cytopathic effects. Immunohistochemistry typically shows viral antigens confined to the cytoplasm, and present in a fine, speckled pattern (Fig. 6.3). With continued *in vitro* passage, however, the virus becomes progressively adapted to growth in cell culture, replicating more rapidly and achieving higher titres.[13,17] Well-adapted variants of the virus that replicate rapidly (i.e. with a replication cycle about 24 h in length) and to higher than usual titres in cultured cells may lead to cytoplasmic vacuolar changes and cell death.[18] Cellular injury appears to arise from the induction of apoptotic pathways leading to programmed cell death.[8,19] These viruses allow the use of conventional plaque assays for viral quantitation. Interestingly, continuous passage of the virus in cell culture frequently results in a reduction in the ability of the virus to replicate and cause disease in primates.[20]

*In vitro* replication is blocked by guanidine and brefeldin A,[21–23] which are both known to inhibit the replication of other picornaviruses.

## Structure of the HAV genome

The positive-strand RNA genome is a single-stranded 35S RNA molecule, approximately 7.5 kb in length, possessing structured 5′ and 3′ termini and a 3′ poly-(A) tail (Fig. 6.1).[1,24] As with other positive-strand viruses, purified genomic RNA is itself infectious and, whether extracted from virions or produced synthetically from cloned cDNA, will produce virus when transfected into permissive cultured cells or inoculated into the liver of susceptible primates.[25,26] However, the recovery of virus from synthetic wild-type RNA is very difficult in transfected cell cultures, and usually requires primate inoculation.

The small VPg protein that is covalently linked to the 5′ end of the genomic RNA is not required for infectivity, and its presence is probably vestigial, reflecting its

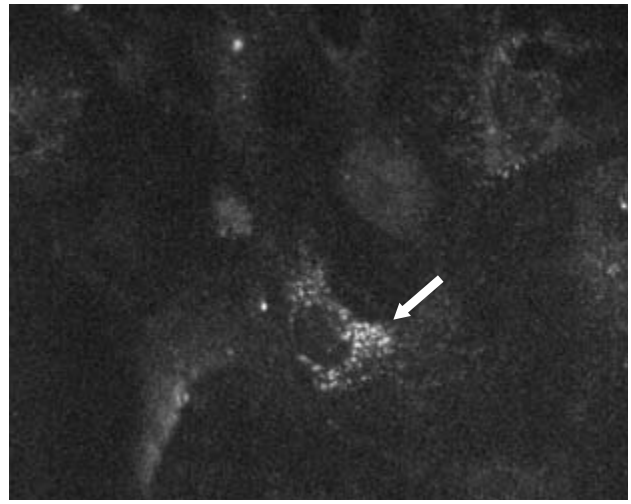

**Figure 6.3** Immunofluorescence staining of viral antigen in African green monkey kidney cells infected with a cell culture-adapted variant of HAV. Convalescent human antiserum was used as the source of antibody. Replication of the virus is evidenced by speckled cytoplasmic fluorescence within infected cells.

role as the protein primer for nucleic acid synthesis (see below).

## 5′ Non-translated RNA segment

The RNA genome contains a relatively lengthy 5′ non-translated region (5′NTR), ~730 bases long, with substantial secondary RNA structure and comprising series of several complex stem-loop structures.[27] RNA secondary structure within this segment of the genome has been mapped by a combination of phylogenetic comparative sequence analyses, functional genetic studies, and direct biophysical and nuclease mapping techniques[27] (Fig. 6.4). Structured elements within the 5′NTR have also been visualized at low resolution by transmission electron microscopy.[28]

The 5′NTR sequence contains several functional domains. These include a 5′ terminal hairpin followed by two RNA pseudoknots that are likely to function in recognition of the viral RNA by the replicase during initiation of RNA synthesis. Although apparently essential for replication,[29] it is not known whether these structures function in the positive-sense molecule, or in the negative-sense RNA intermediate (as replication signals at the 3′ end of this molecule). Both pseudoknots (Fig. 6.4) contain exclusively canonical Watson-Crick base-pairs, and are devoid of non-canonical G:U pairs that are commonly found in other structured RNAs. Thus, they may exist within either sense RNA. Surprisingly, the analogous segment of the poliovirus genome contains a cloverleaf RNA structure that has been shown to be required for initiation of negative-strand RNA synthesis,[30] and not positive-strand RNA synthesis as might be anticipated by the normal direction of RNA synthesis.

The two pseudoknots are followed by a pyrimidine-rich tract of about 44 nts (Fig. 6.4). This segment of the 5′NTR is not essential for either replication or pathogenicity, and has no recognized function.[31] It appears to assume a unique structural conformation,[32] nonetheless, and is likely to provide the virus with some selective survival advantage, given the efficient utilization of the genome that is generally evident among picornaviruses. Analogous and lengthier single-stranded, pyrimidine-rich RNA segments are found in the 5′NTR of other picornaviruses where they contribute to pathogenesis,[33] as well as within the 3′NTR of HCV, where it is essential for RNA replication.[34]

The pyrimidine-rich tract is followed by a highly structured RNA segment that functions as an 'internal ribosomal binding site' (IRES). The IRES facilitates the initiation of viral translation in a 5′ cap-independent fashion, as alluded to above, directing an interaction of the 40S ribosome with the viral RNA at a point close to the initiator AUG for viral protein translation.[35,36] Although IRES elements are found in all picornaviruses (as well as in some other virus families), the HAV IRES

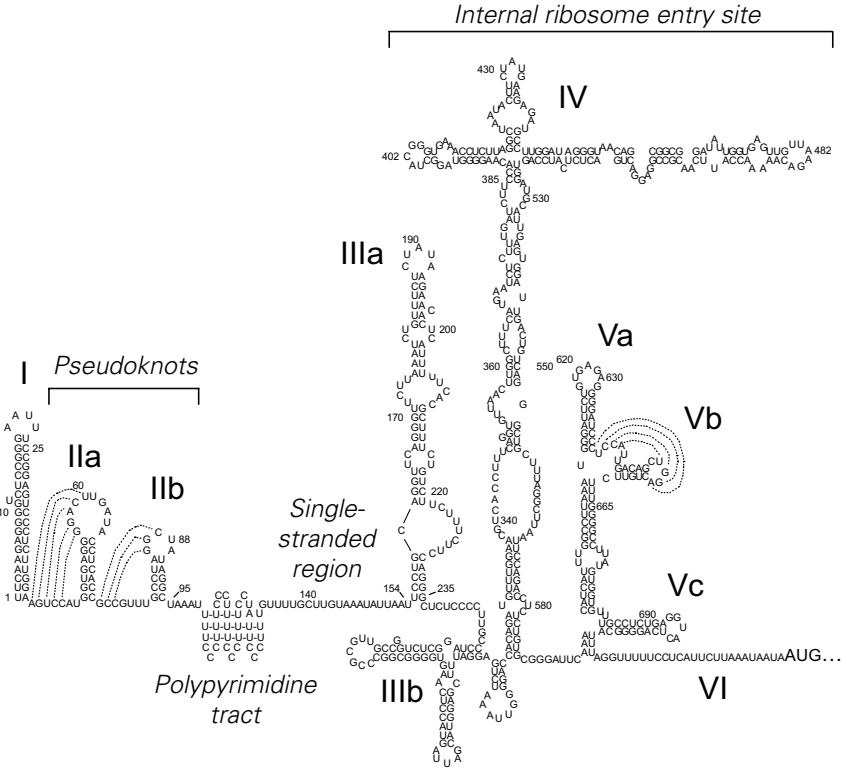


**Figure 6.4** Putative secondary structure of the 5′NTR of the HAV genome.[27,32] The 5′ terminal hairpin (domain I) and pseudoknots (domain II) are likely to function in recognition of the genome by the viral RNA replicase, while the IRES (domains III–VI) control the cap-independent translation of the viral polyprotein. The polypyrimidine tract that is present between these structural elements is not necessary either for replication or translation and is of unknown function. The initiator AUG is large font at the 3′ end of the displayed sequence.

differs from other viral IRES elements in its stringent requirement for cellular translation initation factors. Other picornaviral IRES elements require only a cleavage fragment of eukaryotic initiation factor eIF-4G, but the HAV IRES requires the intact protein for efficient translation initiation.[37,38] It also utilizes one or more cell-specific translation initiation factors,[39–41] and the affinity of the 5′NTR for these and other cellular proteins may play a role in defining cellular tropisms of the virus.[42–45] The strongest evidence relates to the cellular polypyrimidine tract-binding protein (PTB), which stimulates IRES directed translation both *in vitro* and in cultured cells.[40]

Most studies suggest that the IRES of HAV is less active than IRES elements of other picornaviruses, resulting in relatively inefficient translation of the viral message and possibly contributing to the slow growth of the virus.[37,42] The requirement for intact eIF-4G also means that HAV protein translation must compete for initiation factors in cells that are actively supporting ongoing translation of cellular mRNAs. Cellular protein translation shut-off is accomplished by several other picornaviruses (including poliovirus) through the proteolytic cleavage of eIF-4G by a viral proteinase. This does not occur in HAV-infected cells, as discussed above, and it is likely that the need to compete with cellular protein translation further compromises the efficiency with which the viral proteins are translated from the genomic RNA. From an evolutionary viewpoint, the resulting down-modulation of viral RNA replication may provide the virus with a survival advantage. It is likely to facilitate replication at relatively low levels within hepatocytes, such that the infection may fail to induce an active immune response for several weeks before the occurrence of disease, a period during which the virus may be shed and transmitted to other persons.

## Other structured RNA elements

Limited information is available concerning the 3′NTR of HAV, but it is likely to contain conserved secondary and tertiary RNA structure. Studies with other picornaviruses suggest that the 3′NTR is not absolutely required for viral replication,[46] but it nonetheless plays an important role in RNA replication by facilitating recognition of the positive-strand RNA by the viral replicase complex.[46,47]

Small stem-loop structures that function as *cis*-acting RNA replication elements (*cre*s) have been recognized within the genomes of many other picornaviral RNAs. The *cre* is likely to be a universal feature of picornaviruses, as it appears to template the addition of uridyl residues to the viral VPg molecule in a viral polymerase-driven reaction required for protein-priming of viral RNA synthesis.[48–50] HAV is likely to possess a *cre* as well, although it has yet to be specifically identified. The HAV

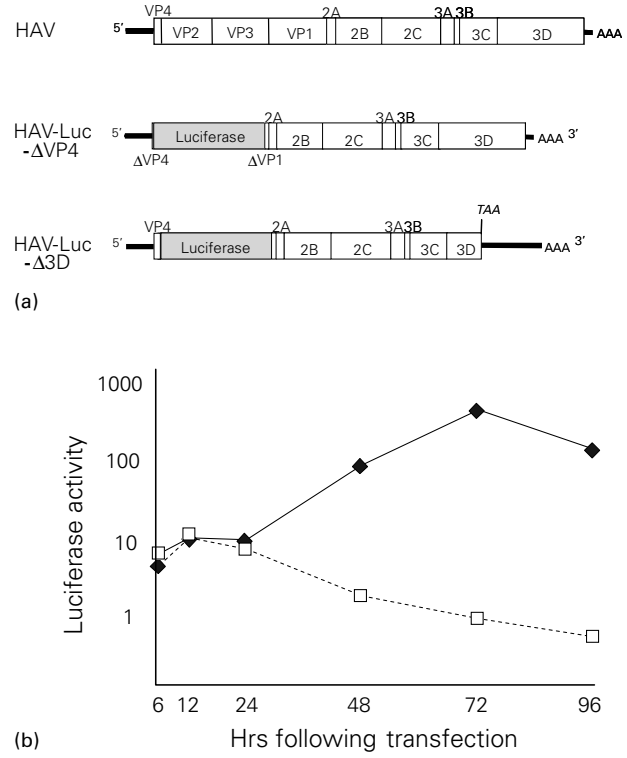


**Figure 6.5** Replication of HAV RNA is not dependent upon capsid protein coding sequence. (a) Genomic organization of HAV and the HAV-LucΔVP4 replicon encoding firefly luciferase (lightly shaded region) inserted in frame, replacing all but the 5′ 12 nts (ΔVP4) and 3′ 39 nts (ΔVP1) of the P1 segment, in an infectious cDNA clone derived from the rapidly replicating, cytopathic HM175/18f virus.[22] Although it contains sequence encoding VP4, HAV-LucΔ3D is otherwise similar to HAV-LucΔVP4 except for a single, lethal nucleotide change resulting in premature termination of translation within the $3D^{pol}$ coding sequence. (b) Luciferase activity (light units) present in lysates of Huh-7 cells transfected with HAV-LucΔVP4 (♦) or HAV-LucΔ3D (□) transcript RNA. Luciferase activity present 24 hours after transfection reflects translation of input T7 transcript RNA, while subsequent increases in luciferase activity (logarithmic scale) are produced from newly replicated subgenomic RNAs.

*cre* does not reside within the RNA segment encoding the capsid proteins, however, as this segment of the genome can be deleted without impairing the ability of the viral RNA to undergo autonomous replication in permissive cell cultures (see Fig. 6.5).[22]

## The polyprotein coding region

As indicated above, the non-coding regions flank a single large ORF encoding a polyprotein that is proteolytically processed into both structural (P1–2A) and non-structural (2BC and P3) viral proteins (Fig. 6.1). The primary cleavage of the polyprotein occurs between these two segments of the polyprotein, under the direction of the

only proteinase encoded by the virus, the $3C^{pro}$ protein.[51–53] The P1 segment encodes four polypeptides (1A–1D or, in order, VP4, VP2, VP3 and VP1) that assemble to form the viral capsid. The 2A protein of HAV lacks homology with any other picornaviral 2A proteins, and does not possess the *cis*-active proteinase activity found in the 2A proteins of other picornaviral genera. As discussed below, it remains attached to some otherwise fully formed virions and is likely to function in viral assembly, most likely as a precursor fusion with 1D (VP1).[54,55] These proteins are discussed in greater detail in the section below concerning viral capsid assembly.

The remaining non-structural proteins probably all contribute directly to the assembly of the membrane-bound viral replicase complex. Proteins 2B and 2C (possibly also the precursor 2BC) are believed to have a role in RNA replication. Mutations in these proteins appear to contribute to attenuation.[56–58] They also appear to be involved in directing the rearrangements of cellular membranes required for replicase assembly.[7] The 2B protein is very hydrophobic, and has been suggested to anchor the replicase complexes to intracellular membranes.[8] 2C, on the other hand, has NTPase activity and contains a helicase sequence motif. The P3 non-structural proteins include a putative RNA-dependent RNA polymerase ($3D^{pol}$), a cysteine proteinase ($3C^{pro}$),[59] which is responsible for most cleavages in the processing of the viral polyprotein,[60] and VPg (3B) which is attached to the 5′ end of both positive- and negative-strand RNAs (see Fig. 6.1).

### Genetic diversity

The genetic diversity of HAV has been investigated by determining the partial genomic nucleotide sequences of large numbers of human HAV strains recovered from diverse sources.[61–63] These studies have shown that circulating human strains of HAV are relatively closely related genetically, especially when compared to the genetic diversity evident among other picornaviruses such as poliovirus. Two major human HAV 'genotypes' (genotypes I and III) have been documented, the sequences of which differ from each other at >15% of the bases studied near the VP1–2A junction. Other human minor genotypes have also been identified. As discussed below, these viruses all belong to a common serotype.

## The HAV virion

Current understanding of the physical structure of the HAV particle is based largely on analogy with other picornaviruses. There are no available high-resolution structural models such as those that exist for other picornaviral genera.[64,65] However, recent yet unpublished cryoelectron microscopy studies suggest that HAV has a structure resembling those of other members of this viral family, with the exception that depressions on the surface of the particle are much less evident than with other picornaviruses (Cheng, H; personal communication).[66]

### Biophysical characteristics

Approximating the physical characteristics of enteroviruses such as poliovirus, the majority of infectious HAV particles sediment at 156S and band at a density of 1.325 $g/cm^3$ in caesium chloride.[67] Also in common with the enteroviruses, hepatitis A is stable at low pH (less than pH 3.0).[68] However, the thermal stability of HAV is considerably greater than that of other picornaviruses.[18,69,70] This characteristic of the virus is likely to contribute significantly to its ability to be spread through the environment, and to cause common source outbreaks of hepatitis.

Incubation of the virus for 4 weeks at room temperature results in only a 100-fold decrease in infectivity. Over shorter periods of time (10 min), significant loss of infectivity does not occur until 60 ºC.[18] The virus capsid is significantly stabilized in the presence of 1 M $Mg^{2+}$, resulting in only a 100-fold decrease in infectivity upon heating to 80 ºC for 10 minutes.[69] However, infectivity is destroyed almost instantaneously by heating above 90 ºC. HAV is highly resistant to drying, and infectious virus has been recovered from acetone-fixed cell sheets. It is also highly resistant to detergents, surviving a 1% concentration of sodium dodecyl sulfate. Solvent-detergent inactivation procedures do not reduce the infectivity of HAV, explaining why hepatitis A transmission has occasionally been associated with the administration of high purity clotting factor concentrates.[71,72]

### Protein composition

Biochemical and genetic studies indicate that the highly stable capsid of HAV is composed of multiple copies of the three major structural proteins, VP1, VP2 and VP3. These designations are based on those of their homologues in the poliovirus capsid, named originally on the basis of relative molecular mass with VP1 being largest. An alternative, perhaps preferable, but less often utilized nomenclature, is based on the position of each within the amino terminal (P1) segment of the viral polyprotein: 1A (VP4), 1B (VP2), 1C (VP3) and 1D (VP1) (see Fig. 6.1).[73] The major proteins are approximately 273, 222 and 246 amino acids in length, respectively.[1,74] VP1, however, has a heterogeneous carboxy terminus,[75] reflecting a unique maturation mechanism (see below). Like other picornaviruses, the viral capsid probably contains 60 copies of each of these major proteins.

In addition to VP1, VP2 and VP3, the existence of a fourth structural protein, VP4 (1A), is predicted by the

genomic RNA sequence. This small polypeptide has a predicted length of either 21 or 23 amino acids depending on which of two possible AUG codons at the 5′ end of the OFR are utilized to initiate translation of the polyprotein.[76] The VP4 polypeptide is incorporated into nascent particles in the form of a VP2 precursor, VP0 (1AB). This precursor protein undergoes a maturation cleavage during the final assembly of the viral particle, leading to production of the mature VP2 and VP4 polypeptides. However, the resulting VP4 product of this cleavage has never been demonstrated directly in preparations of HAV particles,[24] and it is not clear that it remains associated with mature virions, as is the case with poliovirus and other well studied picornaviruses.

### Antigenic structure

Early comparisons of the complete nucleotide sequences of different strains of HAV demonstrated approximately 97% amino acid conservation in the sequences of the major viral capsid proteins.[1,77-80] While only limited information is available concerning the presence of antigenic differences among different strains of HAV, studies with human (HM175 strain) and owl monkey-derived (PA21) isolates of HAV demonstrated that these viruses, representing divergent viral genotypes, elicit antibodies with substantial cross-neutralizing activity and thus belong to a single serotype.[81,82] This appears to be the case for all strains of HAV. Some monoclonal antibodies are capable of distinguishing unique epitopes that are variably present in strains of HAV isolated from humans versus strains isolated from naturally infected cynomolgus and African green monkeys.[83,84] Nonetheless, even simian and human strains of HAV demonstrate substantial antigenic cross-reactivity.

The critical neutralization epitopes of HAV are assembled, conformationally derived structures rather than linear epitopes. Thus, neutralizing murine monoclonal antibodies do not recognize denatured capsid proteins that have been separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels. Furthermore, antisera raised to purified capsid proteins or proteins expressed from recombinant DNA show only weak reactivity with native capsids and have very limited virus neutralization activity.[85] This explains why it has proven difficult to develop a vaccine based on recombinant DNA technology. Although individual capsid proteins expressed from recombinant cDNAs can assemble into antigenic particles *in vitro*, this approach has not achieved commercial success for vaccine production.

Antigenic variants of HAV have been selected in cell culture by continued passage of cell culture-adapted virus in the presence of neutralizing murine monoclonal antibodies. Sequencing of the genomic regions encoding the capsid proteins of these mutants indicated the presence of an array of closely spaced epitopes in polypeptide loops within VP3 and VP1, comprising an immunodominant, conformationally defined neutralization site on the capsid surface.[86,87] These studies suggest that the involved residues of VP1 and VP3 are more closely positioned on the surface of the HAV particle than the analogous residues within the poliovirus capsid,[64,88] but this awaits confirmation by an atomic-level resolution structural model.

Several HAV strains recovered from naturally infected non-human primates demonstrate subtle antigenic differences from human viruses, as discussed above.[83,84] They appear to constitute a biologically distinct group of viruses that are capable of causing liver disease in infected cynomolgus and African green monkeys, but not chimpanzees which, like New World owl monkeys and certain tamarin species, develop acute hepatitis following challenge with human strains of HAV.[89] Conversely, human strains of HAV generally do not cause disease in cynomolgus or African green monkeys. These differences in virus host range could be due, at least in part, to differences in the cellular receptors utilized by these viruses.[82,90]

## Molecular processes in viral replication

### Attachment and entry

Mature virus particles attach to cultured cells and undergo uncoating with release of viral RNA into the cytoplasm by about 4 hours post-infection.[91] However, few other details of cellular attachment, penetration and uncoating of HAV are known. A candidate cellular receptor (huHAVcr-1) has been identified and shown to be a mucin-like glycoprotein.[92,93] This putative receptor protein interacts with the virus particle leading to virus neutralization.[94] It is widely distributed in different tissues but its exact role in the attachment of virus to hepatocytes in humans is uncertain.

### Polyprotein translation and processing

The positive-sense virion RNA that is released by uncoating of the virion acts directly as messenger for the synthesis of the large polyprotein. This probably follows removal of the small virally encoded peptide (VPg) which is covalently linked to its 5′ end.[5] As indicated above, protein translation is initiated in a 5′ cap-independent fashion under the control of the IRES (Fig. 6.4).

The first processing event within the resulting polyprotein is likely to occur in a co-translational manner, is carried out by the virus-specified $3C^{pro}$ proteinase[59] and results in cleavage at the junction of the 2A and 2B proteins.[51–53,95] This results in the release of a large structural protein precursor P1–2A, which is further processed by

$3C^{pro}$ into three capsid protein precursors: VP0 (or 1AB, the precursor for VP2), VP3, and VP1–2A, the precursor for VP1. The non-structural 2BC and P3 polypeptides are co- and post-translationally processed by $3C^{pro}$ to generate all non-structural proteins (Fig. 6.1).[96–98]

## Replicase assembly and RNA replication

The polypeptides located downstream of 2A in the polyprotein play essential, although in many cases poorly defined, roles in RNA replication. Experience with other picornaviruses suggests that many if not all of these proteins may have multiple functions in viral replication. For example, in addition to its role as the viral proteinase responsible for proteolytic processing of the polyprotein, the 3C sequence is likely to play an important role in replicase assembly. In addition, viral precursor polypeptides may have unique functions as partially uncleaved processing intermediates (such as 3ABC) that differ significantly from their functions as fully cleaved, mature proteins.[6,99]

The $3D^{pol}$ RNA-dependent, RNA polymerase of HAV serves as the catalytic core of a large, membrane-bound macromolecular complex that acts as an RNA replicase.[8] The 3A protein is likely to play a role in anchoring this replicase complex to cellular membranes via a hydrophobic sequence located near its amino terminus.[100] Cellular proteins that were shown to bind to 5′ and 3′ NTRs may also play important roles in viral RNA translation and/or replication. Poly-rC binding protein 2 (PCBP2), which interacts with the 3CD protein and 5′NTR of poliovirus, also interacts with the HAV 5′NTR.[41] Surprisingly, glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) has been shown to bind specifically to both the HAV 5′ and 3′NTR sequences, and in doing so may influence translation and/or replication of the viral RNA.[45,101,102] In addition, polypyrimidine-tract binding protein (PTB) and poly-A binding protein (PABP) appear to contribute to the efficiency of picornaviral translation and thus could also contribute in a significant manner to viral replication.

Within the replicase complex, the virion RNA serves as a template for negative-strand RNA synthesis (Fig. 6.2). This negative-strand intermediate then acts in turn as a template for synthesis of new positive-strand RNA molecules, that can either be translated into protein (reinitiating the replication cycle) or packaged into new virions. Studies with poliovirus suggest that 3B (VPg), the small genome-linked protein, is likely to function as a protein primer for viral RNA synthesis in these reactions, as it does in the case of poliovirus.[48] It first must undergo a uridylylation reaction that covalently adds two uridyl residues to VPg in a reaction catalyzed by the viral polymerase and templated by the *cre* stem-loop,[49] a putative RNA structure yet to be identified in HAV (see above). These uridine residues become the first two nucleotides of each nascent RNA strand, and VPg remains covalently coupled through them to the 5′ ends of both strands of RNA. However, although it is clear that the HAV genome is coupled to the 3B protein (VPg) as is the case with poliovirus,[5] there is some evidence that suggests that the priming of RNA synthesis may differ from events occurring during poliovirus replication. In contrast to poliovirus, the infectivity of synthetic HAV RNA is dependent upon the presence of the first two uridine residues in the genome.[103]

RNA amplification is non-conservative and asymmetric, with more of the positive-sense RNA being produced in infected cells than the negative-strand intermediate. The physical nature of the negative-strand RNA during the process of positive-strand RNA synthesis represents an ongoing controversy in the replication of positive-strand viruses. It seems likely that the template for new positive-strand RNA synthesis is actually a duplex molecule, as available evidence suggests that negative-strand RNAs do not exist in a single-stranded conformation and are not physically separated from the positive-strand template following their synthesis. Not much is known about the molecular determinants of RNA replication that probably reside within the 5′ and 3′NTRs of the genome.[104,105] The poly(A) tail following the 3′NTR appears to be elongated during RNA replication in cells transfected with RNA transcripts, indicating that its length may be important for genome replication.[106]

## Particle assembly

Substantial evidence suggests that the mechanisms of HAV particle assembly differ significantly from those operative with other, well-studied picornaviruses. Deletions in the amino-terminal part of 2A have been shown to abolish infectivity.[107] This appears to be due to a requirement for this domain of 2A to be present as a carboxy-terminal extension of P1 in order to facilitate the folding of the capsid protein precursor required for efficient $3C^{pro}$-mediated processing. These cleavages are followed by the assembly of pentamers containing five copies each of VP0, VP3 and VP1–2A. These pentamers subsequently assemble into complete capsids, after which cleavage at the VP1–2A junction releases the mature VP1 protein. This cleavage appears to be mediated by a cellular rather than a viral proteinase, resulting in a heterogeneous VP1 carboxy terminus.[74,75] The amino-terminal 2A extension thus appears to play a critical role in the assembly of the capsid, a feature that is unique to HAV among the picornaviruses.[54,107] Deletions within the carboxy-terminal half of the 2A protein have no appreciable effect on the kinetics of RNA replication, but do alter the VP1/2A cleavage, resulting in accumulation of

uncleaved VP1–2A precursor in virions and appearing to cause a delay in the appearance of infectious particles, as well as a fourfold decrease in specific infectivity of the virus particles.[107,108] A subpopulation of otherwise mature virions contains the VP1–2A precursor, which was referred to originally as the 'pX' protein.[109]

The VP4 proteins of other picornaviruses, such as poliovirus, are considerably larger than the VP4 sequence encoded within the HAV genome. They also are myristylated at their amino terminus, a feature relating to an essential role that they play in the capsid assembly process during replication of these viruses.[110] In contrast, available data suggest that the VP4 molecule of HAV is not myristylated, despite the presence of an internal consensus myristylation site.[76,111] This is surprising, but both the apparent absence of a myristylated VP4 and the functional role of the 2A extension on the VP1 molecule are very unusual features that set HAV apart from other human picornaviruses. They suggest that the structural organization of the HAV particle and its assembly process may be very different from what is known of better studied picornaviruses.

Only the positive-strand RNA is packaged into new virions. Newly assembled HAV particles appear to be transported to the apical plasma membrane of the hepatocytes, where they are released into the hepatic canaliculi, resulting ultimately in the secretion of virus into the biliary system.[23] VP0 (1AB, Fig. 6.1) is cleaved into its two constituent polypeptides following the assembly of the virus particle and packaging of the viral RNA. It occurs by an as yet undetermined mechanism. This is called the 'maturation cleavage', and probably results in increased stability of the virus particle. There is some evidence that this cleavage occurs more slowly with HAV than with other picornaviruses.[112] It has been suggested that this slow maturation of the particle may prevent reinfection of gastrointestinal tract epithelial cells during excretion of the virus. Cells lining the crypts of the small intestine are presumably the very first cells to be infected by the virus following an oral exposure.[113]

## Regulation of viral replication

Replication of the virus is probably regulated at several key steps, including a switch from translation to replication that converts genomic RNA from the support of protein synthesis to the templating of negative-strand RNA synthesis, and a later switch that diverts newly synthesized RNAs from protein production and RNA replication to packaging into progeny viral particles (Fig. 6.2). Although linear in its organization (Fig. 6.1), the genomic RNA of HAV may well assume a circular conformation during active replication. This is supported by studies of other positive-strand RNA viruses,[114] as well as cellular RNAs. Circularization could be achieved by the interaction of cellular and viral proteins with the terminal sequences of the viral RNA, as described above, leading in effect to a protein bridge. Such a conformation would provide unique opportunities for controlling protein synthesis by facilitating re-entry of ribosomes after completion of a cycle of translation, or for controlling the switch from translation to replication.

## Molecular basis of adaptation of HAV replication to cultured cells

The progressive increase in the replication capacity of the virus that occurs upon continued cell culture passage appears to be due to changes in the 2BC sequence and within the viral IRES element.[42,43,56,57,115–117] The molecular basis for these changes remain unknown, but presumably they reflect differences in the cellular proteins available to support replication in cultured cells compared with cells *in vivo*. Mutations within 2BC appear to be particularly important for efficient replication of the virus in cultured cells.[56,57] These mutations directly enhance the efficiency of RNA replication, and not other steps in the virus life-cycle.[22] Mutations within the IRES sequence in the 5′NTR enhance its translational activity in cultured cells, and cause more subtle changes that are cell type-specific.[42,43,118]

Highly cell culture-adapted viruses are often attenuated (or no longer even infectious) when used to challenge susceptible primates and have been studied as candidate attenuated vaccines.[119–121] The attenuation properties of these viruses are closely related to the mutations conferring cell culture adaptation, and have been mapped recently to the VP1–2A and 2C proteins.[58]

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# Chapter 7
# Epidemiology

Mike G Catton, Stephen A Locarnini

The epidemiology of hepatitis A virus (HAV) may be understood in terms of the natural history of the virus and its physical properties. HAV consists of an unenveloped, 27-nm, spherical particle (Fig. 7.1), containing a linear, single-stranded, positive-sense RNA genome, and is classified in the family Picornaviridae genus Hepatovirus.[1] The virus is more robust than other picornaviruses and may remain infectious on surfaces, in the environment and in uncooked foods for significant periods.[2–4]

Infectious HAV has been shown to persist in a variety of experimentally contaminated waters and soils and in sediment after 3 months at 25 °C, when dried onto inert surfaces after 30 days at 25 °C, and in experimentally contaminated cookies after 30 days at 21 °C.[4] Significant quantities of HAV persisted in a faecal suspension on the hands of volunteers after 4 hours, and high levels of infectious HAV were transferred from fingers to fomites over the same period.[5] HAV is relatively heat-stable but generally inactivated by cooking temperatures.[6–8] Significant infectivity of purified virions remained after heating at 60 °C for 60 minutes,[7] and may persist after as long as 12 hours at 60 °C.[6] Infectivity of HAV diluted in phosphate-buffered saline (PBS) was lost after heating at 70 °C for 4 minutes, 80 °C for 5 seconds, and immediately at 85 °C.[8] HAV has also been shown to be relatively resistant to many commonly available hard-surface disinfectants.[9]

Acutely infected human beings are the only significant natural reservoir of HAV, although a number of other primate species may be infected.[10] HAV is secreted from the liver into the bile and is shed in high titres in the faeces.[11] No state of chronic infection with HAV has been identified, and immunity after infection is life-long. Transmission is primarily by the faecal-oral route,[12] most frequently person-to-person, or via contamination of uncooked foods or water. Standards of human hygiene and sanitation, therefore, have an important role in mediating transmission of HAV and are reflected in the variation in hepatitis A epidemiology observed in different populations, and in the changing epidemiology of hepatitis A in many populations over recent decades.

Children experience subclinical infection with HAV much more frequently than adults and may excrete virus for prolonged periods.[13,14] Together with children's generally lower standards of hygiene compared with adults, these factors give children a prominent role in the epidemiology of HAV. For instance, transmission associated with childcare centres is a particularly important feature of the epidemiology of hepatitis A in developed countries such as the United States[15–18] (reviewed by Hadler and McFarland[19]). In many parts of the developing world, asymptomatic seroconversion to hepatitis A in childhood is widespread,[20] and as a consequence morbidity due to hepatitis A in these populations is very low.

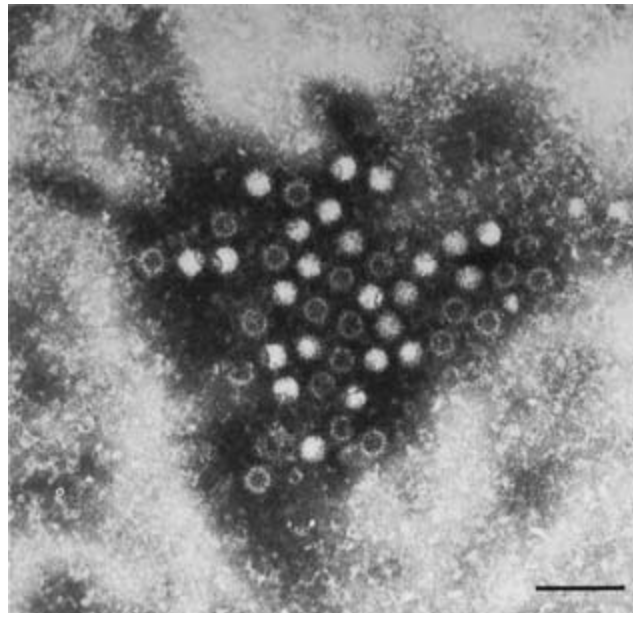

**Figure 7.1** Hepatitis A virus. Bar represents 100 nm. (Electron micrograph provided by Dr John Marshall, Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia.)

A transient viraemia of moderate titre is associated with acute HAV infection,[11] and blood-borne transmission of hepatitis A has been described, both experimentally[12] and in isolated instances after blood transfusion.[21] Recent outbreaks of apparently parenterally transmitted hepatitis A among haemophiliacs[22–26] (reviewed by Vermylen and Peerlinck[27]) have increased awareness of the potential for transmission of HAV by this route.

Normal immunoglobulin was first reported to be effective in the pre-exposure and post-exposure prophylaxis of hepatitis A in 1945.[28,29] Administered within 2 weeks of exposure to hepatitis A, immunoglobulin is 80–90% effective in preventing disease and continues to be the mainstay of post-exposure prevention of hepatitis A[30] (reviewed by Winokur and Stapleton[31]). Immunoglobulin (Ig) has also provided effective pre-exposure prophylaxis of hepatitis A among visitors to endemic areas. More recently, inactivated hepatitis A vaccines have been shown to be safe and effective[32,33] and are replacing immunoglobulin for all but the most short-term pre-exposure prophylaxis among groups at risk of infection with HAV.

## Transmission

### Faecal-oral and person-to-person transmission

Direct person-to-person spread by the faecal-oral route is the most important means of transmission of hepatitis A. High titres of virus are present in the faeces from late in the incubation period, when the patient is maximally infectious, until the first week of clinical illness. Duration of faecal shedding is relatively well characterized, with data available from early studies of human transmission, animal model and laboratory studies[12,34–36] (reviewed by Gust and Feinstone[37]).

During studies at Willowbrook State School,[12] infectivity for humans was demonstrated in pooled faeces obtained from patients with infectious hepatitis 14–21 days before jaundice and was retained until 1–8 days after the onset of jaundice. Faecal samples collected during convalescence have been shown consistently not to be infectious for humans. [38,39] Epidemiological investigations of HAV outbreaks have identified the period of approximately 1 week before onset of clinical symptoms as the period of maximal infectivity.[40,41] Pooling of specimens and the relatively large inocula used in human transmission studies probably explain the slightly greater duration of infectivity observed in these studies than that implied by epidemiological analysis of hepatitis A outbreaks.[42]

The natural history of hepatitis A has been studied extensively in non-human primate models including chimpanzees (*Pan troglodites*), tamarins (*Saguinus mystax*) and owl monkeys (*Aotus trivirgatus*). In experimentally infected tamarins, HAV particles or hepatitis A antigen (HAAg) appears in the faeces several days to a week before the onset of biochemical hepatitis, peaking at about the time of histological evidence of liver damage and quickly declining over several days.[43] More prolonged shedding is evident when viral RNA is measured. HAV RNA reappeared in the faeces of tamarins experimentally infected with the MS-1 strain of HAV on day 4 after inoculation, peaking on day 15, and declining after day 25, roughly coincident with the alanine aminotransferase (ALT) peak, to become undetectable by day 34.[44] In the same faecal samples, HAV antigen was detected between day 7 and 22 after inoculation. The duration of faecal shedding of HAV RNA in this study is consistent with data obtained by virus isolation from experimentally infected owl monkeys using the HM-175 strain[34] and the HM-175/S18 variant.[45] HAV reappeared in stools 4–7 days after inoculation, reached peak concentrations on day 17–26, and declined to undetectable levels by day 39.[34]

Using immune electron microscopy (IEM), HAV-like particles have been shown to be present in human faeces from at least 5 days before the onset of biochemical hepatitis[46,47] until the second week of illness.[35,48] Duration of faecal shedding of HAV was positively related to the presence of icteric hepatitis.[46,47] HAV particles were not detected by IEM or radioimmunoassay (RIA) >14 days after onset of dark urine in 200 consecutive patients with hepatitis A;[34] 45% of faecal specimens collected in the first week after onset of dark urine contained HAV compared with 11% collected in the second week. The estimated concentration of viral particles in faeces was shown to peak within 5 days of onset of dark urine and decline rapidly thereafter.[35] At the peak of shedding, faeces may contain $>10^8$ infectious particles/mL.[11]

The use of nucleic acid hybridization has demonstrated the presence of HAV RNA in the faeces of some patients infected with hepatitis A into the convalescent phase of illness.[14] More recently, polymerase chain reaction (PCR) has been used to demonstrate HAV RNA in faeces of several patients >1 month after clinical onset.[49–51] Few direct data are available regarding the potential infectivity of these patients. However, the weight of human transmission data and epidemiological data from outbreaks suggest that prolonged infectivity in the faeces is unlikely to be common. One study has recently demonstrated intravenous infectivity for two tamarin monkeys of stools collected from a hepatitis A patient 27 days after the ALT peak, and positive for HAV antigen and HAV nucleic acid.[50] Nucleic acid-positive, EIA-negative stools collected days later from the same patient were not infectious for tamarins. This study used virus immunocapture followed by PCR targeting the VPI amino terminus and detection

by gel electrophoresis or nucleic acid hybridization, together with an EIA employing polyclonal animal sera to examine specimens from an institutional hepatitis A outbreak. HAV was detected in the faeces of seven hepatitis A patients and 11 of their contacts who developed hepatitis A despite immunoglobulin prophylaxis. Four of the 11 infected contacts shed HAV nucleic acid for prolonged periods ranging from 25 to 52 days after peak ALT. The patient described above was the only one of these in whom convalescent shedding of HAV antigen was demonstrated, probably reflecting high titre shedding. Convalescent shedding of HAV could not be demonstrated among the seven patients who did not receive IgG prophylaxis. As the intravenous route has recently been shown to be orders of magnitude more infectious for primates than oral inoculation of HAV,[52] further study is still warranted to better define the potential for faecal-oral transmission from patients shedding HAV nucleic acid in the faeces during convalescence.

Shedding of virus in the faeces may also recur during uncommon clinical relapses of acute hepatitis A,[53] but chronic faecal shedding of HAV has not been described. The apparent disappearance of hepatitis A from isolated populations in Greenland and Alaska for long periods, as evidenced by studies of age-specific seroprevalence,[4] and high clinical attack rates during hepatitis A outbreaks among those not yet born at the time of previous outbreaks,[54,55] support the idea that chronic shedding of HAV occurs rarely if at all.

HAAg and HAV RNA may persist in the faeces of infants and young children for longer periods than in adults.[13,14] During the second week of illness, 46% of children had HAAg detected in faeces compared with 14% of adults.[14] During an outbreak of hepatitis A in a neonatal intensive care unit, three of five preterm infants for whom serial faecal specimens were obtained were shown to excrete HAAg for 1–4 months, and HAV RNA for between 4 and 7 months. One of the infants was implicated in transmission of hepatitis A to a staff member 5 months after the initial diagnosis of hepatitis A and coincident with a faecal specimen shown to contain HAV RNA but not HAAg.[13]

Twenty-two per cent of hepatitis A cases reported to the Viral Hepatitis Surveillance Program, Centers for Disease Control and Prevention in 1993 gave a history of personal contact with a case of hepatitis A, which was the most commonly identified risk factor[56] (Fig. 7.2). Other commonly identified risk factors were association with a day-care centre (15.5%), foreign travel (6.3%), homosexual activity (4.9%), injecting drug use (2.4%) and linkage with an outbreak (2.2%).[56] Transmission is usually limited to close contacts of cases,[18] especially to other family members. Attack rates among susceptible household contacts of cases vary between approximately 20% and 80%.[57–59] In one recent small study, 52% of susceptible household contacts were found to be hepatitis A-specific IgM-positive during follow-up. Their relationships to the index case were as follows: mother 40%, wife 57%, brother 60%, son 71% and daughter 80%. None of two susceptible fathers, one husband, two sisters, or three other friends or family acquired infection.[58] In contrast, similar attack rates were observed among susceptible siblings and parents of 96 children hospitalized in Athens with symptomatic hepatitis A, ranging from 20% among fathers to 30.8% among mothers.[60]

Outbreaks of hepatitis A attributable to direct person-to-person spread occur frequently in settings characterized by faecal contamination, close contact, and compromise of hygienic standards such as day-care centres[18,61] (reviewed by Hadler and McFar-

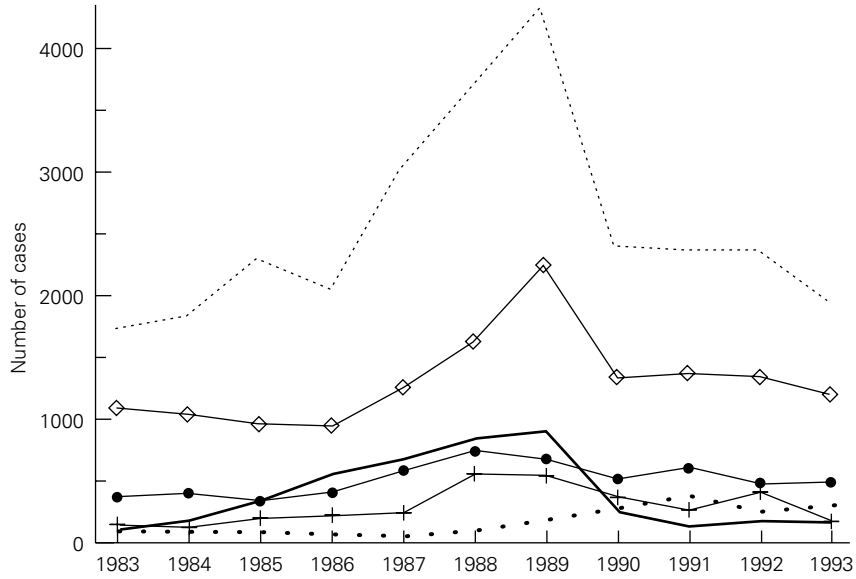


**Figure 7.2** Trends in selected risk factors associated with reported cases of hepatitis A by mutually exclusive groups, United States, 1983–1993. [black line] drug use; [dotted line], homosexual activity; [◊], day care; [dashed line] contact with HAV; [+], outbreak; [●] foreign travel. (From CDC, Viral Hepatitis Surveillance Program, 1993.[56])

land[19]), schools,[62] institutions for the mentally handicapped,[63–65] prisons,[66,67] and among military forces in the field.[68] Patients of institutions for the intellectually handicapped have a higher prevalence of anti-HAV than age-matched controls in the general community,[63,69,70] and prevalence of anti-HAV has been correlated with duration of institutionalization rather than with age.[69,70] Many outbreaks of infectious hepatitis have been reported in this setting,[40,64,65] and high rates of hepatitis have been reported among staff of these institutions.[65]

Young children have a prominent role in spread of HAV because of their relatively low hygiene standards and the frequency of subclinical infection in this age group.[17,19,59,71,72] Hepatitis A infection was asymptomatic in 84% of children 1 and 2 years of age, 50% of children 3 and 4 years of age, and 20% of children over 5 years of age in a large community-wide study of hepatitis A in day-care centres.[18] The importance of day-care centres in transmission of hepatitis A began to be appreciated in the 1970s.[61] Between 1980 and 1981 in the United States, 14% of all hepatitis A cases were found to be day-care-related in counties with large numbers of centres caring for very young children, compared with 1.5% in counties with fewer centres of this type.[15] A large 2-year study established that outbreaks were most associated with large centres operated for profit, centres open for >15 hours per day, and centres with more than 50 children in diapers.[17] Introduction of hepatitis A into the centre was related to the number of children attending the centre at a given time and the hours of operation, whereas the risk of disease spread was related to the presence of diapered children. Like other hepatitis A outbreaks in which children play a central role, those associated with day-care centres are often prolonged and not recognized until several adults have become ill. Parents were found to be most frequently represented among the clinically ill (70–80%), with caregivers (15%) next most often.[73]

Transmission to health-care professionals exposed to hospitalized hepatitis A cases has been relatively infrequent,[74,75] probably because of the rapid decline in faecal shedding after the onset of symptoms. In a prospective study of 248 patients with viral hepatitis hospitalized in Athens, no evidence of nosocomial transmission was obtained despite a lack of strict infection control procedures.[75] Cases of nosocomial hepatitis A transmission are well described, however,[76–80] usually in the context of either a patient incubating hepatitis A and hospitalized for other reasons, or of subclinical infection in a faecally incontinent patient. Several outbreaks in neonatal intensive care units have been characterized by largely subclinical and potentially unnoticed infection of infants brought to light by high rates of clinical disease among their adult contacts.[13,81,82]

Homosexual men have an increased prevalence of HAV-specific antibody and a relatively high incidence of new infection.[83] Many outbreaks of hepatitis A infection among homosexual men have been described since the early 1980s in developed countries,[84–86] including simultaneous outbreaks in 1991 in North America,[87] England,[88] the Netherlands[89] and Australia.[90] These may relate to increases in frequency of sexual activities considered at low risk of human immunodeficiency virus transmission but facilitating spread of other pathogens such as HAV. For example, oral-anal contact,[83,91] digital-rectal contact,[91] and possibly oral-genital sex and handling of condoms after anal intercourse[90] have been identified as risk factors for acquisition of HAV. However, it has not always been possible to link specific high-risk sexual practices to the majority of hepatitis A cases during outbreaks among homosexual men.[84] It is possible that risks for HAV transmission in this setting vary. Further study is warranted.

## Food-borne and waterborne transmission

Transmission of hepatitis A via faecally contaminated food or water is well described and may be associated with outbreaks of disease. Such outbreaks are frequently dramatic because of the explosive nature of spread but currently account for only 2.2% of reported hepatitis A cases in the United States.[56] Food-borne and waterborne transmissions continue to be important routes of transmission in developing countries and are particularly relevant to the susceptible traveller.

Food may be contaminated with HAV during preparation by an infected food handler or before handling, as is typically the case with shellfish. A great many outbreaks of hepatitis A have been reported in association with consumption of raw or partially cooked bivalve molluscs, such as mussels, oysters and clams.[92–95] The availability of specific serology allowed confirmation of the apparent epidemiological association between shellfish ingestion and hepatitis A following a well-characterized family outbreak of hepatitis A as a result of ingestion of contaminated mussels in Victoria, Australia.[93] A dramatic example of such a shellfish-associated HAV outbreak occurred in Shanghai in 1988.[95,96] More than 300 000 cases of hepatitis A were reported and epidemiological studies implicated consumption of raw clams in 90% of cases. HAV and HAV RNA were subsequently detected in clams.[96]

Bivalve molluscs are frequently eaten raw, or after steaming, which suffices to open the shell but does not produce internal temperatures sufficient to inactivate HAV.[97] Bivalves feed by filtering nutrients from large volumes of sea water. HAV present in faecally contaminated water is likewise filtered, and although no replication takes place within the mollusc, HAV may

be concentrated 100-fold relative to the surrounding water and persist for up to 7 days.[98] Shellfish may be grown commercially in shallow waters adjacent to populated areas with potential for contamination by sewage. Shellfish harvested from sewage-polluted waters may be purified for consumption by depuration, which relies on natural elimination of pathogens during a holding period of 2–3 days. However, >10% of input HAV HM175 has been shown to persist in Eastern oysters maintained for 5 days at 12–24 °C in clean flowing sea water.[4]

Food handlers acutely infected with hepatitis A may contaminate food during preparation if basic handwashing practices are not followed. Transmission usually occurs in the late incubation period when the person is asymptomatic and faecal shedding of virus is at its peak. A variety of foodstuffs and beverages have been implicated in transmission of hepatitis A in this manner, particularly sandwiches[87] and salad vegetables,[99] but also bread,[100] pastries,[101] fruit,[102] orange juice[103] and others. Usually the implicated foods are uncooked or have been handled after cooking, as hepatitis A is generally inactivated by normal cooking temperatures. Outbreaks may be sizable and explosive. In 1974, 133 cases of hepatitis A occurred in a single wave of illness among naval recruits in San Diego exposed to salad and grapefruit prepared by an infected food handler,[104] and in 1991, 230 cases of hepatitis A occurred among persons eating at two sandwich shops in Milwaukee staffed by the same infected food handler.[87]

Waterborne transmission is probably relatively important in maintaining endemicity of HAV in the developing world, where faecal contamination of drinking water is widespread. However, accurate data are limited, particularly in view of the potentially confounding effect of hepatitis E. In developed countries contamination of water occurs infrequently, and this route of transmission, although described,[105–107] is of minor importance. However, the large pool of susceptibles in developed countries means that waterborne transmission, when it occurs, has the potential to cause high attack rates.[108]

Contaminated water is probably the most important source of hepatitis A infection among travellers to developing countries, as a consequence of faecal contamination of drinking water, ice and water-based drinks, through swimming in polluted waters, and following consumption of foods prepared with contaminated water such as fruit and vegetables, reconstituted milk products, or shellfish originating from polluted waters.[109] Person-to-person spread, especially from young children, is another likely important source of hepatitis A acquisition by travellers. Adult food handlers in developing countries, on the other hand, are unlikely to be shedding HAV because of the high rates of adult seropositivity in these regions. Few data are available concerning the incidence of hepatitis A among short-term travellers to developing countries. Of hepatitis A cases reported to the Viral Hepatitis Surveillance Program, Centers for Disease Control and Prevention in 1993, 6.3% were associated with a history of international travel.[56] The rate of symptomatic hepatitis A among unprotected, non-immune Northern European travellers visiting developing countries was approximately three per 1000 per month[110] (reviewed by Steffen[111]), the majority of whom were tourists who had stayed in hotels. Among backpackers and others eating and drinking under non-hygienic conditions the incidence of hepatitis A may be sixfold higher.[111] Similarly, hepatitis A has been demonstrated to be a risk for all Canadian travellers to endemic areas, irrespective of the perception of risk.[112] Prolonged travel under poor living conditions was associated with more than a sevenfold greater adjusted risk of acquiring hepatitis A than trips of <2 weeks duration with accommodation in first-class hotels. However, high-risk trips accounted for only 7% of total hepatitis A cases among travellers, the majority of which were acquired during trips in the medium- and low-risk categories.

Long-term travel to developing countries carries a high risk of hepatitis A acquisition among unprotected susceptibles. Among American missionaries with >20 years of service in Africa >90% were hepatitis A seropositive.[113] The incidence of hepatitis A was highest in the first 1–2 years of service, during which 28% of susceptible persons were infected, and continued at a median of 5.4% per annum for the next decade thereafter. A 48% rate of seroconversion to HAV (19/1000/month) was seen among 108 susceptible unprotected French volunteers working in the field in Central or West Africa for 18–35 months.[114]

## Blood-borne transmission

The importance of blood-borne transmission in the epidemiology of hepatitis A remains to be definitively established. The potential infectivity of HAV by the blood-borne route is high. Experimental inoculation studies using tamarins and chimpanzees have recently demonstrated wild-type HAV to be $10^{4.5}$-fold more infectious intravenously than orally.[52] However, transmission of hepatitis A by blood or blood products, while

well described, is infrequent. Among injecting drug users (IDUs) hepatitis A is common and has been associated with large and prolonged outbreaks. Parenteral transmission has not been conclusively established as the major route of transmission between IDUs because of the confounding potential for faecal-oral transmission.

Characterization of the viraemia associated with acute hepatitis A infection has advanced considerably in recent years. Using sensitive PCR assays 73–100% of hepatitis A patients have been shown to be viraemic at presentation.[115–117] Data from patients with hepatitis A[115–120] and animal model studies[34,45,115,121] have shown that the viraemia is longer than previously understood, typically commencing an average of 17 days before the ALT peak, and persisting for between 18 and 79 days after this. An atypical minority of cases have either prolonged or relapsing viraemia and hepatitis.[115–117,122] Human immunodeficiency virus (HIV) co-infection appears to prolong viraemia.[123,124] Viraemia is able to persist even in the presence of high levels of specific anti-hepatitis A IgM antibody.[115,116,122] However, the highest viral loads, and presumably, therefore, the highest risk of blood-borne transmission, appear to occur prior to the ALT peak and the appearance of IgM, being 1000-fold higher than those after the ALT peak.[115] This accords with early transmission studies demonstrating infectivity of pools of serum collected as early as 16–24 days before and up to 3 days after the onset of jaundice.[12,125]

Several studies have used PCR amplification to study human sera collected after the onset of clinical symptoms of hepatitis A. Using nested PCR targeting the 5′-non-coding region (NCR), HAV nucleic acid was demonstrated in the serum of 73.7% of patients at admission. Among patients with typical self-limiting hepatitis, 55.5% were still viraemic between days 11 and 15, declining to zero after day 20.[117] A nested PCR targeting the VP1 region found HAV RNA to persist in the serum for a mean of 30 days (range 5–59) after hospital admission, correlating closely with the duration of abnormal ALT, but not peak ALT.[119] A real-time quantitative PCR targeting the 5′NCR was used to demonstrate HAV viraemia in 100% of 41 acute hepatitis A patients, and to demonstrate the persistence of viraemia in excess of 60 days after diagnosis in six other serially studied subjects.[118] An earlier study using nested PCR to target the 5′NCR had also found a high proportion of acutely infected patients to be viraemic (92% of 38 patients), but a mean persistence of viraemia for only 18 days in 25 serially sampled patients.[116]

Many studies describe subgroups of patients with atypical clinical courses characterized by either prolonged or relapsing biochemical abnormality and viraemia.[115–117,122] Continuous viraemia persisting for as long as 490 days after the onset of jaundice has been described.[122] Recurrence of viraemia and hepatitis for variable periods after resolution of the initial clinical episode is also well described.[115–117,122,126] One early study using slot blot hybridization detected intermittent viraemia persisting for approximately 15 months in a case of relapsing hepatitis A.[126]

A limited number of clinical studies have been able to demonstrate the onset of viraemia. Using PCR, HAV RNA was detected in the serum of two haemophiliacs for 6–7 weeks, from 11 days before ALT elevation in one of the pair.[127] More recently, the onset and persistence of HAV viraemia were studied using archived serum samples from a clinical trial.[115] Nested PCR assays targeting the VP3-VP1 junction, VP1-P2A junction and 5′NCR were used to study 13 subjects with an average of eight serum samples collected at approximately monthly intervals before and after their peak ALT. All subjects were viraemic in specimens collected 15–37 days before peak ALT, and none earlier than this. All subjects were viraemic in specimens collected 18–69 days after ALT peak, declining rapidly thereafter to give a mean persistence of HAV and a viraemia of 79 days.

Data are relatively limited concerning the concentration of HAV viraemia. Using chimpanzee inoculation as an end-point, the titre of HAV in blood has been shown to be moderate, containing between $10^3$ and $10^5$ chimpanzee-infecting doses (CID)/mL.[11] End-point dilution of PCR-positive samples in normal human serum and subsequent PCR has recently been used to estimate HAV virus load before and after the ALT peak, with calibration of the method using cell culture-derived HAV. At the ALT peak, virus load was estimated to be $7.9 \times 10^3$–$7.9 \times 10^5$ copies/mL serum, which correlated to $10^2$–$10^4$ plaque-forming units (pfu)/mL, while titres of $7.9 \times 10^5$–$7.9 \times 10^6$ copies/mL ($10^4$–$10^5$ pfu/mL) were demonstrated in the 20-day period prior to peak ALT, and $7.9 \times 10^2$–$7.9 \times 10^3$ copies/mL (10–100 pfu/mL) after the peak ALT (Fig. 7.3).[115] Before and after this 49-day period, titres in positive samples were estimated to be <400 copies/mL (5 pfu/mL).

Two more recent studies using quantitative RT-PCR assays to study HAV virus loads in hospitalized patients have shown good agreement in detecting peak virus loads after presentation respectively of between $1.8 \times 10^3$–$7.71 \times 10^6$ copies/mL[118] and between $2.0 \times 10^3$ and $3.1 \times 10^5$ copies/mL.[122]

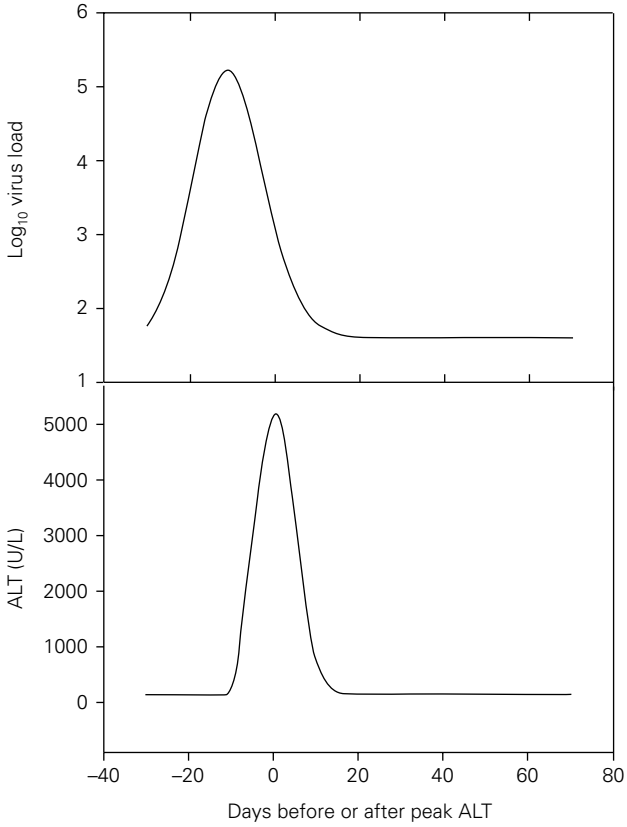


**Figure 7.3** A model of HAV serum viral load relative to peak ALT (based on data from Bower[115]).

Among HIV-infected individuals with acute hepatitis A, the duration of viraemia was significantly longer than in non-HIV-infected patients (median 53 days compared with 22 days), and virus loads estimated by titration were higher (median litre $1 \times 4^{-7}$ compared with $1 \times 4^{-3}$) but the peak ALT was less.[124] There was no correlation of HAV virus load with CD4 count, HIV virus load or HIV therapy. Another study demonstrated a prolonged viraemia of 256 days, and a peak load of $2.1 \times 10^7$ copies/mL in a single HIV-infected individual acutely infected with hepatitis A.[123]

Animal inoculation studies have generally suggested a viraemia of approximately 3 weeks' duration,[34,45,121] although a recent study using nested PCR found longer periods of viraemia ranging from 25 to 91 days.[115] HAV was isolated from the serum of experimentally infected owl monkeys within 11 days of inoculation, and this viraemia continued for a mean of 20.5 days thereafter, peaking a mean of 17.6 days after inoculation and 8.8 days before maximal ALT values.[45] Viraemia significantly diminished in titre but was detectable in the presence of serum-neutralizing antibody in five of six animals.[45] PCR demonstrated HAV RNA in the serum of an experimentally infected chimpanzee for 3 weeks, from 1 week before liver enzyme elevation to 1 week after peak elevation.[121] Nested PCR assays targeting the VP3-VP1 junction, VP1-P2A junction and 5'-untranslated region (UTR) demonstrated viraemias commencing 17–22 days before peak ALT, and persisting for 53–63 days after this in two chimpanzees inoculated with $10^6$ CID of hepatitis A intravenously.[115] Animals receiving lower inocula either orally or intravenously became viraemic 14–18 days before peak ALT, and this persisted for 11–26 days after the peak. Virus loads were also found to correlate with inoculum in this study. Animals receiving $10^6$ CID experienced virus loads of $7.9 \times 10^3$ particles/mL ($10^2$ pfu/mL) prior to the ALT peak, $7.9 \times 10^5$ particles/mL ($10^4$ pfu/mL) at the ALT peak, and 79–$7.9 \times 10^2$ (1–10 pfu/mL) over the following 35 days. Animals receiving 1 CID intravenously had peak viraemias of $10^3$ pfu/mL, and lower virus loads at all time points than chimps receiving larger inocula.

The likelihood of transfusing a unit of blood infectious for HAV appears to be low. Preliminary data obtained using PCR to screen 6000 randomly selected donations of plasma found none positive for HAV.[128] The potential co-transfusion of HAV-specific antibodies to the recipient of multiple blood units and the rising seroprevalence to HAV with age further diminish the risk of post-transfusion hepatitis A. A number of prospective studies have assessed the risk of transmission of hepatitis A by single-donor blood products.[129–132] HAV was not transmitted to any recipients in 15 such studies involving the transfusion of 44 612 units of blood or blood components. Nevertheless, isolated cases of post-transfusion hepatitis A have been described.[21,133] At present, blood donors are not screened for the presence of anti-HAV antibody.

Infrequent outbreaks of apparently parenterally spread hepatitis A have occurred where transmission by multiple-donor blood products has been implicated, albeit usually not conclusively proven. A number of outbreaks of hepatitis A have been described among haemophiliacs receiving factor VIII concentrates (reviewed by Vermylen and Peerlinck[27]), including outbreaks in Italy,[24] Germany,[22,134] Belgium,[27] Ireland[26] and South Africa.[23] Both local unpaid plasma donors[22,24] and products including plasma obtained from paid plasmapheresis donors in the United States[26,44,134] have been involved. In most outbreaks,[22,24] multiple batches of concentrate were implicated, each carrying a relatively low risk of HAV transmission, with severe haemophiliacs requiring intensive treatment most at risk of acquiring HAV. The Irish outbreak saw a higher attack rate and was subsequently attributed to a source plasma pool collected from Irish donors in an area experiencing an outbreak of hepatitis A.[25]

Both ion exchange chromatography and solvent detergent inactivated factor VIII concentrates as well as solvent detergent heparin precipitation purified concentrates have been associated with outbreaks.[23,27] Before

the introduction of solvent detergent inactivation, transmission of HAV by factor VIII concentrates had not been recognized.[135] While the method was highly validated as capable of completely inactivating enveloped viruses in blood products, it has been suggested that this technique may not be sufficient for complete inactivation of non-enveloped viruses such as HAV.[24,85] Furthermore, it has been suggested that the very low concentrations of neutralizing antibody to HAV in final concentrates prepared using ion exchange chromatography or heparin precipitation may diminish the opportunity for neutralization of any residual infectious virus.[23] Study of the Belgian outbreak has also raised the possibility that earlier less pure factor VIII preparations had provided passive immunity to HAV that is no longer present.[136] Significant levels of HAV-specific antibody were demonstrated in now discontinued intermediate purity factor VIII manufactured in Scotland, but were absent from high purity solvent detergent and ion exchange chromatography inactivated factor VIII.[71]

The strength of evidence implicating factor VIII concentrates as the source of hepatitis A among haemophiliacs varies between reports. Italian,[127] South African[23] and German[134] investigations have obtained molecular data in support of HAV-contaminated factor VIII concentrates, the Italian and South Africa investigations also obtaining epidemiological data. In Italy, 52 cases of HAV infection, 81% icteric, were documented between 1989 and 1991 in haemophiliacs receiving organic solvent- and detergent-treated factor VIII at 12 centres.[127] A case-control study showed acquisition of hepatitis A to be highly associated with receiving >2000 units of factor VIII and with receiving factor VIII from one manufacturer, while not associated with other potential routes of HAV acquisition. HAV sequences were subsequently detected by nested PCR in five of 12 implicated factor VIII lots, and sequence identity was shown with HAV RNA obtained from the serum of two recipients available for study.[127] In the recent German investigation, complete sequence identity was shown in the VP3C terminus and VPI/2A junction regions between HAV nucleic acid amplified from a plasma pool, two lots of the subsequent factor VIII preparation and the sera of six haemophilia patients who received it.[134] The virus titre in the infected plasma pool sourced from plasmaphoresis donations collected in the USA was consistent with a single viraemic donation with a titre of approximately $2 \times 10^6$ copies/mL in a pool of approximately 3000 donations. It is not clear why an anti-HAV titre of 6.11 IU/mL in the infected pool did not neutralize and remove HAV as virus–antibody complexes.

In contrast, HAV RNA could not be detected by PCR in several ion exchange chromatography and solvent detergent inactivated factor VIII concentrates manufactured in Scotland and the United States,[128,137] and epidemiological investigations in the United States,[138] France[139] and Brazil[140] failed to demonstrate transmission of HAV by solvent detergent and ion exchange chromatography purified concentrates manufactured in those countries.

Thus far, there have been no reports of successful demonstration of infectivity of factor VIII preparations implicated in HAV transmission.[127,134] This might be due to low virus load and the greater ease of detection of such titres by PCR than by infectivity in animals.

In an *in vitro* study, the HM 175/18f strain of HAV was added to plasma preparations containing varying quantities of HAV-specific antibody and subjected to cryoprecipitation, solvent and detergent inactivation, diethylaminoethyl (DEAE) anion exchange chromatography, and lyophilization.[141] If the reduction in infectivity attributable to each of the above steps was additive, this protocol was potentially able to inactivate 8.6–9.3 log of HAV. Although the magnitude of HAV viraemia and frequency with which viraemic plasma units might be included in plasma pools would be important determinants of the adequacy of such a reduction in infectivity, it was concluded that this preparation method does not provide a significant margin of safety, particularly if the original plasma pool is obtained from a population where HAV is not rare.[141]

A multicentre outbreak of hepatitis A has also been reported among cancer patients treated with interleukin-2 (IL-2) and lymphokine-activated killer (LAK) cells.[142] Pooled human blood group AB serum from paid donors used to supplement the LAK cell culture medium was subsequently implicated as the infectious source. The serum pools underwent complement inactivation (56 °C for 30 minutes) and sterile filtration (pore size 0.22 μm) before use. Attack rates of 85%, 62%, or 50%, respectively, occurred among those exposed to one or other, or both of two implicated pools compared with no cases among susceptibles exposed to other serum pools. Hepatitis A-specific IgM was detected in residual samples from the serum pools when tested at a dilution of 1/2000, but neither serum samples nor LAK cell pools induced evidence of hepatitis A when inoculated into marmosets.[142]

Injecting drug use has become increasingly important in the epidemiology of hepatitis A in many parts of the world. The prevalence of anti-HAV among IDUs was found to be 43% compared with 5% among the general population in Norway and, at 44% among Danish IDUs, was four times the prevalence in the general popula-

tion.[143] Outbreaks of hepatitis A among Scandinavian IDUs have also been reported.[144,145] Increasing numbers of outbreaks of hepatitis A among IDUs were reported in the United States during the 1980s.[146] Between 1983 and 1986, the percentage of hepatitis A cases with a history of injecting drug use rose from 4% to 19% against a background of relatively constant overall rates of hepatitis A. The number of hepatitis A cases associated with injecting drug use peaked in the United States in 1989 and subsequently declined relatively rapidly (Fig. 7.2).[56] Significant outbreaks of hepatitis A have been described among IDUs in England,[147–149] and in inner Sydney[150] and Queensland[151] in Australia. In England the epidemiology of hepatitis A is reported to have changed with most cases occurring in young males and the commonest risk factor being IDU.[147] Fourteen outbreaks were described in England in 2002 alone, all associated with IDUs. In Estonia genotyping of HAV viruses from initial cases among IDUs and other community cases during a 1998–1999 outbreak suggested transmission of the HAV outbreak strain from IDUs into the general population.[152] Needle sharing has not been clearly demonstrated as the mode of transmission in these outbreaks, which have frequently been attributed to poor hygienic conditions among IDUs.[143] Intravenous drug use, frequent injecting, intravenous heroin use and multiple drug use were found to be significantly associated with the acquisition of hepatitis A during a prolonged outbreak among IDUs in Suffolk, England commencing in 2001. While suggesting the potential importance of person-to-person transmission by the blood-borne route, it is equally possible that transmission was via faecal contamination of injecting paraphernalia among this most chaotic group of drug users. Definitive data are not available.

### Perinatal transmission

Isolated cases of apparent perinatal transmission of HAV have been described.[82,153] An infant was isolated from its mother and treated with immunoglobulin when the mother developed symptomatic hepatitis A immediately after delivery, subsequently infecting three other family members.[153] HAV RNA was detected in serum and faeces from the infant, who remained clinically well on days 17 and 32 after birth, but not on day 101. Cord blood did not contain HAV RNA. Hepatitis A-specific total antibody subsequently became positive 6 months after birth. It was concluded that transmission of HAV to the infant occurred via intrapartum exposure to contaminated maternal blood or faeces.[153]

### Primate-to-human transmission

Human HAV naturally infects chimpanzees (*Pan troglodites*) and may be experimentally transmitted to chimpanzees, tamarins (*S. mystax*, *S. labiatus*, *S. nicricollis*, *S. fuscicollis*), owl monkeys (*Atous trivirgatus*), and possibly a number of other non-human primates[46,47,92,154,155] (reviewed by Balayan[10]). Since the description of an outbreak of hepatitis A among chimpanzee handlers at a United States Air Force base in 1961,[156] more than 40 similar outbreaks involving more than 170 human cases have been reported.[10] The epidemiology of these outbreaks suggests that typically the animals were infected after capture from the wild and subsequently transmitted HAV to primate handlers.[37]

## Seroprevalence

### Major seroprevalence patterns

Hepatitis A has a worldwide but uneven distribution between geographical regions and population groups. In many regions, the epidemiology of hepatitis A has been changing as improved sanitation, housing and socioeconomic standards alter transmission patterns.[37,60,157] Our understanding of the comparative epidemiology of hepatitis A is based largely on seroprevalence data. Estimates of HAV disease incidence based on morbidity data are likely to be very inaccurate, although they may provide insight into trends in disease activity within a given region, as deficiencies in data quality will tend to be constant. Shortcomings of hepatitis A morbidity data are attributable to the high frequency of subclinical infection, lack of laboratory facilities to distinguish hepatitis viruses in many regions or the failure to differentiate them in recorded data, and physician underreporting.[71,72]

Comparison of measured seroprevalences in different population groups provides a more reliable picture of the epidemiology of HAV disease. Seroprevalences vary from as low as 13% in Scandinavian countries such as Sweden, to approaching 100% in areas of the developing world such as Ethiopia.[157–159] Three distinct patterns (A, B and C) of age-specific HAV seroprevalence have been described (Fig. 7.4).[13,71,160] These patterns may be descriptive of the epidemiology of HAV in different countries, or in different population groups within the same country. For instance, the pattern of age-specific hepatitis A seroprevalence seen among Aboriginals in the Northern Territory of Australia[161] contrasts with that observed among urban and provincial populations in Victoria.[162]

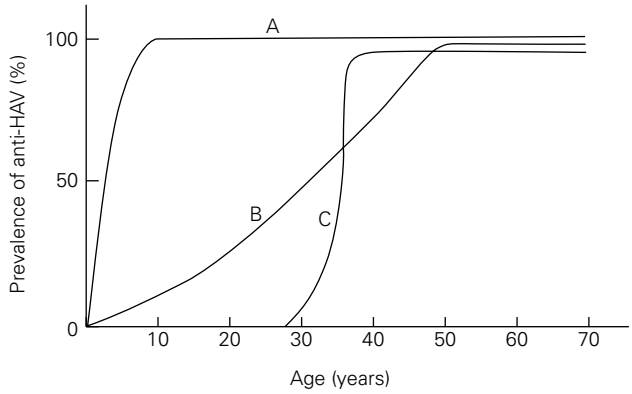


**Figure 7.4** The three major patterns of age-specific prevalence of anti-HAV. (From Gust,[155] with permission.)

Seroprevalence pattern A is typical of HAV epidemiology in developing countries, particularly tropical countries in which HAV may be hyperendemic, including most of Asia, the Pacific islands, Central and South America, and Africa.[159,163,164] Crowded living conditions, poor community sanitation and inadequate water supplies promote high levels of intrafamilial and foodborne and waterborne transmission of HAV within the community in these areas. Exposure to HAV before age 10 years is almost universal in these populations, and seroprevalence among adults approaches 100%.[159,163] Seroconversion among children is usually asymptomatic.[20] In these countries reported rates of hepatitis A are low and outbreaks rare, with most cases of HAV among visitors from lower prevalence areas such as tourists and voluntary workers. In one recent study of long-term American missionaries to Africa, the rate of acquisition of hepatitis A was 28% during the first 1–2 years of service and a median of 5.4% per annum for the next decade thereafter.[113]

Pattern B is typical of most developed countries including Scandinavia, North American, Japan and Western Europe. Exposure to HAV is infrequent among children in these countries, and seroprevalence increases slowly through early adulthood, reaching high or medium levels[162,165–167] among the older age group in a sigmoidal shaped curve (Fig. 7.4). The increase to quite high levels of seropositivity among older age groups is probably a cohort effect, reflecting high levels of HAV exposure present during childhood and subsequent gradual improvement in living standards over many decades. This explanation is suggested by the observed decline in the number of clinical cases of infectious hepatitis among children in the United States, Australia, Denmark, the former Czechoslovakia and Puerto Rico,[168–170] and a decrease in HAV-specific antibody prevalence in sera from the same age group collected many years apart in Australia, Germany, Japan, Italy and Finland.[168,171–174] For example, in Germany the HAV seroprevalence in the 20–29-year-old age group declined from 51% to 11% between 1965 and 1975.[171]

Epidemiological patterns intermediate between the above patterns A and B may occur as living standards improve in countries where HAV was formerly endemic, including eastern Europe, Mediterranean countries, the republics of the former Soviet Union, and parts of the Americas and Asia.[60,175–177] This situation is characterized by a decline in total infection rates and an increase in the mean age of exposure to HAV into older childhood or adolescence. A consequence of the increasing age of first exposure to HAV may be a paradoxical increase in the rate of symptomatic hepatitis A among this new pool of susceptibles. Sizable community outbreaks involving person-to-person spread of HAV may constitute a significant proportion of hepatitis A in such populations along with occasional foodborne outbreaks.[60]

The third seroprevalence pattern, pattern C, is typical of populations in which HAV is not endemic, but into which HAV is introduced from outside, abruptly infecting a large pool of susceptibles before disappearing. This results in an abrupt demarcation between high seroprevalence among those alive during the epidemic and minimal seropositivity among those born subsequently. This pattern has been well described in isolated populations in Greenland[55] and Alaska.[54]

## Demography

The differing age distribution of hepatitis A infection between developing and developed countries is largely a consequence of differing standards of hygiene and sanitation, as discussed previously. In the developing world, HAV infection is almost universal among children, reflecting early widespread exposure. In the developed world, clinical cases generally predominate among adults. Although a slightly greater incidence of cases may be seen among males in developed countries, there appears to be no gender association of HAV disease other than as a reflection of greater exposure risk through occupations, such as sewage workers, or participation in high-risk activities such as frequent oral-anal contact among male homosexuals.[90]

Socio-economic status has been shown to be inversely associated with prevalence of HAV-specific antibody.[167,178,179] Higher and lower HAV seroprevalences, respectively, were found in a lower socio-economic group[178] and in an upper socio-economic group[167] than in middle class populations in the United States. A similar association of HAV seroprevalence and socio-economic

status has been demonstrated in studies performed in Italy[74] and Ethiopia.[159] Other factors increasing the risk of HAV transmission in the Italian study were large families, crowded living conditions, and poor hygienic and sanitary conditions.[180] Higher hepatitis A seroprevalences have been observed in rural areas compared with urban areas in several countries,[175,181] and a higher incidence of hepatitis A infection has been observed among rural Thai children compared with children living in urban Bangkok.[175]

## Temporal patterns

Peaks of disease prevalence occurred every 5–10 years between the early 1950s and 1970s in some temperate developed countries including the United States, Canada, Australia and Denmark.[37] However, this cyclical pattern appears to have broken down as rates of HAV disease have declined in recent decades. The pattern has persisted in some population groups within the United States such as the Sioux Indians, among whom large hepatitis A outbreaks occur approximately every 7 years, predominantly affecting the 5–9-year-old age group.[177,182] A cyclical disease pattern is also seen in some countries currently of intermediate hepatitis A endemicity, such as Mauritius and Thailand, as improving sanitary conditions create a transition phase between endemic disease and sporadic self-limited epidemics.[175,177,183]

Similarly, seasonal peaks of HAV disease, which used to occur in autumn and winter in some temperate countries,[37] are no longer seen except as reflections of travel patterns to endemic areas. For instance, Germany, unlike the United States, retains a seasonal peak of hepatitis A in autumn, more pronounced in children than adults, which has been attributed to summer school holiday travel by children to Mediterranean countries where hepatitis A is still endemic.[158] In contrast, seasonality has never been a prominent feature of HAV epidemiology in tropical countries.

## Molecular epidemiology

Systematic study of HAV genetic variability was facilitated by the introduction of sequencing of HAV nucleic acid amplified directly from crude faecal suspensions by antigen-capture PCR.[184] Genetic analysis of 152 HAV strains obtained from multiple countries subsequently led to the proposal that HAV strains may be differentiated genetically into seven unique genotypes (I–VII). Viruses from four genotypes (I, II, III, VII) were recovered from human hepatitis A cases, and the remaining three genotypes (IV, V, VI) isolated only from cases of hepatitis A-like illness among captive simian species.[185] Nucleotide sequences of strains in the respective proposed genotypes were found to vary at 15–20% of base positions in a 168-bp section of the VP1/2A junction region, whereas within major genotypes, subgenotypes varying at approximately 7.5% of positions were described.[185]

As well as the VP1/2A junction,[184–191] genetic variability of HAV strains has also been studied by sequencing of short regions in the VP3 C terminus,[184] the VP3/VP1 junction region,[186,189,191] the VP1 N terminus[185,187,192–194] and the 5′NCR.[190]

Recently, phylogenetic studies were carried out using the full-length VP1 sequences from 81 HAV strains sourced from Europe and Central and South America between 1983 and 2001.[195] This approach reflects the recognition of the superiority of full-length VP1 over the VP1/2A junction for characterization of other picornaviruses, together with the importance of VP1 as an antigenic determinant both in HAV and other picornaviruses[196,197] (reviewed by Costa-Mattioli[198]). This study revealed five distinct lineages of HAV sequences well supported by high bootstrap values, yet suggested that the existing genotypes II and VII each only represented by a single strain, may in fact be subgenotypes of the same type.[195] Further phylogenetic analysis of the full-length VP2 and VP3 sequences of the genotype II strain 9F94, together with recombination analysis using recombination identification programme (RIP) software, suggests that genotype II may not be a distinct human genotype.[199]

The majority of human HAV infections are due to viruses from either genotype I or III, each of which has two subgenotypes denoted A and B.[198,200] Genotype I strains are the most widespread in the world.[184,185] Genotype IA strains have been identified originating from North, Central and South America, China, Thailand, Japan, the former USSR and South Africa, while genotype IB strains have come from South America, Europe, Australia, Japan, North and South Africa and Jordan[184,185,187–191] (reviewed by Costa-Mattioli[198]).

Genotype IIIA strains have been identified originating from Western Europe, USA, Malaysia, India, Nepal, Sri Lanka, and more recently from France, Italy and Spain.[184–186,198,201–204] In Estonia an outbreak associated with IDUs in 1998–1999 has seen genotype IIIA surplant genotype IA as the dominant HAV strain circulating in the community, the latter having predominated from 1994 to 2001.[152]

There is some evidence that geographically related strains from endemic areas appear to form distinct clusters distinguishable from other isolates of the same genotype from elsewhere.[152,184,185] There is also evidence for co-circulation of imported strains with endogenous strains.[185,186] Association of different HAV strains with shellfish of local and imported origin has been hypothesized to explain heterogeneous co-circulating HAV strains and the prominence of shellfish consumption as a risk factor in outbreaks in France and Italy.[186,201]

In the course of outbreak investigations molecular epidemiological studies provide essential support to traditional epidemiological investigations aimed at identifying chains of transmission and probable sources of infection. A high degree of relatedness has been shown between different viruses from the same outbreak,[152,186,190,201] and between viruses obtained from cases and shellfish identified as the infection source.[190]

Variation observed at the nucleic acid level should not compromise the protective efficacy of HAV vaccines based on genotype I strains (HM175 and CR326), as the close antigenic relationship between the majority, if not all, of human HAV strains is well established.[205] While naturally occurring antigenic variants of HAV have recently been described,[190,195,206] different serotypes have not.

## Conclusion

The general trend in the developed world is towards declining incidence of hepatitis A as standards of hygiene and sanitation continue to improve. In contrast, slow or minimal improvement in living conditions is likely in the foreseeable future in many developing countries, and hepatitis A will continue to be endemic in these areas. Within countries in transition towards hepatitis A eradication, some populations have slower rates of decline in incidence because of special risks, such as residents of institutions, some ethnic groups, IDUs and homosexual men. These will remain potential foci of hepatitis A persistence and may contribute to outbreaks in an increasingly susceptible general population, as may travellers returning from countries in which hepatitis A is endemic. In addition, children in day-care centres represent a large susceptible pool in which hepatitis A may be silently amplified and spread into the community.

With the availability of safe and effective vaccination for hepatitis A, the opportunity arises for intervention in HAV transmission on a population scale. Notably, vaccination could diminish the risk in low prevalence countries of hepatitis A outbreaks arising from persisting or external foci of infection. This might be achieved by targeted vaccination of risk groups, or it may require a broader approach including general childhood vaccination. In contrast, in hyperendemic areas, the morbidity caused by hepatitis A is minimal and competing public health priorities are many. Widespread uptake of hepatitis A vaccination is probably unlikely in such countries in the near future. If vaccine-acquired immunity is found to wane with time, vaccination may be of questionable value in areas where hepatitis A is endemic without improvements in hygiene and sanitation. Otherwise, morbidity attributable to hepatitis A may be increased through creation of a cohort of susceptible adults without altering high level transmission of HAV.

Improved hepatitis A surveillance and monitoring are currently required in both the developed and developing world, along with increased understanding of the duration of vaccine-induced immunity and implementation of public health measures aimed at improving sanitation and hygiene in the developing world. In the longer term, incorporation of hepatitis A vaccination into widespread childhood immunization protocols should facilitate eventual elimination of hepatitis A, as will soon be achieved with poliovirus.

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# Chapter 8
# Natural history and experimental models

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## Historical perspectives

Disease resembling hepatitis A was described in ancient times.[1] Outbreaks of hepatitis were reported among troops in military campaigns of the 17th through the mid-20th Centuries and the disease came to be known as 'campaign jaundice'. Outbreaks of hepatitis were also common among civilians, prompting Cockayne to designate such epidemics of jaundice as 'infective hepatitis'. By the 1940s, a second type of hepatitis, 'serum hepatitis', was recognized, and in 1947, McCollum introduced the terms 'hepatitis A' for infectious hepatitis and 'hepatitis B' for serum hepatitis. Epidemiologic and volunteer studies in adults and children from the 1950s through the 1970s demonstrated the predominantly faecal-oral route of transmission of hepatitis A, the bloodborne and sexual transmission of hepatitis B and the existence of a third hepatitis, designated non-A non-B hepatitis. The development of sensitive serological tests and animal models for each of these viruses during the 1970s and 1980s yielded much new information about the epidemiology and natural history of these agents and provided evidence for two additional hepatitis viruses: hepatitis D virus (HDV) and hepatitis E virus (HEV). Seroepidemiologic studies have shown that virtually everyone in developing countries has been infected with hepatitis A virus (HAV) by age 5–10 years and that this was also true for populations of industrialized countries until the mid-20th Century.[2] Such infections, occurring at an early age, are much more likely to be subclinical than when they occur in older children or adults. Thus, hepatitis A is an uncommon disease (but common infection) in developing countries and such was probably the case in industrialized countries until the early 20th Century. Ironically, the epidemics of hepatitis reported before the early 20th Century were probably not hepatitis A, but hepatitis E, as is still the case in developing countries.

## Clinical features of hepatitis A

The clinical presentation of viral hepatitis is similar, regardless of the aetiology, and the diagnosis must be made on the basis of serologic tests. However, each type of viral hepatitis has clinical features and a natural history that is more characteristic of that agent than of the other hepatitis viruses.

### Clinical spectrum

Infection with HAV may result in a wide spectrum of clinical outcomes, ranging from a completely inapparent infection detected serologically by evidence of a rising titre of anti-HAV, to subclinical disease characterized by limited symptoms and biochemical abnormalities of liver function tests to classical icteric hepatitis, to potentially fatal fulminant hepatitis with hepatic failure and coma. The most important factor determining outcome of HAV infection appears to be age. Clinical hepatitis A is more frequent with increasing age in almost all published reports.[2–12] Less than 5% of children below 3 years of age, 10% of children from 4–6 years of age and 80% of adults develop icteric clinical illness, regardless of the strain of infecting virus. Naturally attenuated and unusually virulent strains of HAV appear to be rare.[12–16] Fulminant hepatitis A occurs most commonly in patients over 50 years of age and death from hepatitis A is also most common in this age group.[8,11,17]

### Prodromal phase

The incubation period in viral hepatitis is often defined

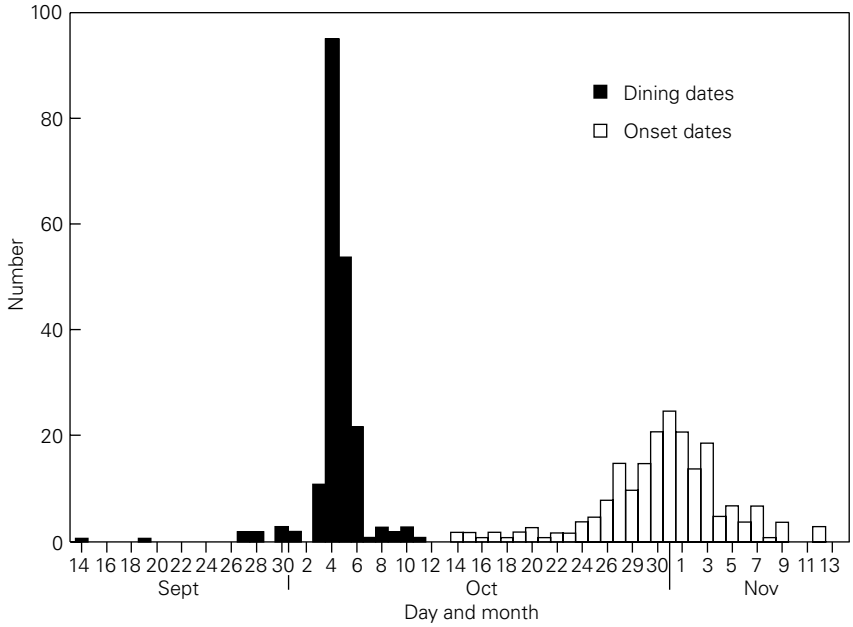


**Figure 8.1** Incubation period of hepatitis A. Number of hepatitis A cases, by date of eating contaminated food and by date of illness onset.[16]

as the time between exposure to the virus and the appearance of dark urine, the first sign usually noticed by the patient. The average incubation period in hepatitis A is approximately 4 weeks (range 2–7 weeks), based on experimental infections and analyses of common-source epidemics[16–20] (Fig. 8.1). Variations in the incubation period may be related to the infectious dose, differences in strain virulence or host factors.[21,22] Patients developing symptomatic disease usually experience a prodromal period beginning several days before onset of illness and characterized by malaise, flu-like symptoms, anorexia and fever.

## Acute disease

### *Major signs and symptoms*

Prodromal signs and symptoms (Table 8.1) may continue into the acute phase, followed by darkening of the urine and lightening of the faeces, but they usually decrease as jaundice, manifest by icterus of the sclera, skin and mucous membranes, develops.[23–26]

### *Laboratory tests*

Hepatitis A viraemia and faecal shedding, detected initially by volunteer studies but now by enzyme-linked immunosorbent assay (ELISA) or reverse transcriptase-polymerase chain reaction (RT-PCR), appears approximately 2–3 weeks after infection, during the incubation period, and peaks before onset of disease (Fig. 8.2).[27–34] As viral titres fall, biochemical markers of liver damage (see below) increase, paralleled shortly thereafter by the appearance of antibody to HAV (anti-HAV), first of the IgM class and then, shortly thereafter, of the IgG class. Anti-HAV of the IgM class can be detected for 3–4 months, but IgG anti-HEV persists, probably for life. Thus, patients with hepatitis A are usually positive for IgM anti-HAV when first seen by a physician and a positive test for IgM anti-HAV is diagnostic of recent infection. With the increasing use of hepatitis A vaccination, cases of modified HAV infection may become more common.[35] These may pose a diagnostic dilemma

**Table 8.1** Frequency (%) of signs and symptoms associated with acute hepatitis A[23]

| Signs/symptoms | Frequency (%) |
|---|---|
| Dark urine | 94 |
| Fatigue/anorexia | 90 |
| Nausea | 87 |
| Weakness | 77 |
| Fever | 75 |
| Vomiting | 71 |
| Headache | 70 |
| Abdominal discomfort | 65 |
| Light-coloured faeces | 52 |
| Myalgia | 52 |
| Drowsiness | 49 |
| Irritability | 43 |
| Itching | 42 |
| Constipation | 29 |
| Diarrhoea | 25 |
| Arthralgia | 21 |
| Sore throat | 20 |
| Nasal discharge | 14 |
| Cough | 7 |

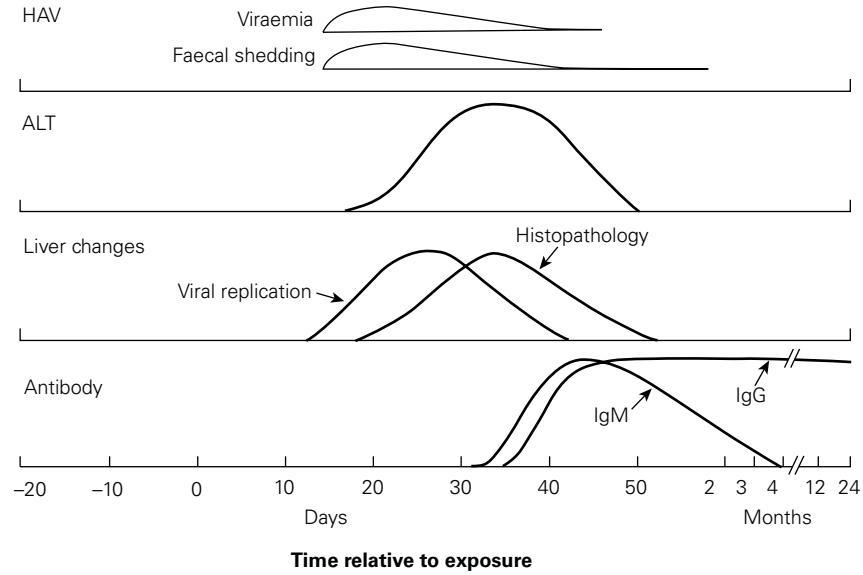


**Figure 8.2** Typical course of acute hepatitis A.

because commercially available tests for anti-HAV cannot differentiate between vaccination and infection. However, tests for antibodies to non-structural proteins of HAV, which appear only after infection, have been developed.[36]

Biochemical evidence of acute hepatitis is indistinguishable from that observed in other forms of viral hepatitis. Serum aminotransferases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) are sensitive indicators of liver cell damage. They rise rapidly during the late incubation period, reaching peak levels (500–2000 U/L) generally within 1 week of the onset of symptoms and fall relatively rapidly after the onset of jaundice in uncomplicated cases. Early changes in the hepatocyte reflect altered permeability of the plasma membrane, resulting in loss from the cell of ALT, found in the cytosol, before loss of AST, which is found in the mitochondria. Thus, serum ALT levels are generally higher than serum AST levels in acute uncomplicated hepatitis A. High levels of AST indicate severe tissue necrosis. Generally, bilirubin reaches peak levels of 170–200 μmol/L shortly after the transferases peak. In uncomplicated hepatitis A, serum alkaline phosphatase and gamma-glutamyl transpeptidase levels are only slightly elevated. Transient elevation of total IgM and total IgG is often present and is more characteristic of hepatitis A than of other types of viral hepatitis. Transient elevation of smooth muscle antibodies may also occur. Changes in blood chemistry, such as minor coagulation defects and depressed fibrinogen levels without bleeding complications may occur, but severe depression of clotting factors is characteristic of fulminant hepatitis. Mild lymphocytosis with atypical mononuclear cells may be present early in the illness and mild pancytopenia may be present during recovery.

## Outcome of disease

### Uncomplicated course

In uncomplicated hepatitis A the duration of illness is generally brief. Clinical and biochemical indices of hepatitis A resolve rapidly and, in most patients, the serum aminotransferase levels have returned almost to normal by week 3 or 4. However, malaise, fatiguability and depression may sometimes persist for several months.

### Prolonged acute hepatitis A and relapse

Although hepatitis A is usually a benign disease of short duration, prolonged elevation of liver function tests has been documented.[17,24,37,38] In three studies of hepatitis A in adults, totalling over 600 patients, serum aminotransferase levels remained elevated for 4–12 months after onset in 14.5% of the patients.[20,39,40,41] Others have documented elevated aminotransferase levels for up to 15 months.[42] In such patients, HAV replication may persist and IgM anti-HAV remains detectable throughout the illness.[27,38,43–45] Although hepatitis A can persist for over a year, and even though histological changes resembling chronic hepatitis have been seen in liver biopsies from such patients, there are no documented cases of true chronic hepatitis attributable to HAV.

Hepatitis A may also recur within a few weeks of apparent recovery, even after liver function tests return to normal.[38,40,46–54] This second bout of disease may be icter-

ic or anicteric but is generally somewhat milder than the initial episode.[55] Rarely, a second relapse of acute hepatitis A has been described.[56] Reactivation of viraemia and/or viral shedding in the faeces can occur during such relapses, or may continue intermittently between episodes.[27,38,57]

### Fulminant hepatitis

Fulminant hepatitis is a rare complication of HAV infection but may be more common in developing countries.[12,58–64] The incidence of fulminant hepatitis and the incidence of fatal fulminant hepatitis both increase with age (Fig. 8.3).[2,8,11,17,65] The disease is characterized by increasingly severe jaundice, deterioration in liver function, especially synthesis of clotting factors, increasing encephalopathy and, ultimately, coma. The treatment of choice in potentially fatal fulminant hepatitis is liver transplantation. In patients who survive hepatic coma caused by acute hepatitis A and who have not undergone liver transplantation, the recovered liver does not contain evidence of fibrosis or cirrhosis.

### Fatal hepatitis

Although fulminant hepatitis A is often fatal, all fatal hepatitis A is not fulminant. The case-fatality rate in a large study of more than 2000 hospitalized patients was <0.2%; mortality increases with age.[65,66] Others have reported a much lower mortality rate of 0.015% among almost 311 000 cases in Shanghai, China in 1988. However, >90% of these cases were between the ages of 20 and 40 years, and older individuals in this population, who would be more likely to have a fatal outcome, were also more likely to have been immune by virtue of exposure to the virus as children.[12] Finally, the strain of HAV causing one waterborne epidemic of hepatitis A in China may have been somewhat attenuated.[67] A study by the Centers for Disease Control of 94 000 cases of hepatitis A in the United States in 1995 reported a case-fatality rate of at least 0.16% and a recent epidemic of hepatitis A among an estimated 555 individuals who were infected by eating contaminated green onions had a case-fatality rate of 0.54%.[16] The reason for these differences is unclear, but may simply reflect differences in the age distribution of the populations at risk. Causes of death from hepatitis A, other than uncomplicated fulminant hepatitis, may include acute hepatitis A superimposed on chronic hepatitis, with or without cirrhosis, caused by HBV or HCV, and other diseases not related to the liver.[8,12,68]

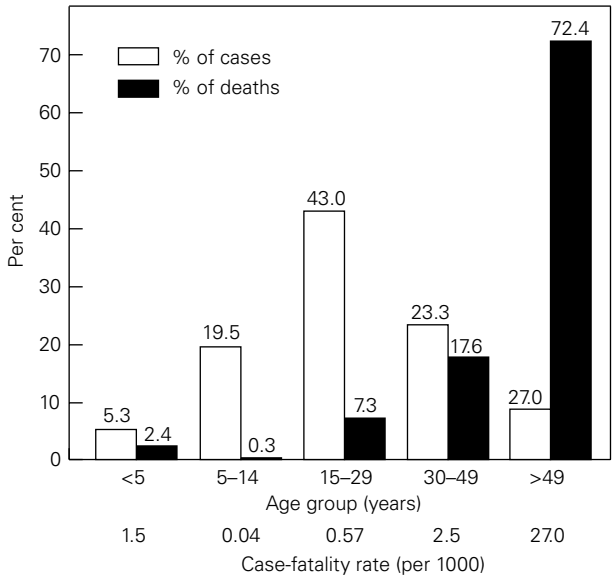


**Figure 8.3** Age-specific incidence of clinical hepatitis A and age-specific incidence of death from hepatitis A. The case-fatality rate is depicted at the bottom of the figure.[2]

### Cholestatic hepatitis

Acute hepatitis A with prolonged cholestasis is a syndrome characterized by pruritus, fever, diarrhoea, weight loss, bilirubin levels >170 μmol/L and a clinical course of at least 12 weeks.[17,55,69] Serum aminotransferase levels tend to be lower but alkaline phosphatase levels higher in this form of hepatitis A. Histologically, the liver of such patients displays centralobular cholestasis and portal inflammation.[69,70] Cholestatic hepatitis A is an unusual form of HAV infection that has a good prognosis and complete recovery. Cholestatic features have also been described in the relapsing form of hepatitis A.[50,52,53]

## Hepatitis A in pregnancy

Unlike hepatitis E in pregnancy, hepatitis A is not associated with increased mortality in pregnant women.[71,72] In the large food-borne outbreak of hepatitis A in Shanghai involving almost 311 000 cases and including >5000 pregnant women, there was no increase in morbidity or mortality associated with pregnancy.[73] Furthermore, there is no evidence that HAV infection causes chromosomal abnormalities or fetal deformities and there are only a few reports of HAV transmission to the newborn.[74–78]

## Hepatitis A in pre-existing liver disease

There is considerable evidence that acute hepatitis A is more severe in patients with pre-existing liver disease, regardless of the aetiology.[68] Thus, 15 of 47 patients who died with acute hepatitis A during the 1988 Shanghai epidemic had underlying chronic active hepatitis B or hepatitis B virus (HBV)-related cirrhosis.[12] In recognition of this, the Advisory Committee on Immunization Prac-

tices in the United States has recommended hepatitis A vaccination for persons with chronic liver disease. The cost-effectiveness of such a policy has been a continuing subject of debate.[79–85] The increased severity of hepatitis A in patients with underlying chronic infection with another hepatitis virus appears to be limited to those with actual disease.[86–91] Inhibition of viral replication in the underlying chronic infection has been reported for some cases of hepatitis A superinfection.[92,93] This probably stems from an interferon-induced antiviral effect of the superimposed infection.

## Hepatitis A and autoimmune liver disease

Autoantibodies are occasionally found transiently in acute hepatitis A and rarely, acute autoimmune hepatitis has been reported following acute hepatitis A. In a prospective study, healthy relatives of patients with type 1 autoimmune hepatitis developed similar disease following acute hepatitis A, suggesting a genetic predisposition for autoimmune hepatitis in such individuals.[94] Protracted HAV infection has been associated with HLA-DRB* 1301, a marker for paediatric autoimmune hepatitis.[95]

## Hepatitis A and extrahepatic disease

Extrahepatic manifestations of hepatitis A are rare. Transient Lupus-like syndrome, arthralgias, arthritis, skin rashes, vasculitis, cryoglobulinaemia and neurological complications, including encephalitis and Guillain-Barré syndrome, have been described.[20,47,96–111] In addition, cardiac involvement, characterized by electrocardiographic alterations (bradycardia, prolongation of the PR interval, T-wave depression), which are transient and resolve during convalescence, have been reported.[112] Haematological changes, including haemolytic anaemia, agranulocytosis, thrombocytopenic purpura, pancytopenia and aplastic anaemia have been reported, although most cases of aplastic anaemia following viral hepatitis are not related to hepatitis A.[113–118] Acute haemolysis associated with hepatitis A has been reported in patients with glucose-6-phosphate dehydrogenase deficiency.[119–121] Other uncommon non-hepatic manifestations of acute hepatitis A include pancreatitis, cholecystitis, glomerulonephritis, interstitial nephritis and nephrotic syndrome.[17,122–132] Lymph node enlargement located near the hepatic hilum, pancreas and small omentum has been described as a unique ultrasonographic feature in acute hepatitis A in children.[133]

## Hepatitis A and human immunodeficiency virus (HIV) infection

In contrast to patients with underlying chronic hepatitis who develop hepatitis A and have a more severe outcome, hepatitis A patients with underlying HIV infection have less severe disease, as indicated by lower serum ALT levels, a higher titre of HAV and a prolonged viraemia when compared with patients not co-infected with HIV.[134,135] In contrast, serum alkaline phosphatase and gamma-glutamyltransferase levels, but not total bilirubin, were higher in the HIV-infected patients. However, hepatitis A in well-controlled HIV patients may resemble disease in immunologically normal patients.[136] Thus, poorly controlled HIV-infected patients with hepatitis A may have a clinical course similar to that of other immunocompromised individuals and such patients may be important in maintaining and propagating HAV in the community.[135,137]

## Other associated diseases

Recently, HAV infections have been linked epidemiologically, but not clinically or causally, to two other diseases: atherosclerotic cardiovascular disease and allergic asthma. In each case, HAV infection is thought to be a contributing (or confounding) factor, rather than the sole aetiologic event.

### Atherosclerotic cardiovascular disease

An infectious aetiology for coronary artery disease has become an increasingly popular hypothesis in recent years.[138–142] Epidemiologic associations between serologic evidence of infection with several pathogens, including *Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus, herpes simplex viruses 1 and 2 and HAV, and atherosclerotic coronary artery disease have been identified. The strength of the association paralleled the cumulative number of pathogens the patients had encountered, but the strongest association was with HAV infection. The associations held not only for coronary disease but also for C-reactive protein levels as a marker of ongoing inflammation. Although a number of hypotheses for this association were put forward, including chronic HAV replication, it is likely that a causal association, if any, is related to the overall pathogen burden or some other environmental factor and that HAV infection is an indirect marker of that association.

### Allergic asthma and atopy

Atopic diseases have increased in prevalence in recent

years in western Europe, the United States and Australia.[143] In 1989, Strachan proposed the 'hygiene hypothesis' to explain these epidemiologic observations.[144,145] An inverse relationship between family size and the development of allergic disease was noted and it was proposed that frequent infections, which were likely to be transmitted among numerous siblings, might modulate the developing immune system through stimulating Th1 lymphocytes and inhibiting the clonal expansion of allergen-specific Th2 lymphocytes.[146–149] Among other associations, such as the household presence of pets and diminished atopy, were associations between serologic evidence of infection with measles, hepatitis A and *Mycobacterium tuberculosis* and a diminished prevalence of asthma.[150] More recently, epidemiologic studies have strengthened the association between the diminished prevalence of serologic evidence of HAV infection and an increased prevalence of asthma.[151,152] HAV is believed to enter susceptible cells via a receptor initially designated HAVcr-1 and subsequently shown to be related to the cell surface receptor TIM-1, a marker that is expressed by activated CD4+ T cells during the development of helper T-cell (Th2) responses and a regulator of cytokine production.[153–155] Close association between Th2 lymphocyte responses and allergy has led some to postulate a molecular association between infection of activated lymphocytes by HAV and modulation of the allergic response early in life.[154] However, as with the direct association between the prevalence of HAV infection and coronary artery disease, the inverse association between the prevalence of HAV infection and atopy is likely to be an indirect marker of an inverse relationship between pathogen burden early in life and the development of allergy, especially asthma.[156]

## Histopathology

Much of our knowledge of histopathological changes in the liver during hepatitis A is derived from experimental infections of chimpanzees, in which periportal liver cell necrosis sparing the perivenular region of the lobule was seen early in the disease.[157–159] Liver biopsies are rarely performed on patients with acute hepatitis, but they have demonstrated that changes in humans are similar to those observed in chimpanzees.[160–162] In one study of 34 patients who were biopsied during different stages of acute hepatitis A, portal infiltration by lymphocytes, plasma cells and macrophages was a prominent feature of biopsies obtained within 10 days of onset of disease, but it was also present in later biopsies when a prolonged course or relapse occurred.[163] The infiltration of the portal triads may spill over into the lobular parenchyma and mimic piecemeal necrosis and chronic hepatitis.[164] Very severe periportal necrosis may lead to portoportal bridging. Other histological changes include iron storage in Kupffer cells.[163] In other large studies of biopsies obtained during the acute phase of infection with patients with hepatitis A, hepatitis B or hepatitis C, hepatitis A was characterized by more pronounced periportal inflammation but less conspicuous parenchymal changes such as focal necrosis, Kupffer cell proliferation, acidophilic bodies or ballooning of hepatocytes.[165] Histological changes also resolved more quickly in hepatitis A, but non-specific changes could still be detected during convalescence.

Following recovery, lobular architecture is intact and there are no chronic changes. In general, histological changes in hepatitis A are not sufficiently distinct to be of diagnostic value and, as liver biopsies are associated with a small but finite risk to the patient, biopsies of acute hepatitis are not useful or warranted.

## Pathogenesis

The pathogenesis of hepatocellular injury in acute hepatitis A is poorly understood. HAV is difficult to adapt to growth in cell culture.[166–168] However, cell culture-adapted strains generally do not produce cytopathic changes, even when virtually every cell is infected, and such strains can be maintained as chronic infections *in vitro* indefinitely. To be sure, certain highly adapted strains of HAV produce a cytopathogenic effect in cell culture.[169] This has been shown to be mediated by apoptosis, but the parent virus does not produce this effect and its significance is uncertain.[170–173] During the incubation period of acute hepatitis A *in vivo*, HAV similarly produces few if any histopathological changes in hepatocytes, even though most hepatocytes at the peak of viral replication are infected.[14] As such *in vivo* infections never progress to chronicity but are terminated more or less in parallel with the appearance of the host's immune response, it is believed that both clearance and disease are the result of immunologically mediated responses.

The humoral response appears to be most important in preventing disease, as antibody alone can prevent or modify infection, and antibody is probably also important in limiting the spread of the virus during the incubation period, because antibody administered shortly after exposure can also modify the severity of the outcome, even after infection is established. However, resolution of hepatitis A is thought to be mediated principally by the cellular immune response. Thus, Vallbracht *et al*. demonstrated lysis of autologous skin fibroblasts infected with HAV by peripheral blood lymphocytes from all hepatitis A patients tested, but not from controls.[174] The cytolytic activity peaked 2–3 weeks after onset of icterus and was mediated by a subset of CD8+ HLA class I-dependent T cells that were also able to produce interferon-γ (IFN-γ).[175] Both functions of cytotoxicity and IFN-γ release were virus-specific. Furthermore, human

T lymphocytes cloned from liver biopsies of hepatitis A patients also belonged to the CD8+ subset and were HAV-specific, HLA class I-dependent, cytotoxic and IFN-γ-producing.[176,177] In one patient, from whom a biopsy was obtained later in the course of illness, CD4+ T-cell clones were also generated in substantial numbers, in addition to the CD8+ clones. This observation is consistent with the demonstration by immunohistological staining of CD4+ cells in the portal tracts of biopsies obtained during acute hepatitis A, although the specificity and functional capacity of these CD4+ cells was not determined.[178,179] Finally, natural killer cells have been shown to be capable of lysing HAV-infected cells *in vitro*, suggesting that they may also have a role *in vivo* in the host response to HAV infection.

However, the actual mechanisms by which HAV is removed from the liver during acute hepatitis A remain to be determined. Clearance of all of the hepatitis viruses during the resolution of viral hepatitis has been thought to be mediated solely by CD8+ T-cell-mediated lysis of infected hepatocytes, but this has proven to be a simplistic view of the pathogenesis of viral hepatitis, at least for hepatitis B and probably hepatitis C.[180,181] Thus, at least in animal models of the disease, clearance of HBV from the liver is effected principally by non-cytolytic mechanisms mediated by CD8+ T cells. The synthesis of the various components of HBV replication is downregulated and, in some cases, disrupted by cytokines, principally IFN-γ and tumour necrosis factor-alpha (TNF-α) released by virus-specific T cells, and removal of hepatocytes by cytolytic T cells is a late, but important, component of viral clearance. This scenario makes sense because, during the peak of viral replication, virtually every hepatocyte is infected and removal by cytolytic means would result in fulminant hepatitis in most infections, rather than the rare case that is actually observed. Indeed, the histologic picture of fulminant hepatitis A is that of massive destruction of virus-infected hepatocytes. Furthermore, viraemia, as measured by serum HAV RNA, is markedly diminished when compared with less severe infections, suggesting an excessive host response.[182] It is not yet known whether such non-cytolytic mechanisms are operative in acute hepatitis A. Antiviral cytokines such as IFN-α, a product of the innate immune response and TNF-α, a product of the adaptive immune response, have been detected during acute hepatitis A, suggesting that non-cytolytic antiviral mechanisms mediated by cytokines are operative in this disease also.[92,183–187]

## Animal models of hepatitis A

Before the development of animal models for viral hepatitis, these diseases were studied by experimental transmission to human volunteers, both adults and children.[18,188,189] Although important information that could not have been obtained by other means was obtained from these studies, they did lead to the death of several adult volunteers and unexpected chronic infections in adults and children, raising serious ethical questions about this research. One positive aspect of the studies in humans was the generation and characterization of clinical materials containing high titres of virus, and these were used to develop and validate serologic tests for HAV and HBV.[190–195] Such serologic tests proved to be the key to identifying animals that were seronegative and therefore might be useful for *in vivo* studies of the hepatitis viruses. The characterized virus pools, of known infectivity, derived from the volunteer studies were also essential for establishing the susceptibility of various animal species to the hepatitis viruses. In turn, the development of useful animal models heralded the end of volunteer studies for ethical reasons. Ironically, ethical reasons are currently put forward as reasons for discontinuing studies in animals. However, despite progress in our understanding of molecular aspects of infection, animals remain essential for the study of pathogenesis and for vaccine development. Neither can be appropriately studied in *in vitro* models.

### Historical perspective

Early attempts to transmit hepatitis A to a variety of laboratory animals were generally unsuccessful.[196,197] This is perhaps not surprising because there were no known infectious pools of the viruses and there were no serological tests with which to distinguish among them or to select susceptible animals. Among the first reports of infection of non-human primates with a hepatitis virus was the report by Deinhardt and colleagues of transmission of hepatitis to white-lipped tamarins (*Saguinus nigricollis* and *Saguinus fuscicollis*) and cotton-topped tamarins (*Saguinus oedipus*) in 1967.[198] One successful transmission utilized an inoculum of serum obtained from a surgeon, whose initials were G.B. and who had developed acute hepatitis without known exposure. Deinhardt believed this virus to be hepatitis A virus, but Parks and Melnick recovered a similar virus from an uninoculated tamarin and provided evidence that the GB virus had physical characteristics that were distinct from those ascribed to HAV.[199,200] This controversy was resolved when Deinhardt's group successfully transmitted bona fide HAV from one of the volunteer studies to tamarins, and the GB agent was subsequently shown to be distinct from HAV.[201–206] Ironically, it was not until 1995 that the mystery of the GB agent was solved, when scientists at Abbott Laboratories resurrected the GB agent from frozen stocks of the original transmission series in tamarins and demonstrated by molecular means that the GB agent was actually two agents, which they designated GB virus-A and GB virus-B.[207] Both are mem-

bers of the *Flaviviridae* family of viruses and, therefore, distant relatives of hepatitis C virus. It is probable that both GB viruses are tamarin viruses that were inadvertently picked up in the earliest animal passages.

Other groups subsequently confirmed the susceptibility of tamarins to infection with HAV, even before serological tests for the virus were developed.[208–210] This was possible because the animals apparently were not exposed to HAV before entry into the laboratory and most, if not all, animals were susceptible.

Such was not the case for chimpanzees. Most attempts to infect chimpanzees with hepatitis virus yielded negative or equivocal results, probably because they were exposed to hepatitis in the wild or shortly after capture.[211,212] Nevertheless, there was epidemiological evidence for susceptibility of chimpanzees. Animal handlers and non-human primates occasionally developed hepatitis after handling newly caught chimpanzees.[211,213–215] The development of sensitive serological tests for antibody to HAV provided a means of screening captive chimpanzees as well as other primates for evidence of prior exposure to HAV. Such tests revealed that most jungle-caught chimpanzees had antibody to HAV, whereas chimpanzees that had been bred in captivity were less likely to have such antibody.[216,217] Attempts to infect seronegative chimpanzees with HAV were successful and confirmed that previous transmission failures probably stemmed from the use of immune animals.[218,219]

Similarly, serosurveys of different species of animals have detected anti-HAV only in non-human primate species.[33] Some, but not all, of these primate species have proven to be useful animal models for the study of hepatitis A.[33,217] These include, in addition to the tamarin and the chimpanzee, the owl monkey and, to a lesser extent, certain macaque species (see below).

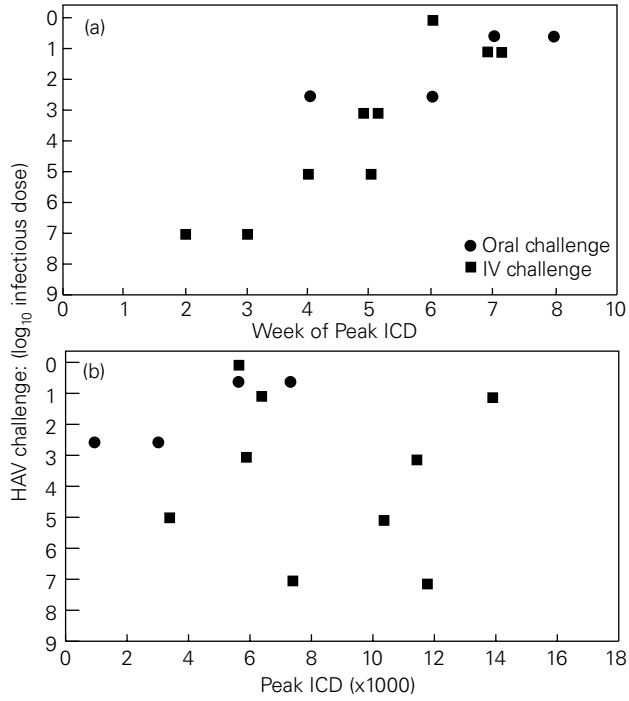


**Figure 8.4** Response of tamarins to oral or intravenous (IV) challenge with different doses of virulent hepatitis A virus. (a) Relationship between challenge dose and interval to peak serum isocitrate dehydrogenase (ICD) level. Challenge by the oral route required $10^{4.5}$ more virus to achieve an incubation period comparable to challenge by the intravenous route. (b) Relationship between challenge dose and magnitude of peak serum ICD level. There was no relationship between challenge dose and severity of hepatitis as measured by peak serum ICD level.[22]

## Course of HAV infection in animal models

Based on data obtained principally from tamarins, chimpanzees and owl monkeys, a picture of the pathogenesis and natural history of hepatitis A in non-human primates has emerged. Exposure of humans to HAV usually occurs via the oral route and such is probably the case for naturally infected non-human primates. However, the virus can be transmitted parenterally to all of these species. Ironically, HAV is more readily transmitted to non-human primates (at least tamarins and chimpanzees) by intravenous inoculation than by oral inoculation.[22] In fact, oral administration of HAV to these two species is 3200-fold less efficient than intravenous inoculation[22] (Fig. 8.4).

After exposure, the first evidence of infection usually is detection of virus in the liver.[220–225] Soon thereafter it can be detected in the blood, bile and faeces. A site of replication in the intestinal tract has been sought but never unequivocally found. Thus, viruses found in saliva, the pharynx and the intestinal tract probably reached those sites via replication in hepatocytes and excretion into the blood and into the bile.[218,220,221,223,226–228] Peak shedding of the virus into the blood and into the intestinal tract via the biliary tract occurs before onset of disease, which is almost always very mild in non-human primates.[30,218,229,230] In fact, there is only one report of serious, fulminant hepatitis in non-human primates (a chimpanzee).[231] Onset of clinical disease usually coincides with diminished replication of the virus and the first detection of the humoral immune response.[232] Thus, as in patients, non-human primates develop IgG and IgM anti-HAV at approximately the time of onset of liver damage, and this is taken as indirect evidence that hepatitis A is an immunologically mediated disease.[232] Viraemia may persist for approximately 1–3 weeks after onset of disease and faecal shedding may persist for 2–3 weeks longer, but both may persist much longer in more protracted infections.[27] Liver damage is detected by liver biopsy and by measuring serum levels of liver enzymes. The most useful enzyme measurement for hepatitis in

humans and chimpanzees is alanine aminotransferase. The most useful enzyme measurement for tamarins is isocitrate dehydrogenase. Histological changes in the liver are not unique to hepatitis A but resemble those seen during the acute phase of infection with the other hepatitis viruses.[146,159] Histological changes consist of focal necrosis, Kupffer cell proliferation and ballooning degeneration and apoptosis of hepatocytes. Necrosis and mononuclear cell inflammation of the periportal tract are seen more frequently in hepatitis A than in the other types of viral hepatitis. Heavy infiltration of inflammatory cells and bile retention are also features of hepatitis A in non-human primates. The severity of acute hepatitis A in non-human primates may range from mild, with focal necrosis, to moderate, with necrosis bridging portal tracts or joining portal areas and central veins. Severity of hepatitis A in non-human primates is not related to the infectious dose (Fig. 8.4).[22] More severe hepatitis is rarely seen in non-human primates but does occur in humans. Non-human primates are almost never symptomatic during experimental HAV infection, and all recover completely.

## Tamarins and marmosets

Tamarins comprise a group of more or less closely related species of small arboreal monkeys that are distributed in Central and South America. They vary in susceptibility to infection with HAV: *S. mystax* and *S. labiatus* (sometimes referred to as '*S. rufiventer*') have proven the most susceptible and *S. nigricollis, S. fuscicollis* and *S. oedipus* somewhat less susceptible. The true marmosets (*Callithrix* species) are still less susceptible to HAV infection but have been used for the study of HAV.[203,210,221,225,234–236]

## Chimpanzees

Chimpanzees are as sensitive to HAV infection as tamarins but have the advantage of being man's closest relative. Thus, chimpanzee proteins are very similar to those of man and reagents developed for studying immunological and other responses in man can be used for experimental studies in chimpanzees.[36,237,238] Similarly, chimpanzee immunoglobulins are closely related to human immunoglobulins, and chimpanzee monoclonal antibodies that neutralize HAV have been derived from combinatorial phage libraries prepared from bone marrow aspirates of chimpanzees convalescent from hepatitis A.[239] Such 'human' monoclonal antibodies may be useful for the prevention and treatment of hepatitis A in humans.

One discrepancy between humans and chimpanzees has been identified. Whereas infections with HBV or HCV or treatment with polyIC polyLC (an inducer of interferon) stimulate the production of type 1 interferon *in vivo* and, at least in HBV infections, this is associated with a demonstrable antiviral effect, the administration of human interferon to chimpanzees does not elicit a demonstrable antiviral effect. The reason for this is unknown.

## Owl monkeys

Locally caught owl monkeys that were introduced into a colony of these animals in Central America developed hepatitis A and a unique HAV strain was recovered from one such infected monkey.[240] The virus was subsequently shown to be endemic in the facility and most animals eventually became infected. Originally, the virus obtained from these animals was thought to be of simian origin but similar strains were subsequently recovered from humans.[240–244] Owl monkeys were shown to be susceptible to other strains of human HAV and the course of infection was similar to that seen in tamarins and chimpanzees.[230,245–247]

## Old World monkeys

The susceptibility of Old World monkey species to infection with HAV has been controversial. Some studies have shown various macaque species to be quite susceptible to infection with HAV, whereas other studies have found them to be sparingly susceptible.[33,248–253] Molecular characterization of strains of HAV recovered from such monkeys (often used for subsequent transmission studies) revealed them to be unique from human isolates.[248,254] Both molecular and epidemiological data strongly suggest that at least three such genetically distinct viruses are true simian strains of HAV.[243,255] Unlike human strains of HAV, these strains are readily transmissible to Old World monkey species and some New World monkey species, but they are sparingly infectious in chimpanzees.[256] Thus, Old World monkey species – principally rhesus, cynomolgus and possibly stumptailed macaques and African Green monkeys – have proven to be excellent animal models if a simian strain of HAV is employed, but the results are less predictable if human strains of HAV are used.

## Bushbabies

Lesser bushbabies (*Galago senegalensis moholi*) have been reported to be susceptible to HAV in transmission studies.[257,258] Clinical hepatitis was not observed but liver pathology, seroconversion, excretion of HAV antigen in the faeces and serum liver enzyme elevations resembled those in human hepatitis A. However, other studies have failed to confirm the susceptibility of bushbabies to

infection with HAV.[217] The susceptibility of this species awaits resolution.

### Guinea pigs

Recent *in vitro* studies demonstrated that, contrary to current dogma, HAV replication was not restricted to cells of primate origin. Thus, HAV replication was demonstrated in guinea pig cells.[259] To determine if HAV can be transmitted to guinea pigs, HAV that had been adapted to growth in monkey kidney cells and a variant that had been further adapted to growth in guinea pig cells were inoculated intraperitoneally or orally into guinea pigs.[260] Guinea pigs that were inoculated with mock-infected cell culture material served as controls. Most of the HAV-inoculated guinea pigs developed infection as measured by RT-PCR amplification of HAV RNA from faeces and/or serum, regardless of the virus or route of administration. Virus was detected as early as 14 days and as late as 52 days after inoculation. Serum liver enzyme tests and bilirubin levels remained normal but histopathological changes were detected in the liver, spleen and intestinal tract. However, none of the animals developed antibody to HAV and histological changes were seen in one or more of the control animals, making interpretation of the study difficult. Additional studies will be necessary to confirm the susceptibility of guinea pigs to infection with HAV.

### Applications

Evaluation of the safety and efficacy of hepatitis A vaccines has been an important use of non-human primates in hepatitis research. Such studies confirmed the effectiveness of inactivation procedures and protective levels of anti-HAV for inactivated vaccines and they have been essential for evaluation of the level of attenuation of candidate live vaccines.[261–264] More recently, they were essential for demonstrating that certain candidate live vaccine strains were genetically unstable *in vivo* and reverted to a virulence phenotype following transmission to tamarins.[14]

Non-human primate species are also important for studies of pathogenesis. Microarray analysis of the host global response to HCV infection has been reported. This revealed a very complex modulation of factors related to the innate and adaptive immune responses as well as to other metabolic pathways.[237,238] Undoubtedly, such molecular analyses of HAV infection of non-human primates will yield comparably illuminating results.

An important tool for evaluation for HAV infections in non-human primates was provided by the development of practical methods for transfecting animals with RNA transcripts of full-length cDNA clones of HAV strains. Such infectious cDNA clones of viruses provide a method for molecular engineering and analysis of the viral genome. For viruses that grow in cell culture, such clones can be tested for infectivity by transfecting susceptible cells.[265] However, for viruses that do not grow in cell culture, transfection must be performed *in vivo*. Initially, this method required laparotomy and direct inoculation of the cDNA or its RNA transcripts directly into the liver.[266] However, percutaneous intrahepatic transfection, guided by ultrasound, has become a simple and repeatable technique for testing the viability of cloned viral genomes and their molecularly modified variants.[14]

Molecular biology has not replaced animals for studies of pathogenesis but it has enriched such studies. New assays for quantifying messenger RNAs of host proteins, either singly or by microarray, and new single and multiplex assays for circulating cytokines and other host entities are yielding much more sophisticated analyses of *in vivo* studies than were feasible only a few years ago. Such studies will probably provide the first comprehensive understanding of pathogenic mechanisms in viral hepatitis.

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# Chapter 9
# Prevention

Beth P Bell

Hepatitis A can be prevented by three general approaches. Because the predominant mode of hepatitis A virus (HAV) transmission is by the faecal-oral route, good standards of hygiene and sanitation, including the appropriate disposal of human waste and provision of clean drinking water, are important contributors to preventing HAV transmission. These considerations currently are most relevant for the developing world, in some parts of which improvements in socio-economic and hygienic conditions have resulted in measurable shifts in the age-specific prevalence of HAV infection.[1–3] In the developed world, where transmission is usually from person to person, good hygienic practices, such as careful hand-washing, can contribute to preventing transmission in settings such as day-care centres and food service establishments.[4,5]

The most exciting new development in hepatitis A prevention in recent years is the availability of safe and effective hepatitis A vaccines for active immunization. In addition to protecting individuals at increased risk of infection, these vaccines, if used broadly, provide the opportunity to substantially lower disease incidence in populations. Indeed, recent evidence of such reductions has been reported from the United States and some other parts of the developed world in which hepatitis A vaccination of children has been implemented widely.[6–9] Because the existence of an animal reservoir has not been demonstrated and there is no chronic infection in humans, strategies of widespread vaccination afford the potential to ultimately eliminate HAV transmission.

A third approach that continues to have a role to play in the prevention of hepatitis A is passive immunization with immune globulin. With the widespread availability of hepatitis A vaccine to prevent hepatitis A before exposure, the most common indication for immune globulin is to provide passive protection to persons recently exposed to HAV (post-exposure prophylaxis), such as household contacts of hepatitis A cases. However, effective post-exposure prophylaxis requires recognition that an exposure has taken place, which occurs in only a minority of hepatitis A cases. In addition, the protection conferred by immune globulin is short-lived.

## Active immunization

Hepatitis A vaccines were developed in a manner similar to polio vaccines. The critical advance came when Provost and Hilleman developed a method for *in vitro* propagation of HAV in cell culture, and produced cell lines suitable for vaccine production.[10] Several years later, Binn and colleagues were the first to produce an inactivated vaccine from HAV propagated in cell culture.[11]

### Inactivated hepatitis A vaccines

A number of inactivated vaccines have been developed by commercial vaccine manufacturers. Two, Havrix (GlaxoSmithKline, Philadelphia) and Vaqta (Merck & Co. Inc., West Point, PA), are approved for use in the United States as well as much of the world.[12,13] Two other vaccines (Avaxim, Sanofi Pasteur, Lyon, France, and Epaxal, Berna Biotech Ltd, Bern, Switzerland) are available in Europe, Canada and selected other countries.[14,15] All are manufactured from HAV that has been propagated in cell culture and is subsequently purified and inactivated by exposure to formalin. All but Epaxal are adjuvanted by adsorption to alum; Epaxal uses a virosome adjuvant. The majority of this chapter will focus on the two vaccines available in the United States and also used widely in Canada and Europe, as these are the vaccines with the most available information and experience to date.

#### *HAV strains used in inactivated vaccines*

Vaqta contains the CR326F strain, initially isolated in Costa Rica and the first strain to be successfully cultivated *in vitro*.[10] CR326F was initially isolated in a fetal rhesus kidney line, FRhK6. After 15 passages, it was transferred to human embryonic lung diploid fibroblasts (MRC-5 cells) for an additional 28 passages.[12] Havrix contains the HM175 strain, isolated from the stool of a

patient in Australia.[16] It was originally adapted to cell culture by a series of 30 passages in green monkey kidney cells followed by adaptation to MRC-5 cells.[13] Avaxim contains the GBM strain of HAV, which was isolated and propagated in human kidney cell culture for 10 passages, followed by adaptation to MRC-5 cells during 20 passages.[17–19] Epaxal contains the RG-SB strain, grown and then harvested from disrupted MRC-5 cells.[14,20,21]

### *Constituents*

All available inactivated vaccines include HAV antigen, but the antigen content is not standardized and the units by which the antigen content is expressed are different for each vaccine. Because of the different assays used and the absence of an international antigen reference reagent, it is not possible to compare the antigen content among vaccines. Based on quantitative analysis of a highly purified standard, the antigen content of the formulation of Vaqta evaluated in early clinical trials was measured as 25 ng of viral protein, and subsequent vaccine lots with equivalent antigenic activity are considered by the manufacturer to contain 25 units (U) of HAV antigen.[22] The viral protein contained in Havrix is expressed in terms of enzyme-linked immunosorbent assay (ELISA) units (ELU), established by the manufacturer according to an in-house reference standard.[13]

Hepatitis A viral particles found in the inactivated vaccines include both 155S particles (intact virions) and 70S empty capsids. The 70S particles appear to have antigenic activity similar to that of wild-type virus.[23] There are differences in the amount of non-virion protein contained in the available vaccines, but these differences have not been found to be clinically relevant. Havrix and Avaxim contain 2-phenoxyethanol as a preservative; Vaqta and Epaxal are formulated without a preservative.

The antigens in Havrix, Vaqta and Avaxim are adsorbed to aluminium hydroxide (alum) as an adjuvant.[13,15,24] Epaxal uses a liposome adjuvant, immunopotentiating, reconstituted influenza virosomes (IRIV), composed of phosphatidylcholine, phosphatidylethanolamine and haemagglutinin from an H1N1 strain of influenza virus.[20,21] It is hypothesized that IRIVs may stimulate both hormonal and cellular immunity by binding to macrophages and other cells primed by influenza virus.[20]

### *Manufacture*

All inactivated hepatitis A vaccines are produced in similar ways. Most vaccines are grown in MRC-5 cell culture and harvested by cell lysis. The HAV in Havrix is concentrated and purified by sterile filtration, ultrafiltration and column chromatography. The antigen is then inactivated by 250 µg formaldehyde per mL for 15 days at 37 °C. The purified/inactivated virus is adsorbed on aluminium hydroxide, and each millilitre contains approximately 0.5 mg of aluminium as aluminium hydroxide.[13]

Vaqta is grown in MRC-5 cells, extracted by organic solvents, concentrated by precipitation in polyethylene glycol, and purified by chromatography. Following inactivation by 100 µg formaldehyde per mL for 20 days at 37 °C, the antigen is adsorbed on aluminium hydroxide, in a concentration of approximately 0.45 mg/mL of aluminium as aluminium hydroxide.[12]

Formalin inactivation conditions have been set empirically by determining the killing kinetics, extrapolating the curve to the zero intercept where 100% inactivation is theoretically achieved, and exceeding that time by a factor of three.[13,24] Because HAV grows slowly in cell culture without cytopathic effect, completeness of inactivation is difficult to prove. Inactivation of these vaccines has been demonstrated by serial blind passages designed to amplify a low level of residual live virus to the point that it would be immunologically detectable.[11]

### *Preparations and dosage (Table 9.1)*

Inactivated hepatitis A vaccines, with the exception of Epaxal, are available in paediatric and adult formulations. Epaxal is prepared in a single formulation. A combination inactivated hepatitis A and recombinant hepatitis B vaccine (Twinrix; Glaxo SmithKline) is available in the United States for persons ≥18 years old; a paediatric formulation is available in Europe, Canada and other parts of the world.

Havrix and Vaqta are licensed in the United States in two formulations, paediatric for persons aged 2–18 years, and adult for persons older than 18 years. Both formulations are given as a two-dose series.[25,26] In other countries, the paediatric formulations are licensed for children as young as 1 year old.[27] The paediatric dosage of Havrix is 720 ELU (0.5 mL) and the adult dosage is 1440 ELU (1.0 mL). Both vaccines are administered in two intramuscular injections, with the second dose given 6–12 months after the first. The paediatric formulation of Vaqta contains 25 U (0.5 mL), and the adult formulation contains 50 U (1.0 mL), with the second dose of each formulation given 6–18 months after the first.

The paediatric formulation of Avaxim consists of 80 'antigen units' and the adult of 160 'antigen units', each in 0.5 mL for intramuscular administration in a two-dose series. The package insert indicates that the paediatric formulation is recommended for children aged 1–15 years old, with the second dose being given 6 months after the first, and the adult formulation for

**Table 9.1** Recommended doses and schedules of inactivated hepatitis A vaccines

| Age (years)* | Vaccine | Dose | Volume (mL) | Number of doses | Schedule (months)† |
|---|---|---|---|---|---|
| 2–18 | Havrix® | 720 ELU | 0.5 | 2 | 0, 6–12 |
| | Vaqta® | 25 U | 0.5 | 2 | 0, 6–18 |
| ≥19 | Havrix® | 1440 ELU | 1.0 | 2 | 0, 6–12 |
| | Vaqta® | 50 U | 1.0 | 2 | 0, 6–12 |
| >15 | Avaxim®‡ | 160 antigen units | 0.5 | 2 | 0, 6–12 |
| 1–15 | Avaxim®‡ | 80 antigen units | 0.5 | 2 | 0, 6 |
| ≥2 | Epaxal®‡ | 24 IU | 0.5 | 2 | 0, 6–12 |
| 1–15 | Twinrix®‡ | 360 ELU | 0.5 | 3 | 0, 1, 6 |
| ≥18 | Twinrix® | 720 ELU | 1.0 | 3 | 0, 1, 6 |

ELU, enzyme-linked immunosorbent assay (ELISA) units; U, units; IU, international units.
*For vaccines licensed in the United States, US-licensed age groups for paediatric and adult formulations are indicated. Recommended age groups vary among other countries in which vaccines are licensed.
† 0 months represents timing of initial dose; subsequent numbers represent months after the initial dose.
‡Not licensed in the United States. Age group for which paediatric and adult formulations licensed varies among countries.

persons older than 15 years, with the second dose being give 6–12 months following the first.[28] However, recommended schedules may be different in various countries.[29]

The dose of Epaxal for adults and children >1 year old is 500 radioimmunoassy (RIA) units of hepatitis A antigen, associated with 10 µg of influenza haemagglutinin and 300 µg of phospholipids.[30] The recommended schedule is two doses at 0 and 6–18 months.

The adult formulation of the combination vaccine, Twinrix, includes 720 ELU of the hepatitis A component and 20 µg of the hepatitis B component in 1.0 mL, 0.45 mg of aluminium in the form of aluminium phosphate and aluminium hydroxide as adjuvants and 5.0 mg of 2-phenoxyethanol as a preservative.[31] The paediatric formulation consists of 360 ELU of the hepatitis A component and 10 µg of the hepatitis B component in 0.5 mL. Both are administered as a three-dose series, with the second and third doses given 1 and 6 months after the first. The vaccine's performance appears to be equivalent to that of each of the single antigen vaccines administered separately.[32–34]

Results of a number of studies indicate that the response of adults vaccinated according to a schedule that mixed the available alum-adjuvanted inactivated vaccines was equivalent to that of adults vaccinated according to the licensed schedules.[35–37] Mixed schedules that include the virosome vaccine have not been studied. Based on results of several studies, the response to a second dose delayed for 24–77 months appears to be equivalent to that after the licensed schedules.[38–41] Schedules with shorter intervals between doses have not been studied using the currently licensed single antigen formulations. Among persons vaccinated according to a three-dose schedule, all study subjects seroconverted, but the peak antibody concentrations were lower among persons vaccinated at months 0, 1 and 2 compared with persons who were vaccinated according to a schedule with a longer time interval between the second and third dose.[42,43] The response of persons vaccinated with the combined hepatitis A/hepatitis B vaccine on a four-dose schedule with doses at 7 and 21 days and 12 months after the first was equivalent to that of persons vaccinated with single antigen hepatitis A vaccine on a two-dose schedule, with the second dose given 12 months after the first.[44]

### *Vaccine stability*

The vaccines should be stored at 2–8 °C. Havrix and Vaqta have been shown to retain potency when kept for at least 2 years under those conditions.[25,26] The reactogenicity and immunogenicity of Havrix after storage at 98.6 °F (37 °C) for 1 week, and the stability profile of Vaqta when stored at this temperature for >12 months did not differ from those of vaccines stored at the recommended temperature.[6,45] Freezing destroys the vaccine, causing aggregation of the alum particles.

### *Immunogenicity*

In extensive studies in children and adults, the inactivated hepatitis A vaccines have been found to be highly immunogenic. The concentration of antibody after vaccination varies with the dose and schedule of the vaccine. However, after a single dose, antibody concentrations are higher than those produced by doses of immune globulin known to be protective. In general, by 4 weeks after one dose of vaccine, 95–100% of children 2 years of age or older and adults respond with concentrations of antibody considered to be protective.[46–58] A second dose 6–18 months later results in a boost in antibody concentration, but the final concentration is

generally lower than those measured after natural infection.[14,46,47,49,50,52,53,55,59–61] This boost in antibody concentration following the second dose is probably important for long-term protection.[14,47,53,55,59,60,62,63] IgM antibody to HAV (anti-HAV) can occasionally be detected by standard assays, primarily if measured soon (i.e. 2–3 weeks) after vaccination.[61,64,65]

Some studies have indicated that, in many subjects, concentrations of antibody above the defined protective level can be measured as early as 2 weeks after one dose.[56,57,62] When neutralizing antibody appears after vaccination is not clear from published reports, owing to differences among assays, dosages of vaccine, and when antibody measurements were obtained. In one study, depending on the assay, 42–100% of children vaccinated with the currently licensed formulation had neutralizing antibody when measured 1 month after a single vaccine dose.[65] In another study, approximately two-thirds of adults vaccinated with a lower dosage than the currently licensed formulation were positive for neutralizing antibody 4 weeks after one dose, and virtually all were positive 2 weeks after a second dose, given 1 month after the first.[67]

Studies in infants and children <2 years old suggest that the vaccine is safe and immunogenic, but the antibody response is blunted by the presence of passively transferred antibody from previous maternal HAV infection.[40,68–70] In studies of infants who received hepatitis A vaccine according to a number of different schedules, those with passively transferred maternal antibody at the time of vaccination responded, but final antibody concentrations were approximately one-third to one-tenth those of infants who did not have passively transferred antibody and were vaccinated according to the same schedule.[48,67,70,71] The clinical significance of these lower antibody concentrations is unclear. One study found that all infants vaccinated in the presence of passively transferred maternal antibody at ages 2, 4 and 6 months responded to a booster dose 6 months later with an anamnestic response, suggesting that they had been primed by the primary series.[72]

As the majority of infants born to anti-HAV-positive mothers have lost detectable antibody by 12–15 months of age, it is likely that any effect of passively transferred maternal antibody on the response to vaccination would be observed primarily when vaccination is begun in infancy.[73–76] This was illustrated by the results of one study in which two groups of infants – those whose mothers were anti-HAV-positive or anti-HAV-negative – were randomized to three different two-dose vaccination schedules, using a currently licensed paediatric formulation of vaccine and beginning the series at ages 6, 12 and 15 months of age. The majority of infants born to anti-HAV-positive mothers and randomized to receive the first dose of vaccine at 6 months of age had detectable antibody at the time of the first dose, and the final antibody concentration after completing the vaccine series was lower than that of the group of infants whose mothers were anti-HAV-negative and who were vaccinated according to the same schedule. In contrast, few infants born to anti-HAV-positive mothers had detectable antibody when administered the first dose of vaccine at either 12 or 15 months of age, and there was no difference in geometric mean concentrations (GMCs) after completing the vaccination series among these children compared to those whose mothers were anti-HAV-negative.[73]

Other conditions that may result in reduced immunogenicity include human immunodeficiency virus (HIV) infection, chronic liver disease and older age. In published reports of hepatitis A vaccination of men with HIV infection, approximately 50–75% had protective antibody concentrations after completing the vaccination series, and final antibody concentrations were considerably lower than those among HIV-negative persons.[77–79] Among HIV-infected men, higher CD+ T-lymphocyte count at baseline was associated with response to vaccination.[77,78] In one study, only one of nine participants with CD4 cell counts <200 at baseline responded to vaccination compared with eight of eleven participants whose baseline CD4 cell counts were ≥500.[78] However, even among participants with CD4 cell counts ≥500, the antibody concentration after completing the two-dose vaccination series was considerably lower than generally reported from studies of persons who do not have HIV infection. Among adults and children with chronic liver disease, seroprotection rates were similar to those observed among healthy adults, but the final antibody concentrations were substantially lower.[80–82] Limited data suggest that the final antibody concentrations achieved among persons >40 years may be somewhat lower than among younger individuals, but response rates were similar.[43,55,83,84] Other factors, such as smoking and obesity, have not been evaluated for the currently licensed formulations.

### *Correlates of protection*

Consideration of how anti-HAV is measured can be useful when interpreting the results of studies of the immunogenicity of hepatitis A vaccines. Anti-HAV concentrations are expressed in milli-International Units per millilitre (mIU/mL), measured in comparison to a World Health Organization (WHO) reference immunoglobulin reagent.[85] The concentrations of antibody achieved after passive transfer by immune globulin or active induction by vaccination are 10–100-fold lower

than those produced in response to natural infection, but the absolute lower limit of antibody needed to prevent HAV infection has not been determined. In one study, the geometric mean antibody concentration of vaccinated children who were protected against hepatitis A was 42 mIU/mL, similar to peak anti-HAV concentrations following receipt of immune globulin.[62,66,86] Concentrations of 10–20 mIU/mL, achieved about 1–2 months after administration of immune globulin, also are known to protect against hepatitis A. Results of *in vitro* studies using cell culture-derived HAV indicate that even antibody concentrations <20 mIU/mL can be neutralizing.[87]

Because no absolute protective antibody level has been defined, in immunogenicity studies generally the lower limit of detection of the particular assay being used has been considered to be the protective level. However, the WHO reference reagent contains in large part high affinity late convalescent antibody, while antibody that develops soon after vaccination appears to have a lower affinity for HAV.[66,85] Thus, caution should be exercised in comparing results of clinical studies of hepatitis A vaccines, as differences in quantitative antibody concentrations may be the result of the configuration of the particular assay being used, even if the WHO 'standard' was used to develop the assay. Clinical studies of Havrix have used levels >20 mIU/mL or 33 mIU/mL, as measured using enzyme immunoassays. Studies of Vaqta have used levels >10 mIU/mL as measured with a modified radioimmunoassay (HAVAB; Abbott Laboratories, N. Chicago, IL).[47,60,72]

Although the minimum level of antibody needed to protect against HAV infection is unknown, serological correlates of protection have been demonstrated using specialized assays, including a radioimmunofocus inhibition test (RIFIT) and an antigen reduction assay (HAVARNA), that measure neutralizing antibody, and a radioimmunoprecipitation assay (RIPA) that measures the capacity of antibody to immunoprecipitate metabolically labelled HAV particles.[66,87,88–90] In one such study, among children participating in two vaccine trials who were tested 4 weeks after receiving one dose of hepatitis A vaccine, the geometric mean titres (GMTs) of anti-HAV measured by HAVAB were 49.3 and 45.2 mIU/mL, but the reciprocal GMTs of neutralizing anti-HAV were 6.5 and 15.0 by RIFIT and 55.6 and 92.0 by HAVARNA, and the GMT of anti-HAV detected by RIPA was ≥401.[66]

Differences between the types of antibody induced by vaccine and those detected in people who received immune globulin (which should be similar to antibody induced by infection) can be identified when results using these specialized assays are compared.[66,67] In one study, when measured 4 weeks after administration, with equivalent titres measured by radioimmunoassay, adult immune globulin recipients had higher neutralization titres, as measured by RIFIT and HAVARNA, but negligible RIPA titres, compared with a group of children who received one dose of vaccine.[66] In another study, the neutralizing GMTs of persons who received immune globulin with hepatitis A vaccine were five times higher than those who received hepatitis A vaccine alone, when measured 4 weeks after administration, using the RIFIT assay.[67] These results provide further evidence that the antibodies produced by active immunization have lower affinity for HAV than those present after passive immunization.[66] However, it is clear that active immunization provides a high level of protection against hepatitis A (see the section on Efficacy below).

In contrast to the humoral immune response to hepatitis A immunization, which has been fairly well characterized, data are limited regarding cellular immune responses following immunization. That re-exposure to the antigen with a booster vaccine dose results in what has been considered to be an anamnestic response suggests that vaccine recipients have immune memory.[72,91,92] However, only a few studies have directly measured cellular immune response. In one study, seven of ten vaccinated adults had evidence of HAV-specific T-cell proliferation by the 10th week after vaccination, and such proliferation could be detected in all vaccine recipients by week 30.[93] Immunization also induced interferon-gamma (IFN-γ) in the three subjects studied. Among 36 vaccine recipients studied 72 months after completing the vaccine series, 19 had *in vitro* lymphoproliferative responses to HAV, and 24 had measurable IFN-γ.[94]

### *Safety*

Experience to date indicates that hepatitis A vaccine has a safety profile equivalent to that of other widely recommended vaccines. After vaccination, local injection site reactions (pain, tenderness or erythema) that are mild and transient have been reported in as many as 21% of children and 56% of adults. Systemic reactions that include fatigue, fever, diarrhoea and vomiting occur in <5% of vaccinees. Headache has been associated with vaccination in up to 16% of adults and 2–9% of children.[15,25,26,28,30,31,51,62,95–97]

Rare adverse events reported post-marketing include syncope, jaundice, erythema multiforme, anaphylaxis, brachial plexus neuropathy, transverse myelitis, encephalopathy and others.[25,26,98] No serious adverse events among children or adults have been identified that could be definitively ascribed to hepatitis A vaccine.[96,99] For events for which incidence rates are available, such as Guillan-Barré syndrome, reported rates were not higher than reported background rates.[6] Based

on limited data, vaccination of immunosuppressed persons does not appear to be associated with a greater risk of adverse events compared to persons with normal immune systems.[77,100]

### *Contraindications and precautions*

The inactivated hepatitis A vaccines should not be used in persons with a history of a severe reaction to a prior dose of hepatitis A vaccine or allergy or hypersensitivity to the vaccine or any of its components. The safety of the inactivated hepatitis A vaccines in pregnancy has not been determined. Because the vaccine is produced from inactivated HAV, the theoretical risk to the fetus is likely to be low.

### *Concomitant use with other vaccines*

Several studies that evaluated administration of inactivated hepatitis A vaccine concomitantly with immune globulin have shown that although the proportion of persons who responded to vaccination was not reduced by co-administration of immune globulin, the antibody concentrations elicited were lower than after receiving vaccine alone.[101–104] However, because the concentrations induced by vaccination far exceed that needed for protection, these reductions are not considered clinically significant. The use of hepatitis A vaccine with other vaccines that might be used for travellers has been studied among adults. There was no effect on either the immunogenicity or reactogenicity of hepatitis A vaccine administered concurrently with diphtheria, polio (oral and inactivated), tetanus, hepatitis B, yellow fever, typhoid (oral and intramuscular), cholera, Japanese encephalitis or rabies vaccines.[105–109] In one study among infants, simultaneous administration of hepatitis A vaccine did not affect the immunogenicity or reactogenicity of diphtheria-tetanus-acellular pertussis (DtaP), inactivated polio and *Haemophilus influenzae* type b (Hib) vaccines.[72]

### *Duration of immunity*

Antibody has been shown to persist in persons vaccinated as adults or children for at least 5–10 years after completion of the vaccination series.[92,110–115] Among children vaccinated during infancy, approximately two-thirds had detectable anti-HAV 4–6 years later.[91,116] It is estimated from mathematical models using data from adults that protective levels of antibody following completion of the vaccination series could persist for 20 years or longer.[110,112–114] Whether other mechanisms (e.g. cellular memory) also contribute to long-term protection is unknown. Because the incubation period for hepatitis A is relatively long and the anamnestic responses observed after the second vaccine dose are rapid and robust, it has been suggested that vaccinees who have seroconverted will be protected even if their antibody levels have fallen below protective levels.[63] However, few such vaccinated cohorts are available to date. In two studies of persons completing the vaccination series 2–6 years after receiving the primary dose, all of those who did not have detectable antibody at the time of the booster responded with a high antibody concentration.[39,117] In one follow-up study of infants who did not have passively transferred maternal antibody at the time of the primary series, all of those who had lost detectable antibody at the time of follow-up had an anamnestic response to a booster dose.[91] However, a small proportion of those who were vaccinated initially in the presence of passively transferred antibody and had lost detectable antibody at the time of follow-up did not have an anamnestic response to the booster dose.[91,116] In 9 years of follow-up, no hepatitis A cases have been reported among children vaccinated as part of a clinical efficacy study in one US community.[118] Additional long-term follow-up studies are needed to evaluate the long-term protective efficacy of hepatitis A vaccine and determine the need for booster doses.

### *Efficacy*

The high efficacy of inactivated hepatitis A vaccines in preventing clinically apparent disease was demonstrated in two double-blind, randomized field trials. In one study, 1037 healthy seronegative children 2–16 years of age in a community with high hepatitis A rates and periodic outbreaks, received either a single dose of 25 U of the inactivated vaccine derived from strain CR326F, or placebo. The mean follow-up period was 103 days. Beginning 50 days after injection, no cases of hepatitis A occurred in the vaccinated group compared with 25 cases in the placebo group, yielding an estimated 100% clinical efficacy, with a lower bound of the 95% confidence interval (CI) of 87%.[39] In the vaccinated group, no cases of hepatitis A occurred with onset ≥17 days after vaccination.[118]

In a large field trial of approximately 40 000 Thai children aged 1–16 years, the vast majority of whom were 3–14 years old, participants were randomized to receive either inactivated hepatitis A vaccine (HM175 strain) or a recombinant hepatitis B vaccine. Measurement began after the second of two doses (360 ELU per dose) administered 1 month apart was given and continued for approximately 1 year. A total of 40 hepatitis A cases was detected, 38 among the controls and two among the hepatitis A vaccine recipients, yielding a clinical efficacy of 94% (95% confidence interval, 79–99%).[97] The two cases among vaccine recipients occurred 257 and 267 days after receipt of the first dose of vaccine, and were relatively mild, with a mean peak

alanine aminotransferase level of 58 U/L and lasting a total of 2–4 days.

The efficacy of the inactivated hepatitis A vaccine formulated with a virosome adjuvant was evaluated in a randomized trial among 274 children aged 1.5–6 years in Nicaragua. Study participants received either a single dose (0.5 mL) of vaccine or a placebo consisting of a virosome suspension, and were followed beginning 1 week after vaccination and continuing for 18 months.[119] Using IgM anti-HAV positivity as the end-point, the efficacy of hepatitis A vaccine among susceptible children was 84.6% (95% CI, 54.7–96.1%). All infections among children in the vaccinated group occurred in the first 6 weeks after vaccination.

### *Efficacy after exposure to HAV*

Several lines of evidence suggest that hepatitis A vaccine may have some efficacy when administered after exposure to HAV, and public health agencies in Canada and several countries in Europe recommend hepatitis A vaccine in this setting.[29,120,121] Hepatitis A vaccine administered soon after exposure prevented infection in a chimpanzee model.[122] Only one small randomized trial in humans has been completed. Among persons aged 1–40 years who were household contacts of hospitalized hepatitis A cases, hepatitis A vaccine was found to be 79% efficacious compared with no treatment in preventing infection when given within 8 days of symptom onset of the index case.[123] However, the CI was wide (7–95%), and the study did not include a comparison group that received passive post-exposure prophylaxis with immune globulin.[124] Because of the demonstrated high efficacy of immune globulin when administered after exposure to HAV (see section on Passive immunization below) and in the absence of more definitive data comparing the efficacy of vaccine to that of immune globulin, immune globulin continues to be recommended by most US advisory groups for post-exposure prophylaxis.[6,125]

### *Effectiveness in populations*

When reviewing evidence of the impact of hepatitis A vaccination in populations, the epidemiology of hepatitis A in the particular population in which it is being used provides an essential context. Transmission patterns are influenced by many factors, including the age-specific prevalence of immunity, prevailing hygienic conditions, and how pervasive HAV is in the environment. It is also important to take into account whether the outcome of interest is control of a community-wide outbreak or a longer-term, sustained reduction in the occurrence of hepatitis A in the population.

#### *Populations with high hepatitis A rates*

A number of studies and demonstration projects have evaluated the effectiveness of hepatitis A vaccine in controlling and preventing hepatitis A in populations with high hepatitis A rates. Populations studied tend to be relatively small, homogeneous, usually rural communities. Examples include indigenous communities in North America and Australia and selected communities in central and eastern Europe and Israel.[7,118,126–132] Hepatitis A epidemics typically occur every 5–10 years in these communities. Few cases occur among persons >15 years old. Seroprevalence data indicate that 30–40% of children acquire infection by 5 years of age, and almost all persons have been infected by young adulthood.[118,127,129,131,133,134] Demonstration projects conducted soon after hepatitis A vaccines became available showed that vaccination of children living in these communities was feasible, and that vaccination of the majority of children, and in some cases adolescents and young adults, resulted in a rapid decline in disease incidence.[126,128,135] For example, a 1992–1993 community-wide epidemic among Alaska Natives in one rural area was ended within 4–8 weeks of vaccinating approximately 80% of children and young adults.[128]

The most extensive information about the impact of ongoing hepatitis A vaccination on hepatitis A rates in these populations over time comes from the United States. In the US vaccine efficacy study site, vaccination of successive cohorts of 2-year-old children has continued since completion of the study in 1991, and in 1998 it was estimated that 76% of 2–16-year-old children were immune. The periodic community-wide epidemics that occurred before vaccination was undertaken have disappeared, while such outbreaks continue to occur in similar neighbouring communities.[118]

In US American Indian and Alaska Native communities, following publication in 1996 of recommendations for routine vaccination of children living in areas with high hepatitis A rates, surveys indicated vaccination coverage of 50–80% among preschool and school-aged American Indian and Alaska Native children, suggesting that recommendations were being implemented.[136,137] National surveillance data demonstrate a dramatic decrease in hepatitis A incidence (Fig. 9.1).[136] By 2000, hepatitis A incidence among American Indians and Alaska Natives had declined by 97% compared with the beginning of the decade, and was lower than the overall US rate.[136] A decline of this magnitude has not been observed in the previous 30 years of surveillance, and suggests a fundamental alteration in hepatitis A epidemiology in American Indian and Alaska Native communities.

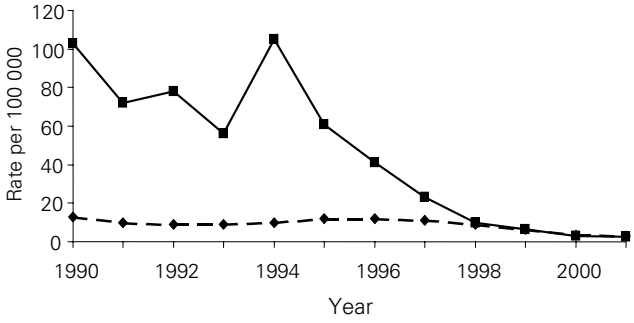


**Figure 9.1** Hepatitis A incidence, United States and Native Americans, 1990–2001. The solid line represents the rate among Native Americans and the dashed line represents the overall US rate (data from Bialek *et al*.[136]).

*Populations with consistently elevated rates*

In some countries, primarily in the developed world, large, heterogeneous communities with hepatitis A rates that are consistently elevated with respect to a national average can be identified. Examples include the Puglia region of Italy, Catalonia in Spain, and the western and south-western areas of the United States.[138–143] Periodic community-wide epidemics can be identified in these communities, but the inter-epidemic period is variable and the majority of the population remains susceptible to HAV infection into at least middle age.[138,139,141] There has been considerable interest in using hepatitis A vaccine to interrupt ongoing community-wide epidemics by vaccinating children in these populations, but the strategy has proven difficult to implement.[140] First-dose coverage has generally been low (20–45%) and the impact of vaccination often has been limited to vaccinated age groups, which may not represent the majority of cases. Because of logistical difficulties, accelerated vaccination to control outbreaks should be undertaken with caution, and this practice is not recommended by many public health authorities.[6,144,145] Efforts are probably better directed towards sustained routine vaccination of children to maintain high levels of immunity and prevent future epidemics.

Results of the longest demonstration project of ongoing, routine vaccination of children, conducted during 1995–2000 in Butte County, California, a community with consistently elevated rates, suggest that this strategy can markedly reduce hepatitis A incidence over time.[138] During the 5 years before the demonstration project, Butte County's average annual hepatitis A incidence rate was 47.9/100 000, and ranged from 122.5 to 11.8/100 000. From 1995 to 2000, children ≥2 years were offered hepatitis A vaccine without charge, and 66% of the almost 45 000 eligible children received at least one dose. The number of reported cases declined 94%, and the four cases reported in 2000 was the lowest number ever reported in the county since hepatitis surveillance began in 1966. Butte County's 2000 incidence rate of 1.9/100 000 was the lowest of any California county (Fig. 9.2).[138]

Several countries, including Italy, Spain and the United States, have implemented routine vaccination of children living in selected parts of the country, and since 1999 successive cohorts of 18-month-old children in Israel have been vaccinated.[6,7,29,141,146] Experience to date using hepatitis A vaccine to vaccinate children routinely suggests that considerable reductions in morbidity can be achieved with fairly modest vaccination coverage, a reflection, at least in part, of a strong herd immunity effect.[7,9,138]

In the Catalonia region of Spain, in September 1998 hepatitis A vaccine was added to an ongoing programme to vaccinate 11–12-year-old children with hepatitis B vaccine, by changing to the combination hepatitis A/hepatitis B vaccine. With an estimated vaccination coverage of 94% in the vaccinated cohorts, the overall average

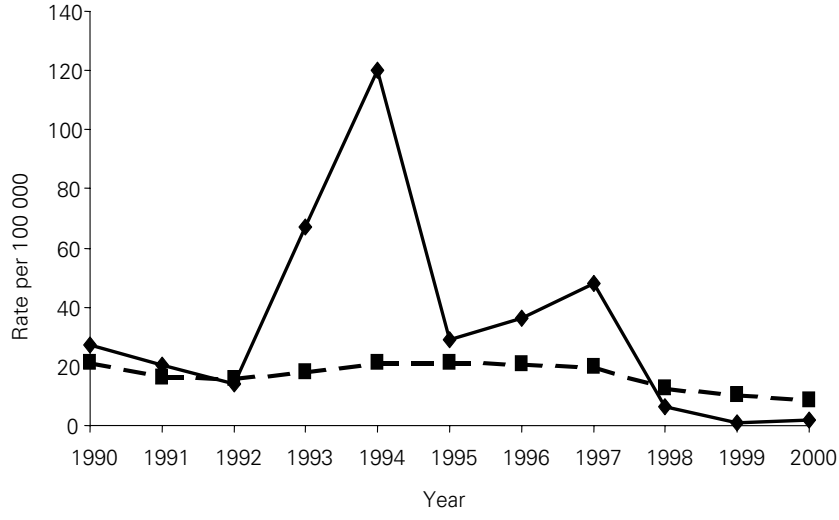


**Figure 9.2** Hepatitis A incidence, Butte County, California, and all of California, 1990–2000. The solid line represents the rate in Butte County, and the dashed line represents the overall rate in the state of California (data from Averhoff *et al*.[137]).

hepatitis A rate in the region declined from 6.2/100 000 during the 3 years before implementation of the vaccination programme to 2.6/100 000 during the subsequent 3 years. The greatest decline occurred in the vaccinated age group, children aged 10–14 years, in which the average rate fell from 10.3/100 000 to 1.8/100 000.[8]

In the United States, vaccination of children living in the western and south-western parts of the country has been recommended since 1999 (Table 9.2).[6] These areas include approximately one-third of the US population, but historically accounted for approximately one-half of hepatitis A cases nationwide.[6] Fairly low vaccination coverage of children in these areas appears to have resulted in appreciable declines in hepatitis A rates (Fig. 9.3).[147] Compared with the average 1987–1997 rate of 22.4/100 000, the 2001 rate in these states declined by approximately 80% to 4.6/100 000, compared with a 39% decrease from 5.6 to 3.6/100 000 elsewhere.[9,147] Rates declined most dramatically among children 2–18 years old. These precipitous declines are reflected in overall national hepatitis A rates. The 2001 overall rate of 3.7/100 000 is a historic low. Because hepatitis A incidence is cyclic in the United States, the precise contribution of vaccination of children to the observed decline in rates is difficult to quantify. However, modelling studies suggest that between 1995 and 2001, an estimated 97 800 hepatitis A cases were averted because of immunization, including 39% of potential cases in 2001.[9]

### *Recommended uses*

Recommendations for the use of hepatitis A vaccine vary considerably among countries. The World Health Organization position on hepatitis A vaccines provides general guidance, including an indication that consideration of the cost-benefit and sustainability of various prevention strategies in the context of the relevant hepatitis A epidemiology is important.[27] In general, hepatitis A vaccination currently has little relevance in countries in the developing world in which hepatitis A is highly endemic and the vast majority of the population develops HAV infection in early childhood.[1] In more developed, industrialized countries, hepatitis A vaccine is being used most widely to protect individuals at increased risk of hepatitis A or its consequences, such as travellers to areas where hepatitis A is endemic.[6,29,119,148,149] However, in some of these countries, such as the United States and Italy, the epidemiology of hepatitis A is quite heterogeneous, and in certain parts of the country large community-wide epidemics occur.[139,150] Areas with consistently elevated rates with respect to the national average can be identified and these areas have been the focus of routine hepatitis A vaccination programmes for children (Table 9.2).[6,141]

#### *Vaccination of children*

Recommendations for routine vaccination of selected populations of children have been made in Israel, Italy and selected other European countries, the United States, and a number of other countries.[6,7,29,125,141,146] In the Puglia region of Italy, a programme to vaccinate 15–18-month-old children with hepatitis A vaccine when they receive MMR vaccine and 12-year-old children when they receive hepatitis B vaccine (using the combination hepatitis A/hepatitis B vaccine) was implemented in 1997.[141] In Israel, all 18-month-old children have been

**Table 9.2** US recommendations for routine pre-exposure use of hepatitis A vaccine*

| Group | Comments |
|---|---|
| Children living in communities with consistently elevated hepatitis A rates | Includes Alaska, Arizona, California, Idaho, Nevada, New Mexico, Oklahoma, Oregon, South Dakota, Utah, Washington and selected areas in other states†‡ |
| International travellers§ | Immune globulin may be given in addition to or instead of vaccine; children <2 years old should receive immune globulin |
| Men who have sex with men | Includes adolescents |
| Illicit drug users | Includes adolescents |
| Persons with chronic liver disease | Increased risk of fulminant hepatitis A with HAV infection |
| Persons receiving clotting factor concentrates | |
| Persons who work with HAV in research laboratory settings | |

*Hepatitis A vaccine is not licensed in the United States for children <2 years old.
†Where the average reported hepatitis A incidence during 1987–1997 was ≥20/100 000 population (approximately twice the national average).
‡Routine vaccination can also be considered for children living in Arkansas, Colorado, Missouri, Montana, Texas, Wyoming and selected areas in other states where the average reported incidence during 1987–1997 was ≥10/100 000 population but <20/100 000.
§Persons travelling to Canada, Western Europe, Japan, Australia or New Zealand are at no greater risk than in the United States.
From US Centers for Disease Control and Prevention.[6]

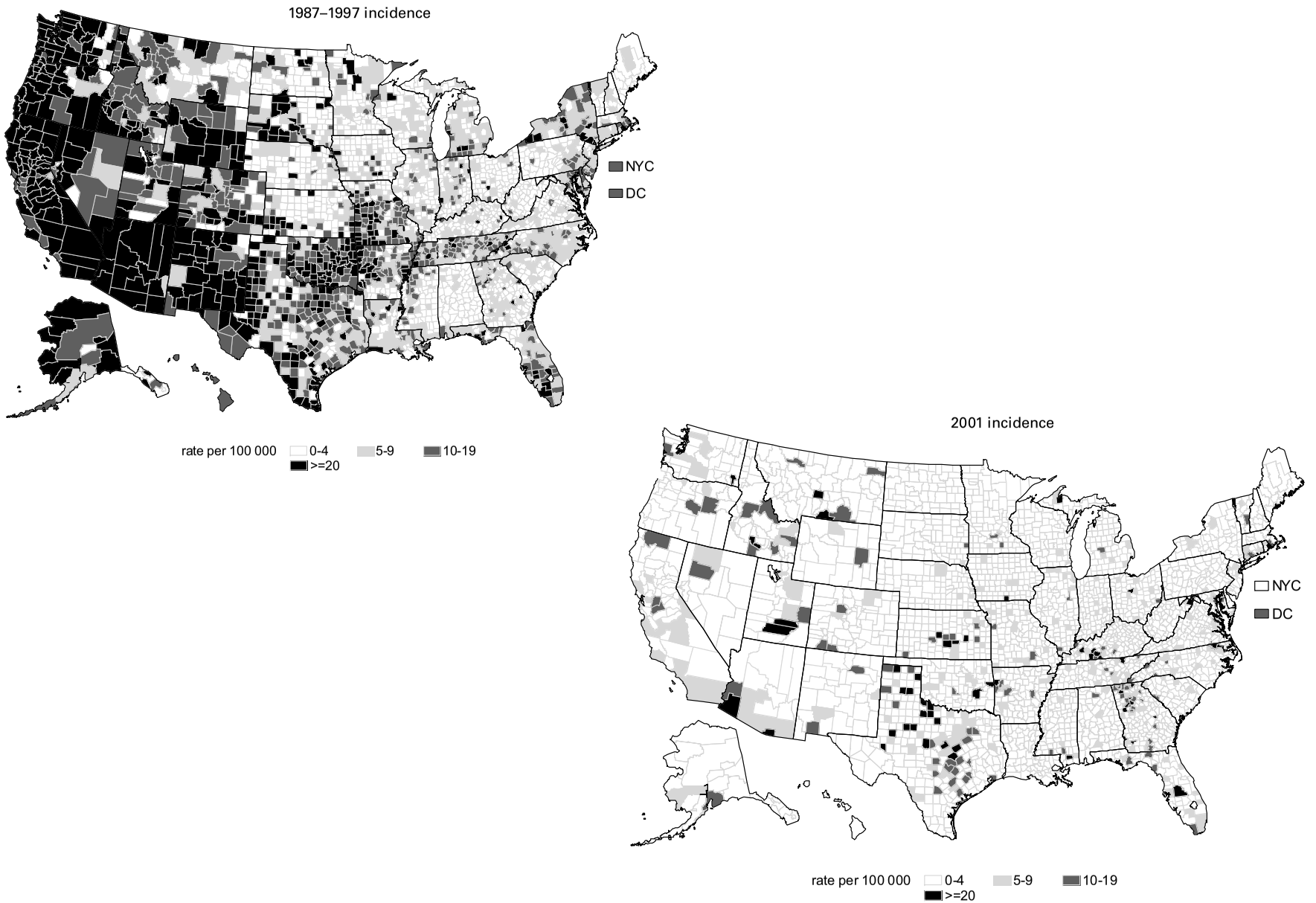


**Figure 9.3** Hepatitis A incidence rates, by county, United States, 1987–1997 (top) and 2001 (bottom) (data from US Centers for Disease Control and Prevention[147]).

vaccinated each year since 1999.[7] In the United States, in 1999 the Advisory Committee on Immunization Practices (ACIP) of the US Public Health Service, the American Academy of Pediatrics (AAP) and other groups recommended routine vaccination of children living in areas where rates of hepatitis A have been consistently elevated, beginning at or after 2 years of age (Table 9.2). These areas include 17 states, located primarily in the western and south-western United States, and comprise approximately one-third of the US population, but historically accounted for approximately one-half of hepatitis A cases nationwide.[6]

*Persons at increased risk of hepatitis A or severe consequences*

Persons from the developed world who travel to countries where hepatitis A is of high, transitional or intermediate endemicity should receive hepatitis A vaccine (Table 9.2).[6,27,63,148] Immune globulin can be used if hepatitis A vaccine is contraindicated or refused. A considerable proportion of these travellers are children, who may acquire hepatitis A while visiting family in the developing world, and transmit it to others when they return.[151–153] Other groups at increased risk of infection for whom hepatitis A vaccination is recommended in the United States and in some other countries include adolescent and adult men who have sex with men and persons who use illegal drugs, because of recurrent hepatitis A outbreaks among persons who report these behaviours; members of the military, persons who work with HAV in research settings; and persons who have clotting factor disorders.[6,29,148,154–156] Vaccination is also recommended for persons with chronic liver disease because of the high case-fatality rate among these persons if they acquire hepatitis A.[6,157–159]

*Other groups and settings*

Although hepatitis A outbreaks occur in child-care centres, in most countries their frequency is not high enough to warrant routine vaccination of attendees or staff to prevent them, and there is little experience of using vaccine to control outbreaks when they occur.[160–162] When outbreaks are recognized, aggressive use of immune globulin is effective in limiting transmission.[163] In areas where routine vaccination of children is recommended, previously unvaccinated children can be vaccinated when they receive post-exposure prophylaxis with immune globulin.[6] In addition, child-care centre attendees can be a readily accessible target population for ongoing routine vaccination programmes.

The frequency of outbreaks in hospitals, institutions and schools is not high enough to warrant routine vaccination of persons in these settings, and there are no data with respect to using vaccine to control outbreaks in these settings. Persons who work as food handlers are not at increased risk of hepatitis A because of their occupation, and vaccination of food handlers has not been shown to be cost-effective.[4,164]

Prevaccination serological testing can be considered to reduce costs by not vaccinating persons with prior immunity. In the developed world, this might include older adolescents and adults born in areas of high hepatitis A endemicity. However, the cost of testing, vaccine cost and the likelihood that the person will return for vaccination should be taken into account.[165,166] In the developed world, prevaccination testing of children is generally not cost-effective. Vaccination of immune people is not harmful. Post-vaccination testing is not indicated because of the high rate of vaccine response. Furthermore, testing methods that can detect the low anti-HAV concentrations generated by immunization are not widely available.

## Alternative approaches to vaccine development

### *Attenuated vaccines*

In theory, attenuated vaccines might have advantages over inactivated vaccines, including potential oral administration, lower cost, fewer doses and possibly a longer duration of immunity. A number of candidate vaccines, including variants of the CR326 and HM175 strains of HAV, have been tested in primates and humans and shown to be safe when given orally or parenterally.[167,168] An attenuated H2 strain vaccine was developed and has been used fairly widely in China, particularly among children.[169–171]

Using the HM175 strain, a full-length infectious cDNA was assembled and a full-length RNA was transcribed from the cDNA that on transfection into permissive cells resulted in complete HAV replication.[172] Many of the mutations responsible for cell culture adaptation and attenuation have now been identified, and appear to accumulate especially in the 5′ non-translated and P2 regions of the genome.[173,174] However, to date, a virus with the ideal characteristics of *in vitro* growth combined with attenuation and immunogenicity in humans has not been produced.[175–177] Unfortunately, the vaccines studied do not induce a satisfactory immune response when given orally, and thus must be given by parenteral injection.[168,178] These vaccine candidates also appear to replicate poorly in humans or chimpanzees, although studies have demonstrated limited faecal shedding of virus following inoculation.[167,179–181] Antibody responses to attenuated vaccines generally have been found to be above those considered pro-

tective, but considerably lower than those measured in recipients of a complete course of inactivated vaccine.[168,169] Whether T-cell responses are also induced is unknown. The efficacy of the CR326/F′ and HM1275 vaccine candidates has not been evaluated in humans. Based on very limited data, the H2 vaccine appears to be immunogenic and to provide clinical protection for Chinese children.[6,171,182]

Two concerns with respect to adverse events related to attenuated vaccines warrant mention. Mild hepatitis has been demonstrated in a small proportion of adults and children participating in clinical trials of the CR326/F′ and H2 strain vaccines, and the worry is that with more widespread use, more severe hepatitis could be observed.[168,169] A second concern, as with any attenuated vaccine, is the potential for the virus to revert to a virulent phenotype. The only available relevant data to date indicate that cell culture-adapted viruses have a stable attenuation phenotype when passed up to three times in primates.[181,183]

### *Recombinant vaccines*

The important neutralizing epitopes of HAV appear to be conformational.[184] For this reason, immunization with synthetic peptides or expressed capsid proteins has not induced an effective neutralizing antibody response.[185–187] Because of the conformational nature of neutralization epitopes, the approach has been taken of expressing the entire open reading frame of the HAV genome in recombinant vaccinia virus or baculovirus expression systems.[187–189] Complete or partial capsid assembly seems to occur in cells infected with the recombinant vaccinia, and antibodies raised to these purified HAV synthetic capsids are neutralizing *in vitro* and protective in animals. Although the immunizing effect of such a vaccine might not be greater than that of inactivated vaccines, theoretically production costs might be lower. However, these types of vaccines have undergone little further development since the early 1990s.

## Passive immunization

Until the licensure of hepatitis A vaccines, immune globulin was the mainstay of prevention of hepatitis A for people who either were likely to be exposed or had recently been exposed to HAV. Over several decades, this product has proven useful for prevention of hepatitis A in travellers, Peace Corps volunteers, military personnel, individuals recently in close contact with a person with hepatitis A, and to control outbreaks in child-care centres.[190–193] For example, the rate of HAV infections among Peace Corps volunteers dropped from 1.6–2.1 cases per 100 per year to 0.1–0.3 cases per 100 per year after the institution of a mandatory programme of immune globulin every 4 months.[191]

### Efficacy

In general, when administered before exposure or within 2 weeks after exposure, immune globulin is >85% effective in preventing hepatitis A.[194–196] Its efficacy was first demonstrated during an outbreak in a summer camp in 1944, when 6% of immune globulin recipients developed hepatitis compared with 45% of persons who did not receive it, an 87% reduction in the attack rate.[196] Similar reductions in hepatitis attack rates were observed among adults in the military and children in institutional settings who received immune globulin. The lowest effective dose and duration of protection were defined in studies that compared various immune globulin doses conducted during the 1950s and 1960s at the Willowbrook State School and other settings where hepatitis was common.[194–198] Results of a number of studies showed that a dose of 0.05–0.06 mL/kg provides protection for 4–6 months. The efficacy of lower doses has not been studied as completely, but it is generally thought that a dose of 0.02 mL/kg protects for about 3 months.[192,194,199–201] Immune globulin has not been effective in controlling community-wide hepatitis A epidemics, largely because its use requires timely recognition of exposure and most infections that occur during these epidemics are not recognized.[143,202,203] Furthermore, the protection afforded by immune globulin is temporary.

Whether immune globulin completely prevents infection or results in asymptomatic infection and the development of persistent anti-HAV (passive-active immunity) probably is related to the amount of time that has elapsed between exposure and immune globulin administration, as well as the concentration of anti-HAV achieved by the immune globulin dose, and the size of the HAV innoculum.[196,204] Evidence of both phenomena has been reported. For example, the results of several studies suggest that the proportion of HAV infections that are anicteric is higher among persons who receive immune globulin.[191,200] However, other studies have shown a very low rate of HAV infection among recipients of immune globulin.[192,205]

### Antibody levels after receiving immune globulin

It is the circulating anti-HAV that results from administration of immune globulin that provides passive protection against hepatitis A. A WHO International Reference Reagent for HAV Immunoglobulin provides a way to standardize anti-HAV levels, and has been used to compare the anti-HAV concentrations of immune globulin lots as well as measure the immune response to passive

or active immunization.[85] One millilitre of this immune globulin preparation has been assigned a value of 100 IU, and the lower limit of detection of commercially available immunoassays, such as HAVAB, is approximately 100 mIU/mL.

Following immune globulin administration, anti-HAV is not detectable by commercially available assays.[206] Studies suggest that peak serum concentrations of anti-HAV following receipt of standard immune globulin doses (0.02–0.06 mL/kg body weight) are approximately 20–45 mIU/mL, when measured using modified immunoassays, such as the modified HAVAB.[62,66,181] In view of the length of protection afforded by a single dose of immune globulin, it is likely that the minimum protective antibody level is extremely low, possibly below a level that could be reliably detected by any assay.

## Manufacture and safety

Immune globulin is a sterile solution of antibodies prepared by a serial cold ethanol precipitation procedure from large pools of plasma, collected from tens of thousands of donors, that has tested negative for hepatitis B surface antigen (HBsAg), antibody to HIV, and antibody to hepatitis C virus (HCV).[207,208] This precipitation procedure has been shown to inactivate HBV and HIV.[207] Since 1995, immune globulin prepared in the United States has been required to be negative for HCV RNA by PCR amplification, or to be produced using a method that ensures additional virus inactivation.

Serious adverse events from immune globulin are rare. Because anaphylaxis has been reported after repeated administration to persons with immunoglobulin A deficiency, these persons should not receive immune globulin.[209] Pregnancy or lactation is not a contraindication to immune globulin administration. For infants and pregnant women, a preparation that does not include thimerosal is preferable.

Commercial lots of immune globulin contain variable concentrations of anti-HAV. In the United States, at least, there is no standard for anti-HAV levels in immune globulin preparations. Because the prevalence of antibody to HAV in the population has been declining, a concern has been raised that declining anti-HAV concentrations in immune globulin preparations might reduce its effectiveness. However, in the United States, no evidence of this has been identified to date.[210]

## Indications

Immune globulin is recommended for post-exposure prophylaxis in selected settings at a dose of 0.02 mL/kg, if it can be administered within 2 weeks of last exposure to HAV. If hepatitis A vaccine is also indicated, the series can be initiated simultaneously with the immune globulin dose. Household and sexual contacts of patients with hepatitis A should receive immune globulin as soon as possible, but no later than 2 weeks after exposure.[6] Casual contacts such as school classmates or co-workers who have not had close physical contact usually do not require immune globulin prophylaxis. Aggressive use of immune globulin is indicated to control hepatitis A outbreaks in child-care centres where a child or employee is diagnosed with hepatitis A and in other settings (e.g. hospitals, facilities for developmentally disabled persons) when outbreaks occur.[5,6] When a food handler is identified with hepatitis A, US recommendations call for immune globulin to be administered to other food handlers at the food establishment and under limited circumstances to patrons.[4,6] Once cases are identified that are associated with a food service establishment, it generally is too late to administer immune globulin to patrons because the 2-week post-exposure period during which immune globulin is effective will have passed.

Immune globulin also may be used for pre-exposure prophylaxis for persons who are travelling to countries with high, transitional or intermediate hepatitis A endemicity, particularly those whose departure is imminent (e.g. within 2–4 weeks), instead of or in addition to hepatitis A vaccine.[6] Immune globulin should be given to travellers younger than the minimum age for which hepatitis A vaccine in licensed (e.g. 2 years of age in the United States), to prevent the rare severe cases that occur and transmission to others after returning from abroad.[6] Economic analysis studies have shown that, in general, hepatitis A vaccine becomes more cost-effective than immune globulin as the number of expected trips during a 10-year period involving exposure to HAV or the duration of each trip increases.[211,212] Immune globulin is also recommended for pre-exposure prophylaxis for anyone with known allergy to the vaccine or a component.

The usual dose of immune globulin is a single intramuscular injection of 0.02 or 0.06 mL/kg. The lower dose is adequate to provide protection for up to 3 months and the higher dose is effective for up to 5 months.[210] Re-administration every 5 months is necessary for extended trips, and hepatitis A vaccine, if not contraindicated, is a better choice for such travellers. Intramuscular preparations of immune globulin should never be given intravenously, and the intravenous preparations of immune globulin are not intended for hepatitis A prevention and are formulated at a lower globulin concentration.

## Future directions in hepatitis A prevention

Experience to date has shown the inactivated hepatitis A vaccines to be highly efficacious and effective in preventing hepatitis A in individuals and in reducing disease incidence in communities. In areas, primarily in the developed world, in which the vaccine has been used with the latter objective in mind, a strong herd immunity effect has been observed, as evidenced by marked declines in incidence with relatively modest vaccination coverage. More widespread vaccination of successive cohorts of children would sustain and extend these observed reductions, providing the opportunity to eliminate HAV transmission. A determination of the cost-effectiveness, feasibility and acceptability of this strategy is needed to inform public policy on this issue.

In the developing world, most would agree that hepatitis A vaccine is not necessary for those countries with high endemicity where infection in early childhood is nearly universal and disease is uncommon. In these areas, reductions in the incidence of HAV infection will accompany improvements in hygienic and living conditions. However, in countries with transitional or intermediate endemicity, a growing proportion of older children, adolescents and adults in selected areas, such as urban centres with good water and sanitation facilities, are susceptible to HAV infection. As the inevitable community-wide epidemics occur, interest in more widespread use of hepatitis A vaccine in these areas may increase. The availability of lower cost inactivated vaccines or alternatives to the inactivated vaccines that offered an economic advantage would make such strategies more attractive in terms of cost-effectiveness and feasibility.

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# Section IV
# Hepatitis B Virus and Other Hepadnaviridae

# Chapter 10
# Structure and molecular virology

Michael Kann, Wolfram H Gerlich

Hepatitis B virus (HBV) was the first human hepatitis virus from which the proteins and genome were identified and characterized. Before discovery of the viruses, two types of hepatitis transmission were differentiated on the basis of epidemiological observations: type A was considered to be predominantly transmitted by the faecal-oral route, whereas type B was transmitted parenterally. In 1963, Blumberg and colleagues, in a search of polymorphic serum proteins, discovered a previously unknown antigen in the blood of an Australian Aborigine (Australia antigen). Four years later, it was recognized that the appearance of this antigen was related to type B hepatitis.[1] Using immune electron microscopy, Dane and co-workers eventually discovered virus-like particles that carried this antigen on their surface, in the serum of hepatitis B patients,[2] and these particles were considered to be the hepatitis B virus. Further unrelated viruses that caused parenterally transmissible hepatitis in humans were discovered subsequently, but HBV retained its name. In 1973 the viral nature of the particles discovered by Dane was confirmed by the detection of an endogenous DNA polymerase activity within their core.[3] This enzyme allowed Robinson *et al*.[4,5] to detect and characterize the HBV genome as a small, circular partially double-stranded DNA molecule.

HBV infection was known to result in acute and chronic liver disease and, based on epidemiological data, it was postulated as early as the 1970s that this virus might represent a cause of liver cancer. This led to a search for an HBV-like agent in woodchucks (marmot-like animals from North America), which had been observed to develop liver cancer. Woodchuck hepatitis virus (WHV) was discovered subsequently[6] and a similar virus was found in seemingly healthy ground squirrels (GSHV),[7] and arctic ground squirrels,[8] which are both distantly related to marmots. In primates, hepadnaviruses could be isolated from Old World and New World monkeys including chimanzees, orang-utans, gorillas, gibbons, woolly monkeys.[9] More distantly related hepadnaviruses were found in avians including Pekin ducks (duck hepatitis B virus, DHBV),[10] grey herons (HHBV),[11] Ross' goose (RGHBV), snow goose (SGHBV),[12] white storks (STHBV)[13] and cranes (CHBV).[14]

HBV and its relatives throughout the animal kingdom comprise a virus family termed *Hepadnaviridae*, the name derived from their hepatotropism and DNA genome.[15] The hepadnaviruses of animals are currently not of particular interest to veterinary medicine, but they serve as important models for human HBV. A highlight was the discovery of reverse transcription of viral RNA into DNA within core particles of DHBV as an essential step of the viral genome replication.[16] This replication strategy is common in retroviruses. Thus, hepadnaviruses, badnaviruses and caulimoviruses (two plant virus families), which use a similar type of replication, are also termed pararetroviruses in contrast to orthoretroviruses, which have an RNA genome.

## Biology of *Hepadnaviridae*

### Immune pathogenesis

To understand the biology of hepadnaviruses, it is necessary to note that the wild-type members of this virus family are not highly cytotoxic *per se*, certainly not to the extent that massive cell death occurs. Acute and chronic hepatitis B are the result of the adaptive immune defence mechanisms against HBV (Chapters 18 and 19), which result in the death of HBV protein-expressing cells. Immunologically deficient or immature individuals, such as newborns, do not develop a typical hepatitis, but they become chronic carriers of the virus in >90% of individuals. In this case, the virus continues to replicate in hepatocytes and, to a small extent, in other sites, causing HBV reinfection after liver transplantation of HBV-infected individuals.

### Viraemia

The infected hepatocytes secrete complete virus particles (virions) resulting in concentrations of up to $10^8$–$10^{10}$ particles per millilitre of serum. The great majority of

the virions in the blood are not cell-bound, but replication was reported in a small number in certain subpopulations of leukocytes and primary bile duct epithelial cells.[17,18] The significance of the persistent viraemia for the virus appears obvious. If these viruses are unable to replicate in cells of the mucosa, they require the bloodstream of the host to leave the site of replication as well as to reach it in a newly infected individual. Blood-to-blood contact is not common between individuals and may lead to exchange of only minute amounts of serum. Thus, long-lasting, high-level viraemia seems to be essential for the survival and spread of hepadnaviruses in the population of its host species.

## Host range

Typical for hepadnaviruses is their narrow host range. Human HBV infects only higher primates such as chimpanzees and, surprisingly, primary hepatocytes of *Tupaia belangeri*.[19,20] The hepadnaviruses of marmot-like animals do not infect other rodents and even the duck virus, DHBV, does not infect all species of ducks. However, the crane HBV seems to have a broader host range including ducks.[14] Important transmission-effective contacts such as sexual activity, squabbles and similar activities would usually occur between individuals of the same species. Thus, a wide host range would not be useful for a virus that is transmitted by blood, unless a vector is involved. It must be noted, however, that replication of hepadnaviruses in arthropods has never been reported, nor have hepadnaviruses been demonstrated in insects. Hepadnaviruses are relatively stable against heat and therefore can maintain infectivity while circulating in the blood. As enveloped viruses having a lipid shell they appear to be sensitive to detergents and additionally to proteases. Thus, passage through the bile to the faeces, if it occurs at all, would not lead to spread of infection as it does so efficiently for hepatitis A virus.

## Antigenaemia

The unusually high level of viraemia is accompanied by antigenaemia. In the typical high-viraemic individuals of all host species, the hepadnaviral surface (HBs) proteins are present at concentrations of 10–1000 µg/mL serum. The HBs proteins are secreted by the infected cells as particles of variable morphology and size (see below), which exceed the number of virions 100–10 000-fold. Moreover, it appears that all hepadnaviruses are able to induce secretion of their core protein (HBc protein) in a non-assembled soluble form that is referred to as HBe antigen. This molecule reaches such high concentrations that it can be detected by a technique as insensitive as agar-gel immunodiffusion, indicating levels of 10 µg/mL and more.[21] The origin and meaning of the 'e' in the name of this protein are an enigma.

## Immune tolerance

It appears that even in immunologically competent individuals who are able to resolve the infection, the onset of the adaptive immune response is delayed by several weeks. However, among HBV-infected humans with seemingly normal immune systems, approximately 5% never develop an effective cytotoxic immune response and proceed directly to persistent infection with high viraemia and antigenaemia. The mechanisms by which hepadnaviruses suppress or escape immune elimination are not fully known, but the HBeAg may play a crucial role in viral persistence. It appears that variability of the surface proteins, as occurs in hepatitis C virus (see Chapters 19 and 21), does not play a major role during the high-viraemic phase. Several serological subtypes and genotypes of HBV are recognized, but variability within these types is very low, even at those genomic sites known to vary most between genotypes.[22] Infection of immune cells has been described,[23] but a general decrease of immune functions has not been observed in HBV carriers.

## Immune complex disease

High-dose tolerance induction may be one possible function of the viral proteins that are excessively produced and secreted. However, this immune tolerance is not complete, as circulating immune complexes of HBsAg or HBeAg and their antibodies are often detectable in viraemic carriers[24] that may lead to immune complex diseases such as glomerulonephritis, periarteritis nodosa and acrodermatitis.

## HBe seroconversion

In HBV carriers, a state of low-level viraemia may often develop after elimination of HBeAg from the blood, whereas HBsAg persists to circulate in the blood. Disappearance of HBeAg seems to indicate that HBc protein-expressing cells are no longer tolerated by the immune system. Once most of the HBV-producing cells are eliminated, the state of 'healthy' HBsAg carrier is reached. However, some latent HBV genomes seem to remain and are able to reactivate infection when the immune system is weakened. Certain variants that cannot synthesize HBeAg may be selected out during immune pathogenesis and lead to considerable viraemia. Such variants are often found in fulminant hepatitis or chronic hepatitis B without HBeAg (see Chapter 11) and occur during treatment of hepatitis B infection with interferon (see Chapter 16). In contrast to HBeAg-positive strains,

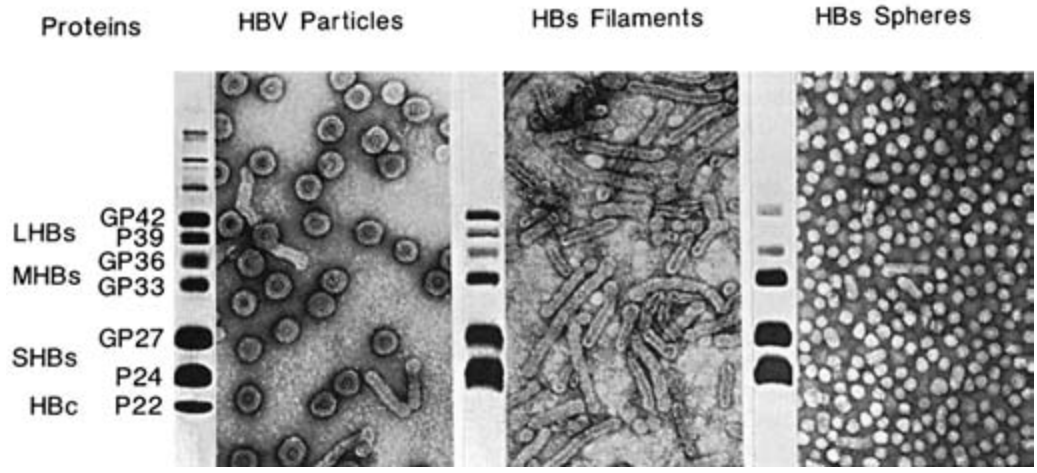


**Figure 10.1** Morphology and protein composition of purified HBV and HBs particles. The particles were isolated from high-titre HBV carrier plasma and separated from each other using ultracentrifugation and gel chromatography. The HBV particles were enriched approximately 1000-fold to about 10 µg/mL, the filaments approximately 10-fold, whereas the HBs spheres were slightly diluted for preparation of these three negatively stained electron micrographs. The same purified particle preparations were denatured by sodium dodecyl sulfate/dithiothreitol (SDS/DTT) and run through a 13% polyacrylamide gel and stained with colloidal silver. The apparent size and the nomenclature of the viral proteins are shown on the left. The viral surface antigen contains a small, middle-sized and large protein (SHBs, M, L), which appear in variably glycosylated forms. The core protein (HBc) forms a single P22 band in gel electrophoresis.

the HBeAg-negative variants have highly variable genomes (see Chapter 11).

### Integration of HBV DNA and oncogenesis

As will be pointed out later, integration of HBV DNA, in contrast to orthoretroviruses, is not an essential step in the replication of HBV. However, integration of fragments of HBV DNA occurs often in chronic carriers. In some cases cells may gain an illegitimate growth advantage by this insertion of viral DNA. Furthermore, viral proteins themselves, if expressed by integrated HBV DNA fragments in a deregulated and/or altered manner, may favour uncontrolled growth. There can be no doubt that certain hepadnavirus species have an oncogenic potential but possibly depend on different mechanisms that vary among species. WHV has the highest oncogenicity, showing typically an integration close to the cellular N-myc oncogen, HBV has intermediate oncogenicity showing a random integration, whereas DHBV and GSHV have the lowest oncogenicity, if any (see Chapter 44).

## Structure of *Hepadnaviridae*

### Virion morphology

Figure 10.1 shows the three types of virus-associated particles present in the blood of HBV-infected persons. Similar particles are also found in the serum of hepadnavirus-infected woodchucks or ground squirrels.[6,25] Using electron microscopy after negative staining, the virus is spherical with a diameter of 42–45 nm, showing a double-shelled structure. The outer protein shell (or envelope) is formed by the HBs proteins.[2] Surface structure details are not reliably visible by negative stain, but cryoelectron microscopy revealed small spikes at the surface of virions.[26] The inner protein shell is referred to as the core particle or capsid.[27] It is composed of HBc protein (also termed capsid- or core protein) and encloses a complex of viral polymerase and DNA, which is often positively stained. Morphologically, there are two different forms of capsids. They consist of 180 or 240 identical core proteins, which form capsids with a T=3 and T=4 symmetry. In cryoelectron microscopy they show diameters of 32 and 36 nm,[28,29] but there is no functional difference known to be related to the symmetry.

### HBs particles

The sera of highly viraemic carriers contain large numbers of non-infectious particles composed of excessive HBs protein (HBsAg). Most abundant are spherical particles of 17–25 nm, which are secreted in 100–10 000-fold excess to the virus, typically reaching numbers of up to $10^{13}$/mL. The spheres seem to be small vesicles with approximately 4-nm thick walls and an interior diameter of 10–15 nm. Less numerous (up to $10^{11}$/mL or 1 µg/mL) are the filamentous (or tubular) particles of approximately 20 nm diameter and variable length. Sera from low-viraemic HBsAg carriers contain up to $10^{12}$/mL HBs spheres and few or no HBs filaments. Neither

of these subviral structures contain the capsid and HBV DNA and thus they are not infective. Avian hepadnaviruses have a virion morphology similar to that of the mammalian hepadnaviruses.[25] The serum from DHBV-infected ducks also contains excessive HBs particles, but their morphology differs in that the spheres have a variable diameter of 40–60 nm. No filaments are detectable.

### HBs particles in the liver

The virion envelope and the HBs particles are synthesized and assembled at the membrane of the endoplasmic reticulum (ER) and bud to the lumen of the ER. From the ER, the particles are transported and excreted by vesicles. However, when the largest of the three HBs proteins (LHBs) is overproduced in comparison to the smaller HBs proteins, filamentous particles are formed, which are retained within the ER. This may lead to the accumulation of HBs protein to the point that the ER becomes dilated.[30] In light microscopy, such LHBs-storing hepatocytes appear opaque like ground glass.

### Core particles in liver

In HBV-producing cells, the core particles are synthesized and assembled independently from the HBs proteins in the cytosol. Thereafter, they probably attach to patches of HBs protein in the ER membrane. By enclosing the whole core particle, the HBs proteins mediate budding of the virions to the ER lumen. Non-enveloped core particles of human HBV, being devoid of the viral genome, are often found in the nucleus where they are obviously stored.[31] In highly viraemic individuals without high disease activity, virtually all hepatocytes may contain considerable amounts of nuclear or cytoplasmic core particles, which can be extracted, purified and visualized by electron microscopy. The appearance of cytoplasmic capsids seems to be related to high replication and high viral load in the serum. A certain HBV-producing cell line, Hep G2.2.15, releases not only virions and HBs particles but also naked core particles.[32] However, no naked core particles are found in the serum, even in that of immune tolerant HBV carriers without anti-HBc.[33]

## Protein composition of HBV particles

### Surface proteins

Virions and large structures such as the HBs or HBc particles are built from protein subunits that are held together by non-covalent interactions and, in the case of HBV proteins, by disulfide bonds between cysteines of different protein molecules. These interactions can be disrupted by the anionic detergent sodium dodecyl sulfate (SDS) and disulfide-cleaving reagents such as β-mercaptoethanol or dithiothreitol (DTT). Moreover, the protein subunit is denatured to form a random coil. For analysis of HBs proteins, gel electrophoresis after treatment with SDS/DTT was applied to separate them by size. Thereafter, the gel slabs were stained with colloidal silver (Fig. 10.1).

In HBs filaments, six protein bands are visible, ranging from 24 to 42 kDa apparent molecular weight. All of these proteins can also be stained specifically by immune blotting with an antibody against the smallest component P24, which demonstrates that at least one epitope of the smallest protein is present in the larger proteins. Four of the proteins contain an oligosaccharide linked to one or two of their asparagine residues (N-linked glycan). These glycoproteins migrate as somewhat larger molecules in electrophoresis than the non-glycosylated forms. Enzymatic removal of the glycan shows that HBs filaments contain only three different HBs polypeptides: (1) the largest (LHBs) is converted by partial glycosylation *in vivo* from P39 to GP42, (2) a middle-sized protein (MHBs) that is either single or double-glycosylated as GP33 or GP36, and (3) a small protein (SHBs) that may be glycosylated as GP27 or non-glycosylated as P24.

SHBs is the most abundant polypeptide in all three HBV-associated particles, whereas MHBs is a minor component in all three. LHBs is more prevalent than MHBs in virions and filaments, but less prevalent in HBs spheres. It appears that the proportion of LHBs determines the morphology of the HBs particles,[34] whereas the proportion of SHBs to MHBs does not significantly alter morphology. A model of the HBs particles is shown in Fig. 10.2.

### Core proteins

In addition to the HBs proteins, purified virions possess a non-glycosylated protein P22, which may be phosphorylated and which builds up the core particle. Naked core particles from liver show the same P22 protein in SDS gel electrophoresis,[35] but virion cores are known to contain the viral DNA polymerase covalently bound to the viral genome.[36,37] Details of structure and functions are described later.

## Genome of HBV

### Endogenous DNA polymerase of HBV

The first hint regarding the nature of the HBV genome was obtained by the discovery of an endogenous DNA polymerase activity in ultracentrifugation pellets from HBV-containing sera.[3] The reaction was dependent on all four desoxynucleotide triphosphates and divalent cations, but no primer or template was necessary. It

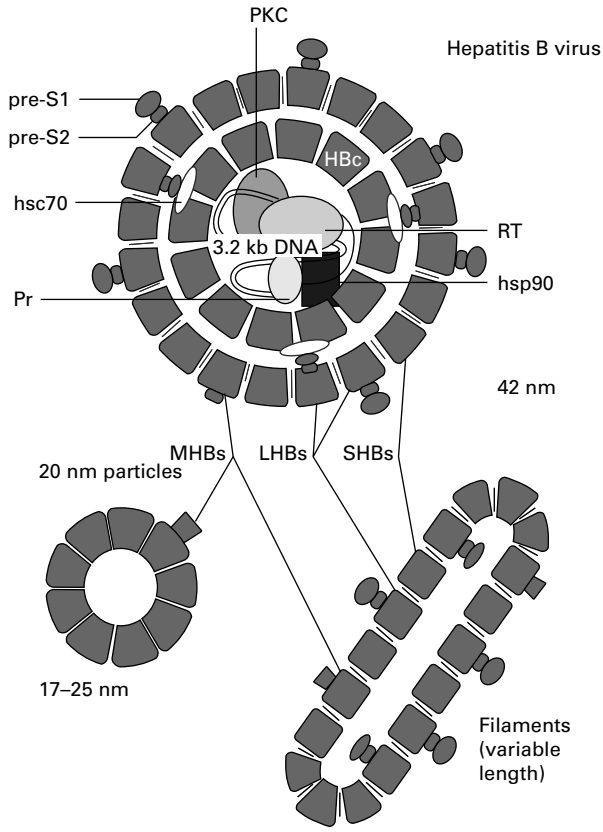


**Figure 10.2** Schematic model of the HBV and HBs particles. The SHBs protein of the viral envelope is identical to the S domain of MHBs and LHBs. MHBs contains a small pre-S2 domain facing towards the outside of the particle. LHBs contains a pre-S domain composed of the pre-S2 and pre-S1 sequences. The viral envelope encloses a symmetrical capsid consisting of 240 HBc protein subunits. These encapsidate the 3.2-kb long DNA genome of HBV to which a primase domain (pr) of the DNA polymerase (pol) is covalently linked. Protein kinase (PK) and the heat shock protein hsp90 are also encapsidated. The filaments consist of the same proteins as the virion envelope. The spheres contain fewer LHBs. The virion envelope and the HBs particles contain small amounts of ER-derived lipid between the protein subunit. Internal pre-S domains of LHBs are associated with the heat shock protein hsc70.

could be inhibited by actinomycin D, which intercalates into DNA templates. The enzyme was precipitable with antibodies against HBc protein if the HBs envelope was removed from virions by treatment with a non-ionic detergent. The DNA template was not accessible to DNase unless the core protein was lysed by SDS or digested by proteinase. The existence and properties of the endogenous DNA polymerase provided evidence that the virus contained a DNA genome.

## Structure of the virion DNA

Using electron microscopy, the DNA of HBV was shown to be circular, partially double-stranded, and 3200 nucleotides long.[4] The endogenous DNA polymerase reaction is possible because one of the DNA strands is incomplete.[38,39] The remaining gap is partially filled by the viral DNA polymerase if the dNTPs are added *in vitro*. *In vivo*, HBV particles are obviously secreted from the infected cell before the double strand is completed. Thus, the incomplete plus-strand has a defined 5′ end but a variable 3′ end (Plate 10.1c, found between p.786–7).

The complete minus-strand has defined 5′ and 3′ ends, with a terminal redundancy of 8–9 bases, resulting in a region in which the genome is triple-stranded.[40] The viral polymerase is covalently bound at tyrosine 63 via a phosphodiester bridge to the 5′ end of the minus-strand.[41] This linkage causes extraction of virion-derived DNA to the phenol phase during classical DNA extraction unless the sample is thoroughly digested with proteinase.[37]

The 5′ end of the plus-DNA strand is formed by an 18-base-long oligoribonucleotide, which is capped in the same manner as a messenger RNA (mRNA).[42] The genome contains two directly repeated sequences of 10 or 11 bases, DR1 and DR2.[43] This genome structure is typical for all hepadnaviridae, but in the case of the avian hepadnaviruses, the overlap and the distance between the DRs are shorter.

The structure of the hepadnaviral genome does not abide by the usual classification criteria for viruses[44] in several respects. It contains both DNA and RNA, and its genome contains partially single-stranded, double-stranded, and even triple-stranded DNA. These unusual features are a direct consequence of its replication mechanism, which will be explained later.

## Definition of open reading frames

From the nucleotide sequence of a double-stranded version of the DNA genome it is possible to derive six different sequences of amino acid encoding triplets of nucleotides (codons). If a sequence of potential codons does not encode a protein, it is randomly interrupted by one of the three possible stop codons, whereas protein-encoding sequences are usually free of stop codons for a distance of at least 50 codons. These stretches of codons are called open reading frames (ORFs). Protein biosynthesis additionally requires a start codon (AUG) in mRNA. By applying these rules, all genomes of mammalian hepadnaviruses contain four ORFs, which are encoded by the same DNA strand (Plate 10.1a, found between p.786–7). The polarity of the viral nucleic acid strands is defined in a way that mRNA has plus-polarity. Thus, the protein-

encoding DNA strand of the HBV genome, which is transcribed into mRNA, has minus-polarity. Additional ORFs can be identified both on the minus- and plus-strand, but they are not conserved, and are probably of minor importance, or generated by statistical sequence variations.

### Compact genome structure of HBV

Most functional ORFs of cellular organisms, as well as those from larger viruses, are arrayed in a linear fashion on the genomes and encode one protein. Moreover, they are interspersed by large non-coding regions (introns), which are removed after transcription by splicing. Regulatory and protein-coding regions are usually well separated. However, the genomes of hepadnaviruses seem to have evolved toward minimal length. Thus, the genome is not much longer than its longest ORF P, which encodes the viral DNA polymerase and its accessory functions (see Plate 10.1a, found between p.786–7). ORF S is completely located within the ORF P. ORF C and X overlap partially with ORF P. Furthermore, HBV encodes more than one protein from one ORF by using internal AUG codons in an ORF as additional start sites for protein biosynthesis. Thus, nested sets of proteins with different amino ends and a common carboxyl end are synthesized. ORF S encodes the three co-terminal HBs proteins.[45] ORF C encodes the HBe protein and the HBc protein.[46] ORF X may also encode more than one protein.[47] Furthermore, the numerous genetic elements that regulate transcription of the viral DNA into RNA, processing of the RNA and translation into protein are placed within coding regions.

Complete HBV genomes are between 3182 and 3221 bases long.[48] The numbering of the bases starts in most publications (as it does here) at the cleavage site for the restriction enzyme *EcoR*I or at homologous sites if a particular genome type does not have such an *EcoR*I site. Other numberings (e.g. beginning at the AUG start codon for the HBc protein synthesis or the first base of the RNA pregenome) (see later) are also in use.

## HBs proteins

### Protein sequence of SHBs

The amino acid sequence of SHBs at the amino and carboxyl ends has been determined biochemically.[49,50] The internal sequences could be only partially analyzed by this approach. However, together with the protein sequence predicted by the nucleotide sequence, it became clear that the sequence of SHBs begins at the third conserved AUG of ORF S, and that it ends at the stop codon of ORF S. The function of the 5′ terminal part of ORF S was originally unknown and was named region pre-S, simply to indicate its location upstream of gene S.[48] It is important to note that proteins containing the pre-S sequence are not precursors of SHBs.

SHBs is rich in hydrophobic amino acids. It has many tryptophans but few tyrosines and thus, unlike most proteins, possesses an ultraviolet absorption spectrum similar to tryptophan. Furthermore, it contains a very high number of 14 cysteines, which are cross-linked with each other.[51,52] At asparagine 146 there is a signal for addition of an N-linked glycan, which is present in approximately half of the molecules.[49] This glycan has two complex antennas with terminal sialic acids. In SDS gel electrophoresis, SHBs has a microheterogenicity, the pattern of which is typical for a virus carrier.[53] The origin of this microheterogenicity is unknown.

### Subtypes

SHBs occurs in stable subtypes that were originally defined by antibodies. Antigen reactivities that were present on all known HBs isolates were considered as determinant *a*. The best known subtypical determinants are *d* or *y*[54] and *w* or *r*. [55] Determinant *d* has a lysine at position 122, *y* an arginine.[50] Likewise, determinant *w* has a lysine at position 160, *r* an arginine.[56] Furthermore, a subdivision of *w* into w1–w4 is possible. These subtype-specific amino acids, however, may occur in quite divergent HBV genomes. Later subtyping has been done by DNA sequencing of the SHBs gene.[57] At least eight genotypes, A–H, which differ by >8% in the protein sequence, have been identified.[22,58–61] Because all SHBs subtypes are able to induce cross-protection after immunization, the significance of serological or other subtyping is mainly of epidemiological and phylogenetic interest. However, the genotypes may have different pathogenicity and epidemiology and have a typical geographic region. Genotype C is reported to cause more severe chronic course than genotype D.[59] In addition, it cannot be excluded that some of the neutralizing antibodies are subtype-specific, as is the case with most other viruses.

### Topology of SHBs

Circular dichroism of HBs spheres suggests that 50–60% of the polypeptide chain are folded to α-helices. Computer programmes used to model the secondary structure of proteins predict that these α-helices are formed by four or five hydrophobic stretches of SHBs (Fig. 10.3)[62,63] Biosynthetic studies suggest that α-helix I is inserted co-translationally into the ER membranes, and that it furthermore is able to translocate amino-terminal sequences to the lumen of the ER if they are not too long.[64] Helix II is also inserted into the ER membrane, but it translocates the carboxy-terminal sequences.[64] The topology implies that the hydrophobic sequence between helix I and II remains cytosolic. After budding of HBs particles

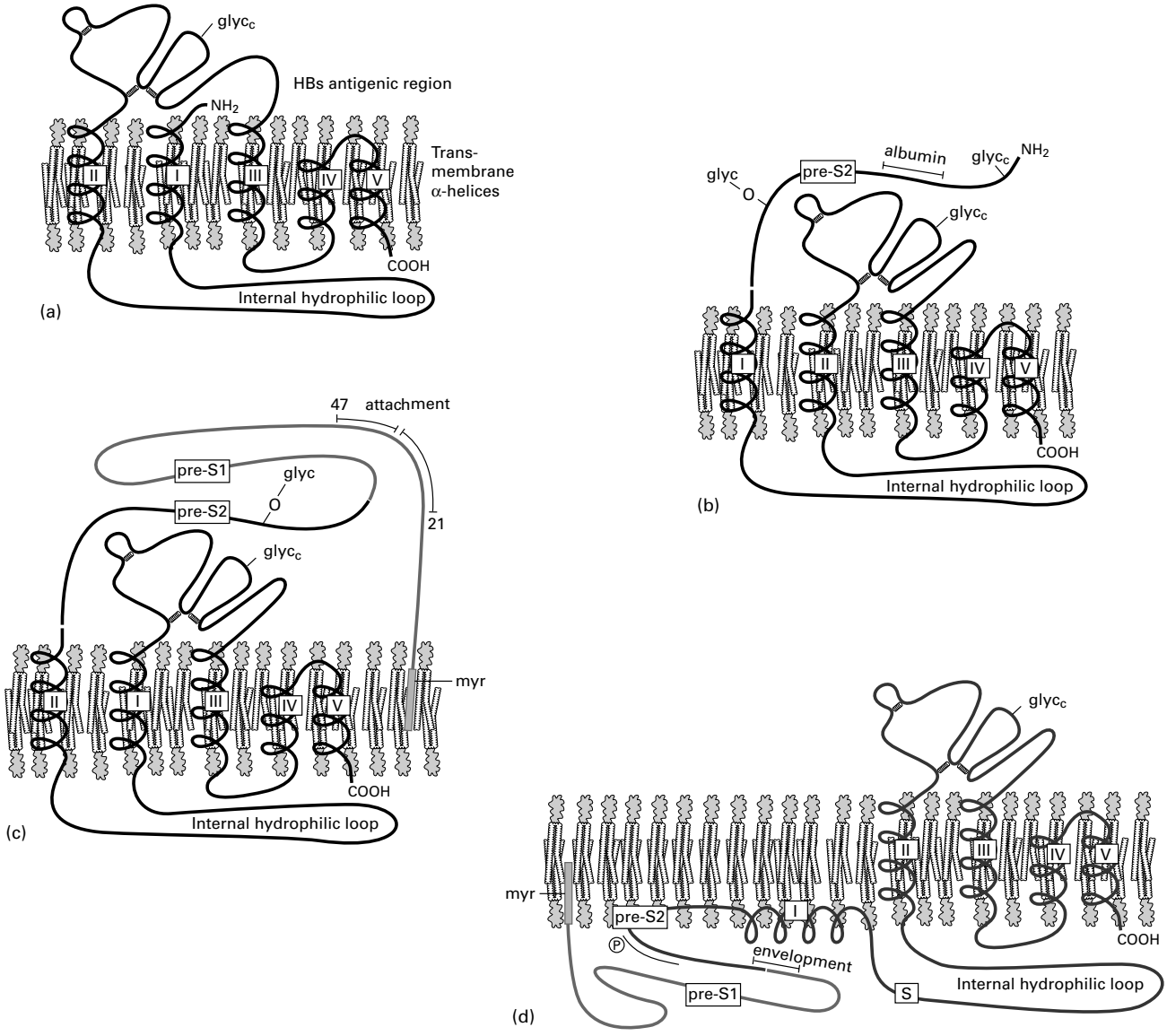


**Figure 10.3** Topological models of the three HBs proteins. The lipid bilayer of the ER membrane is symbolized by the lipid molecules. The top of the figures corresponds to the lumen of the ER or, after multimerization and budding of the HBs protein, to the surface of the virus particles. The bottom of the figure corresponds to the cytosol or to the interior of the particles. It is assumed that the ER membrane is crossed at least four or five times by hydrophobic α-helical segments of the S domain. (a) Structure of SHBs. A complex biantennary glycoside (glyc$_c$) is present in GP27 but not in P24. The HBs epitopes are generated by the complex folding of the external hydrophilic loop between α-helix II and III (as suggested by Chen *et al.*[65]) and are stabilized by cystine bonds the exact nature of which is not known. The structure downstream of helix II is not well understood. (b) Structure of MHBs. The arrangement of the transmembranous α-helices of the S domain seems to be slightly altered in that the amino end is more accessible, and that its hydrophilic loop is less often glycosylated. The pre-S2 domain carries a complex N-linked glycoside (glyc) and a part of the molecules an O-linked glycan. (c) Structure of LHBs in the virion. The structure is in principle similar to MHBs. The pre-S2 domain is not N-, but O-glycosylated. The pre-S1 domain partially covers the pre-S2 domain and is linked at glycine 2 with myristic acid (myr). (d) Presumed structure of LHBs soon after its synthesis in the ER. At the early phase of LHBs folding, it appears that the entire pre-S domain stays in the cytosol. Schematically, the proposed structure would result, but the true folding of the cytoplasmic pre-S domain is not known. At later phases of virion or HBs filament maturation in about 50% of the LHBs molecules, the entire pre-S domain is translocated through the particle membrane to the surface as shown in (c). The binding sites for attachment of the virus and for capsid envelopment are shown.

to the lumen of the ER, this sequence is in the interior of the particles. In agreement with this conclusion, B-cell epitopes have not been found in this region. However, the sequence from amino acids 99–168 is exposed at the particles' surface and forms the HBs antigen region. The composition of discontinuous epitopes was analyzed by a filamentous phage peptide library.[65]

Most of the SHBs epitopes depend on the presence of disulfide bonds. Mild treatment with DTT first destroys subtype-specific epitopes and at higher concentrations also the *a*-epitopes. Completely reduced HBs particles are almost non-immunogenic. Thus, SHBs is virtually undetectable in immunoblots when reduced and denatured proteins are reacted with antisera against native HBs particles.[66]

The exact topology of this region is not known. Binding experiments with antibodies suggest that amino acid 178–186 are surface-exposed.[67] Computer modelling of the following hydrophobic regions suggests formation of two or three membrane-spanning helices (III and V), which may insert post-translationally into ER membrane. Because of their polarity with one hydrophobic and one hydrophilic side, these helices are predicted to multimerize.[62,63] Multimerization may possibly also occur between different HBs subunits, and this event would potentially mediate budding of the HBs particles. In agreement with that presumption, truncated HBs proteins without this region no longer form HBs particles, but remain at the ER.[68] Such truncated HBs proteins have been found in certain hepatoma cells.[69] The most carboxy-terminal part (presumptive helix V) is dispensable for HBs particle formation.[68,70]

## MHBs

This minor component of the virion or HBs particles is composed of the S domain, a sequence that is identical to that of SHBs and of the 55 amino acid long pre-S2 domain.[71] The pre-S2 domain is hydrophilic and does not contain cysteines. It is very sensitive to proteases and can be removed selectively from HBs particles without destroying the S domain.[72] Thus, MHBs is virtually absent in HBV vaccines that contain protease-treated HBs particles from carrier plasma. The asparagine at position 4 is linked with a glycan consisting of two complex chains.[73] The glycosylation at this site is essential for secretion of MHBs-containing HBsAg particles.[74] In addition to N-glycan, pre-S2 of HBV genotypes C or D is linked at Thr 37 with an O-glycan.[73] With the exception of genotype A the corresponding O-glycosylation site is conserved in all other HBV genotypes or primate HBVs. WHV is multiply O-glycosylated in pre-S2.[75]

The pre-S2 domain is located at the surface and partially covers the S domain of MHBs. In mice, it may be slightly more immunogenic than the SHBs antigen. Furthermore, the epitopes are not conformation-dependent and can (in contrast to SHBsAg) easily be generated by synthetic peptides. Peptides with the sequence of the amino-terminal half of the pre-S2 were found to induce protective immunity,[76–78] but recent *in vitro* experiments with *Tupaia* hepatocytes suggested relatively weak neutralization capacity of pre-S2 monoclonal antibodies compared with pre-S1 and SHBs antibodies.[19] However, pre-S2-containing HBs particles from transfected Chinese hamster ovary (CHO) cells have been introduced in some countries as vaccine.[79]

The central part of the pre-S2 domain (amino acid 7–20), which also forms the major epitope, binds a modified form of serum albumin.[80–82] *In vivo*, approximately one in 10 000 serum albumin molecules is able to bind to pre-S2. HBV carriers with >10 µg HBsAg/mL usually have free albumin-binding sites on their particles, whereas at lower HBsAg concentration all binding sites are occupied. The nature of the modification is not known, but it can be mimicked by cross-linking of albumin with glutaraldehyde.[83] Serum albumin of non-primate origin does not bind.[81]

## LHBs

The largest HBs protein contains two domains: pre-S (composed of the pre-S1 and pre-S2 sequence) and S. In the mature virions or HBs particles part of the pre-S domains are accessible for antibodies,[45] receptors[84] and proteases.[72] The S domain and parts of the pre-S2 sequence are hidden by the pre-S1 sequence of LHBs (Fig. 10.3). During biosynthesis, the entire pre-S domain of LHBs seems to stay initially at the cytosol.[85–88] Thus, the asparagine 4 of the pre-S2 sequence is not glycosylated in LHBs,[45] because this modification can occur only cotranslationally in the ER lumen. The amino end of the pre-S domain carries the sequence methionine-glycine, which, together with neighbouring amino acids, serves as a signal for the replacement of the methionine by the $C_{14}$ fatty acid myristic acid.[89] Myristylation is not essential for virion formation but is essential for virion infectivity of HBV[90,91] or DHBV.[92]

During virion or HBs particle maturation, the pre-S domains reconfigure in about 50% of the LHBs molecules and translocate to the surface of the particle. The translocation occurs post-ER but intracellularly, because LHBs contains like MHBs O-linked glycan at Thr 37 of pre-S2. Half of the pre-S domains remain on the cytosolic side, which, after budding into the ER, becomes the internal side of the particle.[85,86] For DHBV, these pre-S domains are associated with the cellular heat shock protein hsc70.[93,94] The significance of the dual topology of LHBs is obvious. Several studies on the attachment of HBV to the target cell showed the necessity of the pre-S region for binding to the cellular receptor (see below),

implying that externally localized pre-S is essential for the viral life-cycle. On the other hand, pre-S seems to be required for envelopment of the core particles by the surface proteins, giving evidence for an essential localization of pre-S on the cytosolic side of the surface proteins.[95] Placement of the pre-S sequence behind a secretion signal peptide allows formation of secreted LHBs-containing particles but prevents envelopment of core particles.[96] Deletion analysis showed that only the sequence proximal to pre-S 108 is required. Shorter pre-S sequences are translocated as in MHBs and thus are not available for interaction with core particles.[86]

Overexpression of LHBs relative to expression of SHBs prevents secretion of HBs particles.[97] Instead, filaments become enriched in the ER[30] or in the intermediate compartment. Expression of MHBs and SHBs is independently regulated from expression of LHBs (see below). Hepatocytes appear to exist that express and secrete SHBs predominantly, whereas for unknown reasons others express more LHBs and store it intracellularly.[98] The degree of hepatic immune staining for HBsAg does not correlate with the level of HBsAg in the serum. Only hepatocytes that express LHBs, SHBs and the other viral components in a well-balanced manner are able to assemble and secrete virions.

The pre-S domain is one of the most variable regions between different hepadnavirus species. One reason may be that the part of the polymerase protein that is encoded by the same DNA region as pre-S1 is not essential for replication. The other reason is that this may be the surface structure that is responsible for host specificity and binding to hepatocytes. However, within a chronically infected person or within a defined chain of infection, pre-S1 is not mutated.[99] Thus, it is not similar to the hypervariable domains of human immunodeficiency virus (HIV) or hepatitis C virus (HCV) envelope proteins.

## Core proteins

### Products of ORF C

The essential product of ORF C is the HBc protein of either 183 or 185 amino acids, depending on the genotype of the virus. In most isolates from highly viraemic carriers, ORF C has 212 or 214 codons, but translation of HBc protein starts only at the AUG 29 codons downstream of the first AUG.[46] The region upstream is termed pre-C for historical reasons.[48] This name is misleading, because the product of the entire ORF C is not a precursor of HBc protein but of the secretory form of the core protein, which is termed HBe protein.

### HBc protein

This protein contains many hydrophilic and charged amino acids. It does not contain lipid or glycan, but if expressed in eukaryotic cells, it becomes phosphorylated.[35,100–104] It is synthesized in the cytosol of the infected cells. As an essential step in the viral life-cycle, it packages its own mRNA and the viral polymerase after formation of the RNA-polymerase complex and assembles into core particles.

The core protein can be separated into two different domains. The 144 amino terminal amino acids are the structural component, essential for capsid formation. They build up five α–helices arranged in an anti-parallel orientation forming a spike between helix 3 and 4.[28,29,105–107] The C-terminal part of this domain forms a flexible array that is located in the interior of assembled capsids.[108] Amino acids 145–185 (183 in some genotypes) are a multifunctional domain. It is essential for RNA packaging, plus-strand DNA synthesis of progeny virion DNA[109,110] comprises a nuclear localization signal (NLS) and serine residues that may become phosphorylated.

The core particles assemble spontaneously after reaching a threshold concentration of 0.8 μM[111] to isometric particle even when expressed in a heterologous system such as *Escherichia coli*, for example. Assembly is strongly enhanced by the presence of RNA, which interacts with the C-terminus of the core protein[110] and thereby becomes encapsidated into the lumen of the capsid.[111]

Assembly of a core particle is initiated by formation of a core protein dimer to hexamers. Apparently, this step is rate-limiting and is followed by a trimerization of dimers. These elements rapidly assemble to particles with a T=3 or – more frequently – T=4 symmetry. The ratio between T=3 and T=4 particles is influenced by the speed of assembly. As in chemical reactions a counter direction exists. This leads to a removal of a hexamer, a process called 'breathing'. However, the balance of the reaction strongly supports the formation of an intact particle.[112,113]

*In vivo*, the cytosolic assembly of the capsids is combined with the encapsidation of a complex formed from the viral polymerase and the so-called pregenomic RNA. In addition, cellular proteins such as some heat shock proteins and a cellular protein kinase become encapsidated (see below). In a coordinated fashion the polymerase converts the pregenomic RNA to viral DNA. This process depends upon the presence of the core protein.

For DNA synthesis of a mature viral genome approximately 5000 nucleotide molecules are required. Therefore, the core proteins do not form a completely closed protein shell but show 2-nm measuring holes that allow the nucleotides to enter the lumen.[28,29] It is believed that

these holes are subjected to a structural change of the capsid upon genome maturation, allowing the C-terminus to become exposed to the surface.[114,115] This conformational change is essential for NLS exposure that facilitates the transport of the viral DNA into the nucleus.[115]

Once core particles are assembled and have left the reducing environment of the cytosol,[116] their structure is stabilized by disulfide bonds, but the conserved cysteines are not required for assembly or envelopment.[117] Although showing the same morphology[29] *E. coli*-expressed and native capsids show different disulfide bonds. *In vivo* the core proteins are linked to dimers at cystein 48, 61 and 185.[118] In contrast, the carboxyterminal cystein 185 becomes linked to the cystein 185 of other dimers when the capsids are expressed in *E. coli*.[119]

## Function of HBc protein

The HBc protein is central in the viral life-cycle. After assembly to core particles and encapsidation of the viral genome, the particles allow envelopment by the HBs proteins and formation of the virus. In addition, the core particles mediate the transport of the mature viral genome into the nucleus of the infected cell, leading to restorage and amplification of HBV DNA in the nucleus of the persistently infected hepatocytes.[114,115]

A certain proportion of the HBc proteins is imported into the nucleus where the HBc units assemble to empty core particles.[35] The significance of the intranuclear core protein is not understood, but core protein associated with nuclear viral DNA was thought to modulate transcription.[120] The observation that the nuclei of rodent and duck livers infected with the corresponding hepadnaviruses are devoid of nuclear capsids suggests that nuclear particles may be a non-essential side-effect of HBV infection. It may be speculated that the assembly to particles is a pathway to dispose of the excess of core proteins that may interfere with the cellular RNA due to their RNA-binding capacity.

## HBe protein

All hepadnaviruses have evolved the ability to express a secretory form of their HBc protein. They achieve this by the 5′ terminal part of the ORF C, called the pre-C sequence. The pre-C sequence encodes a hydrophobic α-helix, which is a secretion signal and allows for translocation of the HBe protein into the lumen of the ER.[121,125] During that process, 19 of the 29 pre-C amino acids are cleaved off by the signal peptidase (Fig. 10.4). The 10 remaining amino acids of the pre-C sequence prevent assembly of HBe to core particles by interaction with the HBc protein sequence.[122]

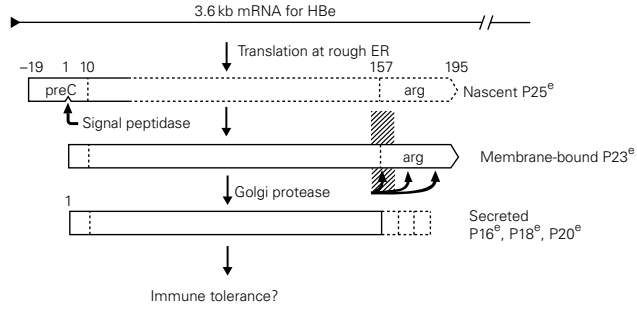


**Figure 10.4** Biosynthesis of HBe proteins. When the more-than-genome-length mRNA starts upstream of the HBe start codon, translation of the pre-C region prevents the necessary folding of ε. Thus, the HBe mRNA does not usually function as a pregenome. It is translated to the HBe precursor P25e, which may be co-translationally cleaved to a membrane-associated P23e. The 10 remaining amino acids of the pre-C sequence prevent folding of P23e to core particles. Secretion requires partial or complete removal of the arginine-rich carboxy-terminal domain by a Golgi protease. Thus, secreted HBe protein may be of variable size between 16 and 20 kDa. The HBe protein is not essential for virion replication but modulates the host's immune response to HBV.

Thus, HBe protein differs in almost all aspects from HBc protein, although the primary sequence of these two molecules is almost identical. Part of the HBe protein is transported to the plasma membrane.[123] Another part is further cleaved within the arginine-rich domain by a Golgi protease and then secreted as a dimeric protein with disulfide bonds between Cys-7 and Cys-61.[124–126] Another part of the HBe protein does not reach the ER lumen and is not cleaved at all. The $P25^e$ protein exposes a nuclear transport signal.[127] Thus, HBe proteins of variable length are found in practically all compartments of the cell and are secreted. Furthermore, uncleaved HBe precursor protein accumulates as phosphoprotein.[128]

## Function of HBe protein

HBe protein is not essential for the viral life-cycle. Variants without functional pre-C sequence and HBe protein arise often during acute or chronic HBV infection (see Chapter 11). Nevertheless, all known hepadnaviruses from duck to humans have an HBe protein, and revertants may occur after cessation of interferon treatment. Using a different expression strategy, murine leukaemia viruses have also developed the ability to produce a secretory form of their nucleoprotein, the glycosylated gag protein.

High levels of secreted HBe protein are found in low-symptomatic, highly viraemic virus carriers. Elimination of HBeAg is usually accompanied by a flare-up of immune pathogenesis and a decrease of viraemia (see Chapters 15 and 16). An HBe-negative variant of wood-

chuck hepatitis B virus was infectious for newborn woodchucks, but it could not induce persistent infection, whereas the HBe-expressing virus results in persistent infection.[129] These observations suggest that HBe protein may somehow suppress the immune elimination of HBV-producing hepatocytes. Indeed, HBeAg was found to induce immunotolerance against HBV-infected cells. Depleting inflammatory HBeAg- and HBcAg-specific Th1 cells that are necessary for viral clearance,[130] HBeAg is thought to support viral persistence.

## Products of ORF P

Although the products of ORF S and C have been identified and well characterized within virions, evidence regarding the products of ORF P is still circumstantial. There is no doubt that such a product or products are expressed during infection *in vivo*, but the modes of transcription, translation and post-translational processing are not exactly known.

### Domains of ORF P

As discussed later, mutational analysis of the 834–845 codon-spanning ORF P, sequence homologies with well-studied reverse transcriptases,[131] and studies on the mechanism of genome replication of HBV show that most parts of the ORF are indispensable for the virus. These studies suggest that ORF P has four clearly distinguishable domains.[132]

**1** The amino-terminal domain that encodes the part of the protein linked to the 5′ end of the minus-strand of virion DNA.[36] This part of ORF P is necessary for priming of minus-strand synthesis and is also termed *primase*.

**2** The next domain has no specific function except as a spacer or tether.

**3** The next domain encodes the RNA- or DNA-dependent polymerase, i.e. the reverse transcriptase (RT). This domain is supposed to have a similar spatial structure to the RT of retroviruses,[133] with fingers of a palm.[134] It is a target of current antiviral drugs against HBV.

**4** The last carboxy-terminal domain is an RNase H, which cleaves the RNA if it is present in hybrids of RNA and DNA. The ORF P of hepadnaviruses differs among other features from the ORF P of retroviridae in that it most likely has no protease and integrase domain, but it has a primase activity that is absent in orthoretroviruses and other pararetroviruses.

Interestingly, the enzymatically active domains of the polymerase are not active *per se*. At least the DNA-dependent DNA polymerase activity depends upon the presence of the core protein.[110] The RNase H is only active on the polymerase-bound substrate,[135] thus only active when incorporated into the viral capsid. However, complementation of primase, RT and RNase H domains on different proteins is possible.[136]

### Products of ORF P in virions

Studies on the viral polymerase have been hampered by its low abundance in viral particles. Thus, most of the results on the polymerase were obtained by introducing mutations in the ORF P. Extraction of an active endogenous DNA polymerase from virion core particles has been difficult, because disruption of cores requires harsh treatment and, thus, destroys the interaction of core protein and polymerase, which appears to be required for regular genome maturation. However, residual activities such as the priming reaction can be observed when expressing the polymerase in cell-free systems[137] or in *E. coli*.[138]

Bartenschlager *et al*.[139] found that the 90-kDa protein encoded by the entire ORF P is predominant in virions. The polymerase protein is packaged together only with the pregenomic RNA within core particles[140] probably caused by sterical changes of the polymerase resulting from its interaction with ε.[141] Because the pregenome contains only one packaging signal[141] and core particles seem to have only a packaging capacity for 3300 nucleotides,[142] probably one polymerase molecule is packaged. In both DHBV and HBV, the polymerase is associated with a dimer of heat shock protein hsp90 and one of its co-factors p23. Hsp90 is necessary for binding to ε.[143–147]

## HBx protein

The 154 amino acid-spanning ORF X is conserved in similar form in the hepadnaviruses of woodchucks and ground squirrels. The seeming absence of an ORF for HBx in DHBV suggests that it does not participate in the mechanisms of genome replication or virion assembly. However, recent studies revealed that duck hepatitis B virus expresses a regulatory HBx-like protein from a hidden ORF.[148] Nevertheless, like the mammalian HBx, the avian one does not interfere with infectivity or *in vivo* growth characteristics.[149]

### Function of HBx

Mutational studies suggest that the HBx protein is dispensable for virus production after transfection of permanent cell cultures *in vitro*.[150] However, transfection of WHV DNA without a functional X ORF into livers of susceptible woodchucks did either not yield infection or caused reversions, which shows that HBx is essential for efficient replication *in vivo*.[151,152] There is no clear indication that HBx protein is a structural component of virions or core particles. The amino acid sequence suggests that HBx does not have a specific intracellular

transport signal. Thus, reports on the occurrence of HBx protein in the serum of virus carriers[153] are difficult to explain. Several studies suggest a predominantly cytoplasmic localization and a minor nuclear fraction.[154] *In vitro*-expressed HBx is highly unstable in animal cells, with a short half-life of 20 minutes, during which time it becomes phosphorylated.[155] Most reports on various biochemical activities of HBx protein seem to be preliminary and need confirmation. It is clear that HBx protein activates transcription of many genes in a somewhat non-specific manner when it is introduced artificially into cells by co-transfection with reporter systems.[156–158] It has been suggested that nuclear HBx interacts with other transcription factors,[159,160] but others suggest activation of cytoplasmic signal transduction factors such as PKC,[161] raf,[162] or NF-κB.[163,164] The number of HBx proteins expressed from ORF X *in vivo* is unknown, but the results of mutational studies suggest that the smaller potential proteins are expressed, having transcription-activating activity. [47]

In addition to the transactivating activity, HBx comprises a second well-established activity. Correlating with transactivation, a pro-apoptotic potential was found to be present in both orthohepadnaviruses and the avihepadnaviral X-proteins. The corresponding domain was localized in the carboxy-terminal half of the different X-proteins.[165]

One of the most significant side-effects of HBx protein may be its tumorigenic activity in premalignant mouse hepatocyte cultures[166] and transgenic mice,[167] but this has not been found in all systems.[168] Particularly, an interaction with tumour suppressor protein p53 has been postulated.[169–171] An association with various other proteins has reported proteasomes and DNA-repair factor[172] being detected more consistently by the two-hybrid system.

## Replication of HBV

### The life-cycle of the virus

As with all other viruses, the life-cycle of HBV and its relatives in the animal kingdom can be divided into several steps: (1) attachment of the virus to the host cells, (2) penetration into the cell, (3) subsequent transport into the compartment in which replication occurs, (4) release of the viral genome, (5) transcription and translation of viral gene products, (6) replication of the viral genome, (7) assembly of virions, and (8) release of the virus. Unlike most other viruses, HBV comprises a further pathway, allowing the entry of newly synthesized viral DNA from the cytosol into the nucleus. The following section describes these various steps, which are shown schematically in Fig. 10.5.

### Attachment

Virus attachment is one of the crucial steps that determines, among other factors, the host range and organ tropism of viruses. Furthermore, blocking of attachment by neutralizing antibodies against surface epitopes is a major component of protective immunity and the basis of many antiviral vaccines. Thus, it is interesting to identify the viral attachment site(s) and the corresponding cellular receptor(s).

#### *Infectivity systems*

A great drawback of many of the studies on the attachment of HBs proteins to cell surface proteins is that target cell lines were not *susceptible* to HBV infection even if they were permissive for HBV replication after transfection. A striking example is the human hepatoma cell line HepG2, which cannot be infected but replicates and secretes infectious HBV quite efficiently after transient or stable introduction of HBV DNA.[32,173]

Obviously, the lack of replication in cell lines is not caused by their missing ability to synthesize progeny virus but by one of the steps required for viral uptake and establishment of infection. Established cell lines are not as differentiated as hepatocytes in their natural environment. Primary hepatocytes were thus used as infectivity systems. In fact, hepatocytes from newly hatched ducklings were shown to be susceptible for the duck hepatitis B virus[174] as well as primary woodchuck hepatocyte cultures[175] and primary hepatocytes of human origin[176–178] are also susceptible for WHV and HBV, respectively. All primary hepatocytes retained their specificity for their natural virus with the exception of primary hepatocytes from *Tupaia belangeri*, which turned out to support infection of HBV and the hepatitis B virus of the woolly monkey.[19,20] However, no *Tupaia* HBV is known.

All primary hepatocytes have in common that they lose their susceptibility to their corresponding virus within a few days after they are taken into culture. It is thought that this phenomenon is caused by an ongoing de-differention process, as the presence of differentiation supporting agents such as DMSO preserves susceptibility for up to 10 days.

Human hepatocytes are not readily available. The need for a readily available cell line allowing study of the entry process of hepadnaviruses and infectivity resulted in extensive research. After years, the problem was recently overcome by a newly established hepatocyte cell line of human origin.[179] This cell line preserved susceptibility to HBV and shows replication comparable to primary hepatocytes, but like all primary hepatocytes the number of progeny hepadnaviruses secrete from the cells is lower

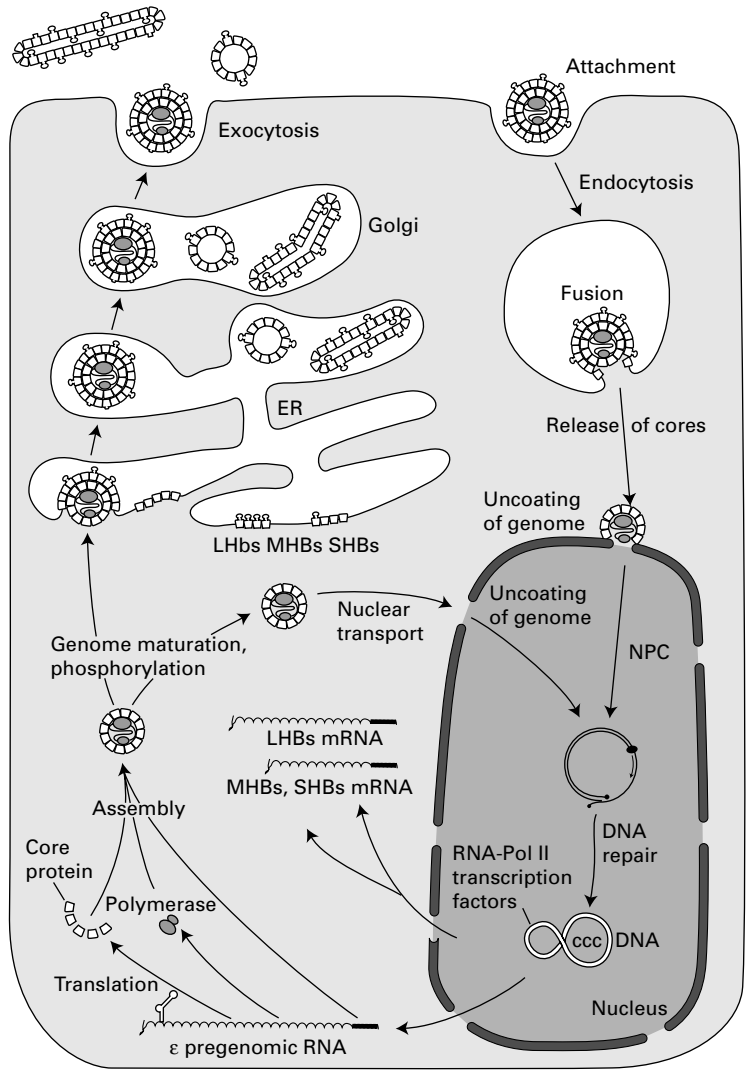


**Figure 10.5** Schematic view of the life-cycle of HBV. In many details, this model is still speculative. It is assumed that the virus is endocytosed after attachment, the nucleocapsid is released to the cytosol, transported via microtubules and binds to the nuclear pore. The capsid is transported into the nuclear basket where the genome is released, converted to cccDNA and transcribed to three essential classes of mRNA. The non-essential HBe and HBx synthesis is omitted for the sake of simplicity. Translation of the core/pol transcript in the cytosol allows for assembly of core particles that contain the pregenome. The transcripts for LHBs and M/SHBs are translated at the rough ER, and the HBs proteins are inserted in that membrane. The HBs particles bud to the lumen of the intermediate compartment, and at least a part of LHBs-rich ER membrane areas envelopes core particles. The HBV and HBs particles are secreted thereafter by the constitutive pathway.

than the number subjected to them.[176–179] This relatively high resistance of hepatocytes in culture contrasts sharply with the high infectivity of HBV *in vivo*.

To achieve high transfer efficiency, hybrids of adenovirus carrying a dimeric HBV genome were constructed, allowing the highly efficient adenoviral transfer.[180] Their use is restricted to the analysis of post-entry steps.

### *Attachment*

The reason for the insusceptibility of long-term hepatocyte cultures to HBV infection is not fully known, but it appears that it is due to a block in early steps of the viral life-cycle. It was thus speculated whether the block of infection is caused by a lack of HBV receptors that are lost when cultivating hepatocytes. However, an organ- and species-specific attachment of serum-derived HBs particles was found by Neurath *et al*.[181] with HepG2 cells, but not with animal liver cells or human carcinoma cells of non-hepatic origin. The binding could be *blocked* by antibodies to the pre-S1 sequence 21–47, and *competed* for by the peptide sequence itself. The relevance of this attachment site was confirmed by studies using natural, purified HBV particles and plasma membrane preparations from surgically obtained human liver specimens.[182] Furthermore, antibodies to peptide pre-S1 (21–47) were able to neutralize *in vitro* HBV inocula, which were no longer infectious.[78] Monoclonal antibodies to this region could block attachment to HepG2 cells or infectivity for primary human or *Tupaia* hepatocyte cultures.[19,183,184]

Avian hepadnaviruses comprise only a single non-glycosylated pre-S region. The involvement of the pre-S region in hepadnaviral attachment was supported by Ishikawa and Ganem using the DHBV system.[185] Pseudotyping heron hepatitis B virus with an envelope containing instead of HHBV pre-S the DHBV pre-S region, or parts thereof, allowed efficient infection of duck

hepatocytes for which HHBV is poorly infectious. Most recent studies indicate, however, that species-specificity is more complex. Replacing – vice versa – the apparently critical DHBV pre-S part by HHBV sequences in a replication-competent DHBV allowed efficient *in vivo* infection of ducks, arguing against the assumption that pre-S is restricting the avian host range (Dallmeier, Schultz, Nassal, unpublished observations).

Pre-S of avian hepadnaviruses and pre-S1 of mammalian hepadnaviruses are essential for viral attachment. It was shown consistently that pre-S of DHBV binds to a membrane-bound member of the carboxypeptidase gene family (carboxypeptidase D, CPD),[186,187] formally termed gp180. Binding to this receptor and infectivity can be blocked by peptides of the pre-S region and the affinity was shown to be extremely high.[188] The mode of pre-S-CPD binding appears to occur in two steps. Initially, a short α-helix in the C-terminus of the receptor binding domain facilitates formation of a primary complex, followed by stabilization, which involves approximately 60 most randomly structured amino acids.[189] Interestingly, the receptor becomes downregulated in infected hepatocytes,[190] which is in accordance with the observation that co-infections of single cells are rare. However, carboxypeptidase D is expressed in a variety of tissues so that the specificity for hepatocytes must be given by additional factors. This may include the fact that to reach the hepatocytes circulating hepadnaviruses have to pass the liver sinusoidal endothelial cells (LSECs) and the space of Disse first. Such a model was proposed from observations showing that DHBV shows a preferential uptake into LSECs. Intracellularly, fluorescent virus particles co-localized with CPD, suggesting receptor-mediated rescue from lysosomal degradation. To comply with the high efficiency by which hepatitis B viruses infect hepatocytes *in vivo*, it was proposed that DHBV is thereafter released to infect adjacent hepatocytes, which are the only cells capable of replicating the virus.[191]

### *SHBs and attachment*

Studies with HepG2 cells,[181] liver plasma membranes[180] and *Tupaia* hepatocytes[19] suggested that SHBs is not involved directly in attachment, but Hertogs *et al*.[192,193] observed attachment of SHBs particles to hepatocyte-bound endonexin II. Furthermore, attachment of SHBs to an altered form of apolipoprotein H has been reported[194] for HBV. The significance of these findings remains unclear. The existence of neutralizing antibodies toward SHBs and of escape mutants to those antibodies (see Chapter 11) suggests that SHBs structures participate in the attachment, entry, or both. Alternatively, such antibodies may mediate aggregation and immune elimination of HBV or hinder sterically the pre-S domains.

## Virus penetration

Three mechanisms are known by which animal viruses can enter a cell. The first is a direct fusion of the viral envelope with the plasma membrane once the virus is bound by the receptor. This process is restricted to enveloped virus such as herpes simplex virus 1, for example.[195] In both other pathways, the virus becomes incorporated into a vesicle after binding to the cell surface. The vesicle – either caveosomes as described for SV40 or endosomes that are apparently the most frequently used pathway – is released from the plasma membrane into the interior of the cell. Within the cell, these organelles are subjected to complex shuttling that is mediated by the cellular microtubule network.

Endosomes are transported towards the microtubule-organizing centre that is located in the periphery of the nucleus. For successful infection, the virus has obviously to escape from the endosomal pathway before the endosome is converted to an acidic phagolysosome. Most viruses using the endosomal uptake make use of the stepwise acidification. The decrease of the pH causes a structural change in the topology of the surface proteins, which allows their insertion into the endosomal membrane, as described for influenza virus. In addition, essential proteolytic modifications can be involved, as demonstrated for adenoviruses, for example. Both strategies result in a pore of the endosome allowing the viral interior to enter the cytoplasm.

The mechanism of HBV entry into hepatocytes is not completely understood. Using primary hepatocytes, an uptake via endocytosis has been shown for DHBV.[196,197] In contrast to most other viruses, this initial step was shown to be independent of acidification. It must be thus assumed that fusion of envelope and endosomal membrane is triggered by receptor binding and/or proteolysis leading to the release of the capsid into the cytoplasm. Consistently, all hepadnaviruses contain a conserved fusion-peptide-like sequence at the amino-terminal end of SHBs that functions in swap experiments with influenza haemagglutinin.[198]

### *Genome release*

It is known that 24 hours after infection with DHBV, DNA occurs in the nucleus and is converted to covalently closed circular molecules.[174] During this time the virus has to penetrate the cell, must be transported through the cytoplasm, followed by the import of the viral genome into the nucleus.

Because of the presence of HBV core particles in the nuclei of infected hepatocytes, it was speculated in the past that the entire core particles were transported into the nucleus. However, recent studies draw a more sophisticated picture of this process, as illustrated in Fig. 10.6.

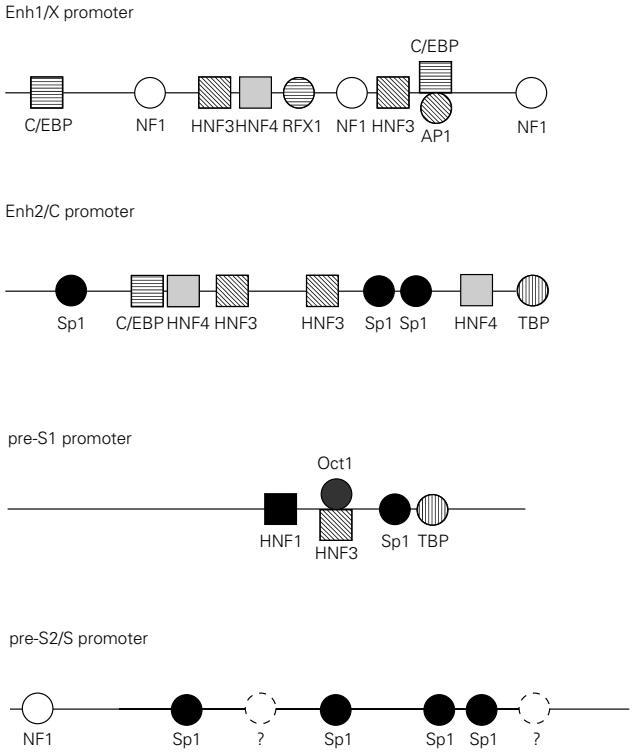


**Figure 10.6** Binding sites for transcription factors in the enhancers and promoters of HBV. C/EBP, CCAAT/enhancer binding protein; NF1, nuclear factor 1; HNF4, hepatocyte nuclear factor 4; AP1, activator protein 1; Sp1, SV40 promoter protein 1; TBP, TATA-box binding protein. Squares represent liver-specific/liver-enriched transcription factors; circles represent ubiquitous transcription factors.

Following the release of the capsid into the cytoplasm (Fig. 10.6 [1]), the capsids are actively transported towards the nucleus. Like other viruses, they make use of the microtubule network, which is the long-range transport system in the cytoplasm (B. Rabe, M. Kann, unpublished observations) (Fig. 10.6 [2]). The interaction with the cellular transport machinery is mediated by the carboxy-terminal portion of the core protein, which becomes exposed to the capsid surface upon phosphorylation within this region.[114] This sequence contains a multifunctional domain. Beside its interaction with cytosolic proteins that facilitate the binding to tubulin, it comprises a nuclear localization sequence (NLS).[114] The NLS causes interaction with importin α, which is an adaptor protein for binding to importin β. The cellular transport receptor importin β mediates binding to the cytoplasmic fibres of the nuclear pores and the subsequent transport through the pore into the nuclear basket (Fig. 10.6 [4, 5]).[199] This structure is a cage-like structure formed by eight filaments at the inner surface of the pore. In fact, electron microscopy after microinjection into oocytes of *Xenopus laevis* showed that HBV capsids entered the basket as entire particles with diameters of 32 and 36 nm (T=3 and T=4 symmetry).[199] This finding was surprising because the maximal functional diameter of the nuclear pore was previously though to be 26 nm. The subsequent re-evaluation of the pore diameter by NLS-coated gold particles consistently showed that cargoes up to 39 nm are able to enter the nucleus.[199]

Another striking result of these studies was that *in vitro* phosphorylated capsids, derived from *E. coli*, entered the nuclear basket but were unable to diffuse deeper into the karyoplasm.[114] In fact, these data obtained by the use of permeabilized cells, which allow a direct addition of capsids into the cytosolic space, are consistent with the observation in HBV-transgenic mice in which the nuclear membrane was shown to be impermeable for nucleocapsids.[200] The data on *E. coli*-derived capsids were confirmed using HBV capsids containing an immature genome[115] (Fig. 10.6). In contrast, virus-derived capsids containing the mature, partially double-stranded genome did not show this arrest but generated intranuclear released viral genomes and intranuclear capsids (Fig. 10.6 [8]), as observed in HBV-infected human livers.

Although the detailed mechanism by which mature capsids release their genomes into the nucleus is still not fully understood, it appears that the mature capsids enter the basket, become arrested as immature capsids but disintegrate during arrest. Consequently, the genome and core proteins that are not subjected to arrest can diffuse into the karyoplasm. The core proteins are thought to reassemble into capsids again once they have reached the threshold concentration for assembly.[111] As unpublished results have shown that the reassembly process is combined with the encapsidation of cellular RNA (Fig. 10.6), it can be speculated that the affinity to RNA – which is known to be higher than affinity to double-stranded DNA[142] – is the driving force for capsid formation.

## Generation of episomal DNA

Recent studies on single DHBV-infected duck hepatocytes revealed that every infected cell contains between 1 and 17 copies of episomal cccDNA showing fluctuation over time.[201] In human livers, copies up to 173 were described as correlated with the activity of infection.[202] It must be assumed that the episomal DNA undergoes degradation, but the half-life was documented to be long. Thus, for persistent infection the pool of the intranuclear viral cccDNA has to be refreshed. Beside degradation, external factors affect the pool of cccDNA. For example, cell division was shown to cause a loss of cccDNA. Most recently, it became evident that this reduction of episomal DNA is triggered by interferon-gamma (IFN-γ)-producing CD8+ T cells in the liver.[203]

To generate the cccDNA, which is the template for transcription and thus generation of progeny virus, the viral genome has to be repaired. This involves (1) the removal of the covalently bound viral polymerase, (2) the conversion of the eight nucleotide-long triple-

stranded region into double-stranded DNA, (3) removal of the RNA primer bound to the 5′ end of the DNA plus-strand, (4) filling the single-stranded gap of the genome and (5) the close of the nicks between the 5′ and 3′ ends of each plus- and minus-DNA strand. After the conversion to the plasmid-like cccDNA, the genome becomes associated with histones forming a mini-chromosome.[204] Interestingly, some viral core protein molecules remain bound to the DNA,[120] raising the speculation that the core protein may be involved in viral transcription by regulating the nucleosomal arrangement of the HBV regulatory elements.

However, most of the conversion processes remain unclear. Based on *in vitro* activities of the topoisomerase I on isolated DHBV virion DNA, Pourquier *et al*. speculated that topoisomerase I activity may be a candidate enzyme for removal of the polymerase.[205] More direct evidence exists for the filling reaction of the single-stranded region. Treating DHBV-infected primary duck hepatocytes with different inhibitors, Köck and Schlicht[206] showed that cellular DNA polymerase and not the viral polymerase is probably involved. However, more recent findings on HBV showed that the polymerase plays an essential role in filling the gap of the plus-strand DNA.[207]

In contrast to the retroviridae, integration of HBV DNA into host cell DNA is not only unnecessary for replication of HBV but even detrimental, because linearization of the circular HBV DNA would disrupt at least one gene. Caused by illegitimate replication, however, a significant proportion of viruses contain a linear viral DNA,[208] thus being a potential source for integration.[209,210] However, depending on the supergenomic size of the functional RNA pregenome, such an RNA pregenome can be transcribed only from linear DNA that is longer than the genome. Such artificial HBV DNA constructs have often been used to transfect cell lines *in vitro*, but integrated replication-competent HBV DNA molecules are not found in HBV-infected liver or naturally occurring hepatocellular carcinomas (HCCs). Integrated HBV DNA often contains fusions between HBV genes and host cell genes. The resulting neoproteins may acquire novel properties and have been found in certain HCCs as potentially oncogenic factors (see Chapter 44).

## Transcription of HBV DNA

### *General aspects of transcription*

Transcription of double-stranded DNA into RNA is a highly regulated process that controls the expression of all genes, whether from the host or the virus, in a timely and structurally ordered manner. Thus, all genomes, including those of the smallest replication-competent DNA elements, contain not only structural genes that encode protein sequences but also regulatory elements. These DNA sequences bind a number of cellular or viral proteins that act as positive or negative transcription factors. Positive factors bind the cellular RNA polymerase II at a more or less defined position of the DNA sequence, where it initiates RNA synthesis. The RNA polymerase transcribes one strand of the double-stranded DNA into RNA until a termination signal for this enzyme in the DNA sequence occurs.

Two types of DNA sequence elements contribute to the binding of transcription factors. Promoters are necessary for binding of essential transcription factors and bring the RNA polymerase into position, where it begins to transcribe only one DNA strand at a defined site. Many promoters contain the so-called TATA box, which binds transcription factor TFIID. This factor binds RNA polymerase II in a way that transcription initiates approximately 30 bases downstream. *Enhancers* are DNA segments that enhance transcription initiation at a given promoter, but they do not need to be in an exactly defined position next to the promoter, and their sequence orientation may even be inverted without impairing their activity. Many enhancers, like those of HBV, may act on expression of several genes.

The rate of RNA synthesis beginning at a promoter is controlled by the amount, type and activity of positive transcription factors available in the host cells and by the accessibility of enhancers and promoters. There are some essential ubiquitous transcription factors, others are organ-specific, and furthermore factors may be inducible by intracellular or extracellular signals. Most regulatory gene sequences also contain negative elements that counteract the effect of promoters and enhancers by binding negative transcription factors. Such sequence elements are termed *silencers*.

### *Promoters of HBV*

At least four promoters and two enhancers have been identified in the HBV genome.[211,212] These promoters direct initiation of RNA synthesis upstream of the HBc/e gene, LHBs gene, M/SHBs gene, and the HBx gene (Plate 10.1a, found between p.786–7). Only the LHBs promoter (often referred to as S promoter I, SPI) has a typical TATA box, which positions RNA-polymerase II via TFIID (transcription factor IID) and only the mRNA-encoding LHBs protein has a sharply defined 5′ end. The TATA-less promoters usually have multiple initiation sites, and this is indeed the case for the HBc/e, M/SHBs and X mRNAs. As a result, the corresponding sets of mRNAs with microheterogeneities at their 5′ ends are able to encode nested sets of co-terminal proteins. The two longer of the HBc/e mRNAs encode HBe protein, the shorter starts four bases after the start codon for the HBe protein. In this case, the ribosome can only use the

next start codon for protein synthesis that defines the amino end of HBc protein. A similar phenomenon occurs in the mRNAs encoding MHBs and SHBs. Only the largest of three mRNAs that are initiated at this promoter (also known as SPII promoter) contains the start codon for MHBs. The other two mRNAs contain only the proximal start codon of SHBs. Whether there are mRNAs that encode only smaller HBx proteins but not full-length HBx protein is not clear.

The relative activity of the four identified promoters and the fine specificity of the heterogeneous 5′ ends depend on the cell type in which the HBV genome or fragments thereof are expressed. It appears that the M/SHBs promoter and the HBx promoter are constitutively active in all mammalian cell lines.[213] The M/SHBs promoter contains, for example, four binding sites for ubiquitous constitutive transcription factor SpI.[214] The relatively weak LHBs promoter requires the hepatic nuclear factor 1 HNF123 and HNF3.[215] The HBc/e promoter overlapping enhancer II also binds liver-specific transcription factors such as HNF3, C/EBP[216] and HNF4[217] in order to be activated. Thus, transcription predominantly occurs in liver cells. It is not completely clear whether the promoter for the HBe mRNA (i.e. the 'precore' mRNA) and HBs mRNA (i.e. the 'pregenomic' RNA) can be sharply divided, but it is evident that core and HBeAg synthesis are not always correlated[218] and are activated by different transcription factors (see below). However, RNA synthesis from the core promoter is thought to represent a major determinant governing the hepatotropism of this virus.[219] In addition, mutations in the core promoter are correlated with the response to interferon-alpha therapy.[220] A survey on some binding sites for transcription factors in the promoter/enhancers and regulatory elements of HBV is shown in Fig. 10.7.

Because the proteins expressed by the corresponding mRNAs are absolutely necessary for genome replication and virion assembly, the liver specificity of the transcription signals in the HBV genome may already sufficiently explain its organ tropism. Replacement of the liver-specific HBc/e promoter by a ubiquitously active promoter such as the immediate early promoter of cytomegalovirus allows replication of the HBV genome and assembly of complete core particles in non-hepatic cell lines.[221] Addition of the SV40 enhancer to the HBV genome also allows for generation of virus-like particles in mouse fibroblasts,[222] but the secreted HBV-like structures lacked HBc and LHBs proteins.

### *Enhancers*

To ensure that mRNA synthesis occurs in an orderly manner enhancers may up- or downregulate the activity of the promoters. In addition to host-specific uptake, these promoters restrict expression of a virus to certain cell types. To date two enhancers (I and II) have been identified on the hepadnaviral genomes. The major stimulating element is the enhancer I that enhances the initiation of transcription by a factor of 10–50 in liver cells. Enhancer I is located between the S and the X gene. It spans approximately 200 nucleotides to which numerous factors can bind. While some of them are ubiquitous (e.g. NF-1, AP-1, NFκB), others (e.g. C/EBP, HNF-4, HBLF[223,224]) are liver-specific, restricting the stimulating effect on the hepadnaviral promoters mainly to hepatocytes.[212,225] However, the liver specificity is predominantly caused by enhancer II, which overlaps the pre-C/C-promoter.[216] Its liver specificity is determined by binding to liver-specific transcription factors such as C/EBP, retinoid X alpha (RXRα), the peroxisome proliferator-activated receptor alpha (PPARα) heterodimer and HNF-4, which act differentially on different promoters and thus modulate viral synthesis.[226] Whereas HNF-4 and the nuclear hormone receptors RXRα and PPARα support HBV replication by supporting transcription of the pregenomic RNA, HNF-3 inhibits pregenomic RNA synthesis compared with that of precore RNA. Downstream of the core promoter located promoters in contrast are stimulated by HNF-3, possibly by interfering with the elongation rate of these transcripts.[226] Corresponding to these observations, the enhancer II does not stimulate all viral promoters. While the S promoter is stimulated 10-fold both *in vivo* and *in vitro*, the LHBs promoter appears to be unaffected in both experimental situations.

### *Other regulatory elements*

In addition to the enhancers, three other regulating elements of hepadnaviral transcription were identified. Addition of dexamethasone activates the glucocorticoid receptor, which then binds to the glucocorticoid responsive element (GRE) of the HBV genome. The GRE is located between enhancer I and II and contains two copies of the hexanucleotide 'TGTTCT'. Binding to the GRE enhances transcription further by a factor of 2–5,[227] but does not interact as an independent enhancer element. The stimulating effect may be also be present *in vivo* because glucocorticoid-treated patients express very high levels of HBV, HBeAg and HBsAg.[228]

Two other *cis*-acting elements were identified on the hepadnaviral DNA. The negative regulating element (NRE) inhibits only the transcription from the core/precore promoters with which the NRE overlaps.[229] The so-called CCAAT element was identified overlapping the S promoter.[230] It negatively effects transcription from the upstream located pre-S1 promoter, at least when the CCAAT binding protein is present in high amounts. In contrast, the CCAAT element enhances transcription of the S mRNA, so it was hypothesized that the element is involved in ordered

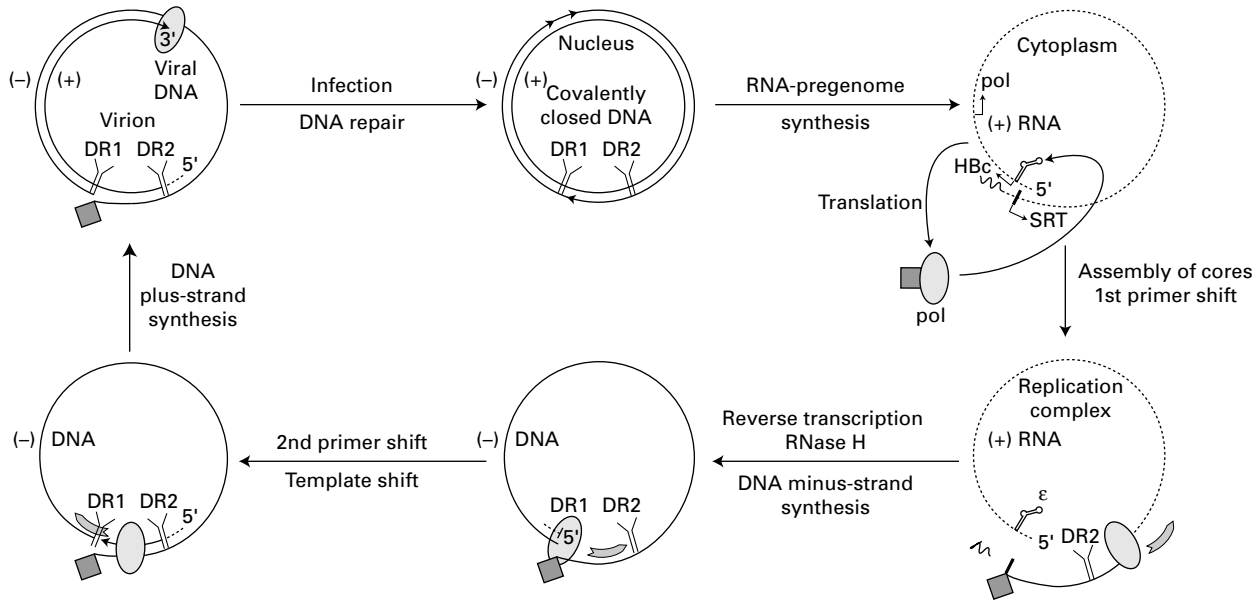


**Figure 10.7** Replication of the HBV genome. The virion DNA (top left, see also Plate 10.1a, found between p.786–7) is brought by the pathway shown in Fig. 10.5 to the cell nucleus and is converted to covalently closed DNA. This episomal DNA is transcribed to various RNAs (see also Plate 10.1a, found between p.786–7 and Fig. 10.5), one of which serves as a template for the polymerase protein (→) and HBc protein (→). These two proteins assemble together with their mRNA to the replication complex. The encapsidation signal ε at its 5′ end governs the packaging of the RNA and the priming of the minus-strand DNA. The redundant part at the 3′ end serves as a signal for the reverse transcription (SRT) after priming. The primase domain of the polymerase (♦) serves as a primer for the reverse transcriptase. Thus, the growing minus-DNA strand (see bottom right) is linked at its 5′ end to the primase. Details of the priming at ε and the first primer shift to the DR1* at the 3′ end of the pregenome are shown in Fig. 10.9. The reverse transcription proceeds until the 5′ end of the RNA template is reached (bottom centre). Thus, a short redundancy is generated in the minus-strand. The RNase H activity associated with the reverse transcriptase degrades the RNA template and leaves at its 5′ end an 18-base-long capped RNA fragment. This fragment has a 6-base homology to the direct repeat DRI and DR2. This weak interaction allows shifting of the fragment and of the reverse transcriptase/DNA polymerase from DR1 to DR2. There, the RNA fragment functions as primer for the plus-strand DNA. The DNA polymerase is able to cross the discontinuity in the minus-strand template because of its short terminal redundancy. Thereafter, the structure of the virion DNA is reproduced. The multiplication effect originates from the fact that one episomal HBV genome can be transcribed to many pregenome molecules by the cellular RNA polymerase II.

expression of,surface proteins to ensure correct virion morphogenesis. Indeed, cloning and expression of naturally occurring mutations of this element were shown to cause retention of the LHBs in the endoplasmic reticulum and one mutation resulted in secretion of malformed viral particles.[231]

### *Viral transcription activating proteins*

All double-stranded DNA viruses and retroviruses contain positive sequence signals for initiation of transcription, but at least in mammalian viruses these *cis*-acting elements are obviously insufficient to warrant highly efficient expression and replication of the viral genome. For most viruses, the first gene products to be expressed are transcription factors that activate promoters of other viral genes in a time-ordered manner. Such products are called immediate early (*i.e.*) genes. The time order of HBV gene expression has not yet been fully analyzed, but it appears that HBx protein is the first protein to be expressed[232] and it has been shown that HBx activates many enhancers (see above). The significance of almost all studies on HBx is hampered by the fact that the effects were observed in cellular systems that either cannot replicate HBV or do not need HBx for expression of HBV from transfected cells. In addition, side-effects of HBx caused by the frequently used high-level transient expression of HBx cannot be excluded.

### *Transcription factors of HBV*

HBx compares with the immediate early proteins of oncogenic DNA viruses such as E6/E7 of papillomaviruses, or EIA/B of adenoviruses. These viral transcription activators are also quite non-specific and also act on cellular growth-controlling genes. It may be noteworthy that the HBx gene contains a weak polyadenylation signal,[233]

which induces a fusion between a truncated HBx protein and polylysine.[234] This HBx variant may be less active in transactivation, but more active in malignant transformation. An effect of an HBV protein on Rb has not yet been observed. However, further transcription activators of HBV have been discovered in HBV DNA-containing HCCs. These are truncated LHBs[235] or MHBs[236] proteins that have lost the sequences necessary for budding and secretion of HBs particles. Although these truncated forms may be rare accidental consequences of the DNA integration, the transactivating effect of the pre-S2 domain[237,238] may also be relevant during normal HBV infection. The transactivating activity depends on the cytosolic localization of pre-S2, which not only occurs with truncated MHBs but also during expression of normal LHBs.[239]

### *Termination of transcription*

As in mammalian mRNAs, all hepadnaviral mRNAs are polyadenylated at their 3′ end. Not only the start but also the stop of transcription is a well-defined and regulated process. It ends at a single polyadenylation signal so that all viral mRNAs are 3′ co-terminal. In all mammalian hepadnaviruses the TATAAA stop signal is located shortly after the start of the HBc gene (Plate 10.1a, found between p.786–7). Thus, the polyadenylation signal for transcription of the supergenomic RNAs encoding for HBeAg, core and polymerase has to be ignored by RNA polymerase II during the first passage. In fact, it was shown that the stop signal needs an additional sequence element upstream of the HBc/e promoter and, furthermore, only becomes active if the initiation site of the RNA is >400 bases distant.[240] In transgenic mice, the recognition of the polyadenylation signal was shown to be tissue-specific.[241]

Due to the leaky stop signal an RNA of supergenomic length is generated, starting upstream or within the pre-C sequence and ending within the core gene. It has redundant ends ('R'), which are essential for the genome replication of hepadnaviridae (Plate 10.1b, found between p.786–7). All other RNAs of HBV are shorter than genome length. Thus, RNAs of 3.5 kb for HBc/e, 2.4 kb for LHBs, 2.1 kb for M/SHBs and 0.8 kb for HBx are formed. They are all 3′ co-terminal and are, with the exception of the X-mRNAs, polycistronic. The existence of several promoters and one common stop signal is an elegant way to express several different translation products from one very compact genome with overlapping genes. This expression strategy is unique to *Hepadnaviridae*.

### *Processing of HBV RNAs*

After transcription, RNAs are modified by addition of methylated bases to the 5′ end (known as cap) and addition of a polyA tail at the 3′ end. Eukaryotic mRNAs are further modified by splicing, i.e. removal of internal non-coding RNA regions (introns) and ligation of the remaining fragments (exons) which finally constitute the mRNAs. The splicing is essential for export of the mRNAs from the nucleus.

However, like many retroviral mRNAs, all known HBV genes are contiguous and, thus, the mRNA precursors mentioned previously must not be spliced, although numerous potential splice signals are present in the RNA. HIV has solved the problem by using the *cis*-acting so-called Rev responsive element (RRE) that binds the viral Rev protein to access the RNA-export pathway Rev mediated by CRM1-mediated nuclear export that is normally used for export of cellular proteins and RNAs (U snRNAs and 5 S rRNA). In contrast, other retroviruses use a constitutive transport element (CTE) for the same purpose. The CTE directly recruits the cellular mRNA export receptor TAP to the viral RNA to override nuclear retention.[242]

Although the molecular mechanism of nuclear export of hepadnaviral mRNAs is not fully understood, a bipartite splicing inhibiting RNA sequence was identified.[243–245] Termed post-translational regulatory element (PRE) it consists of two conserved RNA stem-loops – HPREα and HPREβ – that are required for function.[246] The PRE overlaps the X promoter and most of the X gene. Without the PRE abundance of cytoplasmic HBV RNA is much lower. Thus, the element may be necessary for both splice regulation and RNA transport.

However, recently a spliced RNA has been found in DHBV. This RNA starts at the 5′ end of the pregenomic mRNA and fuses the core with S gene. Mutations of the splice acceptor and donor sites showed, surprisingly, that this RNA appears to be essential for virion production.[247] Spliced HBV RNAs are also found in human HCCs.[248] In chronic HBV carriers a fusion protein of polymerase and an unused ORF was described,[249] inducing antibodies in patients. The appearance of these antibodies was correlated with liver fibrosis.[250]

## Translation

### *General aspects of translation*

Protein synthesis in eukaryotic cells is predominantly regulated by the site and frequency of its initiation at the mRNA. The scanning model of this process applies to most mRNAs. The small ribosome subunit binds to the 'capped' 5′ end of the mRNA and scans thereafter the sequence until an AUG start codon for methionine occurs. Protein biosynthesis starts from this site after binding of initiation factors and the large subunit of the ribosomes. Flanking bases influence the efficiency of protein synthesis at a start co-

don. A purine at the +4 and/or –3 position is usually found in initiation codons, but not in internal codons for methionine.[251] Additionally, a stem-loop structure on the RNA, about 30 bases downstream of the start codon, can enhance the start of protein synthesis.[252] Synthesis stops at one of the three possible stop codons UAA, UAG or UGA, which occurs first in this particular ORF. Thus, the position of the 5′ ends of the mRNAs in the HBV genome determines which gene product is expressed. Exceptions to this rule are internal ribosome entry sites (IRES), leaky scanning for start codons, usage of atypical start codons, read-through of stop codons, frame shifting and reinitiation. Some of these exceptions may apply to the translation of certain HBV proteins.

An important early step in protein biosynthesis is the distribution of the protein synthetic complex between the cytosol and the ER. Nascent protein chains that contain a hydrophobic α-helical stretch of 16–20 amino acids bind to a signal recognition particle that attaches the ribosome to a pore-like structure at the ER. The growing peptide is transported through this pore to the lumen of the ER. The particular structure and number of such hydrophobic signal sequences determine the intracellular distribution, membrane topology or secretion of the protein. Proteins without signal sequence are synthesized by free ribosomes and released after completion to the cytosol. Enveloped viruses with subgenomic mRNAs such as HBV normally use free ribosomes for synthesis of their nucleocapsid proteins, and ER-bound ribosomes for synthesis of the enveloped protein (Fig. 10.5).

### *HBe protein*

The HBe protein is translated from the longest mRNA. As mentioned above, this RNA differs from the pregenomic mRNA in that it comprises an additional start codon at its 5′ end. The translation initiation at this precore start suppresses the use of downstream-located AUGs.[253] This includes the core start codon that is 'in frame' with the precore AUG. In consequence, the primary translation product comprises a 29 amino acid signal sequence amino-terminally of the core protein. The precore targets this 25-kDa protein to the ER lumen where a cellular signal peptidase at the ER lumen removes the first 19 amino acids of the precore region.[125] The remaining 10 amino acids of the precore cause a folding different to that of the core protein, allowing dimerization but no further assembly of these dimers to capsids. Further proteolytic processing of the HBe-protein probably occurs in the trans-Golgi apparatus.[126] This cellular compartment contains proteases that cleave within the arginine-rich carboxy-terminal domain of the HBe protein so that the processed protein has a molecular weight of only 15 kDa. However, as the signal peptide of HBe protein is not particularly strong, a significant part of the HBe protein remains cytoplasmic.[127] The amount of synthesized HBe protein relative to other HBV proteins is probably regulated by the level of mRNA containing the start codon of an uninterrupted HBe gene. Many patients have HBV variants where little or no HBe mRNA is made because of mutations in enhancer II or the HBc/e promoter. Even more frequent are variants with a stop codon in the pre-C sequence (see Chapter 11).

### *HBc protein*

The start codon of HBc protein has two guanosines at position –3 and +4, which is a relatively good but not optimal context for initiation because it is localized within the stem of the encapsidation signal ε. Nevertheless, a highly efficient translation of the HBc mRNA is necessary, because at a critical level of HBc protein and polymerase protein, this mRNA is encapsidated into core particles and is no longer available for protein synthesis.[140] The overlapping use of this mRNA could suggest that core and polymerase are translated from the same RNA species but from different RNA molecules. However, recent data showed *cis*-preferential recruitment of duck hepatitis B virus core protein to the RNA/polymerase preassembly complex.[254]

### *Polymerase*

An intensive search for an mRNA containing a 5′ end slightly upstream of the presumed amino end of the polymerase sequence was not successful, although a very low abundant mRNA for polymerase cannot strictly be excluded. Most members of the orthoretroviridae express their polymerase via a frame shift between the ORFs of gag (i.e. the analogue of HBc protein) and pol, thus generating a gag-pol fusion protein. Mutational analysis excluded this possibility for HBV. Instead, an unusual internal initiation of the pol protein synthesis at the HBc mRNA is implied. This would generate a full-length non-fused HBV polymerase by translation initiation.[132,255] A leaky scanning mechanism appears possible, because an optimal start codon of ORF C seems to reduce the efficiency of pol expression.[256] There are, however, several initiation codons upstream in the HBc mRNA, which should be even stronger. The existence of a weak IRES in proximity to the pol start codon would be another obvious explanation.[257] Ideally, the relative frequency of initiation at the HBc or pol start should regulate the required proportion of HBc to pol protein, which is probably 240:1. However, recent analysis revealed that the polymerase is relatively overexpressed and

that the majority of hepatitis B virus reverse transcriptase in cells is non-encapsidated but is bound to a cytoplasmic structure,[258] as was determined for DHBV. Polymerase was translated at a rate of 10% compared to core, whereas the half-life of non-encapsidated polymerase was very short, indicating that the translation rate of the polymerase is not limiting for encapsidation.[259] The inability of the polymerase to act on exogenous substrates lacking epsilon prevents massive interference with host RNAs.[135,260]

### *LHBs*

Initiation of LHBs protein synthesis follows the usual rules.[261] It occurs initially in the cytosol because the pre-S domains do not contain an ER translocation signal peptide. The signal peptides I and II of the S domain insert into the ER membrane, but the entire pre-S domain is too long to be translocated to the ER lumen during protein synthesis[86] and interacts with cytosolic factors. In agreement with this conclusion is the absence of glycoside in the pre-S2 domain of LHBs. The amino-terminal methionine is substituted by myristic acid. The central hydrophilic part of the S domain, however, is in the lumen of the ER and more efficiently glycosylated than the S domain of MHBs or SHBs.[262] The mRNA for LHBs is not translated into MHBs or SHBs.[261,263]

### *MHBs and SHBs*

These two gene products are usually co-expressed because of the common promoter for their mRNAs. It is not clear which factors control the relative ratio between the mRNAs containing a start for MHBs or only for SHBs. Irrespective of this transcriptional regulation, the ratio between MHBs and SHBs is also regulated at the translational level. The start codon of MHBs does not have the optimal flanking bases, whereas that of SHBs is optimal for initiation of protein synthesis. Thus, mRNAs for MHBs also always express some SHBs unless the start codon of SHBs has been mutated.

Signal I of the S domain is obviously able to translocate the pre-S2 domain of MHBs to the ER lumen, because most of the MHBs is glycosylated in secreted HBs particles.[264] However, the folding of the nascent S domains seems to be slightly different in MHBs and SHBs, because the S domain is only rarely glycosylated in MHBs.[262] Truncation of MHBs at the predicted transmembrane helix III leads to a topology where pre-S2 is not translocated to the ER lumen.[238]

### *HBx*

Translation of HBx protein(s) may occur also from mRNAs that contain or do not contain the first start codon of ORF X,[213] but the size of naturally occurring HBx proteins has not yet been reliably elucidated. Sequence predictions suggest that it is a cytosolic protein. Overexpression of HBx protein using vaccinia vectors suggests that it is a very labile phosphoprotein within the cell, with a half-life of 20 minutes.[155] The high insolubility of HBx protein suggests that it oligomerizes rapidly or binds to other cellular proteins.

## Assembly of HBV

### *General strategy of genome maturation*

The actual multiplication of the HBV genome occurs in the nucleus of the infected cell by the cellular RNA polymerase II, which transcribes the circular HBV DNA to more than genome-length mRNA with redundant ends (see Plate 10.1b, found between p.786–7). All gene products of HBV (probably with the exception of HBx protein) are used to encapsidate that RNA, transcribe it to a circular DNA, and secrete it as an enveloped virion with attachment sites for new target cells. An overview is shown in Fig. 10.8.

### *Encapsidation of pregenomic RNA*

After translation of sufficient amounts of HBc and polymerase proteins, these proteins assemble together with their mRNA, and cellular proteins, including the heat shock protein Hsp90, the co chaperone p23, and additional, as yet unknown, factors.[143–147] Core particle formation includes the encapsidation of a cellular protein kinase, the identity of which is controversially discussed. Encapsidation occurs only when the polymerase interacts with a specific RNA sequence present at the 5′ end (base 1846 to base 1907) of the HBc mRNA.[141] This signal is termed ε (for encapsidation) and acts predominantly in cis to the polymerase. ε is characterized by a secondary structure consisting of a stem, a bulge, a loop and a non-paired U (see also Fig. 10.9),[265–267] but binding of the polymerase alters the structure of ε[268] and binding to ε is required for activation of the polymerase.[260] However, fusions of ε to heterologous RNAs failed to cause encapsidation. This observation initiated a search for additional sequences required for packaging. Indeed, a second region – termed ε II that is 900 nucleotides 3′ of ε I – was found to be essential at least in avian hepadnaviruses.[269] In addition, this observation may explain why only the pregenomic RNA is encapsidated, although the signal sequence ε is present on the 3′ ends of all hepadnaviral RNAs.

### *Initiation of reverse transcription*

In HBV-expressing cells genome maturation (e.g. re-

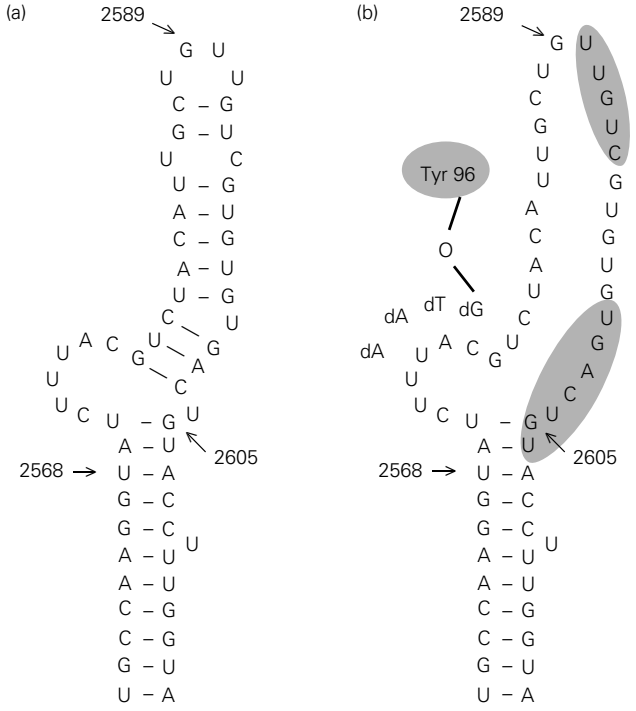


**Figure 10.8** Structure of the DHBV ε as the free form (a) and after binding of the polymerase (b) (modified from Beck and Nassal[268]). Sites of ε interacting with the polymerase are shown as grey shadows. The priming of the DHBV DNA minus-strand starts within the bulge region in the 5′ terminal ε. Priming is initiated by covalent linkage of the first nucleotide with Tyr96 of the DHBV polymerase. After synthesis of the first four deoxynucleotides, the polymerase-oligonucleotide complex switches to DR1 (see Fig. 10.8).

verse transcription to minus-strand DNA and plus-strand DNA synthesis) is a tightly coupled event and seems to occur exclusively within the core particles. However, expression of an active DHBV polymerase in a cell-free system showed that in these systems the first steps of DNA synthesis may occur in the absence of core protein. Using this system, it could be shown that minus-strand DNA synthesis starts in the bulge of the ε-signal. In contrast to that of retroviruses, hepadnavirus replication is initiated *de novo*, i.e. without a nucleic acid primer. Instead, the –OH group of a tyrosine in the amino-terminal domain of the polymerase (Tyr 96 in DHBV,[270] Tyr 63 in HBV[41]) is used for formation of a phosphodiester linkage to the first nucleotide. Due to this mode of initiation, the polymerase becomes and remains covalently linked to the DNA minus-strand.

### *Elongation of DNA minus-strand*

After synthesis of the first four nucleotides within the bulge (DHBV), the polymerase together with its covalently bound nucleotides dissociates from its template and re-anneals with a complementary sequence in the DR1 close to the 3′ end, which was previously named signal for reverse transcription (see Plate 10.1b [found between p. 786–7] and Fig. 10.8). Experiments on the expression of the HBV polymerase confirmed this initiation model for the human hepatitis B virus,[271] although the homology between the bulge and DR1 in all human HBV genomes is restricted to three nucleotides. Because there are many sequences complementary to the three or four initial bases of the minus-DNA strand within the hepadnavirus genome, an additional process must be involved in correct transfer of the polymerase nucleotide complex. Using mutagenesis analysis, a sequence element designated phi located upstream of the 3′ DR1 sequence was identified that is complementary to ε and is important for efficient viral replication. It was hypothesized that this element brings the 3' DR1 sequence into proximity with the three nucleotide primer synthesized at the bulge of ε.[272]

Minus-strand DNA synthesis continues after priming and translocation to the 3′ terminal DR1 of the pregenomic RNA. From mapping the 3′ end of the minus-DNA strand, it appears that this strand nearly coincides with the 5′ end of the pregenomic RNA. Thus, the 3′ end of the minus-DNA strand is specified by 'run off', when the polymerase reaches the end of its RNA template, resulting in a terminal redundancy of the minus-DNA strand of 8–10 nucleotides.

### *RNase H and priming of the DNA plus-strand*

All reverse transcriptases are associated with an RNase H activity, which cleaves the RNA of RNA–DNA hybrids into oligoribonucleotides. Mutational inactivation of the RNase H domain in the HBV polymerase results in a block of DNA plus-strand strand synthesis.[273] However, the RNase H domain of HBV polymerase is not able to cleave the last RNA nucleotides, bound to minus-strand DNA. Thus, an 18-base-long capped RNA fragment from the 5′ end of the pregenome is generated. This fragment dissociates by unknown reasons from its 5′ terminal DR1 in the DNA minus-strand and is translocated to DR2. Here, it functions as a primer for the DNA plus-strand. The DNA begins with the last base of DR2[274,275] and continues towards the 5′ end on the minus DNA strand. Here, the plus-DNA strand synthesis crosses the discontinuity at the 3′ and 5′ ends of the minus-DNA strand leading to a circular DNA genome. Obviously, the specific primer translocation and the circularization processes are complex. In addition to the donor and acceptor sequences, three other *cis*-acting sequences, named 3E, M and 5E, contribute to both processes. By disrupting base-pairing between 3E and M3, and between 5E and M5, evidence was obtained that the ends of the minus-strand template are juxtaposed

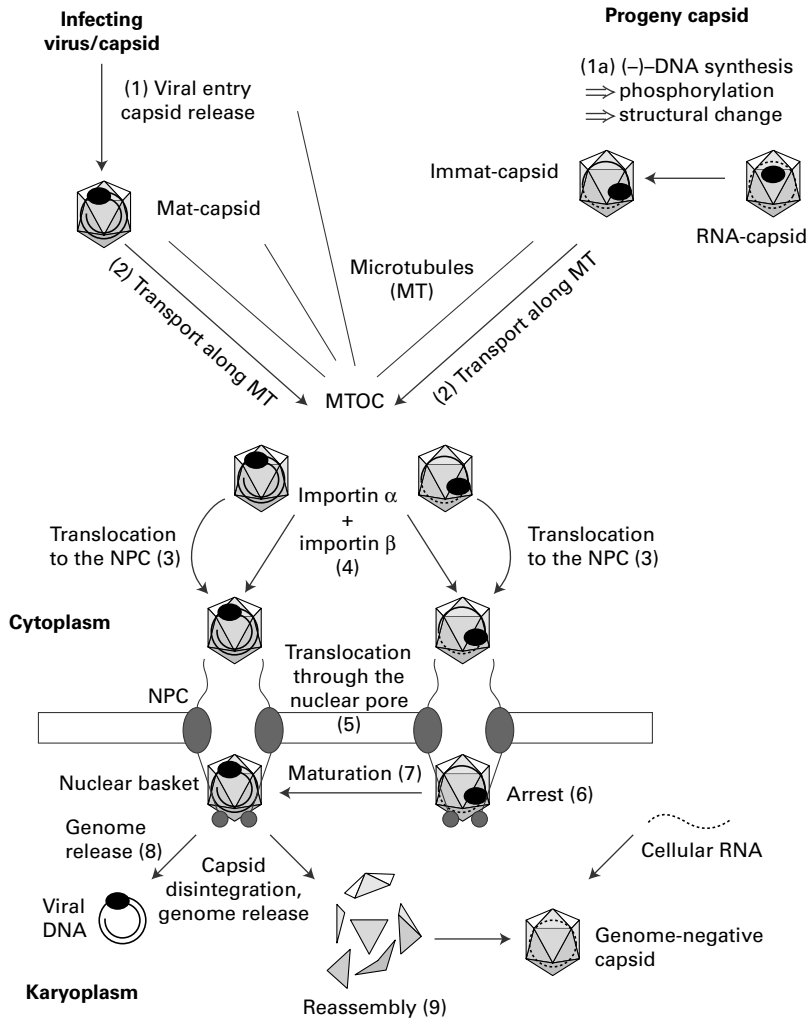


**Figure 10.9** Intracellular transport of HBV capsid and genome. Infection starts with the release of the capsid from the surface proteins (1). The capsid is transported though the cytoplasm towards the microtubules organizing centre (MTOC) using the cellular microtubule network (2), followed by translocation to the cytosolic side of the nuclear pore (3). It exposes nuclear localization signals (NLS) on its surface, allowing the binding of importin β via the importin α adaptor protein (4). Importin β mediates the transport through the nuclear pore into the nuclear basket (5). There, the capsids disassemble and genome becomes released (8). The core proteins can reassemble to capsids (9). At least a part of the newly formed capsids contain cellular RNA. Cytosolic progeny capsids have to undergo phosphorylation of core proteins to induce a conformational change (1a). As infecting capsids, they are actively transported towards the nuclear pore (2, 3). The conformational change allows the binding of importin α and β and the transport into the nuclear basket (4, 5). If the genome maturation has not finished at that time, the capsid remains arrested within the basket (6) and genome maturation can proceed (7). As with the infecting capsids, disassembly and genome release occurs (8).

via base-pairing to facilitate the two template switches during plus-strand DNA synthesis.[276] However, a small terminal redundancy (5′r and 3′r) on the ends of the minus-strand DNA has also been shown to be important, but not sufficient, for circularization.[277,278]

### *Re-entry of mature HBV DNA to the nucleus*

Tuttleman *et al*.[174] showed that the amplification of cccDNA in DHBV-infected primary hepatocytes occurs after the cells have lost their susceptibility for viral uptake. These data were confirmed in the non-susceptible, HBV genome-carrying cell line HepG2.2.15, indicating also that nuclear transport of the genome is not linked to viral uptake. These observations were surprising as they described the first time that a progeny viral particle 'reinfects' a cell without having left the cell. Beside hepadnaviruses, this internal amplification has only been described for other pararetreoviruses such as caulimoviruses and potentially for foamyviruses. In all other viral studied infections, the entry pathway of an incoming virus is strictly separated from the exit pathway of the progeny virion. It must be assumed that in hepadnaviruses this strategy plays an important role in establishment of infection. Early in infection, when viral surface proteins are not yet significantly synthesized, the entry of progeny genomes into the nucleus results in accumulation of the cccDNA.

However, due to the lack of a suitable infection system all data on the nuclear transport were obtained in artificial systems such as the digitonin-permeabilized cells. These experiments show that the progeny capsids harbour the nuclear transport competence and mediate the transport of the progeny genome into the nucleus (Fig. 10.6). Interestingly, exposure of the nuclear localization signal – present on the C-terminus of the core proteins – is associated with phosphorylation of the core proteins.[115] Release of the genome from the capsids was linked to genome maturation, implying a tight regulation.[115] In fact, a defined order of genome maturation, localization and release appears to be essential. If the genome was released outside the nucleus it could not

be transported into the karyoplasms, thus not allowing transcription and further genome multiplication. A premature genome release before genome maturation is finished would prevent synthesis of replication-competent viral DNA as the polymerase depends on the presence of the core proteins for plus-strand DNA synthesis.[110]

Hepadnaviral cccDNA in the nucleus is subject to degradation, with a half-life of 35–57 days *in vivo*.[279] Thus, the pool of intranuclear HBV genomes has to be permanently restored to ensure viral persistence.

### *Envelopment of core particles*

After establishment of infection and sufficient surface protein synthesis, mature core particles are primarily targeted to form progeny virion instead of increasing the pool of intracellular cccDNA. Both pathways overlap as determined for DHBV mutants being incompetent for virion secretion. In these mutants intranuclear cccDNA accumulated up to 50-fold over the natural copy number.[280]

The assembly of core particles occurs in the cytosol, but after assembly and genome maturation HBc protein seems to exhibit an affinity for intracellular membranes, which contain inserted LHBs molecules.[95] It has been shown that significant minus-strand DNA synthesis must have occurred before encapsidation of the core particles.[281] It is presently unsolved whether the conformation change of the particles during genome maturation, which is implied by this observation, is the same as is responsible for exposure of the nuclear localization signal.

For secretion of enveloped virions, an excess of SHBs protein is necessary.[68,282] It appears that the envelope is formed by mixed aggregates of LHBs, MHBs and SHBs, but MHBs seems to be dispensable. For secretion, virions and accompanying HBs particles move from the ER via the Golgi apparatus to the cell surface.[283] During this migration within transport vesicles, the HBs protein is further modified similar to normal cellular secreted proteins. The glycoside side chains of the HBs proteins are trimmed and modified. Covalent disulfide bridges are formed within and between HBc or HBs subunits. It appears that the release of virions and HBs particles from secretory vesicles does not require any specific signal and follows the constitutive pathway of secretion. However, secretion requires trimming of the immature N-linked glycan bound to the HBs protein. Inhibition of that trimming by the glucosidase I inhibitor *N*-butyldesoxynojirimycin also inhibits the appearance of HBV particles in the supernatant of transfected HBV-producing cell lines.[284] The secretion of HBs particles is not as strongly inhibited, probably as a result of the lower contents of glycoside in HBs proteins.

Somewhere between envelopment of core particles and secretion, part of the LHBs molecules is refolded in the pre-S domain from the internal face of the envelope to the surface. Low pH, as it occurs before secretion in the trans-Golgi network seems to favour this refolding, because low pH also induced the *in vitro* appearance of additional pre-S1 epitopes on the surface of HBV particles.[85]

Human hepatoma cell lines such as HepG2 and Huh7 express a suitable ratio of all HBV proteins to allow for virion assembly and secretion. Immortalized mouse hepatocyte cultures[285] are also able to produce HBV. However, *in vivo* not all hepatocytes may be able to express all HBV proteins in a suitable ratio. Thus, in histological specimens from persistently infected liver, only a few cells contain HBV DNA, HBcAg and HBsAg, whereas many cells contain HBsAg or HBcAg alone.

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# Chapter 11
# Epidemiology

Daniel Lavanchy

Worldwide, hepatitis B virus (HBV) is the most common among those hepatitis viruses that cause chronic infections of the liver in humans and represents a global public health problem. In 2000, according to WHO estimates, there were over 5.2 million cases of acute hepatitis B infection. Chronic hepatitis caused by HBV may progress to cirrhosis and death from liver failure, and chronic HBV infection is the major cause of hepatocellular carcinoma (HCC) worldwide.[1,2]

Acute hepatitis B usually runs a self-limited course in adult subjects, with most patients recovering completely. Fulminant hepatitis occurs in 1–2% of acute infections, resulting in the death of most of these patients.

About 15–40% of chronically infected subjects will develop complications, leading to an estimated 520 000–1 200 000 deaths each year due to acute and chronic hepatitis, cirrhosis and liver cancer. HCC prevalence is known to vary widely among the world population, and those areas with higher prevalence of viral infection present the highest HCC rates.[3–8] HBV causes 60–80% of the world's HCCs,[9,10] one of the major three causes of death in Africa, Asia and the Pacific Rim.[11] The incidence of HCC has increased worldwide and nowadays it constitutes the sixth most frequent cancer, representing around 5% of all cancers worldwide.[10,12–15] HBV has been classified by the International Agency for Research on Cancer as carcinogenic to humans.[10] The risk of developing HCC is increased, although currently not precisely quantified, when HBV infection occurs in combination with alcohol intake or co-infection with hepatitis C (HCV), hepatitis delta (HDV) or human immunodeficiency virus (HIV).[16–19]

## Burden of the disease

The economic burden of HBV infection is substantial because of the high morbidity and mortality associated with HBV infection. Even in the early stages of chronic HBV infection, before the onset of advanced liver disease, health-care costs of patients with chronic HBV infection are increased. Costs then escalate with increasing severity of illness. The economic burden should also reflect the loss of productivity due to acute and chronic disease. Such data are lacking in most countries, but particularly in developing countries.

In the USA, it was estimated that HBV-related liver disease accounted for an average of US$ 4000 in patients with compensated liver disease, increasing to more than US$ 20 000 per year as liver function decompensated. Liver transplantation was estimated at a cost of approximately US$ 90 000.[20–23]

The economic burden of hepatitis B in Germany was investigated in a small cohort, and it was estimated that the total HBV-related costs for the country exceeded DM 1200 million (approximately US$ 800) in 1997.[24]

In South Korea the total annual societal cost was estimated to be slightly below US$ 1 billion. The direct costs (prevention and disease-related), amounting to almost US$ 700 million, were equivalent to 3.2% of the South Korean national health-care expenditure for 1997.[25]

Thus, medical costs, in the few countries where they have been evaluated, showed a very substantial burden to society. Due to the lack of data in most countries with higher endemicity for HBV, national cost evaluation studies should be conducted.

## Global distribution of HBV infection

More than 2 billion people have been infected worldwide and, of these, 360 million suffer from chronic HBV infection. The incidence of HBV infection and patterns of transmission vary greatly throughout the world in different population subgroups. It is influenced primarily by the predominant age at which infection occurs. Prevalences of anti-HBs and anti-HBc are much higher than those of chronic carriers – hepatitis B surface antigen (HBsAg)-positives – in all populations. Endemicity of infection is considered high in those parts of the world where at least 8% of the population is HBsAg-positive. In these areas, 70–90% of the population generally have serological evidence of previous HBV infection[26] (Fig. 11.1). Almost all infections occur during either the perinatal period or early in childhood, a fact that accounts for the high rates of chronic HBV infection in these pop-

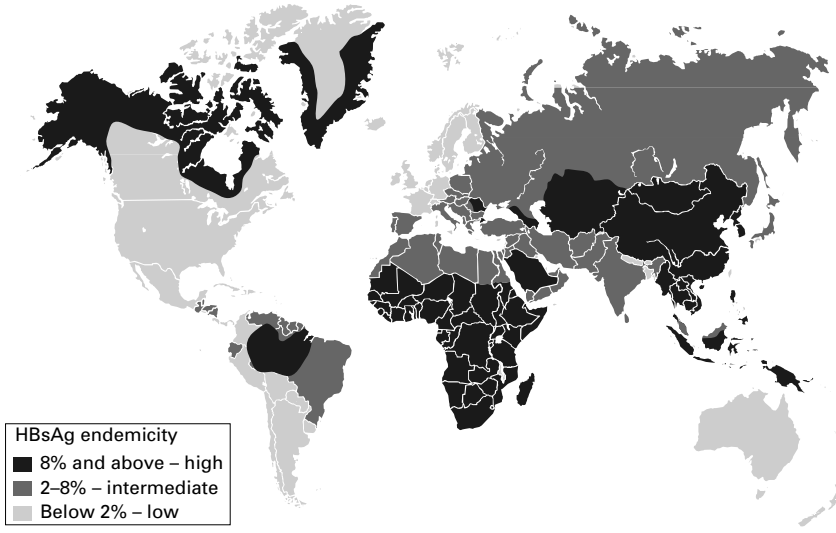


**Figure 11.1** Global prevalence of HBsAg.[26]

ulations. Risk of HBV infection continues after the first 5 years of life, but its eventual contribution to the high rate of chronic infection is less significant.[3]

In high endemic areas such as Asia, sub-Saharan Africa and the Pacific, carrier rates for HBsAg range from 8% to 25% and anti-HBs prevalences from 60% to 85%. Thus, exposure to HBV in high endemic areas, measured serologically, may approach 100%.[27,28]

In areas of the world with an intermediate pattern of HBV infection (eastern and central Europe, the Middle East, the Indian subcontinent and the Amazon basin), the prevalence of HBsAg positivity ranges from 1% to 8%, serological evidence of past infection is found in 10–60% of the population and the lifetime risk of becoming infected with HBV is estimated to be 20–60%.[29,30] In these areas mixed patterns of infant, early childhood and adult transmission prevail.[30]

In most developed parts of the world, the prevalence of chronic HBV infection is <1%, and the overall infection rate is 5–7%.[29]

Overall, approximately 45% of the global population live in areas of high chronic HBV prevalence.[13,28]

## Africa

Sub-Saharan Africa represents a high endemic area for HBV, some countries being among those with the highest prevalence of HBV infection in the world (>8%). This has been explained by a high rate of perinatal and horizontal transmission among children, which in turn has been attributed to promiscuity. Compared with neonatal infection, contamination before the age of 5 but after birth is associated with a rate of chronic infection that is somewhat lower (40–70%), but with a higher proportion of chronic active hepatitis, cirrhosis and HCC among children and young adults.[31,32]

## Americas

In the low prevalence area of North America, the lifetime risk of infection is <20%, and the infection is acquired primarily in adulthood. In most of North America, the epidemiological aspects and public health implications of HBV infection are similar to those in north-western Europe and Australia in terms of prevalence of chronic HBV infection (about 0.2%). The incidence of acute hepatitis B is decreasing as a result of immunization campaigns (routine infant or adolescent immunization) and implementation of measures for controlling HIV infection, transmission through sexual contacts and injection drug use.[32,33] Within these areas most infections occur among adult populations at high risk, including injection drug users, persons with multiple heterosexual partners, men who have sex with men (MSM), and health-care workers. Although the proportion of infant and early childhood infections is low, they can account for a disproportionately high number of chronic HBV infections.[34,35]

The US Centers for Disease Control and Prevention (CDC) estimates that in the United States about 80 000 persons become infected with HBV each year. New infections result in 8400–19 000 hospital admissions annually and in 140–320 deaths from fulminant hepatitis. About 5000 people die every year from liver disease caused by HBV infection.[35,36] There are 1–1.25 million chronic carriers in the United States, many of whom are immigrants from high or moderate prevalence areas. The overall age-adjusted incidence rates of HCC increased from 1.4 per 100 000 in 1975–1977 to 3.0 per 100 000 in 1996–1998. The increase affected most age groups above 40 years, with the greatest increase in the 45–49-year-old age group. White men had the greatest increase (31%) in the time period 1996–1998 compared with 1993–1995. The

incidence of HCC continues to increase rapidly in the United States, with rates increasing the fastest in white men 45–54 years of age. These findings are consistent with a true increase and could be explained by consequences of HBV and HCV acquired during the 1960s and 1970s.[37]

For South America, we need more information on prevalence, sources of contamination (nosocomial), immunization programmes and their impact. There are population groups with different epidemiologies that should be taken into consideration when defining prevalences.[38]

## Asia

Many Asian countries are among those with the highest prevalence of chronic HBV infection in the world (>8%). This is due to a high rate of perinatal transmission from HBeAg-positive mothers to their newborns. Perinatal transmission is associated with chronic infection in >90% of infected children and young adults, explaining the high rate of subsequent neonatal transmission. In patients infected at birth, mild to severe chronic hepatitis develops after a 10–30-year lag phase where the viraemia level is high and hepatitis is mild or absent. In these countries, HBV infection is responsible for most cases of HCC, and represents one of the three major causes of death. Routine immunization of neonates has resulted in a dramatic reduction in the incidence of acute hepatitis and HCC in children in those countries where this has been implemented.[39]

## Australia and Oceania

In Australia the epidemiological aspects and public health implications of HBV infection are similar to those in North America and north-western Europe in terms of prevalence of chronic HBV infection (about 0.2%). The epidemiology differs significantly in the population of Australian aborigines.[39] In Oceania, high endemic areas with carrier rates for HBsAg are also found. Although in some countries (e.g. Papua New Guinea, Solomon Islands) infection rates in infants are relatively low, a rapid increase occurs during early childhood.[39]

## Europe

The prevalence of chronic carriers ranges from <0.1% in north-western Europe to 1–5% in southern Europe. The lifetime risk of infection is <20%, and the infection is acquired primarily in adulthood.

In 1996, it was estimated that more than 1 million people acquired acute hepatitis B infections annually in the 51 countries of the European Region. In Western Europe, incidence rates of <6 per 100 000 were reported from all countries of the European Union apart from France (nine per 100 000) and Luxembourg (13 per 100 000).[40] The estimated number of new cases of hepatitis B has been stable at around 1 million per year. In about 90 000 cases, these infections became chronic. Most cases of chronic hepatitis B are related to the so-called wild-type HBV variant.[30,40,41] By 2001, the overall epidemiological picture had changed little, with low prevalence rates of HBsAg positivity in northern and Western Europe and highest rates in the south and east, in particular the Central Asian republics.

In eastern Europe and the newly independent states, the pattern of risk factors for viral hepatitis is changing significantly, but up until the present, few epidemiological studies have investigated the risk factors for infection using cohort or case-control methodologies. The highest reported rates in the countries of central and eastern Europe, about 20 per 100 000, were in Bulgaria (1998), Estonia and Latvia. Higher rates than that were reported from Kazakhstan, Kyrgyzstan, Moldova, the Russian Federation and Uzbekistan.[41]

In southern Europe, the prevalence of chronic infection is intermediate (1–8%). In these countries, >95% of infections are acquired during adulthood in immunocompetent subjects and are therefore followed by resolution in ~95% of cases. Over half the cases of chronic hepatitis B are related to infection with HBeAg-negative variants. In Italy, up until the mid-1970s, hepatitis B morbidity rates were high, mainly attributed to use of unscreened blood, reuse of inadequately sterilized medical equipment, and increase in intravenous drug use. During the 1980s, HBV infection in Italy began to decrease, mainly due to improvements in health-care delivery, better living standards and the 'AIDS effect' contributing to safer sexual practices. The implementation of a national infant immunization programme in the early 1990s led to a further decrease, transforming Italy into a low prevalence country. A similar, although less marked, evolution could also be observed in Greece and Spain.[42,43]

## Migrating populations

Although travel and immigration are not new phenomena, the impact of migration is most often underestimated. Many countries are facing new situations, with migrants arriving on a mass scale only in recent years. Not only are an increasing number of people on the move, but the demography of these populations is changing. Labour demand patterns have induced a highly gender- and age-specific demography. For the most part, migration populations are highly unstructured, and full access to these populations is often impossible. However, understanding that hepatitis B infection is a highly prevalent disease in some parts of the world is critical to providing the adequate health-care services needed.


**Figure 11.2** HBV genotypes: global distribution (W. Gerlich, personal communication, 2003).

Special migrating populations are infants and young children being adopted, in relation to the fact that international adoption has become increasingly common in recent years. This phenomenon only really started in the second half of the 20th Century. Statistics show that the trend towards international adoption is on the rise. In the United States, for instance, there were 4864 international adoptions in 1979 and 16 000 in 1998. Many children adopted from overseas come from areas where HBV infection is common. Adopted children come often from eastern and south Asia, Africa, South America, and more recently from eastern Europe. In these regions the prevalence of HBsAg ranges from 2% to 15%. Thus, the adopted children have a much higher carrier rate than the general population in most of the countries of adoption, and the figures for hepatitis B infection are alarming within the scope of international adoption.[44–47] Families considering inter-country adoption should be made aware of the risks that the child could be a chronic HBV carrier.

## Epidemiology of hepatitis B serotypes and genotypes

HBV shows substantial genetic heterogeneity. Four serotypes of the HBsAg called subtypes (adw, ayw, adr and ayr) of HBV have been defined by two mutually exclusive determinant pairs, d/y and w/r, and a common determinant a. By subdivision of the four major subtypes in the mid-1970s, nine different subtypes were identified. They show some distinct geographic distributions.[48–56]

Sequencing of viral genomes has now become the major tool used for descriptive virology, and sequence data are now used to reconstruct the phylogenetic history of viruses and to delimit genetic subtypes. Genetic analysis has allowed us to classify HBV into seven distinct genotypes (A–G) that have different geographical distributions (Fig. 11.2), and associations with different risk groups for infection. Genotype A is predominantly found in north-western Europe, North America and central Africa, genotypes B and C are found predominantly in eastern and south-eastern Asia including China and Japan, genotype D is mainly found in the Mediterranean area, genotype E is predominant in western Africa, and the most divergent genotype F is found exclusively among indigenous populations in Central and South America.[57,58] Genotype G has been identified in a few samples in the USA and France. Worldwide genotypes B, C and D are predominant, with an estimated 240 million subjects infected with genotype B/C, and 40 million infected with genotype D. Genotype A has infected approximately 3 million, and genotype E 20 million individuals. The clinical significance of genotypes has not yet been fully evaluated. Some recent data suggest that associations between certain genotypes and HBeAg seroconversions, viral mutations, severity of liver disease and response to treatment may exist. Recent studies from Asia have indicated that HBV genotype B is associated with earlier HBeAg seroconversion than genotype C, thus most likely explaining the less progressive disease in patients with genotype B.[59,60] In the USA, where one study found all genotypes to be heterogeneously distributed among the country, the only significant factors related to genotype epidemiology were ethnicity and place of birth, indicating that population dynamics are an influential causal factor of genotypic distribution.[49–51,53,56,61–65]

## Impact of vaccination on HBV prevalence and liver disease

Data on the decrease of prevalence of HBV markers are accumulating in countries where routine hepatitis B

vaccination programmes were implemented. A decline of incidence of acute cases of disease in paediatric age groups is not easy to demonstrate because hepatitis B is rarely symptomatic in infants and children.[66] Thus, the baseline level of incidence is usually already low before routine immunization. In addition, HBV-related mortality is seen mainly in adult age. However, there are areas with such a high endemicity of infection that it is possible to document the impact of vaccination also by mortality rates.

The impact of mass hepatitis B immunization on the chronic consequences of infection could be demonstrated first in Taiwan. During the 15 years following introduction of mass vaccination, the average mortality from fulminant hepatitis in infants changed from 5.36/100 000 in 1975–1984 (prior to mass vaccination) to 1.71/100 000 in 1985–1998.[67] In parallel, HBsAg prevalence in the population of those under 15 year of age changed from 9.8% (1984) to 0.7% (1999),[68] and in children aged 6–14 years, the incidence of primary liver cancer progressively declined from 0.7/100 000 in the period 1981–1986 to 0.57/100 000 in 1986–1990, and to 0.36/100 000 in 1990–1996.[69,70]

Proof of the decreasing incidence of acute hepatitis B after vaccination is available in Europe from Italy, the first industrialized country to introduce routine immunization in a double cohort of subjects, i.e. infants and 12-year-old adolescents. Data from the Integrated System of Surveillance on Acute Viral Hepatitis (SEIEVA) indicate that during the period 1991–1999, the number of new cases of hepatitis B dropped by about 40%, compared with data from the 1988–1991 period.[42,71] However, the overall incidence of acute hepatitis B was already declining before the introduction of routine vaccination, as it changed from 12 to 5/100 000 between 1985 and 1991, but the decline connected with the improvement of hygienic and living conditions would not have led to such a dramatic reduction in viral circulation without a programme of universal vaccination.[72] As a matter of fact, the incidence in the age group at highest risk (15–24 years) was halved from 1991 to 1994 (12 and 6/100 000, respectively) and continued to decrease thereafter. In Tuscany, a 49% decline of reported cases in the same age group was registered between 1992 and 1996,[73,74] and in a highly endemic area in southern Italy (Afragola), a decline of the incidence from 63/100 000 before implementation of the immunization programme to 3/100 000 in 1997, 15 years after its implementation was reported, and the prevalence of HBsAg dropped from 13.4% in 1978 to 3.7% in 1997 (from 6.8% to 0.7% in children and adolescents).[73] At the present time, the level of acute hepatitis B infection is at an all-time low. However, there are still new infections occurring and there is also a shift in the prevalence of HBsAg-positive subjects towards more advanced age groups. If hepatitis B vaccination coverage rates are maintained at their current levels, elimination of HBV transmission in Italy could become a reality within the next few decades.

In Alaska (an area of traditionally high endemicity), where a programme of vaccination of all infants plus catch-up of susceptibles was initiated in 1983, a 10-year follow-up showed zero HBsAg prevalence in Native Alaskan children aged <10 years, while 16% of those aged 11–30 years were chronically infected.[75]

In Bulgaria, the universal vaccination of neonates introduced in 1991 rapidly reached over 70% coverage. In 1992, a drop of incidence of hepatitis B in infants reached 5.6/100 000 vs. values of 25–35/100 000 during the 1980s.[76]

In South Africa, the prevalence of HBV carriers in 3-year-old children was 12.8% in the pre-immunization years.[77] After 5 years of implementation of HBV vaccination in the framework of the WHO Expanded Programme of Immunization (EPI), none of about 600 vaccinated children showed HBsAg reactivity.[78]

By preventing infections in children through universal immunization programmes against HBV, the number of HBeAg-positive persons declines rapidly over time, because of the inherent clearance of HBeAg in older persons, and this will lead to an ongoing and significant change in the epidemiology of hepatitis B in all those countries where a universal hepatitis B immunization programme has been implemented.

## Transmission of HBV

The incubation period of HBV is long, ranging from 45 to 160 days (average 120). The virus is found in highest concentrations in blood and serous exudates. Percutaneous or mucous membrane exposures to HBV in infectious blood or serum-derived body fluids account for virtually all HBV infections in humans.[44,79,80]

Although HBsAg has been detected in a wide variety of body fluids, only serum, semen and saliva have been demonstrated to be infectious,[81,82] while breast milk and urine remain controversial. The presence of HBeAg in serum correlates with higher titres of HBV and greater infectivity.[83–85] However, HBV strains that have mutations in the precore region of the viral genome that prevent expression of HBeAg (HBeAg-negative/anti-HBeAg-positive variants) also have been associated with transmission.[86]

Infection by faeces, urine, tears, breast milk, bile or pancreatic juice has never been demonstrated even though HBsAg or HBV particles have been detected in such fluids.[87–89] The possibility of transmission by blood-feeding arthropods has been a concern but has never been proven. The hypothesis of bedbug transmission

was tested in the Gambia, where a controlled trial of bedbug elimination in several villages failed to reduce the risk of HBV infection.[90]

## Person-to-person spread

Percutaneous exposures that have resulted in HBV transmission include transfusion of blood or blood products,[91–94] contaminated equipment used for therapeutic injections and other health-care-related procedures,[95–98] injection drug use,[99–105] and needle sticks or other injuries from sharp instruments sustained by hospital personnel.[106,107] In addition, occasional outbreaks of hepatitis B have been associated with tattooing and acupuncture.[108,109] Because HBV is stable on environmental surfaces for >7 days,[110] indirect inoculation of HBV can also occur via inanimate objects. No infections have been demonstrated in susceptible persons orally exposed to HBsAg-positive saliva, although transmission to animals by subcutaneous inoculation of saliva has been demonstrated.[81,82,111,112]

Person-to-person spread of HBV can occur in settings involving non-sexual interpersonal contact over a long period of time, such as among household contacts of a chronically infected person.[113–116] The precise mechanisms of transmission are unknown. However, frequent interpersonal contact of non-intact skin or mucous membranes with blood-containing secretions or perhaps saliva are the most likely modes of transmission.[117] Because of the extremely high concentration of virus in the blood, the number of virions in even very small amounts of blood or body fluids can be quite high. In addition, HBsAg contamination of surfaces is widespread in homes of chronically infected persons,[117] and HBV remains infectious for long periods of time under ambient conditions.

## Sexual transmission

Transmission of HBV from persons with acute or chronic hepatitis B to their sexual partners is an important source of infection,[79,102] and HBsAg-positive adults engaging in high-risk sexual activity are at high risk of transmitting HBV.[118,119] However, most persons with chronic HBV infection are not aware that they are infected. These silent carriers are the most likely source of infection for persons with multiple sexual partners.

In most developed countries, including those in northern and Western Europe, most (80–85%) acute infections of hepatitis B occur among young adults, engaging in high-risk unprotected sexual activity and injection drug use.[120–124]

Historically, men who have sex with men (MSM) were one of the groups at highest risk for HBV infection. Infection in this risk group has been associated with receptive anal intercourse, increased numbers of sexual partners, and number of years of sexual activity (70% of homosexual men were infected after 5 years of sexual activity).[79] Similar factors have been associated with an increased risk of HBV infection among heterosexual men and women, including number of sexual partners, number of years of sexual activity, and a history of other sexually transmitted diseases.[79,125]

## Horizontal and vertical transmission

The risk of perinatal HBV transmission has been well described. This risk is greatest for infants born to women who are HBeAg-positive and ranges from 70% to 90% at 6 months of age. About 90% of these children remain chronically infected.[126] The risk of perinatal infection among infants born to HBeAg-negative mothers ranges from 10% to 40%, with 40–70% of these infected infants remaining chronically infected.[126,127] Children born to HBsAg-positive mothers who do not become infected during the perinatal period remain at high risk of infection during early childhood.[127–132]

A curious, but as yet not fully explained, observation is that perinatal transmission is much more common in Asia than in Africa. The routes of transmission for early childhood infections in Africa are not clearly understood, and there may be significant local variability. In a study of a rural Senegalese community, the prevalence of HBV markers was about 25% at the age of 3 years and increased rapidly after 4 years of age. Even when mothers in Africa are HBeAg-positive, however, their babies do not become HBsAg-positive until 6 months to 1 year of age, whereas in Asia exposed babies tend to become HBsAg-positive by 3 months after birth.[133]

In low HBV endemic countries, a substantial number of children become infected with HBV, many of whom belong to families that have migrated from high HBV endemic countries.[44] As >90% of childhood HBV infections are asymptomatic, the true incidence of childhood disease is not accurately represented by most surveillance data, which in general reflect reported cases of clinically apparent disease.

## Transmission by unsafe medical practices

Transmission occurs through contaminated injection equipment and through inadequately sterilized medical instruments and remains a significant problem because of the difficulty in obtaining disposable needles and syringes and the lack of means to adequately sterilize reusable equipment.[134–136] It was estimated that 8–16 million of new HBV infections may result from unsafe injections each year.[135] In developed countries, episodes of HBV transmission from one patient to another in health-care settings also have been reported.[95,96,134,137–139] In most

cases, these transmissions resulted from non-compliance with recommended infection control practices that were designed to prevent cross-contamination of medical equipment and devices.

In some countries, high HBV endemicity is often connected to the use of non-sterile injection practices. A study in the Republic of Moldova showed that nosocomial transmission was a major risk factor for hepatitis B infection and a study in Kazakhstan in 1998 confirmed this, showing that 52% of acute hepatitis B infections were associated with medical injections. In a study performed in Russia (Moscow) significant associations with invasive manipulations and illicit drug use during the previous 6 months preceding infection with HBV were found. For non-drug users, admission to hospital, tattooing and multiple sexual partners carried an attributable risk of between 3% and 12%.[140]

### Health-care workers

HBV transmission from infected health-care workers (HCWs) to patients is relatively uncommon, but has occurred, especially during exposure-prone procedures, such as invasive surgical, obstetrical or dental procedures.[107,141]

Although HBV infection was recognized as a frequent occupational hazard among persons working in laboratories or exposed to blood while caring for patients,[107] hepatitis B vaccination of HCWs and its wide use, particularly in younger generations of HCWs and in medical and nursing students, determined a clear decline in the incidence of HBV in HCWs.[142] A problem of coverage still exists in older HCWs, who are less compliant with vaccination.[143] Most of the reported cases occurred before hepatitis B vaccination was widely used and before universal infection control precautions were implemented.[144] These mostly involved infected surgeons or dentists who transmitted during the performance of invasive procedures. However, other health-care providers also have been implicated in HBV transmission to patients.[107] The risk of transmission of HBV from HCW to patient during invasive surgical procedures was estimated at values of 2400 per million,[145] much higher than that for HCV and HIV.

In order to prevent HCW–patient transmission, there is consensus that HBeAg-positive HBV carriers should not perform exposure-prone surgery or other treatments at risk of injury for the operator. These personnel should be assigned to a type of work with minimal risk of transmission.

In the long-term perspective, the growing implementation of universal vaccination programmes and the continuing active offer of immunization to HCWs in many countries should diminish the prevalence of HBV carriers in patients and HCWs, thus making hepatitis B transmission in medical settings an uncommon event.[146]

### Transmission of HBV via transfusions and haemodialysis

Transmission of HBV via transfusion of blood and plasma-derived products has been eliminated in most countries through donor screening for HBsAg and viral inactivation procedures, and the introduction in some countries of HBV DNA nucleic acid amplification techniques using mini-pools has further reduced the risk.[147–150]

HBV transmission among haemodialysis patients is consistently associated with the presence of a chronically infected patient, failure to dialyse the infected patient in a separate room using dedicated equipment and staff, and failure to vaccinate patients against hepatitis B. Contaminated environmental surfaces have been a major source of HBV transmission among chronic haemodialysis patients.[92,95,96,151,152]

## HBV and co-infections

Evidence has been generated that HCV co-infection and/or co-infection with HIV are clinically and virologically important co-factors. HBV-positive patients co-infected with HIV tend to have a milder liver disease, but the overall morbidity and mortality seem to be higher than in patients infected with HIV alone. Patients co-infected with HCV tend also to have more severe liver disease and more commonly occurring HCC in comparison with patients having either infection alone.[153,154]

## Disease associations with HBV

The most serious diseases associated with chronic HBV infection affect the liver. However, some uncommon extrahepatic manifestations are caused by immune complexes of hepatitis B antigens and antibodies.[155] The most common ones include chronic membranoproliferative glomerulonephritis and polyarteritis.[156]

## Alcohol and HBV

Alcohol is hepatotoxic and may exacerbate HBV-related liver damage.[157] HCV infection and alcohol consumption appear to be more closely associated with liver damage than HBV infection and alcohol consumption. Chronic alcohol consumption is clearly associated with the development of cirrhosis. Persons chronically infected with HBV and who drink alcohol regularly might be expected to be at higher risk of both cirrhosis and HCC than either HBV carriers who do not drink or drinkers

who are not infected with HBV. Japanese HBsAg-positive blood donors who were moderate or heavy drinkers were at a fivefold to eightfold greater risk of developing HCC than non-drinkers.[158] Other studies have shown either a high, weak or no association.[5,8,159–162]

## Aflatoxin and HBV

Aflatoxins are fungal toxins that commonly contaminate maize, groundnuts and other crops. Aflatoxin B1 plays an important part in modifying the risk of liver cancer associated with hepatitis B. Several studies have shown a strong interaction of chronic HBV infection with aflatoxin B1 exposure and HCC. In some areas of high exposure to aflatoxin, HCCs have revealed a frequent mutation in the p53 tumour suppressor gene in primary liver cancers.[163,164] HBV and aflatoxin B1 may synergistically induce the p53 gene transversion from A G to T at codon 249. The presence of aflatoxin metabolites in urinary excretions increases the risk of HCC about fourfold.[165] Chronic HBV infection increased risk of HCC about seven times, but persons who both excreted aflatoxin B1 metabolites and were HBV carriers had a 30–60 times higher risk than HBV-uninfected, aflatoxin B1-unexposed persons.[164,166,167]

## Conclusions

At the beginning of the third millennium, hepatitis B remains a major public health problem globally. This fact is related to both the continuing occurrence of frequent new infections and to the presence of a large reservoir of persons who are chronically infected, who may develop severe and fatal complications of chronic liver disease. Cirrhosis, HCC, or both, represent a large burden for health-care systems.

Hepatitis B and all the complications resulting from it, as well HDV and its complications, are globally preventable by hepatitis B vaccination, and therefore elimination of HBV transmission and of new acute and chronic infections is a feasible goal. To achieve this, implementation of childhood vaccination programmes against hepatitis B need to be extended to all countries. As of 2002, 154 countries had a national immunization policy.[168]

For the 360 million subjects already suffering from chronic HBV infection, only antiviral treatment can provide help. The currently licensed treatments are expensive, and therefore not accessible to the great majority. Providing safe and effective treatment to those already suffering from the sequelae of chronic HBV infection will reduce the burden of HBV-related morbidity and mortality, but will require significant and coordinated efforts at an international level.

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# Chapter 12
# Avihepadnaviridae

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Members of the Hepadnaviridae family are primarily hepatotrophic, contain relaxed circular (RC) double-stranded (DS) DNA genomes and have a unique method of replication that involves reverse transcription of a greater than genome length RNA. There are two genera within the Hepadnavirus family: the orthohepadnavirus genus that includes viruses infecting mammals and the avihepadnavirus genus including viruses that infect avian species.

The orthohepadnavirus genus includes the human hepatitis B virus (HBV), as well as a number of naturally occurring HBV strains isolated from non-human primates including chimpanzees, orang-utans, gibbons[1–3] as well as the woolly monkey hepatitis B virus (WMHBV).[4] In addition to the human and non-primate strains of HBV, the orthohepadnavirus genus includes the woodchuck hepatitis virus (WHV) from eastern woodchucks;[5] ground squirrel hepatitis virus (GSHV) from Beechy ground squirrels;[6] and arctic squirrel hepatitis virus (ASHV).[7] The orthohepadnavirus genus is phylogenetically classified into seven HBV genotypes, A–G, with an overall sequence homology of 92%.[8] The non-human primate strains of HBV are distinct from genotypes A–G. HBV strains isolated from chimpanzees are most closely related to the HBV genotype G[8] and have an overall homology to human HBV of >94%.[1] However, with the exception of the WMHV, this difference is not great enough to assign these primate isolates as separate species. The non-primate strains, WHV, GSHV and ASHV, are all assigned as separate species and share 70%, 55% and 63% genome homology with HBV.

The avihepadnavirus genus includes the duck hepatitis B virus (DHBV) isolated from Pekin ducks (*Anas domesticus*),[9] heron hepatitis B virus (HHBV) from grey herons (*Ardea cinerae*),[10] Ross goose hepatitis virus (RGHV; GeneBank DHBV-RGM 95589) from Ross Geese (*Anser rossii*), stork hepatitis B virus (STHBV) from white storks (*Ciconia ciconia*),[11] snow goose hepatitis B virus (SGHBV) from snow geese (*Anser caerulescens*)[12] and the Australian maned duck, (MDHBV) and grey teal (GTHBV) hepatitis B viruses (R.J. Dixon, personal communication). The genomes of a number of avihepadnaviruses including the Australian strain of DHBV have been cloned and sequenced from serum[13–15] and have been shown to be infectious.[12–14,16]

The avihepadnaviruses have been classified phylogenetically into 'Chinese' and 'Western' DHBV strains as well as four highly distinct lineages that include the SGHBV, RGHV, STHBV and HHBV.[12,14,17] Nucleotide sequence divergence within the 'Chinese' and 'Western' strains is 5.99 and 3.35%, while divergence between the strains is 9.8%.[17] The SGHBV is the next closest member of the genus to DHBV, varying by 11–13%,[12] while RGHV varies by 17.3–19.1%[14] and STHBV and HHBV vary by 22.2–23.6%[11,12,14] (Fig. 12.1). The larger evolutionary distance between HHBV and DHBV may reflect their distinct host ranges, as HHBV infects only grey herons and not ducks,[10,18] and may have resulted from co-evolution of each virus in its respective host.

All members of the Hepadnaviridae family share unique structural and genomic features including similar virion size and ultrastructure, with an envelope surrounding an icosahedral nucleocapsid composed of core protein containing a RC DNA genome with similar size, structure and organization. A comparison of the physical features, genomes and encoded proteins of HBV, WHV, GSHV and DHBV is included in Table 12.1.[5,6,9,19–25] HBV and DHBV share only 40% nucleotide similarity, while, as explained above, genomic nucleotide sequences are more similar among the members of each genus.

Members of the two genera were originally thought to encode a different number of genes, with the mammalian viruses having a fourth open reading frame (ORF), encoding the X protein, that appeared to be lacking in the original DHBV isolates.[23] However, it was subsequently reported that avihepadnaviridae strains including HHBV[10,26] and later DHBV,[19] also have an X-ORF. In the case of DHBV, the X protein is produced from an unconventional start codon.[19] Unlike the X protein of the orthohepadnaviruses, the X-ORF of DHBV was found to be non-essential for replication *in vivo*.[27] In addition to the X-ORF, hepadnaviruses also have an S-ORF, C-ORF and P-ORF, encoding envelope, core and polymerase proteins (Fig. 12.2).[28]

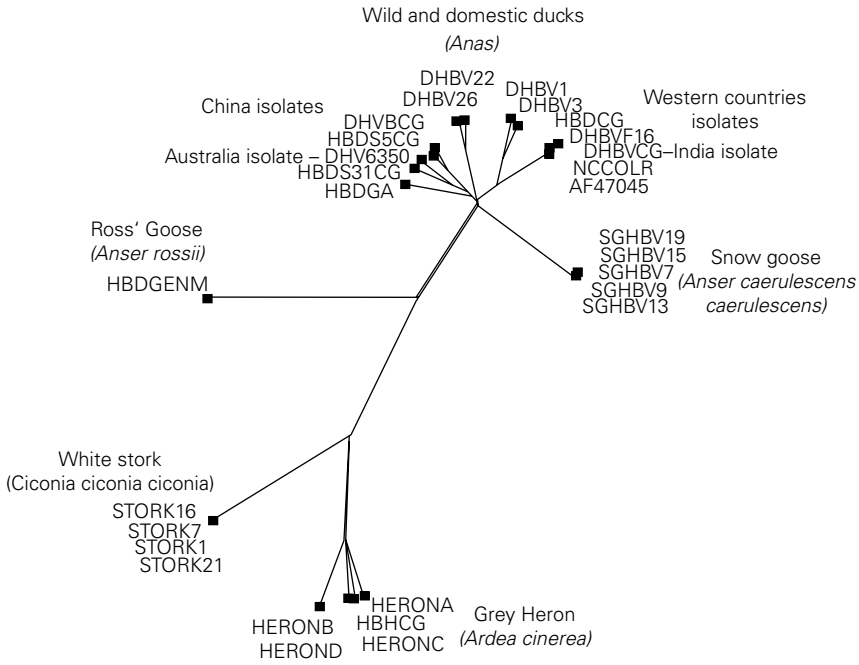


**Figure 12.1** Phylogenetic relationship of avian hepadnaviruses, inferred from the full-length (3018–3027 nt) sequences listed by NCBI number. From Pult *et al.*[11]

Differences in virion structure and the production of non-infectious surface antigen particles exist between the two genera. The mammalian viruses produce 22-nm diameter surface antigen spheres and filaments, while the avian viruses produce pleomorphic, generally spherical surface antigen particles ranging in diameter from 35 to 60 nm and filaments are not produced (Table 12.1). There are also antigenic differences between the two genera. The mammalian viruses show cross-reactivity between epitopes on their core and envelope proteins,[22,25] but not with epitopes of the avian viruses.

Finally, hepadnaviruses all have a narrow host range, which is usually limited to each species. For example, HBV only infects higher primates including humans,

**Table 12.1** Comparison of the different members of the Hepadnaviridae family

| Physical characteristics | Hepadnaviral members | | | |
|---|---|---|---|---|
| | HBV | WHV | GSHV | DHBV |
| **Virion** | | | | |
| Diameter (nm) | 42[5] | 45[5] | 47[6] | 40–45[9] |
| Buoyant density in CsCl (g/mL) | 1.24[5] | 1.225[5] | 1.24[24] | 1.16[9] |
| **Nucleocapsid** | | | | |
| Diameter (nm) | 27[5] | 27[5] | 30[6] | 27[9] |
| Buoyant density in CsCl (g/mL) | 1.34[5] | 1.34[25] | 1.34[20] | 1.34[24] (with spikes) |
| **Surface antigen particles** | | | | |
| Spheres (nm) | 22[5] | 20–25[5] | 15–25[6] | 40–60[9] |
| Buoyant density in CsCl (g/mL) | 1.19–1.20[22] | 1.18[25] | 1.18[22] | 1.14[9] |
| Filaments (22 nm diameter) | Present[5] | Present[5] | Present[6] (long, abundant) | Absent[9] |
| **Genome** | | | | |
| Size (nt)[24] | 3182–3188 | 3308–3320 | 3311 | 3021–3027 |
| Sequence homology with HBV[21–23] | 100 | 70 | 55 | 40 |
| No. of ORFs[21–23] | 4 | 4 | 4 | 4 |
| No. of coded amino acids/ORF[24] | | | | |
| Pre-S | 163 | 204–205 | 206 | 161–163 |
| S | 226 | 222 | 222 | 167 |
| Pre-C | 29 | 30 | 30 | 43 |
| C | 183 | 187 | 188 | 262 |
| P | 838 | 879 | 881 | 786–788 |
| X | 145 | 141 | 138 | 114[19] |

CsCl, caesium chloride; ORF, open reading frame. Superscript numbers are reference numbers.

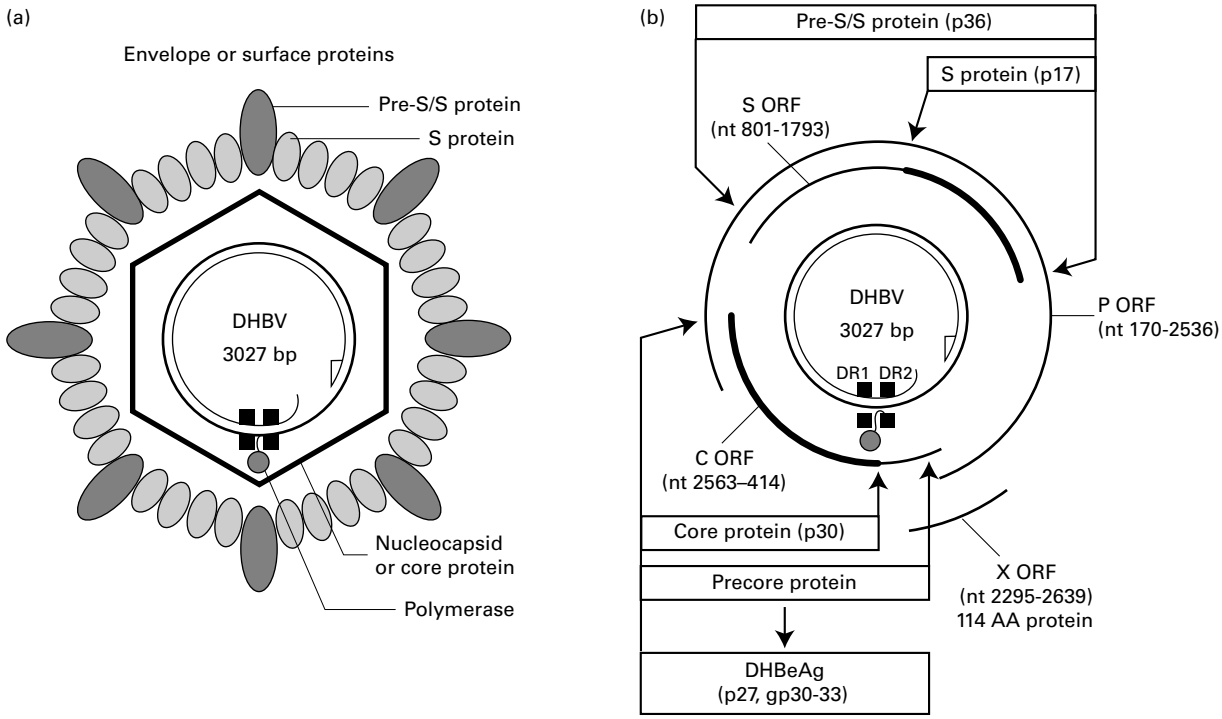


**Figure 12.2** The structure of the DHBV virion (a) and relaxed circular (RC) DNA genome (b), based on the Australian strain of DHBV (14) (DHV6350). (a) DHBV virion showing the viral envelope proteins, pre-S/S and S, the viral nucleocapsid comprised of DHBcAg and containing the RC viral DNA genome and viral polymerase. (b) The inner circles represent the two strands of the DHBV genome, negative (–) and positive (+); the positions of the two 12 nt DR sequences, DR1 (nt 2541–2552) and DR2 (nt 2483–2494) within the 69 nt cohesive overlap region are indicated by closed boxes; and the viral polymerase protein covalently attached to the 5′ end of the negative (–) DNA strand and the polyadenylation signal sequence (nt 2778–2783) are shown. The positive-strand has an 18 nt RNA primer attached to its 5′ end. In virions, the negative-strand is complete, while the positive-strand is less than full length. (Positive-strand completion occurs during initiation of new rounds of infection.) The four overlapping ORFs all present on the negative (–) strand (with production of + sense mRNA) are shown as open arrows with their coordinates and protein products indicated. Modified from Jilbert and Kotlarski.[28]

chimpanzees and gibbons.[1,3] DHBV infection has been found to occur naturally in domestic flocks of ducks and geese but not in other anseriforme species.[29,30] DHBV has also been experimentally transmitted to geese.[29,31,32]

As HBV does not infect non-primate laboratory animals, the discovery of other animal hepadnaviruses has led to the development of alternative systems for studying pathogenesis and replication as well as antiviral therapy. In particular, studies of the WHV and DHBV systems have resulted in important observations that have enhanced our understanding of the replication strategy, natural history and pathogenesis of hepatitis B.

## Replication strategy of avian hepadnaviruses in hepatocytes

The DHBV model was essential in determining that hepadnaviruses replicate via reverse transcription of an RNA intermediate[33] and has also proved valuable in the study of the early events of the hepadnavirus life-cycle.

### Viral attachment and entry into the hepatocyte

Hepadnavirus infection is species-specific and the main cell type infected is the hepatocyte. The steps involved in DHBV infection of a single cell are shown in Fig. 12.3.[28] The species and cell specificity of hepadnavirus infection are determined by the presence of receptors required for virus binding and entry. Cell surface receptors on primary duck hepatocytes have been shown to bind DHBV particles in a species-specific manner,[34] and receptor binding is thought to occur via peptides encoded by the large viral envelope protein. Receptor binding is followed by cell entry via endocytosis.

Recent studies have determined that the ubiquitous protein carboxypeptidase D is capable of binding DHBsAg particles with high affinity.[35] This glycoprotein is found on both internal and surface membranes of the cell. However, transfection of cells with DNA expressing carboxypeptidase D does not confer the ability to be infected with DHBV, suggesting that other co-receptors or mechanisms may be operating.[36] An additional

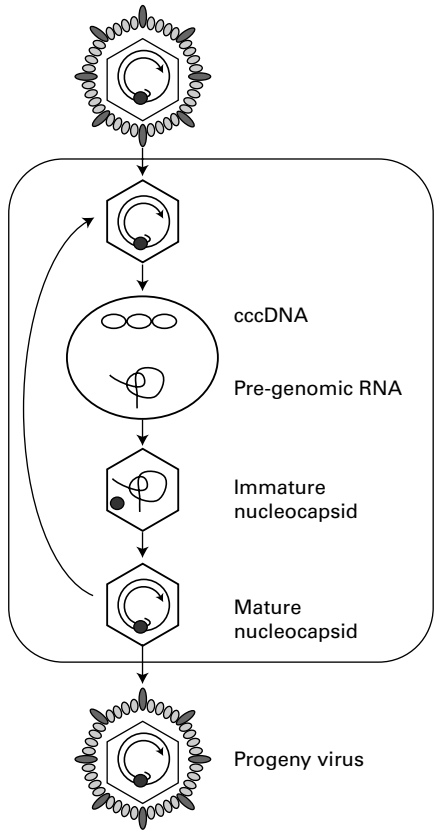


**Figure 12.3** DHBV infection of a single cell. The DHBV enters the cell by receptor-mediated endocytosis and uncoats in the cytoplasm. Virion DNA is translocated to the nucleus, where it is repaired to form cccDNA. cccDNA is then organized into nucleosomes and forms a viral mini-chromosome. Transcription of the DHBV genome produces messenger RNAs that are polyadenylated. The viral core and polymerase proteins are translated in the cytoplasm. The envelope proteins (including p36) are translated in association with the ER (endoplasmic reticulum). The pregenome and polymerase protein are encapsidated in the core particle and viral DNA synthesis occurs in the cytoplasm. Negative- and positive-strand DNA is generated by the viral polymerase. The core particles can be used by two different pathways. The core particles can associate with the p36 and bud into the ER and be secreted from the cell as virions. Alternatively, the viral genomes in the core structure can be translocated to the nucleus via an intracellular conversion pathway to replenish the pool of viral cccDNA. From Jilbert and Kotlarski.[28]

binding protein, glycine decarboxylase, with a size of 120 kDa has been identified.[37,38] Cellular expression of carboxypeptidase D is restricted to the liver, kidney and pancreas, which are the three major organs supporting DHBV replication. These proteins are potential components of the DHBV receptor complex and probably have a role in determining DHBV organ tropism. Several groups using lysosomal-tropic agents have studied the penetration and uncoating of the virus. DHBV uptake is thought to occur by endocytosis in a pH-independent manner.[39]

## Generation of a transcriptionally active hepadnavirus template

Liver cells have a slow turnover in the healthy state, and hepadnavirus infection thus requires a mechanism by which the genome passes through nuclear pores. Phosphorylated HBV nucleocapsids have been shown to bind to the nuclear pore complex (NPC), leading to exposure of a nuclear localization signal (NLS) on the surface of the capsids.[40–42] The NLS is then bound by importin α followed by binding of importin β, allowing translocation of the total complex into the nuclear basket on the nuclear side of the pore. NPCs have been shown to transport particles with a diameter of 39 nm,[43] and HBV capsids can be imported in an intact form through the nuclear pore into the nuclear basket[44] followed by release of the RC viral DNA and capsid protein into the nucleoplasm.

Several modifications of the infecting RC viral genome must be made before transcription can occur. The single-stranded (SS) DNA region of the hepadnavirus genome must be converted to DS DNA. It is known that the endogenous viral DNA polymerase present in the nucleocapsid can complete the synthesis of this region of DNA *in vitro*.[45] In addition to the synthesis of DNA, removal of the terminal protein and oligoribonucleotide attached to the 5′ ends of the viral DNA strands and ligation of the nicks in the RC DNA must occur to generate covalently closed circular DNA (cccDNA) genome. The viral cccDNA is then organized into nucleosomes[46] to form a viral mini-chromosome[32,47] (Fig. 12.3). In DHBV-infected ducks cccDNA has been detected by Southern blot hybridization within 6 hours of virus inoculation, indicating that conversion of RC to cccDNA occurs early in the replication cycle[48] (Jilbert *et al*., unpublished observations). cccDNA provides the transcriptional template for hepadnavirus infection and is present in multiple copies in the nucleus of each infected cell. This intracellular amplification of cccDNA occurs late in the infection process as a result of transport of RC DNA containing nucleocapsids to the nucleus (Fig. 12.3, also see below).

## Transcription of the avian hepadnavirus genomes

Transcription of hepadnavirus RNA from the cccDNA template occurs in the nucleus of the cell using host cell RNA polymerase II. In contrast to cellular mRNA, hepadnavirus mRNAs are exported to the cytoplasm in an unspliced form. Like cellular mRNAs, the hepadnavirus mRNAs are of positive orientation, possess a 5′ cap and are polyadenylated at their 3′ end. The HBV

and DHBV genomes contain a single polyadenylation signal (TATAAA in HBV, AATAAA in DHBV) and have a common 3′ poly A tail.

The avian hepadnaviruses have four ORFs (Fig. 12.2) and produce three major RNA transcripts,[49] which are transcribed from three distinct promoter regions. The core promoter is used to produce the 3.5-kb RNAs, which are terminally redundant and polyadenylated. A second promoter controls the synthesis of the pre-S envelope mRNA and a third, downstream promoter, synthesis of the S envelope protein mRNA. The DHBV envelope mRNAs are 2.3 and 2.1 kb in length and are polyadenylated at the same site as the pregenomic RNA.[50] The viral envelope mRNAs initiate 1100–1400 nucleotides downstream of the core promoter.[51,52] An mRNA species for the DHBV X protein has not yet been described.

Transcription of the greater than genome length pregenomic RNA, is strongly dependent on a *cis*-acting element at the 5′ end of the RNA, which has been called *pet* (positive effector of transactivation).[50] This element suppresses premature termination during the first circuit of the genome by RNA polymerase II. In addition, a negative effector of transcription (*net*) has been identified upstream of the envelope promoter region.[53] The *net* region and polyadenylation signal are essential for transcription termination and polyadenylation. The presence of *pet* allows transcription complexes to read through *net*, permitting production of the greater than genome length pregenome.[53] Once produced, pregenomic RNA is translated in the cytoplasm to produce the polymerase and core proteins and, as described below, is also encapsidated and used as the template for reverse transcription.

The DHBV genome has only one identified enhancer sequence.[51,52] This sequence binds hepatocyte-adipocyte nuclear transcription factor C/EBP, hepatocyte nuclear factor 1 (HNF1), and F3, which resembles the ubiquitous factor EF-C and is essential for full activity.[54] There are also three sites around the enhancer that bind hepatocyte nuclear factor 3 (HNF3) and three other sites that appear to bind GATA factors.[55]

Comparative analysis of HBV and DHBV sequences and transcription studies has shown that similar nuclear binding factors bind to the enhancers of both viruses, but the organization of these binding sites is different. However, in both cases, the result is that efficient transcription of the complete set of viral mRNAs is highly liver-specific.

## Replication of hepadnaviruses

Hepadnavirus replication takes place in the cytoplasm of the cell within nucleocapsids composed of viral core protein (Fig. 12.3). Pregenomic RNA and viral polymerase are packaged together into the nucleocapsids due to binding of polymerase to an RNA packaging signal, epsilon (ε) present within pregenomic RNA (Fig. 12.4a).[56] Once packaged, pregenomic RNA is used by the viral polymerase as the template for DHBV DNA synthesis.[33,57] The negative-strand of DNA is first produced by reverse transcription (Fig. 12.4a–d) followed by production of the positive-strand of DNA (Fig. 12.4e–f).

Pregenomic RNA contains a number of *cis*-acting sequences important for reverse transcription. For DHBV, these include the packaging signals, epsilon and region II,[58–60] the direct repeats, DR1 and DR2,[61,62] and three *cis*-acting sequences 5E, M and 3E.[63] Extensive studies of the role of 5E, M and 3E have shown that they function by base-pairing to each other and contribute to primer translocation and circularization of the genome.[63,64] These results suggest that within DHBV nucleocapsids the ends of the negative-strand of DNA are base-paired, and that this facilitates the template switches that occur during positive-strand DNA synthesis (see below).[64–67]

Two types of replication are identified for hepadnaviruses, legitimate and illegitimate. During legitimate DNA replication (Fig. 12.4a–f), three or four nucleotides in a bulge of the RNA packaging signal, ε, which folds into a stem loop structure, serve as the template for the priming of reverse transcription.[68,69] In the DHBV genome the sequence is UUAC[70] and in the HBV genome it is UUC.[71] Priming of reverse transcription from this site results in production of a complimentary three or four nucleotide DNA strand (Fig. 12.4b)[70] that is covalently bound to the amino-terminal region of the viral polymerase via a tyr residue.[72,73] The polymerase protein remains covalently bound to the RC DNA genome at this site throughout replication and is bound to the RC DNA genome found in virions.

The three or four nucleotide DNA strand and the polymerase then dissociate from the RNA template and re-anneal with complementary sequences of the copy of DR1 at the 3′ end of the pregenome. DNA synthesis then continues towards the 5′ end of the pregenome RNA and generates a short terminal redundancy of approximately eight to nine nucleotides of DNA. During the process of reverse transcription the RNase H activity of the polymerase digests the pregenome RNA template, leaving an 18 nucleotide RNA sequence at the 5′ end of the pregenome, including the 5′ cap (Fig. 12.4e). This capped RNA serves as the primer for positive-strand DNA synthesis.[61] Typically, the primer is first translocated from the end of the newly synthesized minus DNA strand to the DR2 sequence located, for DHBV 69 nt from the (226 nt in HBV), from the 5′ end of the negative-strand DNA (Fig. 12.4b, c), where it serves as a primer for the positive DNA strand synthesis. Synthesis then proceeds from the primer at DR2 to the end of the negative-strand. The eight to nine nucleotide redundancy in the negative DNA strand permits the transfer of synthesis to the 3′

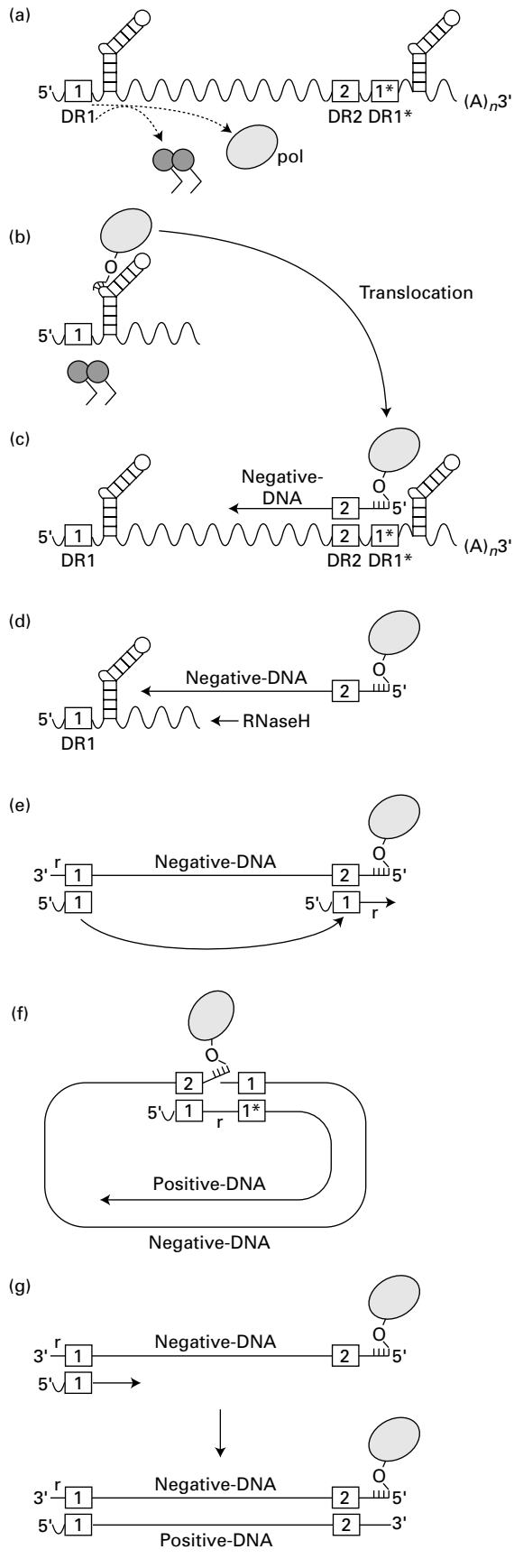


**Figure 12.4** Hepadnavirus replication strategy. (a) The wavy line represents the 3.6-kb longer than genomic length pregenomic RNA, which also serves as mRNA for both the core and polymerase proteins. The encapsidation signal, epsilon (ε) (nt 2566–2622) which is present at both ends of the genome is shown as a symbolic hairpin, and the direct repeat elements DR1, DR2 and DR1* are shown as boxes. (b) Binding of polymerase protein to the 5′ ε and (1) subsequent addition of core protein dimers and hence nucleocapsid assembly, and (2) initiation of synthesis of four DNA nt using the bulge region of ε as template and Tyr-96 of poymerasel as primer. (c) The polymerase–oligonucleotide complex is then translocated to DR1* to initiate synthesis of the negative-strand of DNA that is extended to the 5' end of the RNA pregenome. (d) The RNA template is degraded by the RNase H activity of the polymerase protein, except for a short oligonucleotide at the 5' end containing the DR1 sequence. (e) The 18 nt RNA segment is then transferred to DR2 and used as a primer for synthesis of the positive-strand DNA. (f) Due to an 8 nt terminal redundancy, r, the 3' end of the positive-strand DNA can use the 3' end of negative-strand DNA to circularize the genome and continue positive-strand synthesis. (g) An alternative (illegitimate) replication pathway of positive-strand synthesis where translocation of the 18 nt capped RNA primer to DR2 does not occur but rather positive-strand DNA synthesis is primed *in situ*. Elongation of the positive-strand then proceeds, resulting in formation of a DSL molecule with the 18 nt primer present in an RNA:DNA duplex at the 3′ end of the negative strand. From Jilbert *et al*.[56]

end of the minus DNA strand (Fig. 12.4e, f). This generates a circular genome that, upon further extension of the positive DNA strand, generates the RC DS hepadnavirus genome found in the virion.

Illegitimate replication, in contrast, results from *in situ* priming of positive-strand synthesis without translocation of the 18 nt RNA primer; that is, from the 3′ end of the negative-strand (Fig. 12.4g). This leads to the formation of DS linear (DSL) rather than RC viral genomes. Formation of these DSL genomes occurs at a frequency of about 5–10%.[74] When nucleocapsids with linear DNA are transported to the nucleus, they can undergo illegitimate recombination at their ends to form cccDNA.[74,75] As nucleotides are lost in this process, the resultant cccDNA is often defective in the ability to support virus replication, although some viral proteins may be produced.

Illegitimate DNA replication of hepadnaviruses occurs infrequently. However, mutations that reduce the homology between DRI and DR2 can prevent primer translocation and increase the frequency of *in situ* priming.[76,77] DSL DNA formed due to *in situ* priming has been shown to have a much higher probability than RC DNA of integrating into host DNA during initiation of DHBV infections.[78]

### Assembly of hepadnavirus virions and subviral particles

The DHBV surface antigen ORF has two AUG codons and encodes two forms of DHBV surface antigen, the DHBV Pre-S/S (37 kDa) and S (17 kDa) proteins. The pre-S/S protein contains an additional 163 amino acids (aa) amino-terminal extension in addition to the 167 aa of the DHBV S protein. The amino-terminal extension in the pre-S/S protein contains the receptor binding site[18] and has two alternate transmembrane topologies.[79] In the first topographical form the amino-terminal extension is translocated across the ER membrane, exposing the receptor binding site on the exterior of the virion and resulting in formation of infectious virions. Only 50% of pre-S/S molecules have translocated amino-termini, thus mature DHBV virions contain mixed internal/external pre-S/S topologies, including a partially translocated or intermediate form.[79]

Both the pre-S/S and S proteins contain signal sequences that direct their translocation into the ER and are transmembrane proteins with up to five transmembrane domains. Studies of transmembrane domain 1 (TM1), located within the common carboxy-terminal region of both the S and pre-S/S proteins, have shown it to be essential for stability of the S protein and for particle assembly.[80] The TM1 protein is also involved in fusion of virus particles with infected cells. Recent studies have indicated that some pre-S/S molecules are spring-loaded and undergo a major conformational change analogous to fusion activation.[81] These studies showed that the conformational change exposed TM1 to the virion surface, allowing virions to bind to cell membranes, suggesting that the TM1 of pre-S/S is a fusion peptide. The TM1 domain, common to both envelope proteins, thus has a different functional role in pre-S/S and S and thus a different structural arrangement on the assembled particle.

The envelope proteins have the ability to assemble subviral particles that bud into the lumen of the ER and the Golgi compartments. This auto-assembly process results in the production of a large excess of subviral particles compared with virions in the serum of viraemic individuals. The final step in the assembly of the virions involves the association of the nucleocapsid with viral envelope proteins at the ER membrane. Enveloped nucleocapsids are deposited into the ER lumen, and the particles are secreted via the constitutive secretory pathway.

The DHBV e antigen (DHBeAg) is a secretory protein that is produced from the translational product of the longest of the 3.5-kb viral RNAs.[82,83] It is generated from a precursor polypeptide that contains a signal peptide, which targets this polypeptide to the ER. It is modified by cellular proteases and secreted as a 27-kDa unglycosylated and 30- or 33-kDa glycosylated protein.[82]

### Maintenance of the nuclear covalently closed circular hepadnaviral DNA pool

Cells supporting HBV replication have been shown to contain ~10–50 copies of cccDNA/cell.[84] Similarly, WHV- and DHBV-infected cells contain 10–50 and 6–30 copies of cccDNA per cell, respectively, and up to 500 or more copies of single- and double-stranded replicative intermediates in the cytoplasm.[15,85–87] The copy number of viral cccDNA has been demonstrated, for DHBV at least, to be regulated by the pre-S/S.[88] Viral cccDNA can be produced from the infecting virion, and also from genomes that are produced via the reverse transcriptase pathway and are transported into the nucleus.[15,87,88] Thus, mature intracellular nucleocapsids containing RC DNA proceed along one of two alternative pathways. Early in the infection process, the newly replicated virion DNA within mature nucleocapsids is delivered to the nucleus, resulting in amplification of cccDNA. Later in the infection process, production of the pre-S/S inhibits viral cccDNA amplification, redirecting viral nucleocapsids into envelope virus particles that are exported from the cell. Importantly, hepadnaviral cccDNA appears to be a major viral replicative DNA species that is resistant to inhibition by conventional antiviral agents[89,90] (see below). This reflects the fact that it does not itself replicate, except via the reverse transcription pathway, and functions solely as a template for viral RNA synthesis.

## Pathogenesis of avian hepadnaviral infections

### Transmission and outcome of infection

The predominant route for the natural transmission of hepadnaviruses varies significantly between the mammalian and avian hepadnaviruses. Mammalian hepadnaviruses are transmitted via parenteral contact with body fluids, such as blood and lymph.[91] Transmission also occurs via perinatal exposure of infants to carrier mothers, which typically results in persistent infection of the infant.[91] In contrast, avian hepadnaviruses are primarily transmitted *in ovo* where persistent infection develops at a frequency of 100%.[85,92–94]

The ability of individual ducks to resolve DHBV infection is linked to the age of the duck at the time of inoculation and the dose of inoculated virus.[95] Titration of stocks of the Australian strain of DHBV in newly hatched ducks (1–3 days old) has shown that it is possible to establish persistent infection by inoculation of pooled serum from congenitally infected ducks containing the equivalent of one virus genome. This result

indicates not only that newly hatched ducks are highly susceptible to the development of persistent infection, but that a 1:1 ratio of infectious viral particles to DNA genomes occurs in congenitally infected duck serum.[96] Similar infectivity experiments have been performed in Australian ducks infected with both the German DHBV 3 strain[19] and the USA strain DHBV16,[23] and similarly high ratios of infectious particles to DNA genomes have been demonstrated.[27,97]

In older ducks, the outcome of infection is also linked to the dose of virus inoculated. For example, inoculation of $1 \times 10^6$ genomes of DHBV into 14-day-old ducks leads to a persistent infection, while inoculation of $4 \times 10^4$ genomes results in transient infection.[95] After 4–6 weeks of age, it is harder, although possible, to establish persistent DHBV infection. In one study in 4-month-old ducks, one of three animals inoculated with infectious serum containing $2 \times 10^{11}$ DHBV genomes developed persistent infection, while all animals inoculated with serum containing $10^3$, $10^6$ and $10^9$ DHBV genomes had transient infection.[95] The failure to develop persistent infection in older ducks is not due to a loss of receptors or to an inability to infect hepatocytes in the liver, as the ducks receiving $2 \times 10^{11}$ genomes had DHBV infection in >95% of hepatocytes at days 6, 9 and 12 after inoculation.[95] In contrast, it is thought that older ducks have an increased ability to generate effective immune responses, resulting in production of virus neutralizing antibodies and cytotoxic T lymphocytes that lead to resolution of DHBV infection (reviewed in Jilbert and Kotlarski[28]).

Characterization of the DHBV model has enabled some basic questions about the biology of hepadnavirus to be asked, including the kinetics of growth in the liver, with the idea that the prolonged incubation period reported for HBV infection might be due to slow virus spread. Instead, DHBV infection in young ducks spread rapidly throughout the liver with a doubling time of 16 hours, resulting in full infection of the liver from a single virus particle within 4 weeks.[96] The rate of spread of DHBV in the liver of 3-day-old ducks infected with $1.5 \times 10^3$ $ID_{50}$ of the Australian strain of DHBV is shown in Fig. 12.5.[96] The number of cells expressing DHBV surface and core antigen increase exponentially, due to cell to cell spread of the virus, resulting in infection of >95% of hepatocytes by day 13–15 after infection. The DHBV model has been used in a similar way to study the kinetics of growth and behaviour of viral mutants *in vivo*, including DHBV strains with mutations in the X-ORF[27] and cytopathic mutants of DHBV with specific mutations in the viral pre-S/S protein[97–99] (Meier *et al*., unpublished observations).

Studies of the immune response during DHBV infections have been hampered by the lack of reagents for studying the immune system of the duck. However, recent progress has been made with the development of *in vitro* stimulation assays for detection of viral antigen-specific T-cell responses,[100–102] monoclonal and polyclonal antibodies for duck T-cell markers[103,104] and ELISA assays for DHBV surface antigen and anti-surface and anti-core antibodies (reviewed in Miller *et al*.[102]). The recent cloning of cDNAs for the duck cytokines, interferon (IFN)-$\alpha$[105] and IFN-$\gamma$[106,107] and the duck T-cell markers, including CD3, CD4, CD8, MHC I and II by David Higgins and colleagues at the University of Hong Kong (GenBank AF378704; T-cell surface glycoprotein CD4 precursor mRNA, GenBank AF378701; T-cell antigen CD8 alpha mRNA, GenBank AF378373; MHC class I antigen alpha chain mRNA, GenBank AF393511; MHC class II antigen beta chain mRNA, GenBank AF390589), now make studies of the immune response during DHBV infection highly feasible. In addition to the reagents above, anti-duck CD4 and CD8 monoclonal antibodies are now available (Ursula Schultz, University of Freiburg, unpublished data) to allow further studies.

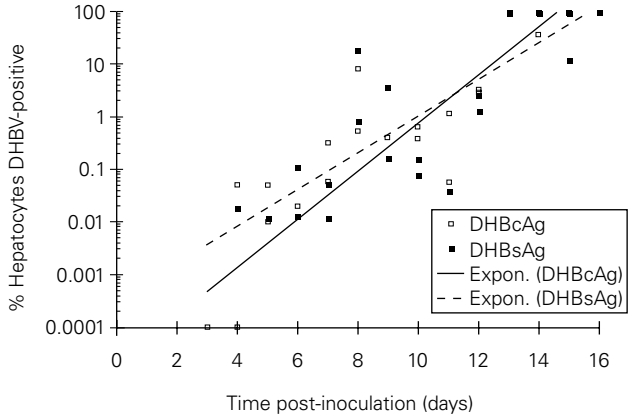


**Figure 12.5** The spread of DHBV infection from cell to cell in the liver of a group of 28, 4-day-old ducklings inoculated intravenously with $1.5 \times 10^3$ $ID_{50}$ of DHBV. DHBV infection was also monitored from days 3 to 16 post-inoculation by immunoperoxidase staining of DHBcAg and DHBsAg in sections of fixed autopsy liver tissue. The percentage of DHBcAg and DHBsAg-positive hepatocytes was determined by cell counts in sections counterstained with haematoxylin and the calculated line of best fit for exponential increase in the number of DHBcAg and DHBsAg-positive hepatocytes is shown. From Jilbert *et al*.[96]

The ability to modify the outcome of DHBV infection and reproducible kinetics of DHBV infection following experimental inoculation have made the DHBV model a valuable and reliable experimental system for studying aspects of acute and chronic hepatitis.[28,85,95,96,102,108,109] Studies are in progress to further define the role of the immune response during the resolution of transient DHBV infection and in assessing the protective and therapeutic efficacy of various vaccine strategies against DHBV infection.

## Pathology of infection

The study of the animal hepadnaviruses has made it possible to determine whether differences in liver pathology following infection are proportional to the amount of viral replication, or are the result of host-specific responses and/or environmental factors.

Acute infection with HBV can be asymptomatic or can result in severe and fatal hepatitis. Recent studies of the resolution phase of transient WHV infection have demonstrated that extensive turnover of hepatocytes occurs with the equivalent of at least one entire liver of hepatocytes being destroyed and regenerated during resolution.[110] Analysis of the levels of integrated WHV DNA indicated that the recovered liver was populated to a large extent by previously infected hepatocytes that had undergone cell division, consistent with the idea that loss of virus infection from hepatocytes required turnover of infected cells.[110]

In agreement with this, resolution of transient WHV infection was also accompanied by histological evidence for activation of Kupffer cells, of marked apoptosis and for a substantial amount of cell death. Remarkably, during resolution the Kupffer cell population in the liver increased from 5–9% (weeks 0–4) to 25–29% of total lobular cells (weeks 8–12). Kupffer cells also increased in size due to the accumulation of PAS-diastase-resistant material. The high apoptotic index and high fraction of PAS-D-positive Kupffer cells supported the idea that a major fraction of hepatocytes had been destroyed by the host immune response (Jilbert *et al*., unpublished data). Preliminary studies of Kupffer cells in the liver of ducks undergoing resolution of DHBV have shown similar levels of Kupffer cell activation, suggesting that cell death is also a significant feature of the resolution of DHBV infection (Jilbert *et al*., unpublished data).

Chronic HBV and WHV infection are associated with various levels of liver disease that can vary from minor inflammation in the liver to severe hepatitis, cirrhosis and, ultimately, hepatocellular carcinoma (HCC). Chronic hepadnaviral infection is associated with the development of HCC in WHV, HBV, GSHV and DHBV infections in descending order of risk.[111]

In contrast, chronic infection with the avian hepadnaviruses including DHBV generally results in a mild hepatitis that does not usually progress to cirrhosis or HCC.[29,112] This type of infection is similar to the 'healthy' carrier status of humans infected with HBV.

The failure to detect HCC in avian species may be linked to the timing and mode of transmission of these viruses, as they are usually transmitted vertically by *in ovo* transmission, resulting in congenital infection with immune tolerance and an absence of liver disease.[29,85,112] In support of this idea, experimental infection of 4-month-old ducks with DHBV leads to the development of chronic DHBV infections with mild and marked liver disease,[95] and these animals may have a greater chance of developing HCC. The ability of avian species to develop HCC may also be affected by their shorter lifespan. Chronic infection of Pekin ducks has been followed longitudinally for many years without the development of tumours.[113–116] HCC has only been found to occur in ducks from a single province in China,[112,117,118] where environmental aflatoxin exposure may have been prevalent. In summary, pathogenesis does not appear to correlate directly with the amount of virus replication but rather, with the host immune response to the infection. Environmental factors also appear to play a role, particularly in the development of HCC.

## Extrahepatic DHBV infection

Hepadnaviruses primarily replicate and cause cellular injury in the liver. A large number of studies have provided evidence for DHBV infection in extrahepatic sites as well.[9,119] Despite the large number of studies performed, the significance of extrahepatic viral replication to infection outcome remains unclear.

Most studies of extrahepatic DHBV infection have been based on the *in situ* detection of viral proteins, DNA and RNA replicative intermediates, and the natural history of hepadnaviral infection of extrahepatic sites has been best described in the DHBV model *in vivo*.[112,120–122] Embryonic studies of DHBV replication have identified that endodermal yolk sac cells support virus replication before hepatocytes.[123] This endodermal cell layer is oncogenetically and functionally related to the liver. Furthermore, pancreatic endocrine and exocrine cells, kidney tubule cells and glomeruli have also been shown to support viral replication in duck embryos.[85,124]

A systematic study of the course of viraemia and tissue tropism in ducks congenitally infected and experimentally infected with DHBV was performed for 6 months.[122] During this study, experimentally infected ducklings had detectable virus infection in the endocrine and exocrine pancreas. Over time, the number of infected acinar cells declined. However, there was a corresponding increase in the number of pancreatic islet cells that expressed DHBV DNA and DHBsAg. Earlier studies by Halpern *et al*.[119,125,126] had found that DHBV DNA infection was present in both α-cells (glucagon-secreting) and β-cells (insulin-producing), with the former having a high percentage of cells expressing viral antigens.

DHBV infection has been demonstrated in kidney tubules[119] and some glomeruli.[119,120,122,127] Viral DNA was found in foci of adjacent glomeruli[120] and proximal tubules[119,120] using the technique of *in situ* hybridization.

Viral antigen accumulation in glomeruli may in part be bound within immune complexes or due to the excessive antigenic burden produced by chronic infection with the respective hepadnavirus.

Small clusters of DHBV antigen-positive cells were detected in adrenal glands of young. These cells were identified on the basis of histological criteria and found to be adrenal cortical cells.[125] However, no evidence was obtained for DNA replication in the adrenal gland. The pancreatic islet cells and the cells of the adrenal gland secrete hormones involved in the regulation of carbohydrate metabolism and are susceptible to DHBV infection.[128] The implications of this are presently unknown, and further investigations are required.

DHBV DNA is not usually found in the spleen of chronically infected ducks but has been observed during acute infection,[121,129] specifically in splenic mononuclear cells.[129] The distribution of DHBsAg in the spleen was found to correlate well with the presence of DHBV DNA, although a proportion of splenic red pulp cells, which were shown to contain lower levels of DHBV DNA by *in situ* hybridization, were negative for DHBsAg. Only viral DS DNA has been found within germinal cells of the spleen of ducks and humans.[129] The DS nature of the viral DNA in spleen cells may also be related to the age of the ducks examined. Earlier studies had noted an age-associated transition from SS to DS DHBV DNA in the spleen of infected ducks.[121]

Within the liver, DHBV replication has also been found to occur in a population of cells other than hepatocytes. Bile duct epithelial cells have been reported to contain DHBV proteins and DNA.[85] DHBV replication has been convincingly demonstrated using primary cultures of bile duct epithelial (IBDE) cells isolated from ducklings. The primary IBDE cells formed duct-like structures of multi-layered IBDE cells, which were identified as bile duct cells using the anti-keratin marker, CAM 5.2, and were negative for duck albumin. Primary IBDE cells were shown to be susceptible to DHBV infection with production of DHBV cccDNA, replicative intermediates and viral surface and core proteins. These cells might form a reservoir for DHBV infection in the liver and are a clear example of DHBV replication in cells other than hepatocytes.

## Development of antihepadnaviral agents

The mechanisms that determine the final outcome of the interaction between HBV and its host are multifactorial. As chronic HBV infection can cause serious liver damage, elimination of persistent infection before the development of cirrhosis, liver failure and/or malignancy is an important clinical therapeutic strategy.[91] Thus, the primary goal of antiviral therapy is to eradicate HBV infection from the host and thereby prevent the consequences of persistent infection, namely chronic liver disease and its further clinical progression.[130]

### The experimental model of DHBV

The relatively low response rate to the existing approved antiviral therapies for the treatment of chronic HBV infection clearly supports the necessity for the ongoing development of alternative therapeutic regimens.[130] As all hepadnaviruses share a common replication strategy, animal models such as DHBV have provided useful preclinical systems for the development of novel agents or improved treatment protocols. Two major classes of therapeutic agents are currently used for the treatment of chronic HBV: immune modulating agents (cytokines such as IFN-α) and antiviral agents (such as nucleoside analogues). More recently, these agents are also being trialled in various combination strategies.

The use of the DHBV model of hepatitis B in the assessment and development of therapeutic agents and protocols has proved particularly useful for the purine nucleoside analogues such as ganciclovir,[89,90,131] penciclovir,[132] adefovir (PMEA),[133–135] entecavir (ETV)[136,137] and FLG,[138,139] as well as the pyrimidine analogues including emtricitabine (FTC),[140] an agent very similar in antiviral profile to lamivudine,[141] clevudine (L-FMAU) (Aguesse-Germon) and β-L-Fd4C[141] (see Table 12.2)[51,86,89,90,131–133,135,137–140,142–155] (reviewed in Shaw and Locarnini.[153]). Such detailed studies in the duck model have highlighted the value and reinforce the need for extensive preclinical testing of an agent before it enters phase I/II clinical trials. In this way, both predictable and unpredictable toxicity should be avoided.[89]

Early attempts to screen potential anti-hepadnaviral compounds measured antiviral efficacy by the amount of endogenous viral DNA polymerase activity or viral surface antigen in serum. This approach had limited applications and did not attempt to define intrahepatic effects of the agent on the overall hepadnaviral replication. Present antiviral evaluations *in vivo* have become much more sophisticated and typically include a detailed intrahepatic viral protein and nucleic acid analysis,[92] pharmacokinetics[90] and drug toxicity assessments[151] as part of the standard evaluation protocol (Table 12.2).

Because of the limited success achieved with monotherapy, several different approaches and new protective strategies and treatments for persistent HBV infection are being developed by combining an antiviral treatment with a nucleoside analogue to reduce viral load, and vaccination with DNA vaccines expressing the DHBV antigens. A recent combination study of antiviral and DNA vaccine study has used ETV treatment and found reduced levels of viral replication and antigen expression in the liver and serum.[136] However, the

**Table 12.2** Antiviral compounds tested for activity against chronic DHBV infection *in vivo**

| Compound | Viral assay | Comment | Ref. no. |
|---|---|---|---|
| Phosphonoformate | DNA (S,L) | Treatment reduced viral DNA levels in serum and liver in a dose-dependent manner | 154 |
| Adenine arabinoside (ara-A) | DNA (S,L) | Dose-dependent inhibition of viral DNA in the serum and liver. Higher doses than given in human studies were required to demonstrate an antiviral effect. cccDNA levels in the liver were unaffected by therapy | 147 |
| Dideoxycytidine | DNA (S,L) | Reduction in serum DNA and DNA polymerase activity and >90% reduction in DHBV DNA in the liver. Some antiviral activity was sustained after therapy had ceased | 71 |
| Azothymidine (AZT) | DNA (S,L) | No activity | 145 |
| Ganciclovir | DNA (S,L) | Reduction in serum and liver DHBV DNA levels. No effect on viral cccDNA in the liver | 90 |
| | Viral proteins (S,L) | Reduction in viral envelope and core proteins in hepatocytes. No reduction in serum antigenaemia | 89 |
| Cyclobut-A, Cyclobut-G, AZT | DNA (S) | The purine analogues reduced serum DHBV DNA levels. AZT has no activity | 152 |
| Dideoxyadenosine (ddA)/ dideoxydiamino purine | DNA (S) | Dideoxydiaminopurine reduced serum DHBV DNA more effectively than ddA | 149 |
| 2′CDG | DNA (S,L) core protein (L) | Virus production was inhibited >50-fold. Viral DNA in the liver was reduced, including the viral cccDNA, by >70%. The number of infected hepatocytes declined after 3 months of therapy | 144,151 |
| FTC | DNA (S,L) viral proteins (L) | FTC inhibited viral DNA replication in the serum and the liver. Viral cccDNA was reduced by >50%. A high proportion of hepatocytes remained infected at the end of treatment | 140 |
| PMEA (adefovir) | DNA (S) | PMEA reduced serum viral DNA levels during treatment, but relapse occurred in the follow-up period. No effect on cccDNA. Reduction of replication in bile duct epithelial cells | 133, 135, 146 |
| Penciclovir/famciclovir | DNA (S,L) | Penciclovir, and its orally active form famciclovir, inhibited viral replication in the liver and virus secretion into the serum. No effect on viral cccDNA levels after 12 days of treatment;[51] however, PCV therapy reduced cccDNA levels by >50% after 4 weeks[143] | 133, 151, 156 |
| Entecavir (ETV) | DNA (S,L) | Very potent inhibitor of replication. cccDNA reduced but not eliminated | 137 |
| L-FMAU (Clevudine) | DNA (S,L) | Potent inhibitor of replication | 148 |
| FLG (Fluoroguanosine) | DNA (S,L) | Potent inhibitor of replication | 138,139 |
| β-L-Fd4C | DNA (S,L) | Potent inhibitor of replication. Minimal effect on cccDNA | 141 |

*The viral products were detected either in serum (S) or in the liver (L). Modified from Luscombe and Locarnini,[131] with permission and Shaw and Locarnini.[153].

administration of five doses of DNA vaccine expressing the DHBV S, pre-S/S and core antigens did not prevent the rebound of DHBV infection after withdrawal of the drug or lead to significant long-term effects on DHBV infection. Similar findings were observed with the combination approach of adefovir (ADV) plus DNA vaccination, except that more of a sustained antiviral effect was achieved.[156]

In attempts to enhance the immune response to DNA vaccines, approaches used by others for eliciting cell-mediated immune responses are being tested in ducks. These include whole-cell DNA vaccines using primary duck embryonic fibroblasts (PDEF) expressing DHBcAg, an internal antigen of the virus, and DNA vaccine prime, followed by fowlpox virus boosts (Miller and Jilbert, unpublished data).

## Challenges for development of therapies for chronic HBV: the role of cccDNA inhibitors and animal model testing

From the results of such testing as discussed above and outlined in Table 12.2, the most important observation found to date is that conventional antiviral therapy has a minimal effect on the intrahepatic viral cccDNA levels as well as viral RNA production.[157–159] Complete inhibition of viral polymerase activity by a nucleoside analogue should prevent replenishment of the cccDNA pool by blocking synthesis of its RC DNA precursor. However, the resistance of chronic HBV infection to treatment with nucleoside and nucleotide analogues such as lamivudine and adefovir indicates that the cccDNA pool in infected cells is relatively stable. If production of new

virions could be completely blocked, cccDNA should eventually be eliminated as a result of either death of infected hepatocytes or intracellular decay. The lifespan of infected hepatocytes appears to be variable, depending on the severity of liver disease, and intracellular turnover of cccDNA is probably similarly variable.

The presence of functional hepadnaviral mini-chromosomes in hepatocyte nuclei is essential for viral replication and establishment of persistence. Therefore, their complete elimination is necessary in order to affect a lasting cure. Because it is replicated via an RNA intermediate, viral cccDNA is not directly affected by deoxynucleos(t)ide analogue inhibitors of DNA polymerase activity. These inhibitors are only virustatic. The long *in vivo* half-life of cccDNA during chronic infection and low viral replication fidelity make it likely that drug-resistant virus will emerge before cccDNA is completely depleted.

One approach has been to attempt to destabilize cccDNA and prevent its formation by treatment with compounds that either interact directly with viral cccDNA or that inhibit specific enzymes required for its formation and processing. The first group includes intercalators and compounds that bind to DNA. The second are mostly inhibitors of topoisomerases, the enzymes that catalyse interconversion of DNA topoisomers by introducing transient single- or double-strand breaks via formation of covalently enzyme-DNA intermediates. Stabilization of topoisomerase-DNA 'cleavable complexes' by inhibitors and cross-linking agents blocks progress of polymerases, ultimately causing permanent strand breakage and has been shown to work as a 'proof of principle' in primary duck hepatocytes infected with duck HBV.[160] Compounds shown to have antiviral activity included adriamycin, tenoposide and camptothecin, which have been used clinically as anti-cancer drugs, but which are clearly too non-specific and too toxic to be used long-term in patients with chronic HBV. An alternative approach might involve the use of a new generation of antineoplastic drugs that interfere with histone and DNA modification.[160–163] Another extension of this approach is to use combinations of conventional nucleoside analogue therapy with topoisomerase inhibitors[164] as well as antiviral cytokines.[165] These studies indicated that the recalcitrant nature of the cccDNA molecule as an antiviral target could at least be partly reversed and should act as a stimulus for further studies in the duck model. Whether or not these compounds have clinically useful antiviral activity awaits evaluation, but their use in animal models such as the DHBV model will ultimately generate useful information about the structure and function of hepadnaviral mini-chromosomes that could eventually lead to their therapeutic elimination and 'cure' of chronic HBV.

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# Chapter 13
# Woodchuck hepatitis virus

Michael Roggendorf, Michael Lu

## Introduction

The Eastern woodchuck (*Marmota marmota*) is naturally infected by woodchuck hepatitis virus (WHV) which was discovered in 1978.[1] WHV was found to be closely related to hepatitis B virus (HBV)[2] and classified as a member of the genus orthohepadnavirus, family Hepadnaviridae. However, these viruses differ in several aspects, e.g. regulation of transcription.[3] The family Hepadnaviridae was still growing in recent years.[4,5] Infections of woodchucks with WHV have been shown to be endemic in the mid-Atlantic states of the USA, e.g. Delaware, Maryland, Pennsylvania, whereas in the states of New York and New England woodchucks are rarely infected with WHV. The molecular characterization of WHV and experimental infection of woodchucks with WHV has been of value in modelling several aspects of hepadnaviral infection in humans. Many questions of hepadnaviral infection have been addressed in this model, e.g. natural course of infection,[6–9] immunopathogenesis,[9–15] host and viral factors associated with development of chronicity,[15–17] development of hepatocellular carcinoma (HCC),[18,19] and rise and significance of viral mutants in acute and chronic infections.[20–28] From a medical point of view, the woodchuck model has been used to develop new strategies for prevention of infection[29,30] and therapy of chronic hepadnaviral infection including: nucleoside analogues,[31–45] non-nucleoside analogues,[46] therapeutic vaccination[47–50] and gene therapeutic approaches for treatment of HCC.[51] Liver transplantation has recently been established for woodchucks to study early events in reinfection and adoptive immune transfer.[52,53] Previous reviews have covered the natural history of WHV infection,[54] aetiology of HCC,[19] antiviral treatment by nucleoside analogues[55–57] and pathogenesis of hepadnaviral infection.[9] Lately, significant progress has been made in this model of hepadnavirus natural infection, which demonstrates that non-cytotoxic and cytolytic antiviral immune responses are needed for elimination of the virus from hepatocytes or to at least control viral replication. This review therefore focuses on the recent development of tools to study humoral and cellular immune responses to WHV and the immunopathogenesis of WHV infection, prevention of infection by protein and DNA vaccines and immunomodulatory approaches to treat chronic WHV infection. These new insights into the immune response to WHV will enable the development of new strategies to treat chronic hepatitis B virus (HBV) infection.

## New tools to characterize the immune response to WHV

The woodchuck is an ideal model for hepadnavirus infection in humans, as WHV infection in woodchucks results in a number of different outcomes which are similar to HBV infection in humans, ranging from a subclinical or acute transient infection to chronic infection progressing to HCC. Unfortunately, at present no inbred animals are available to standardize infection experiments. For an understanding of the underlying mechanisms responsible for different outcomes of infection, detailed *in vivo* studies on humoral and cellular immune responses to WHV are required. Assays for antigen and antibody detection have been available for many years.[58] However, until recently little information about the cellular immune system was available.

### Characterization of virus-specific T cells in the woodchuck

For many years, studies on the cellular immune response to WHV were hampered by the lack of effective proliferation assays for peripheral blood mononuclear cells (PBMCs). Thymidine uptake by woodchuck PBMCs stimulated by mitogens or specific WHV proteins was very low compared with other cell systems such as mouse or human, although mitogen clearly induced blast formation of woodchuck cells, as confirmed by microscopic examination. Based on these negative results reported so far the experimental conditions were re-evaluated to measure antigen-induced PBMC proliferation.[59] The lack of incorporation of [$^3$H]-thymidine into cellular DNA by PBMCs was due to the absence of expression of thymi-

dine kinase (TK) gene in the woodchuck lymphocytes. However, the TK gene is present in the woodchuck genome and is probably expressed in other cell types.[60] Transfection of woodchuck cells with mouse TK gene demonstrates that TK1 is transcribed and a functional TK protein can be expressed.[60] Using 2[$^3$H]-adenine as an alternative labelled nucleoside, we could demonstrate a significant incorporation into cellular DNA and partially RNA in proliferating PBMCs.[10] In addition to 2[$^3$H]-adenine, a non-radioactive proliferation assay for woodchuck PBMCs using 5-bromo-2′-deoxyuridine (BrdU) as thymidine analogue was established.[61] However, measurement of PBMC proliferation induced by mitogens and WHV core protein (WHcAg) by the incorporation of BrdU was less sensitive than the assay using 2[$^3$H]-adenine. Another alternative assay to determine T-cell proliferation was established by measuring secretion of interleukin (IL)-2 of activated T cells.[47,62] In this assay, woodchuck IL-2 secreted in supernatants from stimulated PBMCs was tested in an IL-2-dependent murine cell line, CTLL-2, indicating a cross-reactivity of mouse and woodchuck IL-2. Viral proteins for *in vitro* stimulations were obtained by purification of WHsAg from plasma and recombinant WHcAg in *Escherichia coli*. Using overlapping and non-overlapping peptides derived from WHcAg, a number of T-cell epitopes on WHcAg could be identified (see below). These modified and optimized proliferation assays made it possible to perform studies on T-cell immune response, specifically to WHV proteins in acute and chronic infection and immunized woodchucks.[61] It remained to be proven that stimulated T cells are actually CD4-positive T-helper cells because of the lack of specific antibody to the woodchuck homologue of CD4. A cross-reacting anti-CD3 antibody from swine binds to these cells.[10,11] It can be assumed that proliferating CD3-positive T cells correspond to the T-helper cell population which expand under similar experimental conditions in the mouse system. Despite many efforts, demonstration of cytotoxic T cells has not been achieved for the woodchuck model. Using DNA vaccination in mice with plasmids expressing WHcAg, specific CTLs to WHcAg could be demonstrated in the mouse model (Isogawa *et al*., unpublished results).

## Cloning and characterization of components of the woodchuck immune system

In recent years, a number of cytokines and surface markers of immune cells have been characterized. Proteins related to immune response such as antigen processing (TAP-1, TAP-2) and presentation, e.g. major histocompatibility complex (MHC) class I and class II, proteins involved in signal transduction, and cell surface markers like CD3, CD4 and CD8 present in cells of the immune system have been cloned and sequenced. In Table 13.1, the components of the woodchuck immune system characterized at the molecular level so far are summarized.[15,29,63–69] In general, cDNA fragments were amplified by reverse transcription polymerase chain reaction (RT-PCR) with RNA from woodchuck lymphocytes. Primers were designed according to aligned sequences and chosen from regions well conserved among human and other mammalian species.

### *Cytokines*

The complete coding sequences of tumour necrosis factor-α (TNF-α),[63,67] interferon-α (IFN-α),[66] IFN-γ,[63] IL-6,[63] IL-10,[23] IL-12[29] and IL-15,[15] GMCSF,[68] lymphotoxin-α (LT-α) and -β (LT-β)[67] were obtained. Partial sequences of IL-2, IL-4, Fas ligand[69] and others were cloned. The

**Table 13.1** Cloned cytokines of woodchuck

| | Size | Homology (aa) Human/mouse (%) | Biologically active | Accession no. | References |
|---|---|---|---|---|---|
| TNF-α | 233 aa | 80/84 | + | Y14137 | Lohrengel *et al*. (1998),[63] Lohrengel *et al*. (2000)[64] |
| IFN-γ | 166 aa | 60/43 | + | Y14138 | Lohrengel *et al*. (1998),[63] Lu *et al*. (2002)[65] |
| IFN-α | 167 aa | 62/58 | + | AAG27516 | |
| | | | | AAK19944 | Salucci *et al*. (2002)[66] |
| IL-2 | Partial | | | AF082496 | Lu *et al*. (unpublished results) |
| IL-4 | Partial | | | AF082495 | Lu *et al*. (unpublished results) |
| IL-6 | 207 aa | 49/46 | ND | Y14139 | Lohrengel *et al*. (1998)[63] |
| IL-10 | 178 aa | 80/72 | ND | AF012909 | Li *et al*. (2000)[67] |
| IL-12 p35 | 223 aa | 62/51 | + | X97018 | Garcia-Navarro *et al*. (2001)[29] |
| IL-12 p40 | 325 aa | 78/65 | + | X97019 | Garcia-Navarro *et al*. (2001)[29] |
| IL15 | 192 aa | 79/70 | + | AY426605 | Wang *et al*. (2003)[15] |
| GM-CSF | 138 aa | 63/49 | + | AF255734 | Wu *et al*. (2001)[68] |
| Lymphotoxin-α | 202 aa | 78/80 | + | AF095586 | Li *et al*. (2000)[67] |
| Lymphotoxin-β | 306 aa | 67/69 | ND | AF095587 | Li *et al*. (2000)[67] |
| Fas ligand | Partial | | ND | AF152368 | Hodgson *et al*. (1999)[69] |

TNF, tumour necrosis factor; IFN, interferon; GM-CSF, granulocyte macrophage colony-stimulating factor.

length and homology of woodchuck cytokines to human and mouse cytokines are given in Table 13.1. In general, the woodchuck genes are more closely related to human than to mouse counterparts. The structure of woodchuck cytokines as compared to human cytokines seems to be well conserved, e.g. the comparison of woodchuck TNF-α to the human sequence demonstrates that two cysteine residues, cys69 and cys101, that are known to be involved in an intermolecular disulfide bridge are conserved. Other amino acid residues like leu29, arg32, ala143 and ser145, which were found to be important for receptor binding of the human protein, are also conserved in the woodchuck TNF-α.[63] For IFN-α it could also be shown that four cysteine residues which form intramolecular disulfide bridges are conserved.[66]

TNF-α, IFN-α, IFN-γ, IL-12 and LT-α have been tested for their biological activities. The entire open reading frame (ORF) of IFN-α was cloned under control of the cytomegalovirus (CMV) promoter into the expression vector PVIJ.[66] Supernatants of HeLa cells transfected with this construct protected woodchuck cells from vesicular stomatitis virus (VSV)-induced cytopathic effect (CPE). The biological action of IFN-α was also demonstrated by induction of expression at MxA-protein. Finally, the biological activity of IFN-α was tested in WHV-infected woodchuck hepatocytes. Woodchuck IFN-α reduced WHV surface antigen expression in a dose-dependent fashion.[66]

The biological activity of TNF-α and LT-α was tested in a cytotoxicity assay using woodchuck A2 cells. The specific activities for both woodchuck cytokines TNF-α and LT-α were significantly higher in woodchuck cells than in mouse L9 to 9B and human HepG2 cells.[23,63]

We expressed recombinant woodchuck IFN-γ (wIFN-γ) in *Escherichia coli* and mammalian cells. wIFN-γ protected woodchuck cells against infection with murine encephalomyocarditis virus in a species-specific manner. It upregulated the mRNA level of the woodchuck major histocompatibility complex class I (*Mamo-I*) heavy chain in permanent woodchuck WH12/6 cells and regulated differentially the gene expression.[65] However, the level of the replication intermediates and specific RNAs of WHV in persistently WHV-infected primary woodchuck hepatocytes did not change despite treatment with 1000 U of wIFN-γ per mL or with a combination of wIFN-γ and wTNF-α (Fig. 13.1). Rabbit antibodies to wIFN-γ were able to block biological activity of IFN-γ.

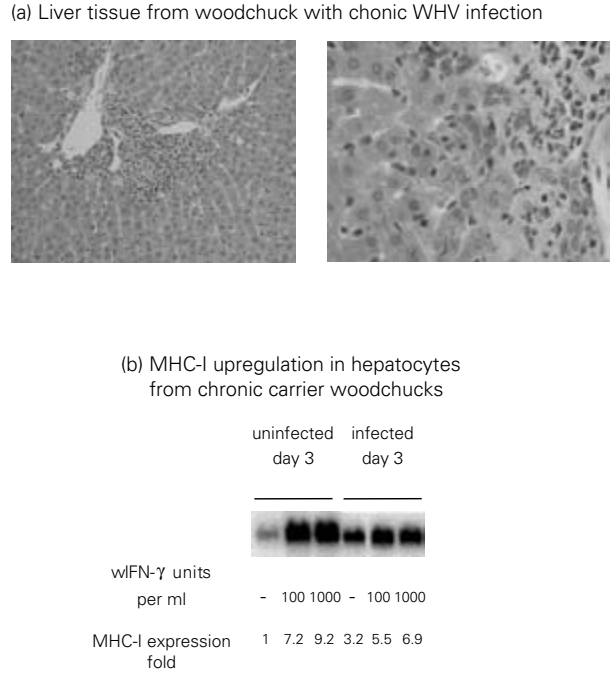


**Figure 13.1** (a) The persistent viral infection leads to infiltration of lymphocytes into the liver. Recent studies showed clearly that the intrahepatic production of wIFN-γ continuously occurs in chronic carriers and leads to stimulation of cellular gene expression. (b) MHC-I expression in response to the stimulation with IFN-γ. The woodchuck MHC-I cDNA is expressed only at a low level in naïve hepatocytes (uninfected), but showed an enhanced expression in hepatocytes derived from chronically infected liver (infected). The expression of MHC-I increased up to 9.2-fold with treatment with IFN-γ at a concentration of 100 or 1000 units/mL in cultures. Notably, the expression of MHC-I in infected hepatocytes did increase only slightly after stimulation with IFN-γ.

## Intrahepatic expression of wIFN-α and -γ in chronic WHV infection

wIFN-α and -γ were cloned into adenoviral vectors for gene therapeutic approaches to treat chronic WHV infection.[57,70,71] *In vitro* transduction of woodchuck hepatocytes with adenoviral vector resulted in secretion of biologically active cytokines. In subsequent experiments, both vectors have been used to treat chronic WHV infection *in vivo*. The transduction of livers of WHV carriers with HD-AdwIFNα or HD-AdwIFNγ induced levels of biologically active IFN, which could be measured in the sera of these animals. Expression of wIFN-α in the liver significantly reduced intrahepatic WHV replication and WHV-DNA in sera. However, transduction with HD-wIFNγ reduced WHV replicative intermediates only slightly. The data demonstrate for the first time the successful HD-Ad-vector-mediated transfer of genes for IFN-α and IFN-γ *in vivo* and timely limited reduction of WHV replication by wIFN-α, but not by wIFN-γ.[70] Similar experiments with a second generation of adenoviral vector expressing wIFN-γ and wTNF-α reduced WHV core-positive hepatocytes and cccDNA. However, this may be due to the immune response to the vector.[57]

## Cell surface markers

Characterization of T-cell markers is a prerequisite to define T-cell subpopulations and to functionally analyze

these cells in the course of acute and chronic infection. By designing primers chosen from regions conserved among humans and other mammalian species, fragments of CD3, CD8 and the complete sequence of CD4 have been cloned (Table 13.2).[15,28,69,72–75] The complete coding region of woodchuck CD4 has a length of 1365 bp encoding a protein of 455 amino acids (aa) (Fiedler, unpublished results). The cDNAs of woodchuck CD28 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) have been cloned and sequenced.[74] These two molecules are known to play important roles in the regulation of T-cell activation by delivering the co-stimulation signals. Woodchuck CD28 showed a similarity of 70–80% to its mammalian homologues according to the deduced amino acid sequences. Woodchuck CTLA-4 has a higher similarity of 74% to the corresponding mammalian CTLA-4 molecules. The strict conservation of critical amino acid residues like cysteine and asparagine residues in woodchuck CD28 and CTLA-4 suggests that both molecules may structurally resemble their human or mouse homologues. A hexapeptide motif, MYPPPY, which has been supposed to be essential for the interaction with CD80, is present in both woodchuck CD28 and CTLA-4.[74]

Sequence information of woodchuck CD3, CD4 and CD8[77] has been used to determine the kinetic of influx of T cells into the liver. Either sequences of wIFN or wTNF-α were suitable to determine cytokine expression during the incubation period and acute or chronic WHV infection.[7,77,78] In week 2 post-infection, an influx of CD3+ lymphocytes could be observed and reached higher levels before and during the recovery phase. The peak level of CD4+ and CD8+ T cells coincided with recovery. Monoclonal or polyclonal antibodies to CD3+, CD4+ and CD8+ of woodchuck are not available yet. One monoclonal antibody to the conserved region of CD3 from swine was found to cross-react with woodchuck lymphocytes. The antibody was used to identify T cells in the liver during acute and chronic WHV infection. During transient infection, T cells can accumulate in the liver and reach up to two-thirds the total number of liver cells.[7]

## MHC class I

The full-length cDNAs of woodchuck *Mamo-I* genes were cloned using cellular mRNA isolated from peripheral blood mononuclear cells and liver tissues of woodchucks.[79] DNA sequence analysis of *Mamo-I* cDNAs revealed that the coding regions of *Mamo-I* genes were about 1080 bp long, encoding 359 amino acid residues.[74] The deduced amino acid sequences of *Mamo-I* showed structural features like leader, α1, α2, α3, transmembrane and cytoplasmic domains, similar to their homologues in humans and other mammals. Analysis of five full-length clones from unrelated woodchucks indicated a polymorphism within the α1 and α2 domains of *Mamo-I* heavy chain and a high conservation within the α3 and the transmembrane/cytoplasmic domains. Amino acid residues of the α2 and α3 domains, which are supposed to be involved in the binding of MHC class I to CD8 molecule, were largely conserved among

**Table 13.2** Clonal cell surface marker and other genes related to the woodchuck immune system

| Gene | Sequence information | Homology (aa) Human/mouse (%) | Accession no. | Reference |
|---|---|---|---|---|
| CD3 gamma | Partial | | AF082493 | Nakamura *et al.* (1997)[72] |
| CD3 epsilon | Partial | | AF232727 | Michalak *et al.* (2000)[73] |
| CD4 | 455 aa | | AF082497 | Zhou *et al.* (1999),[28] Fiedler (unpublished results) |
| CD8 | Partial | | AF082499 | Zhou *et al.* (1999),[71] |
| CD28 | 221 aa | 76/74 | AF130427 | Yang *et al.* (2003)[74] |
| CTLA-4 | 223 aa | 86/85 | AF130428 | Yang *et al.* (2003)[74] |
| MHC-I (*Mamo-I*) | 359 aa | 81-83/78-79 | AF146091 usw. | Yang *et al.* (2003)[79] |
| MHC II (WLA) | 266 aa | 87/80 | | Viazov (unpublished results) |
| B2 microglobulin | Partial | | AF232726 | Michalak *et al.* (2000)[73] |
| TAP-1 | Partial | | AF232724 | Michalak *et al.* (2000)[73] |
| TAP-2 | Partial | | AF232725 | Michalak *et al.* (2000)[73] |
| 2′-5′oligoadenylate synthetase | Partial | | AF082498 | Zhou *et al.* (1999)[28] |
| Stat4 | Partial | | AY177676 | Wang *et al.* (2003)[15] |
| Stat6 | Partial | | AY177677 | Wang *et al.* (2003)[15] |
| T-bet | Partial | | AY177675 | Wang *et al.* (2003)[15] |
| GATA-binding protein 3 | Partial | | AY177678 | Wang *et al.* (2003)[15] |
| Peml | Partial | | AY494083 | Fourel (unpublished observations) |
| Perforin | Partial | | AF298158 | Hodgson *et al.* (1999)[69] |
| Tumour suppressor p53 | 391 aa | 86/77 | AJ001022 | Feitelson (1997)[75] |

*Mamo-I* genes. Phylogenetic comparison of woodchuck MHC class I genes to other mammals indicated a close evolutionary relationship between woodchuck and squirrel MHC class I.[74] Among these 14 alleles identified so far, *Mamo-A**01 and *Mamo-A**09 were of the highest frequency of about 21.5% and 14.5%, respectively.

To prove the allelic nature of *Mamo-I* genes by classical genetics, like the segregation analysis, allelic diversity of *Mamo-I* in two three-generation woodchuck families consisting of 15 members was analyzed. Alleles were identified by the sequencing of *Mamo-I* genes and immunoblotting of *Mamo-I* proteins after one-dimensional isoelectric focusing (1D-IEF). A typical Mendelian segregation of *Mamo-I* genes and antigens was observed in the families studied.[56] Our study established *Mamo-A* as a classical MHC class I locus by the polymorphic and allelic nature of *Mamo-I* gene in the woodchuck. Full-length cDNA of *Mamo-I* has been transfected to mouse p815 cells and peptides bound to MHC-I were eluted from the transfected p815 cells and analyzed. The potential anchor sequence of eluted peptides has been identified for *Mamo-I* (Stephanovic, unpublished results). This information will be used to generate peptides of WHcAg and WHsAg, which are potential epitopes for CTL in woodchuck with the *Mamo-I* allele.

MHC class II sequence has also been identified. The putative protein has 266 aa and typical domains like signal peptide $P_1$Domain, $\beta_2$Domain, transmembrane and cytosolic domain. So far, 14 alleles have been identified. One of these has a high frequency of 69% in our woodchuck cohort. The overall homology of MHC clade II of woodchuck is 87% to the human analogous DRB (S. Viazov, unpublished results).

## Characterization of T-cell response during the incubation period and acute phase of WHV infection

Currently, the major issue in the pathogenesis of hepadnavirus infection is how the immune system contributes to the elimination of the virus from the liver and which viral and/or host factors determine the recovery or chronic outcome after an acute phase of hepadnavirus infection. There is controversial discussion on the contribution of cytokines secreted by natural killer (NK) cells, T-helper cells and cytotoxic T cells for downregulation of hepadnavirus replication at an early stage of acute infection.[80,81] At a later time-point of acute infection cytolytic function of CTLs seems to be responsible for elimination of infected hepatocytes and for the ultimate recovery from HBV infection.[82,83]

In several studies, the course of WHV infection after experimental infection has been investigated either intravenously or subcutaneously. After inoculation of adult woodchucks with a medium titre of WHV ($10^5$–$10^8$), viraemia can be detected at week 2.[16] Maximal titres of WHV are observed in the serum during weeks 6–8. WHsAg and anti-WHc are detected in weeks 3–5 and 4–6, respectively (Fig. 13.2). Inoculation of low titre WHV resulted in a very short and late viraemia in some animals. The only marker of infection was seroconversion to anti-HBc 8–10 weeks after inoculation. In neonatal subcutaneous infections, viraemia seems to be delayed as compared with intravenous infection of adult animals.[16] Low titre infection of neonate woodchucks is also associated with lower carrier rate.[6] Highest levels of WHV DNA always precede the peak of succinyl hydrogenase (SDH), which is a good marker of hepatocyte

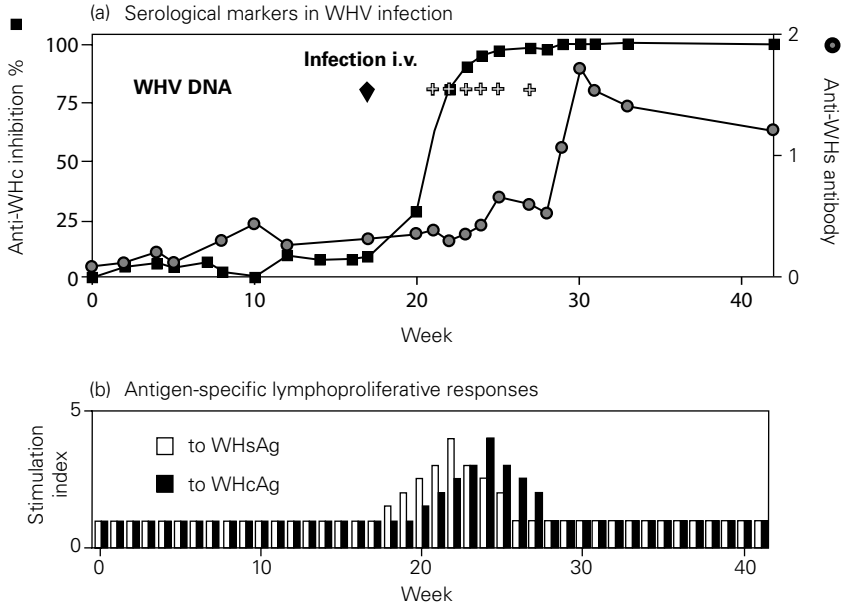


**Figure 13.2** Experimental infection of woodchucks. (a) Experimental infection of woodchucks is initiated by intravenous injection of WHV. The WHV infection can be monitored by detection of WHV DNA using spot-blot hybridization and detection of WHsAg by using specific ELISA antibodies to WHcAg and WHsAg. (b) The lymphoproliferative response to the WHV proteins is usually measurable during the viraemic phase. The WHsAg-specific response appears early in the course of infection, while the response to WHcAg peaks later and is coincident with the appearance of anti-WHsAg.

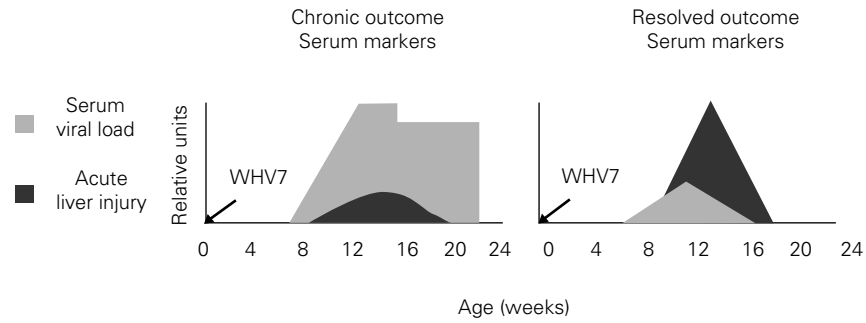


**Figure 13.3** Schematic diagram of relation of serum viral load (based on WHV and WHsAg) and liver injury response (based on succinyl hydrogenase, SDH) to outcome of neonatal WHV infection.

lysis (Fig. 13.3).[11] It has been demonstrated that influx of CD8-positive T cells in the liver of woodchucks reaches maximal levels when viral DNA replication and expression of core protein are already reduced,[7,84,85] similar to the situation during HBV infection in chimpanzees and patients. These findings indicate that non-cytolytic mechanisms are involved in downregulation of WHV replication, and most probably CTLs are responsible for elimination of infected hepatocytes, as shown in chimpanzees,[86,87] transgenic mice[83] and patients.[88]

The problem of elimination of virus without lysis of the majority of infected hepatocytes in a very short time window has been elucidated in a recent study by Summers *et al*.[78] They used the presence of viral DNA sequences uniquely integrated into the DNA of a small fraction of infected hepatocytes as genetic markers for the population of infected cells to follow the fate of the infected cells during viral clearance. They found that after recovery from the infection, there was no discernible reduction in the number of copies of integrated viral DNA in the liver, indicating that the uninfected cells of a recovered liver were derived primarily from infected cells.

## T-helper response in acute and chronic WHV infection

Experimental infections of woodchuck provide the opportunity to study the detailed kinetics of WHV-specific T-helper cells during the early incubation phase in the peripheral blood and the liver. This has not yet been tested in chimpanzees and humans. The strength of T-helper response may be important for the clinical outcomes of infection (see below). The establishment of methods to determine T-cell proliferation[8,11,59,61] has made it possible in recent years to monitor the dynamics of T-cell response to WHsAg, WHcAg and derived peptides after experimental infection during the incubation period, acute and chronic disease under well defined conditions in detail. The response of woodchuck T cells to WHsAg recombinant WHcAg and WHcAg-derived peptides was monitored by 2[$^3$H]-adenine assay, which had a higher sensitivity than that assay using BrDU incorporation.

The first T-cell responses against WHsAg were detected 3 weeks after WHV inoculation (Fig. 13.2). The maximum T-cell responses to WHsAg occurred when WHsAg was detected in serum and decreased upon seroconversion to anti-HBs. The development of anti-WHs, which is regarded as a virus neutralizing antibody, was associated with the elimination of WHsAg from the serum. These findings suggest that T-helper cell responses to WHsAg occur very early in the infection before liver damage takes place, as shown by the presence of normal SDH levels in the serum. The T-helper cell response to recombinant WHV core protein was seen in week 4, generally 1 week later than HBsAg-specific T-cell responses. In independent experiments, CD4-positive T cells were detected in the liver at week 3.[7] However, it has not been determined whether these cells were specific to WHV epitopes. Similar to the early response in the WHV-infected woodchucks, an early CD4+ T-cell response could be observed during the incubation period in a limited number of patients.[88] In chimpanzee experiments, the application of anti-CD4 antibodies at week 6 did not alter the course of infection. The interpretation of this has been that T-helper cells are no longer needed after week 6. However, elimination of CD8+ T cells through a specific antibody results in a prolonged viraemia in these chimpanzees. The virus was eliminated after rebound of specific CD8+ T cells, indicating that specific CD8+ T cells are essential for virus elimination.[86,87]

## Mapping of T-helper epitopes

The T-cell responses to the peptides derived from WHcAg measured in different studies indicate that at least 10 different epitopes located throughout the entire core protein are recognized in woodchucks. Different subsets of WHc epitopes were recognized by different animals (at least four in each animal) during acute self-limited infection.[12] The large number of epitopes may be explained by the fact that outbred animals were used in these studies which might have had different alleles

of MHC class II. Some peptides, on the other hand, like peptide 97–110 (100%), 110–119 (86%), 112–131 (55%) and 1–20 (53%), were recognized by the majority of woodchucks with acute self-limiting infection, which strengthens previous findings that some immunodominant epitopes may be promiscuous (Fig. 13.4). The importance of the immune response to the epitope 97–110 has been demonstrated in subsequent experiments. Woodchucks immunized four times with this peptide showed a specific T-cell response and were protected from WHV infection, as no WHV DNA was detectable in serum and liver. We observed a specific and increasing T-cell response to peptide 97–110 and rWHcAg following immunization. The predominant T-cell response to WHV antigens during immunization and after challenge suggests that protection was primarily based on the cellular response (Th cells and/or CTLs). Likewise, immunization of mice with peptides derived from the nucleoprotein of lymphocytic choriomeningitis virus resulted in protection without detectable virus neutralizing antibodies.[89,90] Further experiments are needed to elucidate the mechanism of protection. Animals that developed chronic infection showed only a weak T-cell response. The majority of them were unable to recognize peptide 97–110, but developed T-cell response to other epitopes which are present at a low frequency in animals with acute resolving WHV infection. The appropriate immune response during the incubation period of WHV infection has to be considered as the critical period of virus–host interaction. Further studies are needed to characterize virus-specific CD8+ T cells in the woodchuck system in the peripheral blood and the liver to determine their contribution to elimination of virus replication and WHV-infected cells with cccDNA.

## Failure of appropriate immune response in the incubation period and early acute infection results in chronic carriership

In chronic HBV/WHV carriers, only weak or no T-helper responses are detected. The question arises whether the chronic outcome of hepadnaviral infection is related to failure to mount an early T-helper response or to loss of T cells during the acute phase.

Studies on the early immunological events following HBV infection in humans are usually limited due to the time interval between infection and onset of clinical symptoms. Only very small numbers of patients could be tested so far.[88] As the majority of these cases represent self-limiting courses, there are only few opportunities to examine the T-cell response in the acute phase associated with subsequent development of chronic HBV infection. The question whether chronic outcome is associated with an absent or suboptimal

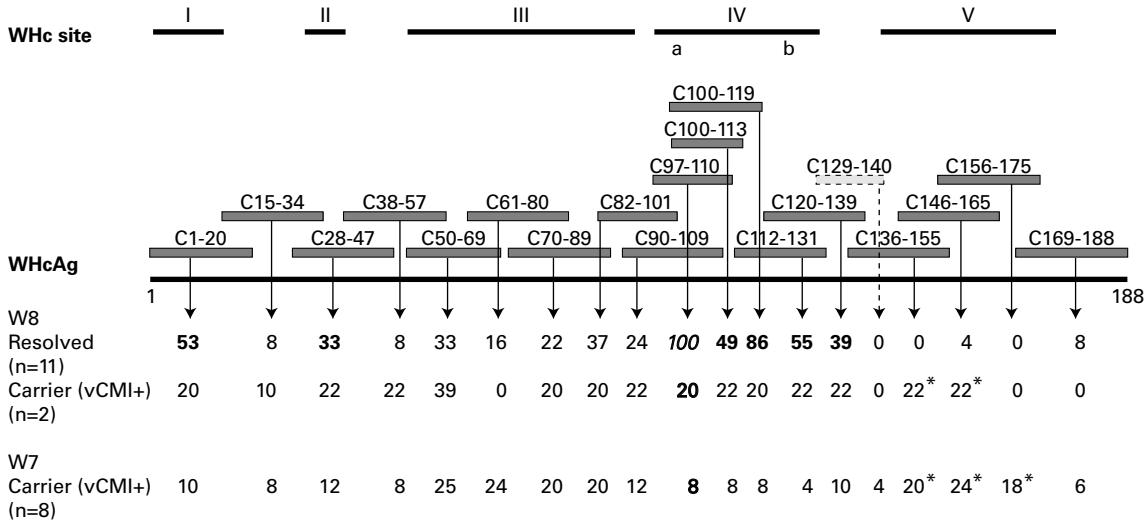


**Figure 13.4** Summary of specific lymphoproliferative responses to WHcAg-derived peptides in woodchucks with resolved WHV infection and in carriers with positive acute phase lymphoproliferative responses.[12] Values for the strain W8 and W7 outcome categories were expressed relative to the most frequently recognized WHcAg peptide, C97–110, in the resolved strain W8 infection group (i.e. 51% in Table 13.3 was set at 100%, italic, here). Other values were calculated accordingly. Sites I–V were demarcated on the basis of differential and similar response frequencies for the present groups of neonatally infected woodchucks and also on other published information. In site IV, a and b represent at least two non-overlapping epitopes, one of which (C97–110) has been shown previously to be protective in experimental challenge studies. Sites I, II and IV appeared to be preferred recognition sites for woodchucks with resolved infections (bold numbers). Site III appeared to be recognized similarly in both outcome groups. Site V may be recognized preferentially by neonatal carriers (numbers are marked with an asterisk).

**Table 13.3** Percentage of neonate woodchucks responding to and frequency of PBMC samples positive to WHV antigens and WHc

| Outcome of infection | % of woodchucks responding to | | | | % of PBMC samples positive to | | | |
|---|---|---|---|---|---|---|---|---|
| | rWHcAg | WHsAg | rWHxAg | C97-110 | rWHcAg | WHsAg | rWHxAg | C97-110 |
| Resolved | 100 (11/11) | 82 (9/11) | 91 (10/11) | 100 (11/11) | 59 (32/54) | 34 (18/53) | 34 (17/50) | 51 (28/55) |
| Chronic | 39 (9/23) | 22 (5/23) | 26 (6/23) | 17 (4/23) | 6 (16/262) | 3 (6/242) | 4 (10/242) | 2 (5/265) |

Data from Menne *et al*.[12] Eleven woodchucks recovered from infection and 23 developed chronic hepatitis.

cellular immune response during the incubation period or early acute infection has been addressed in the woodchuck. WHV infection of neonate woodchucks led to the development of a high carrier rate. The rate of chronic outcome depends on age at time of infection, viral strain and viral titre of inoculum. WHV strains W8 and W7 are closely related and differ from each other by a few positions on their genomes.[91] They have identical precore and core proteins, identical small and middle surface proteins, and there are only two amino acid substitutions in each of the X-protein and pre-S1 region of the envelope protein and nine amino acid substitutions in the polymerase protein. Inoculation of neonatal woodchucks with strain W8 resulted in 11 resolved infections and four chronic carriers out of 15 infected woodchuck neonates.[12] In a second experiment, neonates were infected with strain W7, which consistently produces higher rates of chronicity. Inoculation of 19 neonatal woodchucks with strain W7 produced 19 chronic carriers. The reasons why the strains produce differing chronicity rates are not known. Infection of neonates and subsequent monitoring of cellular immune response may provide information as to whether animals that developed chronic disease show initial lack of immune response or weak immune response as compared with animals which resolve WHV infection. Wang *et al*.[14] found that woodchucks which resolved infection had lower titres of WHV DNA in serum than animals developing chronic infection (Fig. 13.3). Woodchuck with acute resolving infections had a robust acute phase T-cell response against WHV antigens, to WHcAg (100%), WHsAg (82%) and WHX protein (91%) (Table 13.3). They recognize at least four epitopes in three distinct regions of WHcAg, including the protective epitope C 97 to 110.[12] T-helper response in neonates was seen significantly later than in experimentally infected adult animals. This finding may be explained by the different route of infection. Neonates were infected subcutaneously with $10^5$ particles from adults which were infected intravenously with $10^{10}$ particles. The chronicity may be interpreted as an outcome of neonatal WHV infection which resulted from a complete or partial deficiency of primary T-cell response to WHV proteins; 56% of carriers appeared to be unresponsive altogether to WHV antigens during the early stage of acute WHV infection. This indicates unequivocally that acute phase T-cell response is required for resolution of acute infection. Failure to respond to specific viral proteins was not due to some general unresponsiveness of PBMC, as PBMC samples from such carriers responded normally to mitogens in a similar manner to woodchucks in which infection was resolved or uninfected control woodchucks. Suppressing T-helper cells by cyclosporin during the incubation period of WHV infection resulted in a high WHV carrier rate.[92,93] This underlines the importance of functional T-helper cells for appropriate immune response.

The lack of significant T-cell responses in carriers could be the result of an early induction of tolerance. Possible mechanisms for early tolerance may include complete or partial deletion of virus-specific T-helper cells, partial deletion of such cells to below the level of detection in the *in vitro* proliferation assay, clonal energy, clonal exhaustion due to rapid increase in viral load or an abnormally high level of T-cell apoptosis. Absent or incomplete early T-cell response was associated with increasing viral load and with the onset of chronic infection (Fig. 13.3). It is remarkable that a WHcAg-derived peptide C 97–110 was recognized in all animals with acute-resolving infection but not by animals developing chronic infection, indicating that the T-cell response to this epitope is relevant for clearance of viral infection. In the animals that became carriers, only 17% recognized peptides C 97–110, whereas 100% of animals with resolved infection showed a T-cell response in 100% (Fig. 13.3). In humans only a small number of patients could be systematically analyzed for T-helper response in the incubation pe-

riod. However, these data indicate that patients who developed a chronic infection also have an impaired primary T-helper response.

## Development of prophylactic and therapeutic vaccines in the woodchuck model

Many efforts have been made to improve the current vaccine against hepatitis B with respect to the enlarging humoral and cellular immune response to reduce the number of revaccinations required for low and non-responders and expand time intervals for revaccination. HBV vaccines that are used in humans are subunit vaccines consisting of 22-nm empty subviral particles composed of the envelope proteins of HBV. Despite the existence of a safe and efficient vaccine to prevent infection with HBV in humans, DNA-based vaccines have been evaluated and might be used for preventive or therapeutic vaccination in the future.

The need for new types of prophylactic vaccines against HBV is not only a priority in the developed countries, but efforts are also being made to improve health care in other less developed countries. In contrast, therapeutic vaccines against chronic HBV infection are of general interest, as regards both the basic science and clinical use. Through the progress on the characterization of the woodchuck immune system and the development of specific immunological assays, the woodchuck model became an informative animal model for vaccine development. Among the new types of vaccines, genetic vaccines based on purified plasmid DNA provide a series of new features in contrast to classical protein vaccines and seem to be the most promising candidates for future development.[94,95] The woodchuck is an excellent model in which to assess prototype prophylactic or therapeutic vaccines, as efficacy can be tested by challenge experiments or viral elimination in chronic carriers, respectively.

### DNA vaccines against WHV infection

DNA immunization is a powerful method to induce protective immune responses to viral infection, particularly with the option to induce cellular immune responses.[94,95]Plasmids expressing HBsAg or HBcAg are able to induce specific antibody and CTL responses to HBsAg or HBcAg in mice, respectively.[96–98] In addition, results of DNA immunization in chimpanzee and duck models have demonstrated that DNA vaccines prime the specific immune responses and lead to control of HBV infection.[99,100]

In woodchucks, DNA vaccines are able to prime the immune response to WHcAg and WHsAg and confer protection against WHV challenge.[30] Similar to immunizations with HBcAg and HBsAg, the intramuscular injection of plasmids expressing WHcAg and WHsAg (pWHcIm and pWHsIm) induced specific anti-WHcAg and anti-WHsAg antibodies in mice, respectively, with the dominance of an IgG2a subtype. However, despite the high immunogenicity of WHcAg, 1 mg of plasmids expressing WHcAg were necessary to induce a low, transient anti-WHcAg antibody response in woodchucks. After three immunizations, woodchucks developed specific humoral and cellular immune responses to WHcAg, but still at a low level. Woodchucks that received three immunizations with 1 mg of pWHcIm remained negative for markers of WHV replication after virus challenge, whereas unimmunized or pcDNA3-immunized woodchucks developed acute WHV infection. Anti-WHsAg antibodies became detectable in week 5 after challenge. Apparently, the WHV replication was efficiently suppressed by DNA-primed immune responses. This could be interpreted in such a way that the immune responses to WHcAg did not prevent the infection of hepatocytes by input viruses but limited the virus spread. Thus, woodchucks became positive for anti-WHsAg in response to WHsAg produced by infected hepatocytes. Similarly, woodchucks that received immunizations with 1 mg pWHsIm per injection did not develop a measurable anti-WHsAg response but were protected against the subsequent virus challenge. An anamnestic anti-WHsAg response developed in week 2 after virus challenge, while no significant anti-WHcAg antibody response was measured in these woodchucks.[30] Altogether, immunization with plasmids expressing WHcAg and WHsAg induced protective immune response in woodchucks.

A disadvantage of DNA vaccination is the need for large amounts of plasmid DNA to induce adequate immune responses in humans or large animals. Thus, we explored the option of using IFN-γ as molecular adjuvant and evaluated the potential of a single, low-dose DNA vaccination by co-delivery of the expression vectors pWHcIm for WHcAg and pWIFN-γ for woodchuck IFN-γ.[101] Animals were immunized with 10 μg pWHcIm only, or with pWHcIm and pWIFN-γ using a gene gun. These experiments demonstrated clearly that only the animals vaccinated with pWHcIm combined with pWIFN-γ developed a significant proliferative response against WHcAg and were protected against virus challenge. Two out of three animals vaccinated with pWHcIm only were negative for WHV DNA by dot-blot analysis, but positive by PCR after challenge, indicating that a single-shot intradermal immunization using 10 μg of pWHcIm reduced viraemia but did not protect all animals. In a similar experiment, IL-12, a potent inducer of IFN-γ-production, has been shown to enhance the protective immunity induced by DNA vaccine.[29] All vaccine

**Table 13.4** Vaccine studies in the woodchuck with protein and DNA vaccines

| Vaccine | Animals | Application | Challenge | Protection rate | References |
|---|---|---|---|---|---|
| Protein vaccine | | | | | |
| WHV core | 2 | Intramuscular | 20µ $10^8$ | 2/2 | Roos *et al.* (1989)[102] |
| WHV core | 7 | Intramuscular | $10^6/10^5$ | 7/7 | Schödel *et al.* (1993),[103] Menne *et al.* (1997)[10] |
| WHV core live recombinant *S. typhymurium* X4064P | 3 | Intraperitoneal | | 1/3 | Schödel *et al.* (1993)[103] |
| HBV core | 6 | Intramuscular | $10^6$ | 4/6 | Schödel *et al.* (1993)[103] |
| Peptide 97-110 of WHV core | 2 | Intramuscular | | 2/2 | Menne *et al.* (1997)[10] |
| DNA vaccine | | | | | |
| WhsAg | | Intramuscular | | | Lu *et al.* (1999)[30] |
| WHV core | 3 | Intramuscular | | 3/3 | Lu *et al.* (1999)[30] |
| WHV core | 3 | Gene gun | $10^6$ | 0/3 | Siegel *et al.* (2001),[101] |
| | 4 | Gene gun | $10^{10}$ | 1/4 | Garcia-Navarro *et al.* ( 2001)[29] |
| WHV core + IFN-γ | 3 | Gene gun | $10^6$ | 3/3 | Siegel *et al.* (2001)[101] |
| WHV core + IL-12 | 5 | Gene gun | $10^{10}$ | 4/5 | Garcia-Navarro *et al.* ( 2001)[29] |

studies to prevent WHV infection are summarized in Table 13.4.[10,29,30,102,103]

An interesting approach is to fuse bioactive domains like L-selectin and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) to an antigen. Such fusion antigens may be expressed *in vivo* and directed to the immune cells by the specific bioactive domain, and therefore possess a great potential to induce and modulate antigen-specific immune responses. In a new study, this approach was tested for the immunomodulation against hepadnaviral infection in the woodchuck model (Lu *et al.*, unpublished observations). Plasmids expressing the nucleocapsid protein (WHcAg) and e antigen (WHeAg) of WHV only, or in fusion to the extracellular domain of the woodchuck CTLA-4 and CD28, were constructed. Their ability to induce a specific immune response to WHcAg was tested by immunization of mice. While immunization with plasmids expressing WHeAg or WHcAg only led to a specific antibody response with IgG2a as the dominant IgG subtype, fusions of WHcAg to the woodchuck CTLA-4 and CD28 induced an antibody response with both IgG1 and IgG2a at comparable levels. Furthermore, the specific IgG1 to WHcAg/WHeAg was developed after just a single immunization with the CTLA-4-WHcAg fusion antigen. Moreover, woodchucks were immunized with plasmids expressing WHeAg or CTLA-4-WHcAg fusion antigen and then challenged with WHV. Results of these experiments indicate that the protective efficacy of this plasmid vaccination was demonstrated.

DNA vaccines against hepatitis D virus were tested in chronically WHV-infected woodchucks. However, immunizations with HDV p24 antigen did not protect the woodchuck against virus challenge.[70,75]

## Therapeutic vaccination against chronic hepatitis B in the woodchuck model

To date, several attempts to stimulate the specific immune response to HBV to reach control over HBV in chronic carriers have been undertaken but showed only limited success.[104–108] Woodchucks with chronic WHV infection provide excellent opportunities to test the effectiveness of new options for therapeutic vaccinations (see Table 13.5).[49,50,58,109]

**Table 13.5** Therapeutic vaccinations

| Vaccines | Application | Outcome | Reference |
|---|---|---|---|
| WHsAg and Th-peptide | Intramuscular | Transient anti-WHs antibody response<br>Two woodchucks died | Hervas-Stubbs *et al.* (1997)[109] |
| WHsAg in adjuvant | Intramuscular | Antibodies to the pre-S region of WHsAg | Lu *et al.* (2003)[49] |
| WHsAg in combination with L-FMAU | Intramuscular | Stimulation of T-cell responses to WHV proteins, anti-WHs antibody response | Menne *et al.* (2002)[50] |
| WHsAg-anti-WHs immune complex and DNA vaccines in combination with lamivudine | Intramuscular | Stimulation of anti-WHs antibody and suppression of WHV titre | Lu (unpublished results) |
| WHcAg | Intramuscular | Viral elimination in one of six animals | Roggendorf and Tolle (1995)[58] |

No antiviral effects were achieved in chronically WHV-infected woodchucks during the early immunization trials with WHcAg only or in combination with famciclovir.[58] In subsequent experiments, a T-helper cell determinant peptide FISEAIIHVLHSR encompassing amino acids 106–118 from sperm whale myoglobin (termed FIS) was added to the vaccine preparation, in an attempt to circumvent the T-cell unresponsiveness to WHV proteins.[47,109] This approach was promising, as a combined immunization with HBsAg and FIS induced anti-HBsAg in SJL/J mice, a non-responder strain to immunization with HBsAg.[109] Indeed, PBMCs from FIS-immunized woodchucks produced IL-2 upon restimulation with FIS *in vitro* IL-2. Seven chronic WHV carrier woodchucks immunized with WHsAg in combination with FIS produced anti-WHsAg.[109] However, two woodchucks developed high titres of anti-WHsAg and suffered severe liver damage. Thus, foreign T-helper cell determinant peptides might provide help to overcome the deficiency of specific T-cell responses to viral proteins in chronic carriers.

In another immunization experiment, we treated a group of chronically WHV-infected woodchucks with plasma-derived WHsAg adsorbed to aluminium salt with monophosphoryl lipid A.[49] Anti-WHsAg antibodies were detected in all immunized woodchucks and persisted for a time period of up to 2 years after immunization. The major portion of anti-WHsAg antibodies was directed to the WHV pre-S region. However, neither WHV DNA nor WHsAg titres in immunized woodchucks changed significantly. Sequence analysis of the WHV pre-S and S genes of WHV isolates from these woodchucks showed that no WHV mutants emerged after the induction of anti-WHsAg/anti-WHpreS antibodies. These results indicate that immunization with WHsAg could partially induce specific B-cell responses to WHV proteins in chronically WHV-infected woodchucks. However, additional components for the stimulation of T-cell responses are necessary to achieve therapeutic effects against chronic hepatitis B.

The high replication level of hepadnaviruses in chronic carriers may maintain the immunotolerance to viral proteins. The T-cell response to HBV was successfully restored in patients treated with lamivudine or IFN-α. Thus, a reduction of the viral load by antiviral treatments may enhance the effect of therapeutic vaccines. Recently, Menne *et al*. reported that a combination of an antiviral treatment and immunization with WHsAg induces WHV-specific lymphoproliferation in chronic carriers.[110] For the antiviral treatment against HBV, a potent drug (L-FMAU) was applied for 8 months and led to a dramatic decrease of viraemia. Although subsequent immunization with WHsAg led to lymphoproliferative responses to WHV proteins in both treated and untreated woodchucks, the antiviral treatment led to a broader spectrum of specific T-cell responses to different WHV proteins core and X-proteins. Immunization with WHsAg consistently induced a low level of anti-WHsAg in chronic carrier woodchuck. This finding provides hints as to the usefulness of a combination of antiviral treatment and immunizations.

A novel prototype therapeutic vaccine was developed by Wen and co-workers.[108] Therapeutic vaccination using an antigen–antibody complex has been successfully tested in transgenic mice and is now under clinic phase I evaluation. In the woodchuck model, a combination of antiviral treatment with lamivudine and therapeutic vaccination with DNA vaccines or antigen–antibody complexes was carried out to evaluate their efficacy (Lu *et al*., unpublished observations). Ten woodchucks chronically infected with WHV were treated with 15 mg of lamivudine per day for 4 months. Six weeks after the start of lamivudine treatment, one group of woodchucks was immunized with pWHsIm, a plasmid expressing WHsAg, and a second group with WHsAg-anti-WHsAg complex and pWHsIm. Two woodchucks treated with lamivudine only served as controls. Blood samples were taken every 2 weeks and tested for WHV DNA, anti-WHsAg and lymphoproliferative responses to WHV antigens. The treatment with lamivudine led to a marginal decrease of WHV DNA concentrations in woodchucks. Immunization with pWHsIm did not lead to any change in the carrier status in woodchucks. Interestingly, three woodchucks immunized with WHsAg–anti-WHs complexes and pWHsIm developed anti-WHs antibodies and showed a further decrease of serum WHV DNA and WHsAg concentrations. The anti-WHs antibody persisted in two woodchucks for a period of 8 weeks. These results indicate that immunization with antigen–antibody complexes is an effective treatment against chronic HBV infection.

## What we learnt from the woodchuck model

In recent years, the immune control of hepadnaviral infection has been studied intensively in animal models.[7,82,86,87] Particularly, the HBV-specific immune T-cell response was analyzed in the transgenic mouse model.[80,81] In addition, immune transfer and immunization experiments in the mouse model provided interesting results, indicating the important role of antiviral cytokines IFN-γ and TNF-α as major mediators of the antiviral action of specific CTLs. Several findings in the animal models support the direct antiviral activity of cytokines, as follows. (1) In experimentally infected chimpanzees and woodchucks, decline of replication of WHV/HBV has been observed prior to cell destruction as determined by elevated transaminases. (2) There is a direct correlation of intrahepatic expression of increased levels of

IFN-γ and reduction of viral replication. (3) In the transgenic mouse model, antibodies to IFN-γ can completely abolish reduction of replication of HBV DNA. (4) Elimination of IFN-γ producing the specific CTLs by an anti-CD8 antibody is correlated to persistent replication until CD8+ cells reappear at later time-points. These findings led to the hypothesis that cytokines suppress viral replication as a first step of viral clearance, and on the other hand chronic HBV/WHV infection may be caused by a low expression of cytokines like IFN-γ. This hypothesis raises the question as to whether direct application or *in vivo* induction of cytokines could actually be used for the therapy of chronic HBV infection.

Our experiments in the woodchuck model with chronic virus infections revealed some aspects that had not yet been considered in the interpretations of the results from the transgenic mouse model.

It is not surprising that inflammatory cytokines including IFN-γ are expressed in liver tissues of chronically WHV-infected woodchucks. A number of IFN-stimulated genes were shown to be induced in liver tissues. The expression of IFN-γ expressed in this context is, however, not sufficient to terminate WHV infection. It remains to be investigated whether the IFN-γ expression contributes at all to limitation of WHV replication during the chronic course of WHV infection.

The results of therapeutic use of the cytokine wIFN-γ in the woodchuck model are rather disappointing. wIFN-γ was applied to WHV-infected primary woodchuck hepatocytes, but showed no significant antiviral effect even at cytotoxic concentrations, and no significant suppression of WHV replication was achieved by *in vivo* application by adenoviral vector-mediated expression in the liver. Nevertheless, wIFN-γ has been clearly demonstrated to be biologically active in terms of induction of interferon sensitive gene (ISG) expression in primary hepatocytes and *in vivo*. Taken together, these findings raise the question as to whether an established WHV infection renders hepatocytes strongly resistant to wIFN-γ, indicating that chronic hepadnavirus infection is a result of viral resistance to cytokines or could be due to the changed cellular responsiveness to cytokines.

In the therapeutic approaches, interesting results were generated in the woodchuck model. It became clear that the induction of antibodies to WHsAg is possible in chronically infected individuals. In particular, immunization with immune complexes appears to be an effective method to stimulate antibody responses with antiviral action. These exciting findings raise the hope of developing a useful protocol of immunotherapy for chronic HBV infection.

Thus, future investigations will have the major advantages of the woodchuck model as an authentic model of infection. No other animal model is available that mimics the chronic course of hepadnaviral infection and presents the features in pathogenesis and virus–host interaction in a satisfactory way, as compared with the woodchuck model.

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# Chapter 14
# Hepatitis B surface antigen (HBsAg) variants

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Viruses are under constant selection pressure and as a consequence undergo continual sequence variation (Fig. 14.1). This process has had a number of effects on many genes of the hepatitis B virus (HBV), described here and in Chapter 15. Some of these are clinically relevant.

The anti-HBs (antibody to HBsAg) response following natural infection or after immunization comprises mainly antibodies that recognize the major hydrophilic region (MHR) of the protein. This comprises amino acids (aa) 99–160 that encompass the group-specific 'a' determinant, an epitope recognized by a variety of antibodies. Isolated cases of infection with HBV variants bearing substitutions in this region, which are predicted to escape neutralization (as there is no *in vitro* or *in vivo* model, it is impossible to be certain) by antibody, have been described. There have also been cases of infection that have been missed because of failure of current serological assays to detect some variant forms of HBsAg. Variants that escape neutralization by monoclonal and polyclonal anti-HBs have been selected during immunoprophylaxis against HBV during liver transplantation and in vaccinees receiving passive/active immunization after birth to HBV-infected mothers. Moreover, in recent years, variants resistant to nucleos(t)ide analogues as a result of mutations in the polymerase encoding gene have been described. Such variants may affect the antigenicity of the S gene that encodes HBsAg, as it is overlapped completely by the polymerase open reading frame (ORF).

Here, we describe HBs antigenic variation, possible geographical differences and variation under human-induced selection pressures and its relevance to diagnosis, vaccination and treatment.

## The surface gene and subtypes

A single ORF that occupies more than one-third of the HBV genome encodes the three HBsAg-containing polypeptides. These proteins are highly conserved at the amino acid level, with regions of variability.[1] The

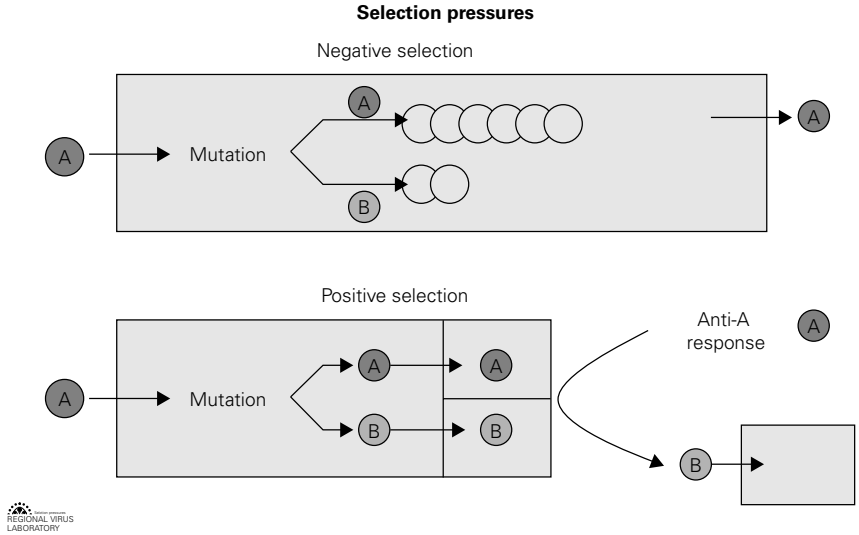


**Figure 14.1** In the top diagram, a virus A mutates randomly to virus B, but virus A out-competes virus B and therefore virus B disappears. In the bottom diagram, viruses A and B replicate equally efficiently, and so go on to infect further cells independently. However, the immune response against virus A (which was pre-existing) destroys cells containing virus A (or viruses derived from cell A are neutralized by the humoral response) so virus B predominates.

domain within pre-S1 (aa 21–47) which is involved in binding to the hepatocyte[2] is highly conserved (see Chapter 10). Based on antibody-peptide binding and chimpanzee inoculation experiments as well as *in vitro* mutagenesis,[3,4] a model was proposed[5] of the antigenic region of the small envelope protein that contained two loops formed by disulfide bridges between cysteines 124/137 and 139/147.[6,7] It is well-documented that the antigenicity of HBsAg is dependent on tertiary structure. The affinity of an anti-HBs-positive human serum for the linear peptide from aa 139 to 147 is higher than for the peptide from aa 124 to 137.[6] If the former peptide is cyclized between the terminal cysteines, affinity is increased.[6] This region, particularly between aa 139 and 147, is highly conserved (Fig. 14.2). Studies using phage epitope libraries[8] indicate that only the second loop is likely to be real, and that the first loop is incorporated within a larger loop from aa 107 to 138. There may also be a small loop between cysteines 121 and 124[8,9] (Fig. 14.3). The importance of the highly conserved cysteines in the S protein to secretion and antigenicity has been discussed elsewhere.[10,11]

Before the sequence of the HBV genome became available, a number of antigenic determinants in HBsAg had been identified. The *a* determinant is common to all strains, but its limits are poorly defined. In the latter case, studies have been based on the use of overlapping peptides, in the absence of *in vitro* or *in vivo* models of neutralization. All subtypes of HBsAg contain the common *a* determinant and are defined using appropriate monospecific antisera (monoclonal antibodies are also available). Geographical variability has most likely come about by population pressure over the millennia, probably under the influence of major histocompatibility complex (MHC) antigen type and other immunogenetic factors (Basuni, unpublished observations).[12] This is further discussed below. There are two pairs of mutually exclusive subtype determinants, *d* and *y*, and *w* and *r*.[13] There are nine subtypes (because there are other sub-subtypic determinants), but the major ones number four (*adw*, *ayw*, *adr*, *ayr*). Rare examples of unusual specificity, such as *adwr* or *adyr*, may be the result of mixed infections. Although there are clear differences in the geographical distribution of the subtypes,[14] there is no obvious correlation between subtype and virulence. The presence of lysine or arginine at aa position 122 confers *d*/*y* specificity: *adw* and *adr* subtypes have lysine at this position, whereas *ayw* and *ayr* have arginine (Fig. 14.2).

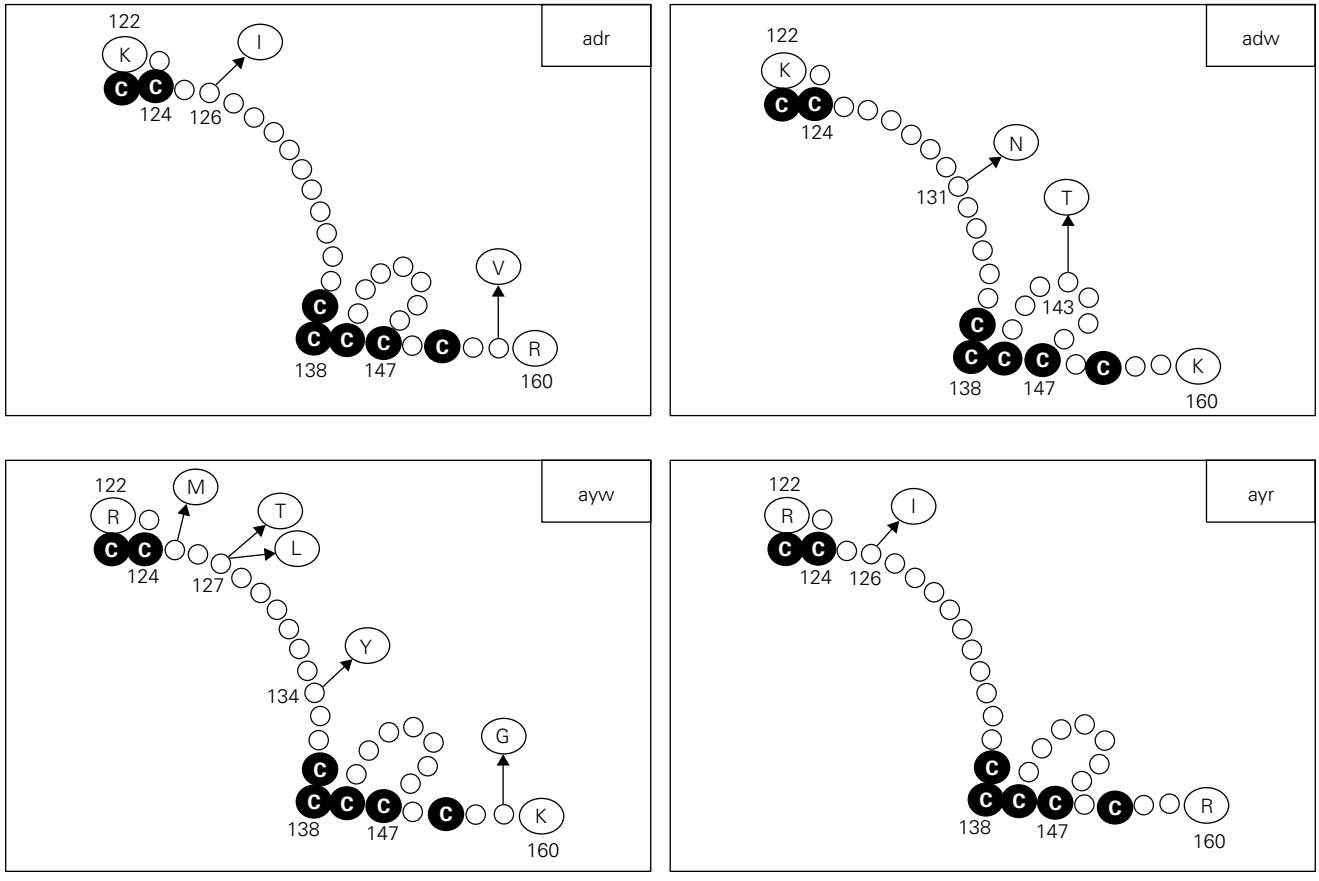


**Figure 14.2** Variability within the four standard subtypes. The two loops of the new model are shown in an expanded format. Well-described variability, which distinguishes each subtype, is indicated. Note that the first loop is less variable than the second.

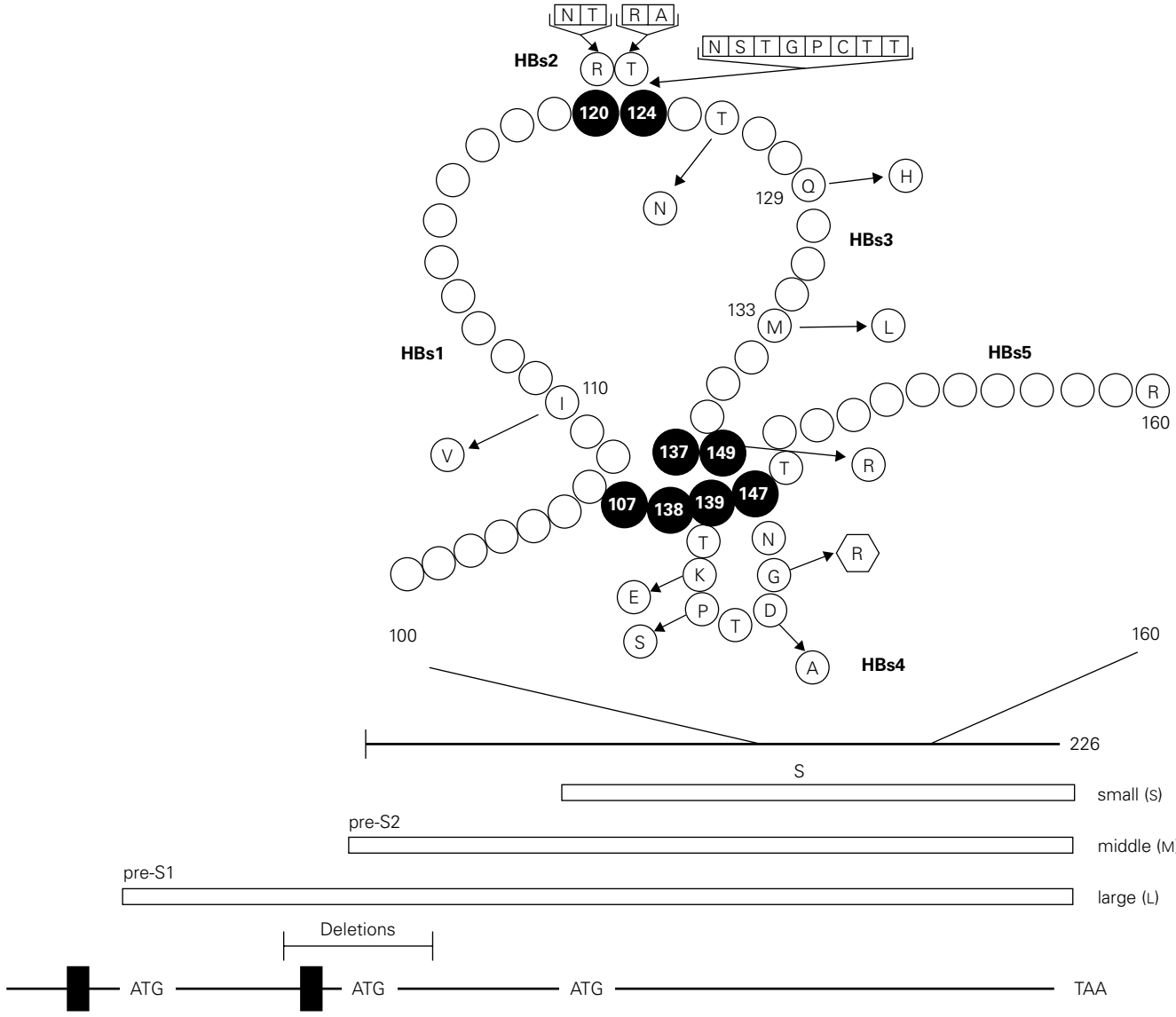


**Figure 14.3** Surface gene antigenecity: variants associated with vaccination, and insertion variants. There are three ATG (initiation codons) and one TAA (stop codon). Thus, three envelope proteins can be translated: L (large), M (middle), and small (S) or HBsAg. The region between aa 100 and 160 (or thereabouts) is termed the major hydrophilic region (MHR); this is found on LS, M and L proteins. Deletions of pre-S1 (between the first and second ATG), which often overlap the pre-S2 (beginning of M protein) ATG lead to biological effects (see text). Two major loops and one minor loop are proposed in the MHR, defined by multiple potential disulfide bridges, between aa 107/137 (or 138), 139/147 (or 149), and 121/124. Reseach workers have suggested that there may be five antigenic regions termed HBs1-5. Vaccination-associated variants are shown in circles. Note that sG145R is by far the commonest, seen in all studies, and that most are clustered into HBs4. Insertions in HBsAg have only been seen in Far East patients and are all between aa 121 and 124.

Similarly, residue 160 seems to be central to *w/r* specificity: *adw* and *ayw* have lysine at this position, whereas *adr* and *ayr* have arginine (Fig. 14.2). In addition, the residues at positions 113 and 134[3] and those at residues 144 and 145[15] constitute subdeterminants of *d/y* specificity. Codon 126 also correlates with *w/r* specificity: *w* isolates have threonine and *r* isolates have isoleucine at this position (Fig. 14.2). Minor subtype specificities have also been defined that correlate with sequence.[15] Mutations at aa positions 120, 143, 144, and 145 can result in poor reactivity with *d/y* subtyping monoclonal antibodies.[15,17] As this specificity is defined by the residue at aa 122, this indicates the discontinuous nature of many of the epitopes in this antigenic region. There are linear and discontinuous B-cell epitopes, and some regions are part of more than one epitope. Subtype-related variation can

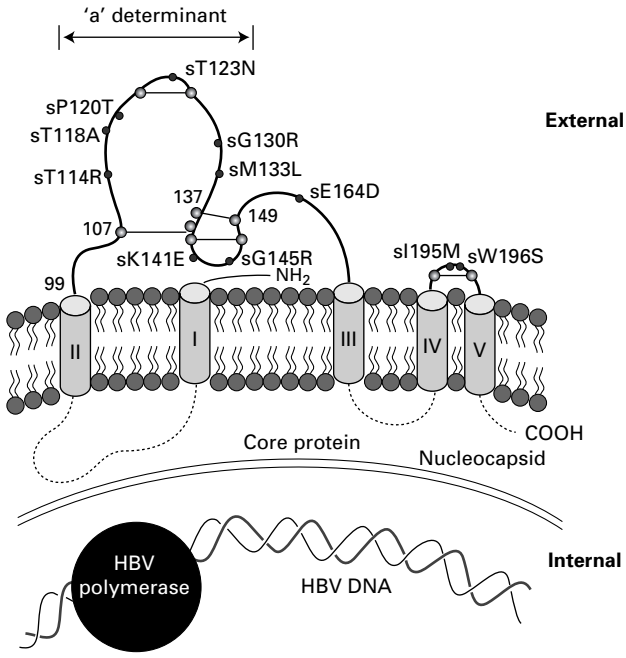


**Figure 14.4** Topology of the major hydrophilic region (MHR) of HBsAg, in relation to the lipid bilayer forming the envelope of the HBV virion. Cysteine residues are shown as shaded circles and disulfide bridges are indicated as thick lines. Alpha-helices traversing the lipid bilayer of the envelope are shown as cylindrical structures. Taken from Torresi.[76]

alter binding of antibodies to regions linearly distant. Because of this interleaved and complex nature of the antigenicity of HBsAg, we will not refer to the *a* determinant herein, but will consider the MHR in its entirety.

Of course, antigenia subtypes have become less important with the advent of cheap large-scale sequencing. Genotypes are now the primary classification of HBV variability. Some subtypes can be found within more than one genotype (Fig. 14.5). Genotypes of HBV are discussed in more detail in many reviews.

## Geographical and ethnic variability

There are a number of viral systems in which the geographical location (and thus ethnic background) of the host has been shown to be linked with specific subtypes or strains of an infectious agent. Human immunodeficiency virus (HIV), hepatitis C virus (HCV), human T-cell lymphoma/leukaemia virus (HTLV) and mumps are all examples. Recent work has expanded on this principle in HBV by examining the inter-relationship between south-east Asian and Pacific Islander sequences. As can be seen in Plate 14.1 (found between p.786–7), the island of Kiribati has predominantly genotype D (of unknown origin), whereas the rest of the Pacific is genotype C. The strains of D that appear on these other islands are related to those in Kiribati. The genotype C sequences in the Pacific are clearly discrete compared with the rest of Asia (Plate 14.2, found between p.786–7). Indeed, they are more diverse as well, implying that genotype C may have arisen in the Pacific and then migrated back to south-east Asian countries. Within the Pacific islands, there are distinct motifs that define the local strain (Plate 14.2 [found between p.786–7] and Table 14.1). It is likely that this represents human migration patterns. Each movement of people from island to island (as tribes moved on in search of better habitats) took with it a distinct strain. Vanuatu, which is relatively isolated, has an almost homogeneous and specific strain of virus.[18]

It is also possible to define genotype and subtype-defining motifs for the core gene (Table 14.2), although the distinction is less marked because of the reduced variability (and therefore options for change) in this gene.

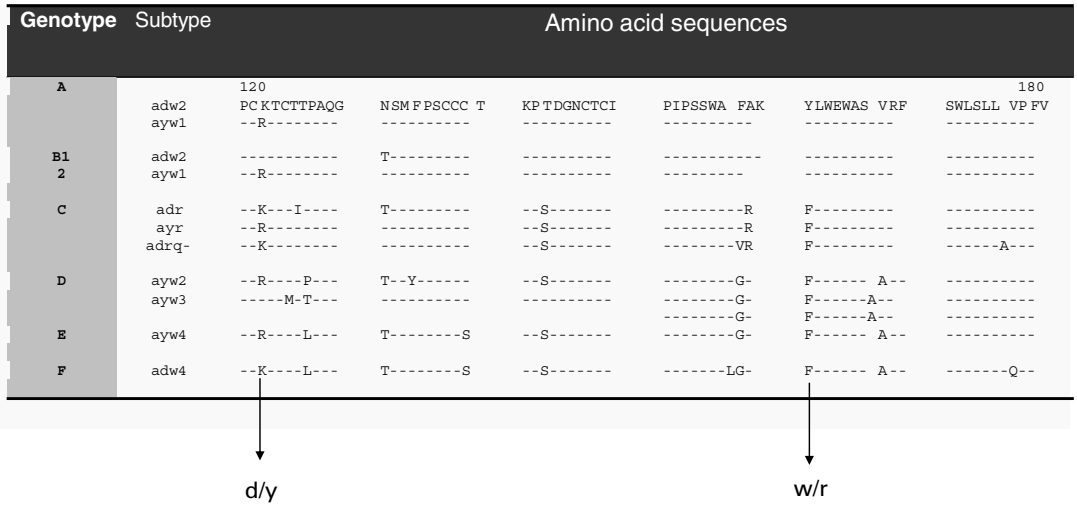


**Figure 14.5** HBV genotypes A–F and allotted subtypes based on S gene variability between amino acid residues 120–180.

**Table 14.1** Surface gene, amino acid and nucleotide differences between prevalent strains in Fiji, Tonga and Vanuatu.

| Amino acid position | Number with each variation | | |
|---|---|---|---|
| 44–47 aa pair | Vanuatu | Fiji | Tonga |
| G/V | 13 | 3 | Nil |
| E/R | 5 | 12 | 8 |
| E/V | 2 | 5 | 12 |
| **Nucleotide position** | **Number with each variation** | | |
| 13 A>G | 1 | 2 | 10 |
| 203 T>C | 2 | 3 | 12 |
| 213 C>T (S) | 12 | 2 | Nil |
| 246 C>A (S) | 4 | 1 | Nil |
| 282 G>A (S) | 1 | 3 | 11 |
| 339 A>C (S) | 3 | Nil | 13 |
| 348 C>A (S) | 1 | 3 | 11 |
| 384 T>A (S) | 13 | 2 | Nil |
| 524 T>C | 14 | 2 | 2 |
| 551 T>C | 1 | 1 | 12 |
| 552A >C (S) | 1 | 3 | 10 |
| 594 G>T or A | 5 (T) | Nil | 1 (A) |

Of especial interest (Table 14.3) is that one can define an amino acid motif which seems to prevail in either Asian or Caucasian peoples, irrespective of the genotype infecting the patient. If confirmed, this would imply that there is extremely strong selection pressure on HBV based on the ethnic background of the person, overriding the 'push' for diversity by the virus. This would most likely be immune-based.

It is of interest to look at sequences in the Asian population of the west coast of the USA. The sequences observed are highly related to the ethnic origin of the person (e.g. China, Singapore, etc.) rather than being distinctive to the USA. This would be in keeping with recent migration patterns and supports the observations on Pacific Islands which represent past migrations. It has become possible to distinguish with some accuracy the origin of a virus which may aid forensic investigation (e.g. outbreak tracing), and would also help to inform public health authorities and vaccination policy on the origins of viruses that are causing chronic hepatitis. For example, in many Western European countries, imported HBV poses a much greater threat than locally acquired infections: this can readily be studied using data to identify the origin of the isolate.

**Table 14.2** The most frequent differences in HBcAg amino acid sequences between genotypes and antigenic subtypes in B and T cell epitopes.

| | | Variant amino acid positions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CD8 | CD4 | | B cell | | CD8 (CTL) | | | |
| Subtype | Genotype | 27 | 59 | 67 | 74 | 83 | 87 | 91 | 97 | 116 |
| adw2 | A | V | I | T | N | D | N | T | I | L |
| adw2 | B | I | I | N | S | E | S | V | I | L |
| ayw1 | B | I | I | N | S | E | S | V | I | L |
| adr | C | I | I | N | S | E | S | V | I | L |
| adrq- | C | I | V | N | S | E | S | V/I | I | L |
| ayw2 | D | V | I | T | G/T/V | D | S | T | F | L/I |
| ayw3 | D | V | I | T | G/T/V | D | S | T | F | L/I |

**Table 14.3** Specific amino acid motifs in HBcAg found in south-east Asian and Caucasian populations

| | | Amino acid variant position | | | | | |
|---|---|---|---|---|---|---|---|
| Ethnicity | Subtype/genotype | 12 | 27 | 67 | 74 | 83 | 91 |
| South-east Asian | | | | | | | |
| China | adr/C | S | I | N | S | E | V |
| | adw2/B | S | I | N | S | E | V |
| Korea | adr/C | S | I | N | S | E | V |
| Japan | adr/C | S | I | N | S | E | V |
| Vietnam | ayw/1/B | S | I | N | S | E | V |
| Pacific | adrq-/C | S | I | N | S | E | V |
| Caucasian | | | | | | | |
| USA | adw2/A | T | V | T | N | D | T |
| Italy | adw2/A | T | V | T | N | D | T |
| | ayw2/D | T | V | T | V | D | T |
| Scotland | adw2/A | T | V | T | N | D | T |
| | ayw2/D | T | V | T | V | D | T |

Specific amino acid motifs are found in specific ethnic backgrounds.

## Variants associated with active immunization

The emergence of variants of HBV can be due to selection pressure associated with extensive immunization in endemic areas,[5,19–25] but at present there is little evidence of this on a population-wide basis. Amino acid substitution within the MHR can allow replication of HBV in vaccinated persons, as much of the antibody induced by current vaccines does not recognize critical changes in this surface antigen domain.[26] These variants are summarized in Fig. 14.3 and Table 14.4. The first published study is instructive. Responses to the plasma-derived vaccine were studied in 1590 subjects, mostly infants from two regions of southern Italy, where the prevalence of HBsAg is >5%.[24] A total of 44 of the vaccinees became HBsAg-positive, 32 with additional markers of HBV replication in the presence of anti-HBs. Sera from several of these cases were investigated further by polymerase chain reaction (PCR) sequencing of the region of the MHR.[5] One case was a child of an HBeAg-positive mother who was given hepatitis B hyperimmune globulin at birth and at 1 month and a course of vaccine at 3, 4 and 9 months. Despite immunoprophylaxis, the child became chronically infected with HBV. HBsAg present at 11 months and at 5 years (when anti-HBs was no longer detectable) showed reduced reactivity with a panel of monoclonal antibodies known to bind to the MHR, although binding to HBsAg from the mother was normal with two of the monoclonal antibodies and only slightly reduced with a third. The sample taken from the child at 5 years had sG145R. The mother had sG145.

Additional studies of the prevention of mother–infant spread using hyperimmune globulin and vaccine showed similar results (Table 14.5). sG145R has been described from Singapore, Japan, United States, Venezuela, India, China and elsewhere.[22,27–34] The mutant is not generally found in the virus population of the mother, but in a US study, it was present as a small minority component in a couple of cases. If it is present, the sG145 virus in the inoculum from the mother may have been neutralized by the hyperimmune globulin, leaving the variant to become established. Recent studies in the United States indicate that 0.8% of infants born to HBV-infected mothers develop persistent infection with the sG145R variant in spite of adequate passive/active vaccination. Because neonatal transmission is responsible for 75% of HBV transmission worldwide, this could result in a significant reservoir of HBV variant infection. While the vertical transmission of HBsAg mutants (in particular sR145) has been observed from mothers to their vaccinated infants, few horizontal transmissions have been reported so far for sG145R[35] and D144A.[36]

Other mutations have been noted in vaccinated children. Amino acid 144 replacement is frequently encountered with or without G145R.[17,37–39] In two siblings from Japan, sT/I126N was selected,[32] but unlike the sG145R cases, this was also seen in the majority (12 of 17) of clones in the mother. The grandmother had a mixture of strains at aa 126 (although no s126N), indicating a slow selection process over three generations. Apart from vaccinated individuals, HBsAg variants have also been reported in blood donors (positive for anti-HBc alone) and after interferon (IFN) therapy.[40] In no case has horizontal transmission of a vaccine-associated variant to a vaccinee been documented, although this must be regarded as a potential problem, as anti-HBs-positive vaccinees may be susceptible to infection. In the ongoing Gambian study, emergence of sK141E has been seen in children from two villages that were infected horizontally.[41] It is not known whether this strain has become predominant or whether, with a higher rate of vaccina-

**Table 14.4** The most frequent medically selected amino acid changes in HBsAg.

| Amino acid position | Wild-type | Mutant | Cause |
|---|---|---|---|
| 120 | P | E-S-T | V-L-HBIG |
| 123 | T | N | HBIG |
| 124 | C | R-Y | mAb-HBIG |
| 126 | I/T | A/N/S | V-HBIG |
| 129 | Q | H/L | V |
| 130 | G | D/R | L-HBIG |
| 133 | M | L | V |
| 134 | F/Y | N/R | HBIG |
| 141 | K | E/I | V-mAb |
| 142 | P | S | V-mAb |
| 144 | D | A/E | V-HBIG |
| 145 | G | R | V-L-HBIG |
| 146 | N | S | V |
| 148 | T | I | V |
| 149 | C | R | V |
| 157 | A | D/R | V-L |
| 158 | F | Y | L |
| 164 | E | D | L-Fam |
| 175 | L | S | L |
| 179 | F | Y | L |
| 181 | Q | H | V |
| 182 | V | * | L |
| 183 | F | C | V |
| 184 | V | A | V |
| 193 | F | S | L |
| 194 | V | A | V |
| 195 | I | M | L |
| 196 | W | S | L |
| 198 | M | I | L |
| 204 | N | S | V |
| 207 | Q | R/S | L |
| 208 | I | T | V |

The causal selection pressure is V, vaccine; L, lamivudine; mAb, monoclonal antibody; HBIG, hepatitis B immune globulin; Fam, famciclovir.

tion coverage, it may infect more of the children in these villages. Horizontal spread of variants to previously vaccinated individuals is less probable than vertical spread to a neonate who receives vaccine simultaneously with exposure. Nevertheless, 1560 health-care workers from Sardinia were studied some years after vaccination;[42] 13 were anti-HBc-positive. Of these, nine were available for further study, four were PCR-positive, and one had a dual variant, sI110V and sC149R. The importance of the former is unknown, but mutation of sC149 is known to have major antigenic effects. In many studies, mutations in vaccinated children were preferentially located in the 'a' determinant,[40,43] in contrast to the more randomly located mutations in unvaccinated children.[44] In general, replacement in the presumed second 'a' determinant loop, including amino acid positions 144 and 145, are more often associated with anti-HBs immune pressure than mutations in other epitopic domains of the MHR.[45] On the other hand, second 'a' determinant loop mutations are unusual in the absence of anti-HBs pressure.[46]

Whether mutants of the HBV 'a' determinant may pose a potential threat to immunization programmes is still controversial. Studies on infected vaccinated children with 'protective' anti-HBs titres reveal some with only mutants in the MHR.[44,47,48] The potential for takeover by variants in vaccinated children has been modelled,[49–51] showing that variants could become the dominant viral strains, even in the epidemiological setting of horizontal transmission (Fig. 14.7). In this model, the rate of emergence of vaccine escape mutants (for example sG145R) was predicted by combining previous models under a baseline parameter set, as estimated for the Gambia.[52] This two-strain, age-structured model also incorporated the strong age dependency in the probability of becoming a carrier of HBV.[52] sG145R is most likely to appear in vaccinated individuals, suggesting that sR145 out-competes sG145 best under the selective advantage of being able to evade vaccine-induced immunity. The model assumed that the fitness of sR145, in the absence of vaccination, as measured by the basic reproductive rate ($R_0$),[53] was lower than that of sG145. If the fitness is identical (and there is no reason to believe otherwise), then the following predictions are minimalist. The predictions of the model were robust to uncertainty in the parameter estimations, with the exception of two parameters, for which there is no reliable information. These are the cross-immunity of the HBV vaccine, c (the reduction in protection provided by the vaccine against sR145 as opposed to sG145), and the mutation term, Q (the prob-

**Table 14.5** Incidence of infection and variants in vaccinated infants born to HBeAg-positive mothers

| Country | Vaccine regimen | Incidence of infection (anti-HBc/HBsAg) | Proportion of variants |
|---|---|---|---|
| UK | S vaccine + hyperimmune globulin | 6% | 12% |
| USA | S vaccine + hyperimmune globulin | 9% | 23% |
| Taiwan | S vaccine + hyperimmune globulin | – | 33% |
| Indonesia | S vaccine | 23% | ND |
| Singapore | S vaccine + hyperimmune globulin | 12% | 39% |

ND, not done.

ability that an infectious contact, between a susceptible and an individual infected with s145G, will produce an infection with s145R). Q is obviously a function of the rate at which sG145R mutations arise during viral replication, the number of replicating viruses in an infectious individual, and the number of virions that constitute an infectious dose.

Figure 14.7 plots the time taken for sR145 to numerically out-compete sG145, at different values of c and Q. A notable feature is the time predicted for the emergence of the mutants. This is because, although highly contagious, HBV is not very infectious compared with measles or influenza. The spread of HBV is dependent on chronic infection generating new infections over a long time, so the incidence of new infections is low and the mutant takes decades to spread through the population. This seems to be the case whatever the values of c or Q. However, just because the change in HBV type is slow does not mean the mutants are unimportant. If they are allowed to attain high prevalence, it will also take several decades to eradicate them, even with cross-reactive vaccines, because of the chronic nature of the infection. Chronic infection of children may go undetected for decades while imposing a risk of liver disease and hepatocellular carcinoma (HCC) and the possibility of onward transmission. It is highly relevant that, of the vaccine escape variants so far described, sR145 and 144 are the most persistent and are not lost in favour of standard strains over time. Thus, they may have the capacity to cause chronic hepatitis B in a vaccinated population. This model does not take account of vertical transmission (which is the most common setting of emergence), or of a baseline prevalence in the unvaccinated population (which has been clearly shown). Thus, these predictions fall at the latest point of a range of time periods. However, there is little evidence at this stage that transmission is occurring horizontally, especially among vaccinated individuals. Further, experiments in chimpanzees reveal that 'standard' vaccine is protective against an sG145R inoculum.[54] Other mutations located beyond the MHR[22,29,42,55–57] also show altered viral antigenicity, but they are rare in studies of vaccinated infants. A correlation between HBV subtypes and emergence of variants has been reported in several studies.[19,58–60] Chen and Oon.[61] described a close association of vaccine-related HBsAg mutants with the *adw* subtype in Singapore compared with subtypes *adr, ayw* and *ayr*.

## Variants associated with passive immunization

Patients with end-stage liver disease attributable to HBV often undergo transplantation and are treated with human monoclonal anti-HBs or hyperimmune globulin to prevent infection of the graft. In some cases, HBsAg again becomes detectable in serum, and a number of substitutions have been observed in this virus compared with pretransplantation virus (Fig. 14.8 and Table 14.4).[45,57,62–68] In one reported series of transplantation patients treated with monoclonal antibody,[69] three of six patients exhibited reactivation of HBV in the graft, and the surface gene of the virus was sequenced. sG145R was seen alone in one and as a mixture with sG145 in a second. Other mutants were also present simultaneously as a quasi-species in this patient: sT140S and sC124Y. In the third patient, sC137Y was observed to emerge. It is interesting that loss of cysteines has also been observed after vaccination (see above) and hyperimmune globulin therapy (see later). There are no natural strains of HBV without these cysteines that are proposed to be involved in the loop structure of the MHR. Whether these

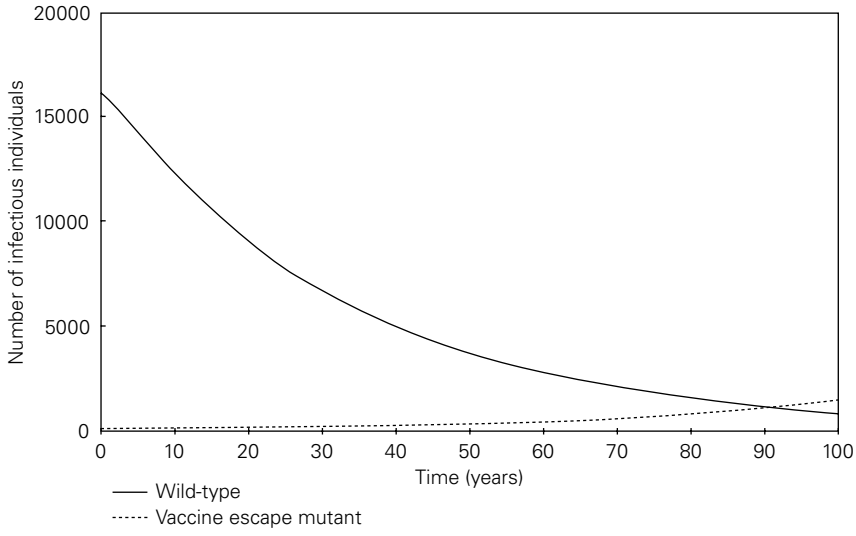


**Figure 14.7** The impact of mass vaccination on the prevalence of standard sequences and vaccine escape variants. The model was run to equilibrium before vaccination and then mass vaccination was simulated with 80% coverage. The number of infectious individuals is plotted on a non-logarithmic scale.

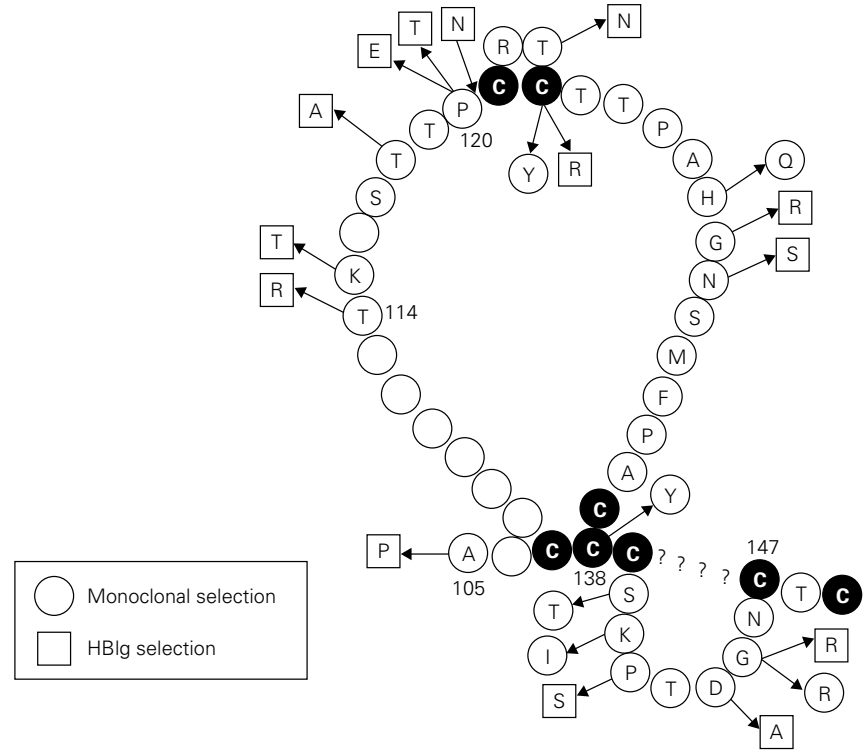


**Figure 14.8** HBsAg mutants selected during monoclonal or polyclonal anti-HBs therapy after liver transplantation. The latest model of the MHR of HBsAg, expanded for clarity, identifies a selection of variants observed in a number of studies. Amino acids in the loops are the 'usual' sequence, and those at the arrowheads are the mutants that emerged.

are independently viable strains was not addressed by this study, and in some cases a premature stop codon was noted in the polymerase ORF.

That monoclonal antibody can select HBsAg mutants is not surprising. However, polyclonal antibody in the form of hyperimmune globulin can also lead to different strains after therapy (Table 14.4).[62,70–73] On the withdrawal of hyperimmune globulin, the mutants are often lost and the wild-type again becomes dominant.[45] Only one study has controlled for immunosuppression in the absence of hyperimmune globulin pressure. sG145R has appeared in four of these five studies, but not in all cases. Hyperimmune globulin does put selection pressure on HBsAg. There is a report on the post-transplant patients who received hyperimmune globulin that mutants persisted despite the withdrawal of hyperimmune globulin administration for 4–5 years.[63] The variants are not present as a minority species before therapy; not all the mutations are within the MHR (although the majority are); within the MHR, they tend to accumulate within and around HBs2 and HBs4, but mainly HBs2.[74] However, there are discrepancies in this story. McNair *et al*.[72] found no changes between pretransplantation and post-transplantation samples in seven treated patients. Immunosuppression alone usually does not lead to HBsAg selection. It is possible to be viraemic in the absence of detectable HBsAg (such a case had loss of a critical cysteine – sC124R). Overall, in one study, 80% of the nucleotide changes led to amino acid changes (implying selection). There is some doubt about the significance of these results because some of the changes seen after therapy were seen before therapy in other patients. Although the data indicate that variant selection is a factor in graft infection, there are probably other factors such as inability to fully neutralize virus in extrahepatic sites. Without an *in vivo* model of neutralization, it will be difficult to ever be sure. Pre-S variants can be lost, but seldom gained, during hyperimmune globulin therapy,[75] implying either that these strains are less replication efficient or that they are viral dead ends.

## Variants associated with anti-viral therapy

As a result of the overlap of the S and P ORFs (Fig. 14.9),[76] HBV isolates with P gene mutations that are selected during the course of antiviral nucleoside analogue therapy may carry altered neutralization epitopes within the HBsAg. The classic lamivudine resistance 'signature mutation' in the HBV polymerase is the substitution of either valine or isoleucine for methionine in the highly conserved tyr-met-asp-asp (YMDD) motif, which comprises part of the active site (domain C) of the viral reverse transcriptase (Fig. 14.9). These YMDD mutations also result in amino acid substitutions in the HBsAg (Table 14.4). The B domain of the HBV polymerase coincides with amino acids 150–175 of HBsAg. This domain contains most of the S gene mutations observed

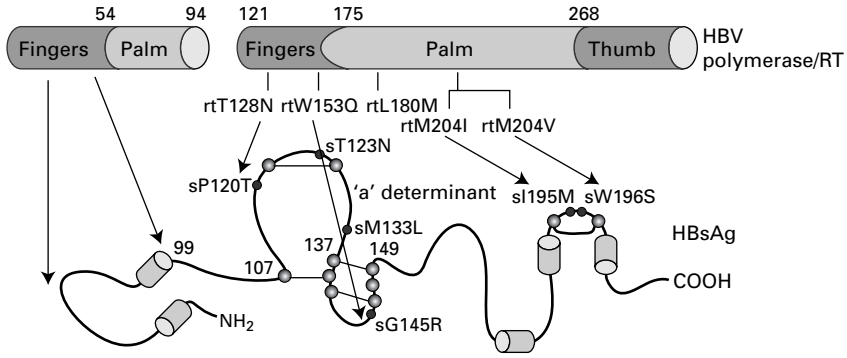


**Figure 14.9** Overlap of the HBV polymerase/RT with the HBsAg protein and the MHR. The HBV polymerase/RT is subdivided into fingers, palm and thumb domains. The vaccine-escaped mutations are shown together with the overlapping lamivudine-associated changes downstream of the 'a' determinant. The positions of key cysteine residues are shown as shaded circles and the important disulfide bridges in the HBsAg protein are indicated as broad lines. Alpha-helices in the HBsAg protein are shown as cylindrical structures. Taken from Torresi.[76]

in the presence of lamivudine[61,66,77–79] (Table 14.4). These drug resistance isolates may have the potential to become vaccine-escape mutants.[76]

Torresi *et al*.[80] investigated the role of lamivudine in selecting HBV mutants with antigenically altered HBsAg protein using pooled individual vaccine sera. They found that vaccine sera had reduced binding to HBsAg mutants selected during lamivudine treatment to a varying degree (Fig. 14.10).[80] In an enzyme immunoassay (EIA), binding of HBsAg mutants was reduced relative to the binding to the wild-type HBsAg (Fig. 14.10a and b). However, the potential of nucleoside analogues to select HBV mutants which reduce the efficacy of the current HBV vaccine has yet to be established. On the other hand, it has been shown that the changes in the S gene overlapped with the P protein have a compensatory effect that increased *in vitro* viral DNA levels in both intracellular replicating HBV core particle and extracellular virus.[81]

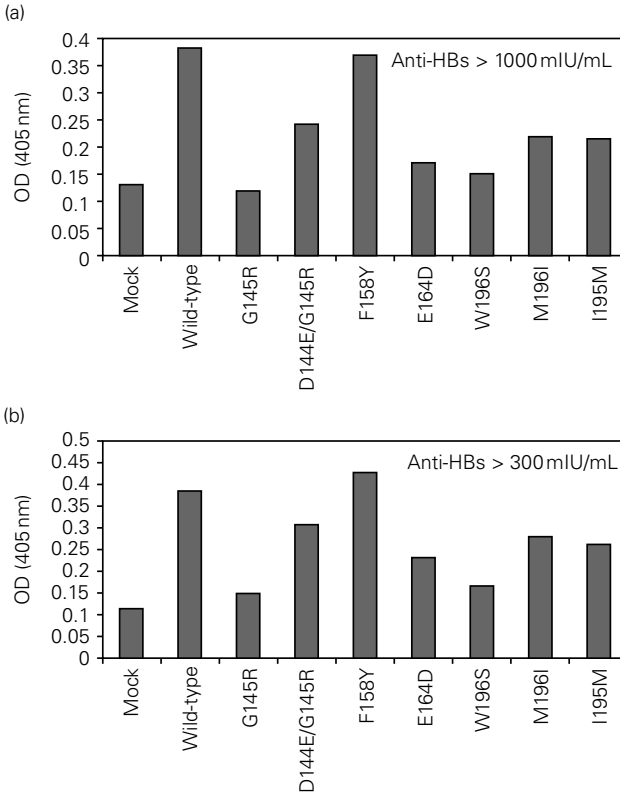


**Figure 14.10** Binding of individual vaccinee sera to wild-type and recombinant yeast-derived HBsAg. Enzyme immunoassay (EIA) plates were coated with 5 µg/ml of recombinant HBsAg and incubated with individual human vaccinee sera. The binding pattern of two representative sera (a and b) to the mutant proteins is shown. Taken from Torresi *et al*.[80]

## Diagnostic problems

A wide variety of commercial assays is available for the detection of HBsAg. Some use polyclonal anti-HBs and others use multiple monoclonal antibodies. Clearly, the sensitivity of an assay to detect a variant is dependent on the anti-HBs used. Thus, it is not surprising that there are examples of variants that cannot be detected by all assays. Clues to their existence are provided when a low-level signal is either not confirmed or is found to give a much higher signal by a second assay. There are accumulating data from Europe, Asia and Africa about HBsAg variants which are not recognized by either monoclonal or polyclonal antibodies specific for wild-type group 'a' determinant, but positive by DNA PCR.[17,30,31,82–85] Table 14.6 provides examples of this situation from a recent series from Papua New Guinea.[42] Sera with isolated anti-HBc (i.e. no HBsAg or anti-HBs) have been known to be infectious. Clearly, there are two possible explanations for lack of HBsAg positivity: a very low level of antigen or an antigenic variant. Of 104 sera from Sardinia with this serological picture, 21 (of the 75 available) were PCR-positive. However, only one of these

**Table 14.6** Variation in sensitivity of HBs antigen detection by various assays (samples from Papua New Guinea)

| Case | pAb-ELISA (S/CO ratio) | AUSZYME (S/CO ratio) | AUSRIA II (S/CO ratio) | PCR | | |
|---|---|---|---|---|---|---|
| | | | | Core | Surface | Sequence of S gene |
| PNG1 | 3.67 | 0.55 | 152 | + | – | N/A |
| PNG2 | 24.39 | 0.49 | 30722 | + | + | T1311 (ayw) |
| PNG3 | 4.04 | 0.84 | 129 | + | + | T1311 (ayw) |
| PNG4 | 4.89 | 0.19 | 320 | + | – | N/A |
| PNG5 | 2.00 | 0.24 | 481 | + | + | (adr) |
| PNG6 | 6.90 | 0.23 | 144 | + | + | P135S (adr) |
| PNG7 | 5.12 | 0.49 | 174 | + | + | (adr) |
| PNG8 | 24.39 | 0.25 | 550 | + | + | (adr) |
| PNG9 | 3.99 | 0.08 | 149 | – | + | (ad) |
| PNG10 | 24.39 | 0.36 | 1635 | + | + | M133T (adr) |
| PNG11 | 2.85 | 0.03 | 158 | + | + | (adr) |
| PNG12 | 3.89 | 0.73 | 61 | – | + | T116S (adr) |
| PNG13 | 6.17 | 0.36 | 62 | – | – | N/A |
| PNG14 | 2.66 | 0.44 | 50 | – | – | N/A |
| PNG15 | 2.39 | 1.46 | 60 | + | + | S155Y (adr) |
| PNG16 | 30.30 | 0.51 | 80 | – | + | S154T |
| PNG17 | 5.89 | 1.47 | 67 | + | + | S117R,T118S, S155P |

S/CO ratio <1 = negative; N/A, not applicable; pAb, polyclonal antibody-based assay. Sequences refer to subtype in parentheses and observed rare or unique variants. (From Carman *et al.*[42] with permission.)

PCR-positive samples had a clinically relevant variant. Other studies have shown that 'a' determinant variants may be responsible for 'anti-HBc only' positive individuals.[37,85,86] Still others can occur in patients who have HBsAg and anti-HBs simultaneously. A good example of the last[87,88] had sD144N, as well as changes at aa 126, 131, and 133. The patient also had a large deletion and other mutations in pre-S1. Another series of cases with co-existing HBs/anti-HBs came from Japan.[46] Five cases had either sR145, sN126 or an eight amino acid insertion in the tight loop between cysteines at 121 and 124. These show that sR145 can occur naturally, which gives it an added importance in an increasingly vaccinated world. However, not all series of HBs/anti-HBs-positive sera have variants to explain this situation. This is perhaps not surprising considering that this antigen and antibody are often complexed, and that detection of one or the other alone merely indicates dominance at that time. Also, with sensitive commercial assays, both can usually be detected, as was the case with the first infected vaccinated neonates.[24] Patients who have cleared their infection by classical criteria (anti-HBc/anti-HBs-positive),[89] usually do not have viraemia by PCR. An exception is within the first year after successful interferon (IFN) therapy.[89] Thus, it must be very unusual for a person to have a variant that is not detectable using standard HBsAg assays in this situation.

As already described, variants are not always simple but can have multiple changes within the MHR. Another good example of a complex variant, which also exemplifies the situation of disparate results in two commercial assays, was a patient who developed fulminant hepatitis B (FHB) after withdrawal of cytotoxic therapy for lymphoma.[20] No anti-HBs was administered, nor was this patient proven to have been vaccinated. He was positive by a polyclonal antibody-based assay but negative by a monoclonal antibody-based assay. sR145 as well as an insertion of N and T between aa 123 and 124 was found. Similar insertions at the same site have been seen in Chinese and Japanese patients.[46,90] Thus, all the insertions have been found in Far Eastern people and all within HBs2. Another example of a complex strain was from an Arabian man who was positive in a polyclonal assay but negative in a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA). Sequencing revealed sK145 plus other changes at aa 133, 134, 142 and 144. Finally, an example of a complex variant with undetectable HBsAg by a number of commercial and laboratory-based assays has recently been described in an agammaglobulinaemic patient, who had in addition substituted the sybtype determining amino acid at position 122 to isoleucine.[91]

## Antigenic characterization

### Immune escape at B-cell level

Most companies have either assessed their current diagnostic assays or produced new versions in order to better detect a range of variants, both natural and clini-

cally selected. The introduction of sensitive techniques for detection of HBV DNA has highlighted this issue, as a significant proportion of persons can be DNA-positive in the absence of HBsAg. Although currently available HBsAg assays are superior to their predecessors, there is a need for further development with ongoing assessment of assay ability to detect a wide range of HBsAg variants. It is obvious that the ability of an assay to detect a variant depends critically on the choice of anti-HBs used. However, the fundamental difficulty in the *in vitro* characterization of all this variation is that it is difficult to quantify the expressed HBsAg in a way that does not depend on its antigenicity. Several studies compare the sensitivity of modern assays for the detection of HBsAg variants.[65,74,92–96] In a study by Ireland and colleagues,[74] various cloned variants from 13 diagnostically relevant cases of hepatitis B patients were tested by seven commercial diagnostic assays. They compared assay performance of the variants that reacted at a level ≥10% of the standard HBV sequence (Table 14.7).[74] The assays detected between seven and 11 of the variants. In general, they found that the use of a polyclonal antibody in the capture and/or detection phases was associated with higher detection rates; assays 1, 2, 5 and 6 detected 11, 9, 10 and 9 of the 11 variants, respectively. The exception was assay 7 (detected seven variants), which used a sheep polyclonal antibody in the capture phase and mouse monoclonal antibody for detection. Assays 3 and 4, which detected only seven and eight of the variants, respectively, used monoclonal antibody in both capture and detection phases. However, having a similar, if not identical, amount of 'standard' antigen to compare the variant to is crucial for the interpretation of results. Use of an antibody that binds to a common region away from the variant domains being tested is one approach, but how can one be sure that the conformation is not affected? Another approach is to include an epitope tag within HBsAg. Basuni and co-workers developed an epitope tag system for standardization of the number of variant particles, independently of HBsAg antigenicity. An influenza epitope was inserted at both ends and the particle numbers could thus be standardized. Subsequently, the HBsAg was measured after capture by an anti-influenza monoclonal antibody. They found that single epitopes of up to 15 amino acids can be inserted at either end of the HBsAg protein without affecting HBsAg reactivity (Basuni, unpublished data). Counting of particles by electron microscopy is too labour-intensive and variable (Carman, unpublished data). Production in yeast has been successful for studying the role of the cysteines in the MHR.[3,4,10,97] Constructing HBsAg variants, particularly sG145R, in yeast led to markedly reduced binding by monoclonal and polyclonal antibodies,[98,99] indicating that this change alone may be important in the cases of vaccine escape described to date. However, naturally occurring sR145 variants are often detectable with monoclonal antibody-based assays,[74,100] albeit at reduced sensitivity. Whether these cases also had very high levels of serum HBsAg or whether there are antigenic subtleties after expression in yeast is unclear. It appears that variants can have different antigenic expression depending on their genotype/subtype background.

## Immune escape at T-cell level

The humoral response to HBsAg is T-cell-dependent. At least four regions within HBsAg present epitopes for MHC class II restricted $CD4^+$ T cells.[101,102] The effect of HBsAg T-cell epitope variants on the cellular immune response has been studied in individuals vaccinated against HBV by Bauer *et al*.[103] Six of 23 different variants in two HBsAg Th epitopes were shown to be responsible for inadequate T-cell reactivity.

Similarly, the MHC class I-restricted $CD8^+$ CTL response plays a key role in suppressing HBV infection. Schirmbeck *et al*.[104,105] showed that in $H\text{-}2^b$ mice even small changes in amino acid residues within two different CTL epitopes that mimic natural variants of adw2, ayr and adr, completely eliminated the immunogenicity of each epitope.

## Pre-S/S variants and chronic hepatitis

It was mentioned earlier that variants of HBsAg can be selected at or around the time of HBs/anti-HBs se-

**Table 14.7** Number of variants detected by each assay at a level of 10% of the standard HBsAg (GlyY/GlyD)

| | Assay 1 | Assay 2 | Assay 3 | Assay 4 | Assay 5 | Assay 6 | Assay 7 |
|---|---|---|---|---|---|---|---|
| ay subtype variants | 5/5 | 5/5 | 2/5 | 2/5 | 4/5 | 4/5 | 2/5 |
| ad subtype variants | 6/6 | 4/6 | 5/6 | 6/6 | 6/6 | 5/6 | 5/6 |
| Total variants detected | 11/11 | 9/11 | 7/11 | 8/11 | 10/11 | 9/11 | 7/11 |
| Capture antibody | Guinea pig pAb | Guinea pig | Mouse mAb | Mouse mAb | Mouse mAb | Goat pAb | Sheep pAb |
| Detection antibody | Goat pAb | pAb | Mouse mAb | Mouse mAb | Goat pAb | Mouse mAb | Mouse mAb |

Taken from Ireland *et al*. [74]

roconversion. Ongoing mutant selection with the development of new anti-HBs populations persists until functional constraints on HBsAg do not allow further mutations. This can also occur after IFN therapy. Loss of proline at aa 142 and sR145 has been observed in patients who successfully cleared HBe and HBs antigens many years earlier. These patients are HBsAg-negative, DNA-positive with elevated transaminases.

Mutations in the pre-S region of the surface ORF have been described in many patients from different countries with chronic hepatitis B or HCC.[22,75,106–111] There is evidence to suggest that these mutants may be found with certain HBV genotypes. They have been more frequently found in genotypes C and/or B than in other genotypes.[107,110,112] Serum HBV DNA is not always detected by nucleic acid hybridization. A plethora of nucleotide substitutions and deletions have been observed in this region. A deletion of 61 amino acid residues from the carboxy-terminal half of the pre-S1 domain and of the promoter for the mRNA for the middle and small envelope proteins has been seen more than once. The authors also presented electron microscopic evidence of aberrant forms of virions. The region of pre-S1 from aa 21 to 47, believed to be responsible for hepatocyte attachment,[2] is always conserved. In most cases, a full-length gene is simultaneously present in serum, implying that complementation is necessary for viability. Pre-S1 deletions can arise after IFN therapy.[113] These probably affect the upstream transcription factor binding sites, explaining the lower production of HBsAg. Furthermore, decreased secretion of encapsidated mutant viral genomes, which could be trans-complemented by co-transfection of the S ORF, has been observed. There is also some *in vitro* evidence that deletions render the virus cytotoxic. Retention of S protein-containing particles in vesicles and relative overexpression of pre-S1 occur.[114] Cytopathic effects of pre-S1 mutants have been shown in several studies with 'ground glass-like' hepatocytes in transgenic mice and in ducks.[115,116] However, ground glass-like appearance also has been shown in samples with pre-S2 mutants.[117] Cells transfected with plasmid constructs bearing these deletion mutations were often destroyed, implying that the effects of these mutations were directly cytotoxic. It is of interest that a case of fibrosing cholestatic hepatitis (FCH) (that is characterized by intracellular retention of viral proteins) has been described (see above) with mutations in the S promoter.[75] There is also an assumption that pre-S1 regulates viral cccDNA levels via negative feedback,[118] redirecting the nucleocapsid into the virion formation pathway.[119] Variations in the pre-S2 region may thus lead to the accumulation of HBcAg in the nucleus, and newly synthesized genomes may be converted into cccDNA, thus increasing viral DNA transcription and replication.

Patients with HBeAg-negative viraemia can have pre-S2 deletions as well as precore mutations. Which appears first and whether there is any special functional significance or interaction is not clear. In Koreans who seroconvert to anti-HBe, precore and pre-S variants appear almost simultaneously.[120] Deletions in the pre-S2 region often include the pre-S2 initiation codon, thus preventing M protein synthesis.[107,121] Internal deletions[107,110] occur within the spacer region of the polymerase ORF and do not affect virus viability. As the M protein is not obligatory for virus production, such variants can clearly exist without complementation with full-length genes. Both pre-S1 and pre-S2 internal deletion variants increase the intracellular retention of envelope proteins as well as reducing the secretion of the S envelope protein. However, unlike the pre-S1, the effects of pre-S2 internal deletions on reducing the secretion of the small envelope protein are modest, and also, no apparent cytotoxicity in cell cultures transfected with pre-S2 internal deletion variants, has been detected.[110]

## Conclusion

HBV has evolved in humans into genotypes, antigenic subtypes and other variants, some antigenically important, others not. Virus strains can clearly be shown to be restricted geographically, due to random mutations linked to immune pressure. Further pressures can be mounted by medical intervention such as antibody therapy, drugs or vaccines. The swarm of genetic variants that can be found in any patient infected with HBV tend to present a mixture of viruses with varying replication fitness and selection advantages under the changing conditions of host cell type, immune response or pressure from drug treatment. The fitness of the predominant population changes in response to changing selection pressure.[26] There is good evidence that these variants are important causes of vaccine failure in infants born to HBeAg-positive mothers and that lamivudine can occasionally lead to variants that are not detected in HBsAg assays. Most manufacturers have altered their assays to better detect common mutants of HBsAg.

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# Chapter 15
# Molecular variations in the core promoter, precore and core regions of hepatitis B virus, and their clinical significance

Peter Karayiannis, William F Carman, Howard C Thomas

## Introduction

The advent of the polymerase chain reaction (PCR) in the late 1980s revolutionized our understanding of virus genetics. Amplification of viral nucleic acids and their sequencing either directly, or after cloning, has added significantly to established sequence databases, thus permitting detailed genomic and proteomic analyses to be carried out. In the case of hepatitis B virus (HBV), sequencing studies have led to the identification of a number of stable virus variants normally found during the natural course of infection, or arising as a result of preventative or therapeutic intervention. Examples are the hepatitis B e antigen (HBeAg)-negative phenotype and the basic core promoter (BCP) variants, others affecting the enhancer II (EnII) region, as well as various deletion variants found in immunocompromised individuals. These variants will be reviewed in detail in this chapter. Variants of the hepatitis B surface antigen (HBsAg) and polymerase genes, arising after vaccination or treatment with monoclonal or polyclonal antibodies (anti-HBs), and treatment with nucleoside analogues respectively, are reviewed in Chapters 14 and 21.

## Virus biology and evolution

Hepatitis B virus isolates exhibit significant variation that may involve up to 12% of the virus nucleotide sequence. Natural variants exist, which give rise to the well-recognized serological subtypes and genotypes of the virus.[1] Multiple additional variants can also be found in a single host as a mixture of genetically related viral strains. This genetic diversity within an isolate is nowadays referred to as quasi-species variation. Such quasi-species populations are particularly evident in RNA viruses[2] because they have a high rate of nucleotide mis-incorporation during RNA replication or reverse transcription, coupled with the inability of the enzymes involved to correct such mistakes, as they lack proofreading capacity. Although HBV is a DNA virus, it replicates through an RNA intermediate (pregenomic RNA, pgRNA) which is reverse transcribed, thus rendering it liable to a high mutation rate.[3,4] As a consequence, HBV exhibits a higher mutation rate than would otherwise be expected for a DNA virus, amounting to $2 \times 10^{-4}$ base substitutions per site per year.[5] However, the potential for variation is constrained by the compact genomic organization of the virus, featuring overlapping open reading frames (ORFs) and the existence of regulatory elements of transcription, replication and encapsidation within these ORFs. For example, the surface ORF (S) is entirely overlapped by that encoding the polymerase (P). Any nucleotide change that leads to loss of function of either protein will be lethal or deleterious to the virus and therefore lost (*negative selection*). However, individual point mutations frequently have no phenotypic effect, either because of the redundancy of the genetic code (the nucleotide change may not result in an amino acid change) or because the change does not affect higher level structures of the RNA or protein. Some of these phenotypically silent mutations may become incorporated into the quasi-species pool.[6] The major force for evolutionary change within a virus population is *positive selection* by the host immune response. This subject has been covered in more depth elsewhere.[6–8]

### Biology of the precore/core gene

The precore/core ORF has two in frame initiation (AUG) codons, which lead to the synthesis of two co-terminal proteins. Initiation of translation at the first AUG results in the synthesis of the precore/core protein, the precursor of HBeAg (Fig. 15.1), while the second initiation codon is utilized for the synthesis of the nucleocapsid or core protein (HBcAg). The two proteins are synthesized from different messages known as the precore

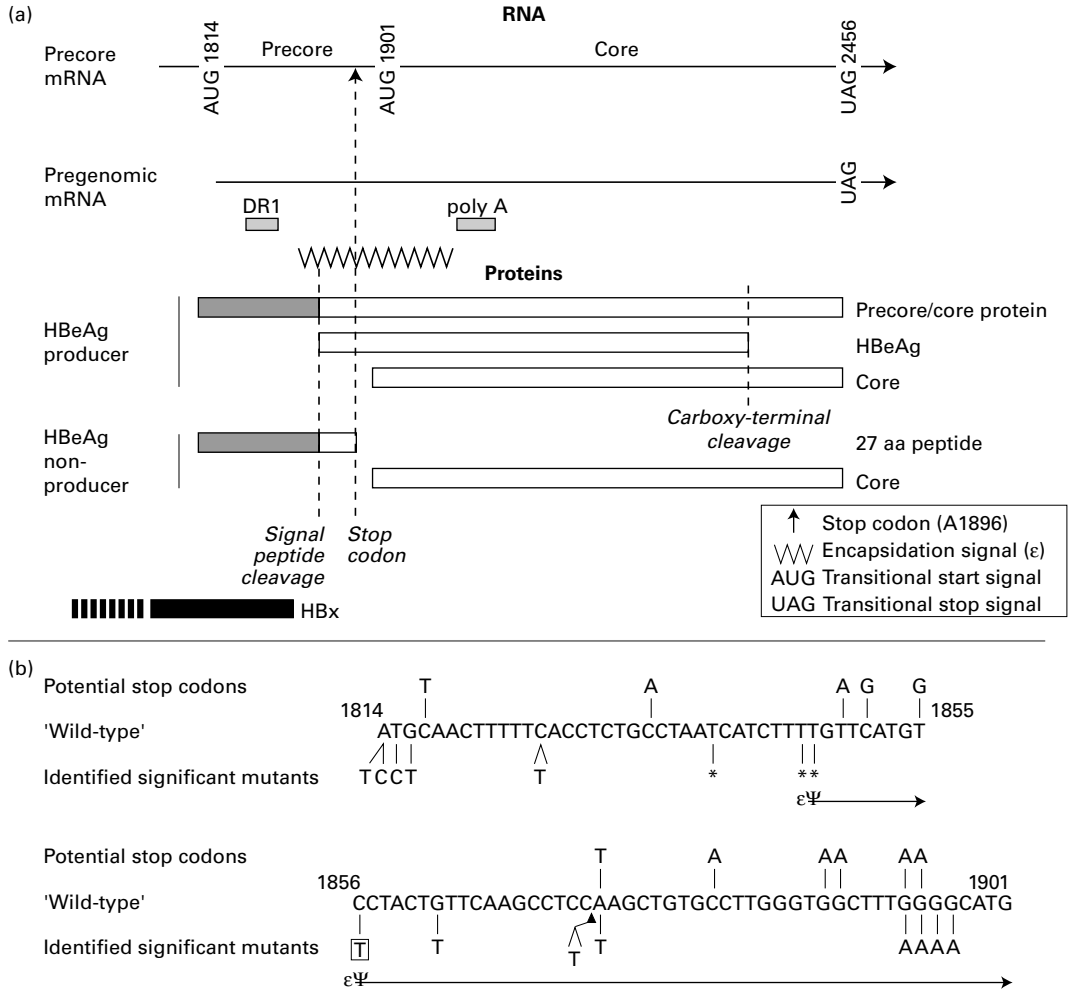


**Figure 15.1** Precore/core biology and nucleotide variation. (a) There are two in frame translation initiation codons (AUG) and one termination codon (UAG) in this gene. The precore/core protein translated from the first initiation codon is the precursor of HBeAg. This is generated by proteolytic cleavage of the first 19 amino-terminal amino acids (to remove the signal peptide) and the last 34 carboxy-terminal ones (codon 149 onwards). Translation from the second initiation codon produces HBcAg, the nucleocapsid protein of the virus. A G to A substitution at nucleotide 1896 (A1896) within the precore generates a stop codon that leads to abrogation of HBeAg production. HBcAg production is unaffected. Note that the X open reading frame (ORF) overlaps the 5′ end of the precore. (b) All the potential stop codons that could be generated by single mutations are indicated above the precore sequence. Nucleotide substitutions or insertions of variants that have been described are shown below the sequence. Note that there are some positions at which the potential stop codons have not been observed.

mRNA and pgRNA, respectively. Once synthesized, the precore/core protein is targeted to the endoplasmic reticulum (ER) by a 19 amino acid signal peptide at its amino-terminus.[9] Cleavage of this peptide releases the remainder of the protein into the ER lumen, where further processing of the carboxy-terminus results in the removal of about 34 amino acids from this end. What remains is HBeAg, which is released from the cell. HBeAg retains 10 amino acids from the precore region and shares the remaining 149 with HBcAg. The finding of antigenic epitopes that are different between the two proteins, even though they share most of their amino acid sequence, is explained by conformational changes in HBeAg related to the 10 amino acid precore peptide at its amino-terminus and the retained carboxy-terminal arginine-rich domain of HBcAg.

HBeAg is conserved within all members of the hepadnavirus family. It is secreted from the infected cell but is not part of the virion structure and does not have any known role in replication. In the neonate born to an HBV-infected mother, it has been suggested that HBeAg crosses the placenta and induces tolerance.[10,11] HBeAg may also have an immunomodulatory function during infection in adult life. Low molecular weight soluble proteins, such as HBeAg, induce a state of immune non-responsiveness, at the T-helper (Th) level. HBeAg-in-

duced tolerance results in T-cell non-response to HBcAg, as well as HBeAg-derived epitopes, so that elimination of HBV-infected cells does not occur. In transgenic mice, this non-response to nucleocapsid proteins can be broken by the injection of peptides bearing single amino acid substitutions. If such substitutions occur spontaneously during persistent infection, they may account for spontaneous HBeAg/anti-HBe seroconversion.[12] HBeAg probably also influences the host response by down-regulating cytokine (IFN-β) production.[13]

HBcAg forms the nucleocapsid of the virus, which has icosahedral symmetry, as demonstrated by cryoelectron microscopy.[14] The core protein dimerizes readily, and the dimers assemble spontaneously into particles that simultaneously encapsidate the pgRNA with a copy of the viral polymerase. The arginine-rich motifs at the carboxy-terminus of HBcAg are responsible for binding the pgRNA, and also the newly synthesized DNA strands of the virus. Encapsidation is triggered by the binding of the polymerase to a stem loop structure at the 5′ end of the pgRNA known as the encapsidation signal or ε (Fig. 15.2), with strict requirements of structure and sequence.[15] This structure, which is formed by the precore region sequences, is in turn recognized and bound by the polymerase. The latter uses the side-bulge of ε for the synthesis of a short primer for minus-strand DNA synthesis. Thus, any variation in this region may affect the stability of the signal and compromise virus viability.

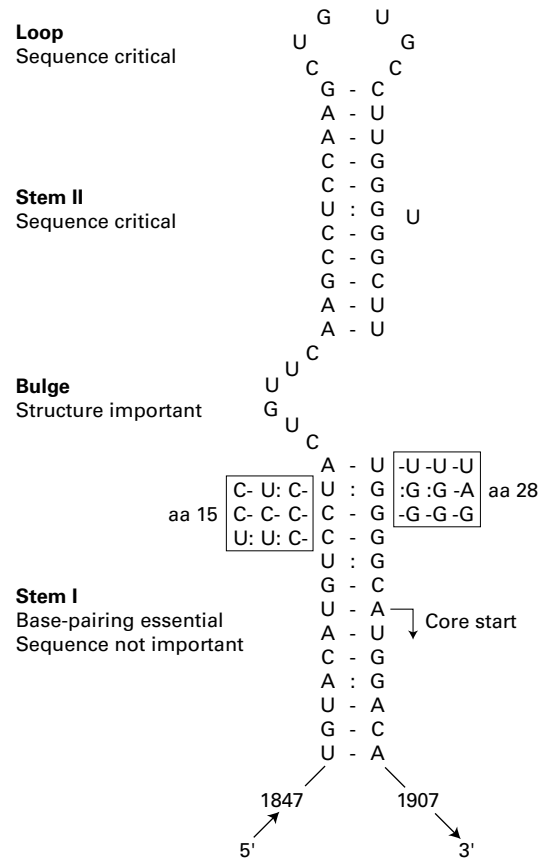


**Figure 15.2** Secondary structure and some variants of the pregenomic RNA encapsidation signal or ε. The 5' end of the pregenomic RNA, which starts just upstream of the precore AUG, folds into a stem loop structure as a result of base-pairing of complementary nucleotide sequences. This secondary structure is necessary for encapsidation of the RNA into nascent core particles. Unpaired regions form into a bulge and a loop. Note that nucleotides 1858 (third base of codon 15) and 1896 (second base of codon 28) are opposite each other on the lower stem. Sequences 1 and 2 (on the left side of the lower stem) code for serine, while sequence 3 and CCU code for proline. Sequence 6 is the stop codon variant, A1896. If there is imperfect base-pairing, as for sequences 1 and 4, a further mutation, for example sequence 2 or 6, will lead to instability of the stem and the virus will not survive.

## Biology of the core promoter region

The core promoter (CP) of the viral genome has a pivotal role in the replication and morphogenesis of the virus. It directs initiation of transcription for the synthesis of both the precore and pgRNAs. The CP consists of the BCP and upstream regulatory sequences, containing both positive and negative regulatory elements that modulate promoter activity.[16] The BCP overlaps with the 3′ end of the X ORF and the 5′ end of the precore region, and contains *cis*-acting elements that can independently direct transcription of the precore mRNA and pgRNA, both of which are about 3.5 kb in length (Fig. 15.3).[17] The pgRNA starts 3′ to the precore AUG and is translated into the core and polymerase proteins, but in addition serves as the template for the synthesis of the negative DNA strand of the virus by reverse transcription after encapsidation within the core particle.[3] The precore transcript, which is slightly longer than the pgRNA and is initiated upstream of the precore start codon, is the template for the translation of the precore/core protein that is proteolytically processed to produce HBeAg, as already described.

The enhancer II (EnII) element regulates the activity of the CP and partially overlaps with it and its upstream regulatory sequences. This whole region contains in addition nucleotide motifs that constitute transcription factor binding sites, imparting at the same time liver cell specificity for optimal function of these elements.[18–20] This interaction between the *cis*-acting elements with ubiquitous and liver-specific transcription factors is absolutely necessary for liver-specific expression from the CP. Moreover, the interaction between these *trans*-acting factors and the *cis*-acting elements allows the virus to co-ordinately or differentially regulate the transcription of the two mRNAs.[17]

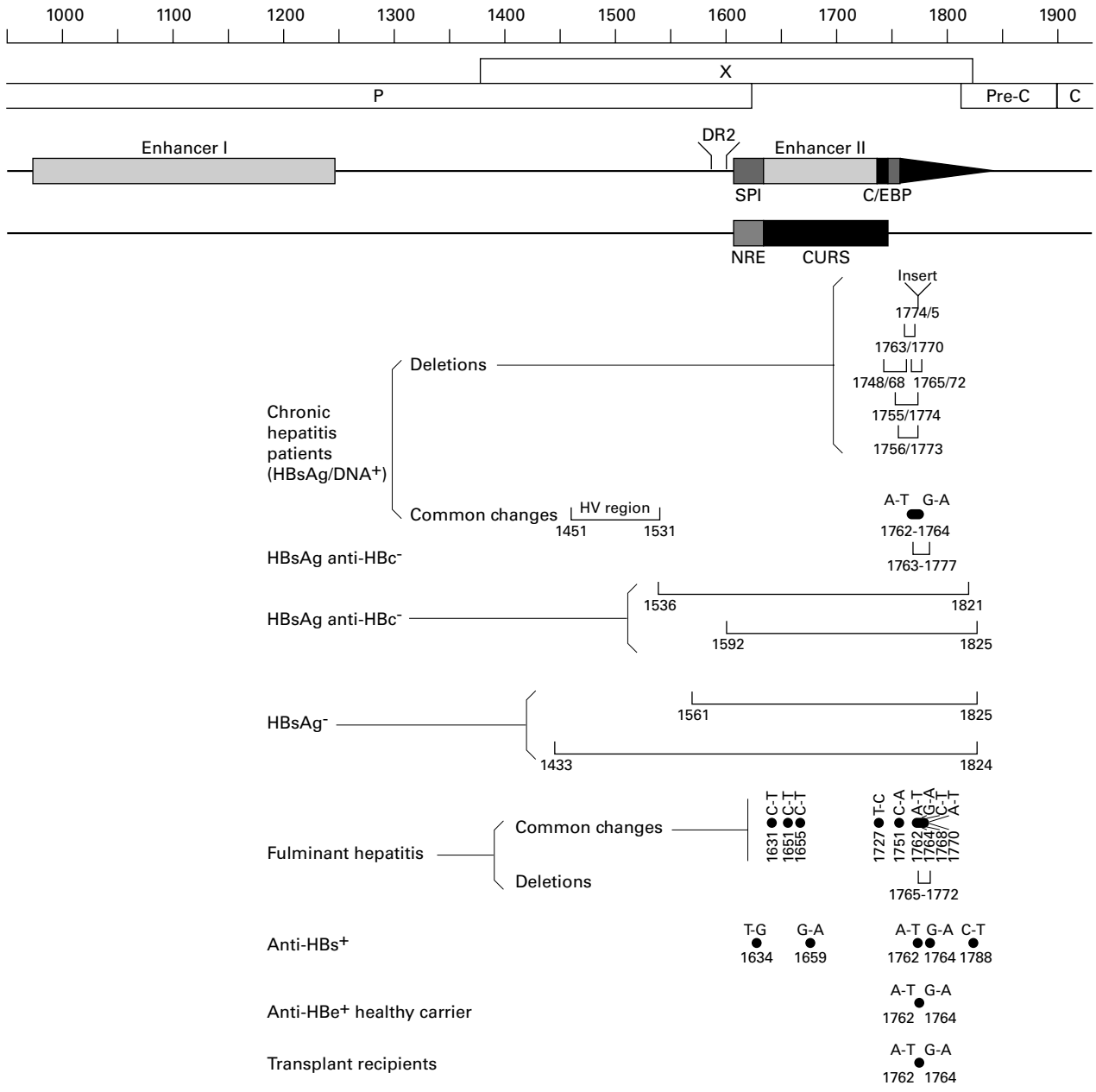


**Figure 15.3** Transcriptional regulatory elements – the X gene and variants. The promoters and enhancers involved in HBV replication are indicated. Note that they are upstream of the precore/core gene and, in the case of the BCP, overlap the 5′ end. The X gene is embedded within these elements. Thus, any nucleotide changes in one element may affect the amino acid sequence of HBx protein. Variability in a number of clinical scenarios is given. It is likely that deletions of X are not viable unless accompanied by a full-length gene. NRE, negative regulatory element; CURS, core upstream regulatory sequence; BCP, basal core promoter; DR, direct repeat. The ruler at the top is the nucleotide numbering.

## Precore variants

A molecular explanation for the HBeAg-negative phase of hepatitis B viraemia, first described in Mediterranean patients,[21,22] became apparent when a variant of HBV with mutations in the precore region was described by Carman *et al*. in 1989.[23] This variant had a G for A substitution at position 1986 (denoted as G1896A or A1896) (Fig. 15.1) which converted the TGG codon for tryptophan (codon 28) to TAG, a translational stop codon. This is by far the commonest substitution encountered in anti-HBe-positive patients.[23–27] A1897, seen less often, converts TGG to TGA, also a translational stop codon. Other mutations that have the same phenotypic effect are loss of the precore/core protein translation start codon (ATG to ACG or CUG),[25,28,29] mutation of the second codon to a stop codon, and frameshifts and deletions resulting in the synthesis of nonsense proteins.[27,29–34]

Although there are several additional positions within the precore sequence where stop codons could be generated by point mutations, these are rare, possibly because such mutations would destabilize the secondary structure of ε (Fig. 15.2). Precore amino acid substitutions have been detected in HBeAg-negative strains,[25,35] sometimes associated with A1896. The run of four Gs between nucleotide positions 1896–1899 is therefore a mutational hot spot and all four can be substituted by A. Two of these, A1898[35] and A1899,[23,36] are of unknown significance but are strongly linked with T1856 and A1896, respectively. These associations are likely to be related to encapsidation signal secondary structure requirements, but whether they have clinical relevance remains unclear even after years of inquiry. A1899 is primarily seen (with rare exceptions) together with A1896, implying that a second selection pressure is exerted on precore after emergence of A1896. In early studies, it was linked with severe disease, but subsequent work has failed to confirm this association.[37]

A significant proportion of chronic hepatitis patients in Mediterranean countries[21,38,39] and in the Far East[40] are infected with HBeAg-negative variants. It appears that this clinical picture has become the most common new presentation of chronic hepatitis B in Italy and Greece. In the Gambia and Brazil,[41,42] as well as northern Europe, there is a low prevalence of the precore stop codon mutation in anti-HBe-positive patients. These findings may relate to the prevalent HBV genotypes that circulate in these populations. Precore variation, not all of which affects translation of HBeAg, is distributed in particular genotypes. The stop codon mutation is not favoured in genotype A, C and F isolates, because they possess C at position 1858. This forms a canonical Watson-Crick pair with the G at position 1896 seen in wild-type strains. Inspection of the likely secondary structure of the encapsidation signal (Fig. 15.2) reveals a highly conserved lower stem that includes nucleotides 1856, 1858, 1896 and 1899. Mutagenesis experiments have shown that base-pairing is important in this region,[15] and various groups have confirmed these findings on clinically observed variants.[34,43–45] Viruses that have unstable stem loops do not encapsidate their pgRNA and do not compete successfully with other strains. Mutual exclusivity of A1896- and T1856-containing strains is explained by stem instability, as C1856T is always found with C1858, which is opposite A1896 on the stem. Of interest is that in anti-HBe-positive patients, C1858 strains appear more pathogenic than those with A1896.[46] A1896 is uncommon in genotype A patients as already mentioned.[34,47] Woodchucks may also exhibit precore variants of the woodchuck hepatitis virus, usually in the equivalent positions to HBV.[48]

## Chronic hepatitis

Following acute HBV infection, 5% of adults and almost 95% of children born to chronically infected mothers become chronic carriers of the virus, probably because of failure of induction or activity of cytotoxic T lymphocytes (CTLs). Viraemia continues for many years, and initially there is very little inflammatory necrosis of infected hepatocytes (Fig. 15.4).[40] As the years go by, this immune-tolerant phase gives way to increased hepatitic activity, presumably reflecting immune activation, perhaps as a result of viral antigenic variation caused by transcriptional errors as already mentioned. The role of CD4-positive lymphocytes in this process is supported by the observation of an increased response to the nucleocapsid proteins as HBeAg/anti-HBe seroconversion

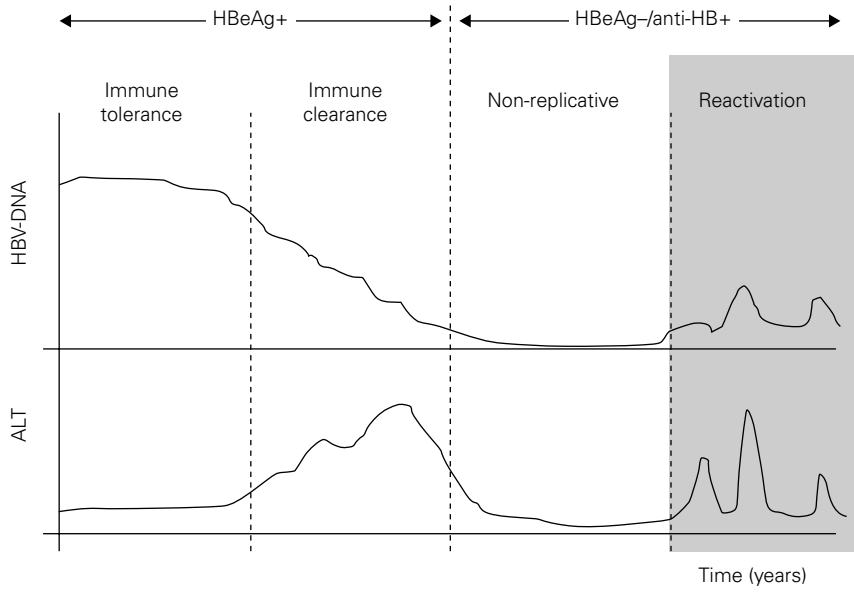


**Figure 15.4** Diagram of the natural history of HBV infection showing the immune-tolerant, immune clearance and non-replicative phases of disease. In some patients, the latter phase may be followed by a reactivation phase characterized by increased viral replication and liver damage (shaded area). Precore and BCP variants are the main isolates seen in such patients during this latter phase.

approaches.[49] These cells presumably provide help to CTLs and B lymphocytes. Either because of CTL lysis of infected cells,[50] or of cytokine production by CD4-positive lymphocytes, which inhibit virion production,[51] cells supporting productive HBV infection are cleared during the seroconversion hepatitis. About 5% of HBeAg-positive chronic infections may spontaneously seroconvert to anti-HBe per year. In the majority of these patients, HBs antigenaemia persists predominantly because of the presence of small numbers of cells containing integrated HBV sequences.[52] However, by PCR, it is possible to detect small amounts of HBV DNA in the serum of these HBsAg-positive, anti-HBe-positive subjects with normal liver function tests, indicating that viral replication is still occurring at low level.[23]

In some patients, particularly those infected at birth or in the early years of life from infected family members, there is emergence of an HBeAg-negative variant. This virus, along with the HBeAg-positive strain, can be detected in virtually all patients after HBeAg/anti-HBe seroconversion.[23] The A1896 variant is present in very small amounts during the latter period of the HBeAg-positive phase of the disease and is selected at, or after, seroconversion to anti-HBe, while the HBeAg-producing strain is gradually being cleared.[28] This process can take years, during which time a mixture of both strains is usually seen. HBeAg-positive cases with active hepatitis (in the immune clearance phase) have mixtures of HBeAg-producing and non-producing strains during this phase.[44,53] In contrast, anti-HBe-positive cases with a mixture usually have mild hepatitis.[23,54] Those in whom HBeAg and anti-HBe are intermittently detected tend to be infected with an HBeAg-producing strain during this period.[35] Similarly, in cases that reactivate after some years, the precore sequence tends to be similar to previous samples. For example, if cases had G1896 earlier, most likely that would be the dominant strain in later samples, even if the patient had become anti-HBe-positive.[55] Why in some patients the virus then emerges and replicates at high levels, and why patients then go on to develop further inflammatory liver disease,[56] is currently unknown. Clearly, some component of the immune response is responsible for control of the virus during the anti-HBe-positive phase. This is most probably a Th/CTL response to nucleocapsid proteins. Mutations within epitopes recognized by sensitized T cells involved in control of the infection, or immunosuppression, may tip the balance in favour of the virus re-emerging. It is noteworthy that during this phase of the infection, amino acid substitution in the core protein is frequent (see below). It seems likely that the important selection pressure is the presence of anti-HBe in the absence of an adequate CTL response, as most of the variants so far described emerging in the late phase of the infection have had a common phenotype, namely an inability to produce HBeAg.

## Fulminant hepatitis

There is strong epidemiological evidence linking A1896 with fulminant hepatitis B (FHB),[57–61] suggesting that such a severe outcome may be due to viral factors rather than being solely dependent on a strong immune response to the infecting viral strain.[61] A substantial body of evidence shows that those who are HBeAg-negative at the time of sampling are usually found to be infected with the A1896 variant.[57–60,62–64] As the source of the infection usually has minimal chronic hepatitis and is infected with a precore mutant of similar or identical sequence to that found in the FHB patient,[65–69] it is evident that the disease has been caused by the new host mounting a vigorous immune response to the particular strain of HBV. Although many FHB cases are associated with infection by precore variants, FHB may also be associated with HBeAg-positive HBV infection being infected with HBeAg-producing strains.[57,63] Furthermore, even in anti-HBe-positive cases, the link to A1896 is far from clear. This variant has not been found in many anti-HBe-positive FHB patients from Britain, Hong Kong,[70] France[71] or the United States.[72] It has therefore been hypothesized that precore variants may replicate rapidly, spread throughout the liver and precipitate a fulminant course only if the host mounts a strong lytic immune response. It is suggested that the absence of HBeAg-mediated immune modulation contributes to the rapid lysis of large numbers of HBV-infected cells. The development of FHB in neonates infected from HBeAg-negative mothers, and the observation that transmission of A1896 strains to children seldom gives rise to chronic carriage,[73] may also be partially explained by the absence of immunomodulation by HBeAg.

The picture has become clearer, although also more complex, with the discovery that variation within the *cis*-acting elements of the basic core promoter (BCP), that overlaps with the HBx protein encoding region (see later), is also associated with FHB. The commonest variants contain a double mutation (T1762/A1764) in the BCP. The working hypothesis is now that there are several routes to enhanced replication that, in a genetically predisposed host, may result in FHB. Although A1896 is often found, this does not necessarily mean that it is the variant *per se* that is causing the fulminant hepatitis. In fact, there appear to be multiple strains with A1896 associated with different outcomes. Transmission of an A1896 strain (with T1762 and A1764) to chimpanzees[74] resulted in more severe hepatitis than would be expected, although this was not accompanied by a higher than average level of viraemia. However, it has been dem-

onstrated that the precore region of HBV is involved in inhibition of HBV replication.[75–79] and it follows therefore that BCP mutations in the presence of A1896 may result in higher levels of viral replication. Furthermore *in vitro* experiments have shown that, although an A1896 genome did not have higher replication efficiency, a genome from an FHB patient with A1896, T1762 and A1764 produced very high levels of HBV DNA.[80] The major effect was on encapsidation. Variability at nucleotides 1766 and 1768 seems to be important in this process, implying that there is another region outside ε involved in encapsidation, perhaps indirectly.[81] Detailed phylogenetic analysis that included 20 epidemiologically unrelated FHB sequences,[82] revealed six clusters of fulminant strains each with distinct mutational patterns. Mutations were again clustered in the *cis*-acting regulatory regions and HBx protein, but no unique variant was specifically associated with this disease outcome. Rather, the combination of nucleotide variation in the enhancer/promoter regions plus amino acid substitution in HBx was almost uniquely associated with this disease. There was statistical evidence of a heightened replication rate of the FHB genomes.[82] Finally, oligonucleotides containing variant sequences failed to bind to some nuclear transcription factors, implying that an additional pathogenetic mechanism in FHB might be loss of inhibition of transcription. Clusters (viral lineages) and transcription efficiency were both strongly linked to mortality and rapidity of progression to fulminant hepatitis after infection

Viral selection during the course of FHB has also been demonstrated.[83] For example, in one infant–mother pair, the mother had a mixture of two subtypes and a mixture of quasi-species within each subtype, yet the infant was only infected by one subtype and a reduced number of quasi-species within that subtype. There is no evidence that this is of pathogenetic significance.

### Fibrosing cholestatic hepatitis

Fibrosing cholestatic hepatitis (FCH) occurs after liver transplantation,[84] occasionally after renal transplantation[85] and in human immunodeficiency virus (HIV) infection (personal observation). The common factor is clearly immunosuppression. These patients undergo a fulminant course and the hepatocytes are packed with HBs and HBc antigens. Although precore and HBc variants are often associated with FCH, it is difficult to link these causally with intracellular accumulation.[85,86] The issue has been raised whether cytopathic effect, rather than immune lysis, is primarily responsible for this form of hepatitis. In particular, it is possible that mutations that result in truncated proteins that have lost secretory signal sequences or exhibit altered cellular localization may cause cellular toxicity. The picture has been made more complex with a recent description of an S promoter variant in an FCH case.[87] The issue of direct cytotoxicity justifies further exploration.

### Acute non-fulminant hepatitis

The incidence of the precore mutation during acute, non-fulminant, hepatitis is presently unclear. Selection of A1896 strains does occur during acute infection[63,88] consistent with anti-HBe exerting an initial selection pressure followed by clearance of the HBeAg-negative virus by other immune mechanisms, possibly CTLs. However, other groups were unable to find evidence for this process during acute hepatitis,[28,58,89] finding instead that years were sometimes needed for emergence of precore variants. Using an amplification-created restriction site method, A1896 was shown to dominate in both acute and chronic hepatitis cases of various severity in Taiwan (58% in acute versus 70% in chronic),[90] but not so in a recent study from Japan.[89] Acute hepatitis in neonates born to anti-HBe-positive mothers is well described.[73] The description of a chronic carrier transmitting A1896 in a pure form and resulting in typical acute hepatitis with seroconversion to anti-HBe without there having been an HBeAg-positive phase, shows that anti-HBe is cross-reactive with HBcAg epitopes and that A1896 can lead to the same serological disease profile as G1896 viruses. [91]

## Basic core promoter variants

Mutations in the core promoter region may have repercussions on viral gene expression and/or replication, with a concurrent impact on viral pathogenesis. A double mutation in the BCP leading to substitution of A for T, and G for A, at positions 1762 and 1764, respectively, has been described in various disease states or settings of HBV infection.[26] The biological significance of the double A1762T/G1764A mutation is still under investigation, particularly in relation to the precore stop codon mutation. However, the puzzle is beginning to come together in spite of initial conflicting results. The double BCP mutation has been shown to be associated with HBeAg negativity in some studies, [26,92,93] but not in others.[94–101] These discrepant findings may be due to the fact that these are cross-sectional studies performed at different stages of chronic liver disease, coupled with incomplete data on mutations elsewhere in the genome, including that of the precore. However, the presence of the double mutation is clearly associated with downregulation of HBeAg production,[64,94,102,103] as demonstrated by transfection studies.[104–108] There is evidence to suggest that the double BCP mutation results in decreased levels of the precore mRNA and therefore diminished production of HBeAg. Laras and co-workers demonstrated that this is indeed the case using a transcript-specific PCR ap-

proach and liver biopsy material from HBV carriers with the double mutation. In all cases, there was absence or low levels of precore mRNA without a significant effect on total CP-directed transcription.[109] Conversely, it has been suggested that the double mutation may result in increased viral replication as a result of upregulation of pgRNA production, promoting encapsidation and core protein production.[104,154,107,108] Others, however, reported decreased precore mRNA levels, but wild-type levels of replication and gene expression.[106,110–112] This latter finding is supported by measurements of viral load in patient sera, which do not show any significant increases in the presence of the double BCP mutations.[99,102,107,113,114] A clear link between severity of liver disease and HBeAg negativity with the BCP mutations has been found in some studies,[93,103,115–117] but not in others.[114]

The differences seen in transcript levels in the presence of the BCP mutations are thought to be due to the conversion of a nuclear receptor binding site to one for hepatocyte nuclear factor 1 (HNF1).[111,118] This effect has been investigated further in HepG2 cells and in the woodchuck animal model.[119,120] Mutations in the HNF1 binding site decreased dramatically the synthesis of woodchuck hepatitis virus pgRNA, thus suggesting that the HNF1 site is essential for this activity. The double mutation in addition converts L to M at position 130 and V to I at 131 in the overlapping X-ORF gene product. In the work by Li *et al*.,[118] it was shown by transfection studies in Huh7 hepatoma cells that the removal of the nuclear receptor binding site had no effect on the transcription of HBV mRNAs, the two codon change in the X-protein suppressed both transcripts, and that the creation of the HFN1 site restored the pgRNA level. What is more, analysis of revertants with either one or the other of the BCP mutations showed that the T1762 change is critical for the mutant phenotype.[107]

Chan and co-workers found that the double A1762T/G1764A mutation was significantly more common in genotypes that have C at nucleotide position 1858, while in contrast the precore stop codon mutation was found in patients with a T at the same position.[95] Nevertheless, the double BCP and the precore stop codon mutations are far from being mutually exclusive.[95,116] The BCP mutations as mentioned before have been found in patients regardless of HBeAg status. However, in anti-HBe-positive patients the double mutation was often accompanied by a change at position 1753, from T to C or G.[101] In addition, other point mutations upstream and downstream of T1762/A1764 have been described, occurring either alone or in combination with the double mutation, and in different settings, including chronic hepatitis, FHB, hepatocellular carconoma (HCC) and liver transplantation. [64,65,74,80,92,94,100,103,115,121,122] It was recently shown that CP mutations other than those at positions 1762/1764 could have a major impact on viral replication and HBeAg expression.[123] Transfection studies with full-length infectious clones reveal that CP mutants with additional mutations at 1753 (T to C) and/or 1766 (C to T) replicated at high level. Site-directed mutagenesis shows that combined mutations at 1762/1764/1766 and 1753/1762/1764/1766 result in higher replication rates and lower HBeAg expression than 1762/1764 mutations alone, whereas the 1753/1762/1764 variant was not much different from the double BCP mutant. Deletions within the CP region varying in length from 1 to 21 bp have been reported once again in different settings. These include fulminant hepatitis,[124] chronic hepatitis,[78,125–128] asymptomatic infection,[92,129] serologically silent infection,[129–132] HCC,[133,134] renal dialysis,[125,126] liver and renal transplantation,[135,136] as well as in patients who have survived haematological malignancies or solid tumours.[137,138] Such deletion variants are often characterized by low viraemia levels,[139] and may need help from the wild-type virus for survival. Transfection studies with clones having an 8-bp deletion in the BCP (nt 1768–1775), demonstrated reduced levels of transcription and progeny virus production.[140] In contrast, three BCP deletion variants (either nt 1758–1777 or 1749–1768) from Singaporean chronic carriers had high viraemia levels.[140] It appears, therefore, that the strain genetic background in which the BCP and A1896 mutations arise, in relation to additional ones, determine replication rate, expression of HBeAg and pathogenicity.

Another variant with a G1862T substitution affecting codon 17 has been described in chronic carriers,[33,142] HCC[47] and in patients with fulminant hepatitis.[31] In the latter study, this substitution was found in association with G1862T and G1899A, in the absence of the double BCP and A1896 mutations. All patients, being Chinese, were infected with genotype B. The G1862T mutation leads to substitution of valine for phenylalanine, affecting the –3 position in the signal peptidase recognition motif. As demonstrated by functional studies, this results in impaired processing of the precore/core protein into HBeAg.[31] It was previously suggested that since the G1862T change affects the side-bulge of ε involved in DNA primer synthesis, this may in turn interfere with replication.[47] However, the detection of the variant on its own and in the absence of wild-type virus argues against this hypothesis.[31] What is more, it suggests that the primer for negative-strand DNA synthesis is likely to be only three nucleotides long.

Strains carrying the C1856T variation that leads to substitution of proline for serine at codon 15 of the precore may behave in a similar manner to the G1862T variant. This amino acid change affects the signal peptide also, and may therefore affect production of HBeAg from the precursor precore/core, but there are no experimental data supporting this proposition at the moment. This variant was initially found by chance while sequencing

the complete HBV genome of an HIV-positive patient with a strange serological profile,[143] and then in Hong Kong Chinese patients with chronic hepatitis.[35] T1856 or A1896 was found in about half of each of the studied Chinese population, but because of their positions on opposite sides of the stem of the encapsidation signal, these variants are mutually exclusive. T1856 was found both in the HBeAg- and the anti-HBe-positive phases of the illness, quite unlike the usual picture with A1896. On long follow-up during the anti-HBe-positive phase, no selection for A1896 was seen in those with T1856. Further analysis of the core gene showed that these strains are independent lineages[144] that can be transmitted within families.[145]

Recently, an additional class of variants has been described in black South Africans with substitutions in the –5, –3 and –2 positions upstream from the AUG codon of the precore ORF, which affected the Kozak sequence. Triple mutants exhibited severely impaired HBeAg expression, while –5 and –2 double mutants had reduced HBeAg expression to levels seen with the double BCP variants.[146]

### Chronic and fulminant hepatitis patients

The double mutation has been detected with increased frequency in patients with fulminant hepatitis including children,[62,64,68,69,74,81,82,92,96,98,100,122,124,147–149] HBeAg- and anti-HBe-positive chronic hepatitis,[68,92,98,101–103,127,150,151] and HCC,[94,121,152,153] but less so in asymptomatic chronic carriers.[26,94,97,102,103,121,151,152] In addition, they have been detected in immunosuppressed,[97] liver transplant[100] and seronegative patients.[130,131] There are also reports in which the BCP double mutation has not been seen in patients of the same disease groups as above. For example, the 1762/1764 mutations were rarely seen in patients with FHB in the United States, even though these are common in chronic carriers.[98] Similar results have been reported from Brazil.[41] This once again may relate to differences between prevalent genotypes in these areas.[154]

### Immunosuppressed patients

In patients with severe recurrent disease following liver transplantation, it was shown that although pre- and post-transplant sequences in the BCP and precore/core region were very similar in each patient, in those with severe recurrence there were more nucleotide and amino acid substitutions in the precore/core encoding region, but not in the core promoter, than in patients with mild recurrence. There was also a link between severe disease and genotype D isolates.[100] However, additional studies indicated that immunosuppressed patients with severe liver disease, or end-stage cirrhosis, had HBV isolates with deletions, insertions and/or base changes in the BCP region,[98,135,155] leading to the appearance of new transcription factor binding sites (HNF1, HNF3) or duplication of others (C/EBP, HNF4).[135] Moreover, such variants also had other deletions in the core gene and/or deletions in the pre-S1/2 regions, the former of which are discussed later.

## Emergence of variants during chronic infection

There is still controversy as to which one of the two groups of variants is the most important with respect to disease activity and which one of the two appears first. Studies from the Far East (Japan) suggest that the BCP mutation is the one that appears first.[156–159] However, both can be present during the HBeAg-positive phase in mixtures with the wild-type virus.[28,159] Contingent with the appearance of these mutations is a reduction in HBV DNA levels. The mutations, as well as the reduction in HBV DNA levels, are associated with HBeAg seroconversion.[160,161] It seems, therefore, that reduced HBeAg expression (BCP mutation, and other mutations described above that result in reduced expression) or complete abrogation (precore stop codon) are a prerequisite for successful seroconversion to anti-HBe. The reduction in the level of HBeAg may remove a tolerizing effect on the immune system, allowing seroconversion from HBeAg to anti-HBe, clearance of the wild-type virus and selection of the variants, which become the dominant species. The liver disease in many patients enters a quiescent phase with little, if any, virus replication and levels of viraemia $<10^3$ copies/mL, and minimal changes in the liver. It is the cellular immune response to HBV that appears to hold the virus under control.[162] In some patients, however, the virus continues to replicate at moderate levels, biopsies show increased histological activity and amino acid substitutions begin to accumulate in the core region. In some patients, there are frequent acute exacerbations accompanied by increased viral replication and alanine aminotransferase (ALT) levels rise, which on occasion may result in fulminating disease. In such patients, progression to cirrhosis or development of HCC at a faster rate than carriers with milder disease is not unusual. The immunological mechanisms that may be involved during these stages of disease are discussed later.

## Core gene variants

### Amino acid substitutions

The finding that precore mutants are found in those with both mild and severe disease after seroconversion led to the investigation of changes in other genes. Variation in the core gene is related to genotype, and thus there is ethnic/geographical variation in incidence. There are

well-defined epitopes in HBcAg that are recognized by antibodies, Th cells and CTLs (Table 15.1). A number of groups have now described mutations that are selected in the core gene of patients with progressive chronic hepatitis. In HBeAg-positive patients from the Far East with severe hepatitis, greater clustering of substitutions was noted between amino acids 84 and 101, compared with those with mild disease[163–167] (Fig. 15.5). In Korean patients who seroconverted to anti-HBe, clustering was again seen between amino acids 84 and 101 of HBcAg, indicating that this is a consistent region of immune pressure in Far Eastern patients. A further analysis by this group indicated clustering of mutations in B-cell and Th epitopes.[168] Chinese patients also select more variants during anti-HBe seroconversion than during the immune tolerant phase.[169] In anti-HBe-positive Mediterranean patients with minimal hepatitis, very few changes were noted. [170] In contrast, a mean of five or six substitutions was seen in an anti-HBe-positive group with progressive disease. In a cross-sectional analysis, these were statistically significantly clustered in B-cell and Th epitopes.[170] A sequential study confirmed this finding for B-cell epitopes but revealed a weaker association with Th epitopes.[171,172] Of interest is that, in those who seroconverted from HBeAg to anti-HBe and went into clinical remission, multiple substitutions occurred at amino acids 50–69, a Th epitope. The contrast with progressive disease was thus striking. HBcAg mutations also accumulate in Th epitopes in Chinese patients with HCC.[173] However, in Spanish patients, there were many substitutions in and around the HLA-A2 class I epitope from aa 11 to 27 and the B-cell epitope from aa 74 to 83.[174] It is clear that studies of variation in presumed T-cell epitopes must be related to major histocompatibility complex (MHC) phenotype. One example is instructive. In Mediterranean patients, an amino acid change from threonine to serine at codon 12 of the core protein (T12S) is significantly associated with progressive disease. Although this change is very close to an HLA-A2-restricted CTL epitope, it has no association with human leucocyte antigen (HLA) type. These patients selected T12S only after they had seroconverted to anti-HBe and selected A1896.[170,174] Yet, in nearly all HBeAg-positive Japanese patients, serine was found at this position. The implication of these conflicting results from different geographical areas is either that the host is an important factor in viral evolution or that functional constraints in some isolates from Japan do not allow this variant to be selected. This finding of a temporal association between mutation in the HBeAg encoding gene and HBcAg, the former preceding the latter, suggests that HBeAg production must stop before selection pressure falls on HBcAg epitopes. It is of interest that this variation in HBcAg occurs around the time of A1896 selection.[170–172,175–177] In some patients with acute exacerbations during the anti-HBe-positive phase, there is clearance of the pre-existing strain and emergence of new viral variants with amino acid substitutions in epitopes suggestive of immune selection.[171,177] In contrast, asymptomatic carriers without hepatitis have invariant amino acid sequences. It seems that, once A1896 has been selected, because HBeAg shares common T-cell epitopes with HBcAg and the immune modulating effect of HBeAg has been lost, immune activity puts selection pressure on HBcAg.

Finally, the sequence of HBcAg from FHB cases is quite variable compared with strains from patients with chronic hepatitis.[61,65,66,178] This variation tends to cluster within epitopes and, as for chronic hepatitis, this is significantly associated with pre-selection of A1896.

## Core deletion variants

As mentioned earlier, genomic deletions affecting regions other than the BCP have been described for the core and pre-S coding regions. Deletions within the core gene are variable in length and usually affect the central region, although carboxy-terminal deletion variants have also been described. [128,175,179–182] Such deletions can

**Table 15.1** Statistical analysis of correlation between mutation and B-cell and Th epitopes in core protein*

| | | HBeAg to anti-HBe and into remission | | | Continuously anti-HBe-positive with severe disease | | |
|---|---|---|---|---|---|---|---|
| Epitope | T/B | Odds ratio | 95% confidence interval | *p* value | Odds ratio | 95% confidence interval | *p* value |
| 1–20 | T | 0.81 | 0.13–3.74 | 0.99 | 0.7 | 0.31–1.50 | 0.33 |
| 50–69 | T | 5.64 | 2.12–14.85 | 0.00045 | 1.09 | 0.56–2.07 | 0.78 |
| 74–83 | B | 0 | – | – | 2.59 | 1.39 | 0.00084 |
| 76–89 | B | 0 | – | – | 0.85 | 0.36–1.90 | 0.67 |
| 107–118 | B | 1.42 | 0.02–6.69 | 0.65 | 1.31 | 0.61–2.71 | 0.45 |
| 128–135 | B | 1.04 | 0.02–7.06 | 0.99 | 3.03 | 1.58–5.68 | 0.00015 |
| 130–138 | B | 0.92 | 0.02–6.18 | 0.99 | 2.56 | 1.35–4.80 | 0.0014 |

*The odds ratio and 95% confidence intervals represent the likelihood of amino acid substitutions occurring in the epitope in comparison to the remaining core sequence. *p* values are calculated using the $\chi^2$ test or Fisher's exact test. T/B, T- or B-cell epitope

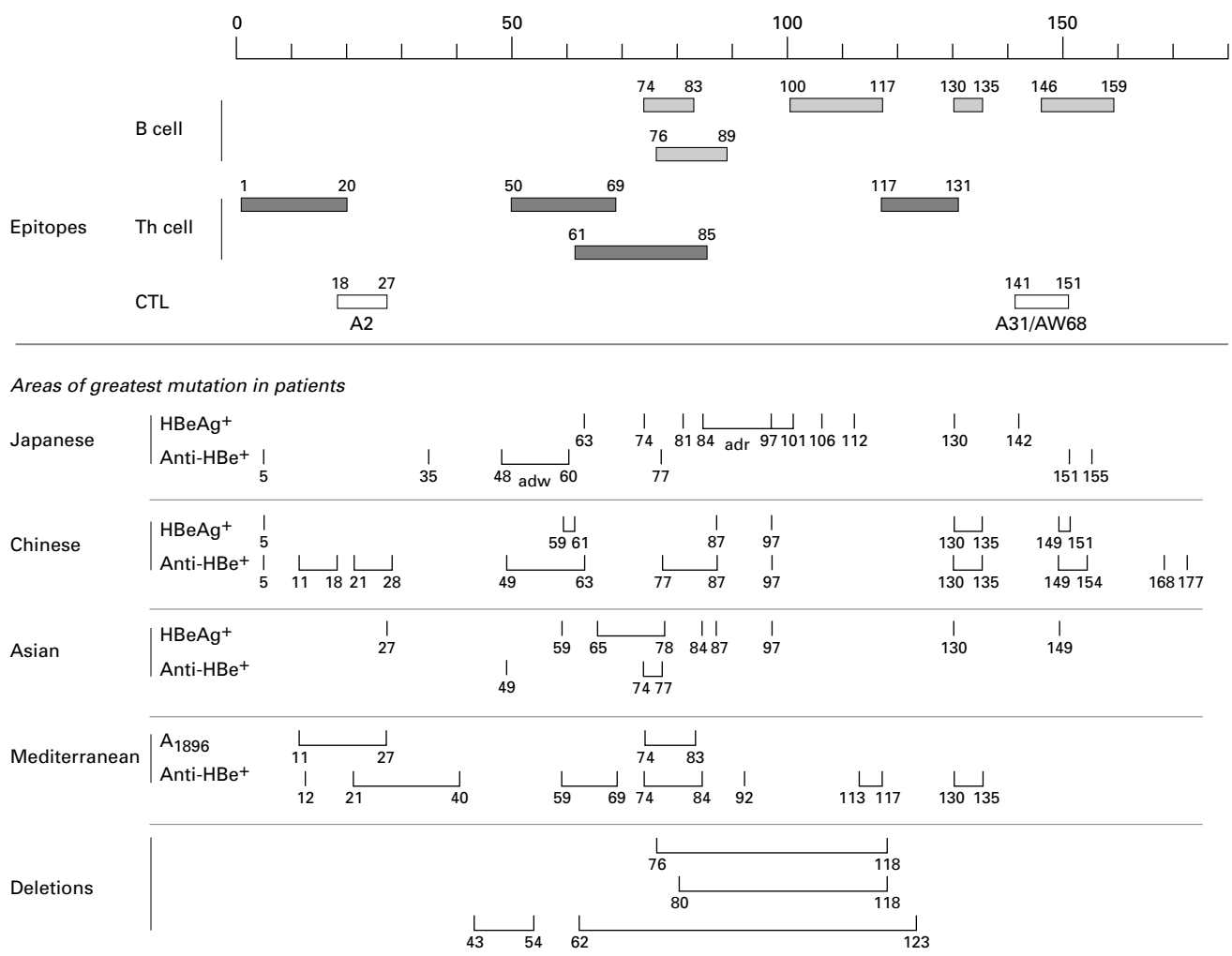


**Figure 15.5** HBcAg variability correlated with immunologic epitopes. The Th, B-cell and CTL epitopes are shown above. Below are shown areas of greatest variability highlighted by the authors or, if not provided, our subjective interpretation. Note that there is little consistency in these results. This may reflect methodological differences such as clinical parameters and a variable definition of consensus sequences against which results are compared, as well as ethnic and HLA profiles of the patients. Alternatively, it may simply be that there is no correlation. Deletions are also indicated; note that they cluster around the middle of the protein.

be in frame, resulting in the production of truncated core species, or out of frame, terminating core synthesis prematurely. There is experimental evidence to suggest that deletion variants are unable to produce capsids or that any capsids formed are unstable. Such variants therefore co-exist with the wild-type virus that provides the core protein for their encapsidation.[155,183,184] In view of this, the suggestion has been put forward that these deletion variants may act as defective interfering particles, involved in a cyclic interference-and-enrichment phenomenon between the variants and the wild-type.[183–186] Core deletion variants have been reported both in immunocompetent[181,187] and immunosuppressed individuals.[180,188] In the former individuals, the detection of core internal deletion variants was associated with lower levels of viraemia and early seroconversion to anti-HBe, with concurrent disappearance of the variants.[181,187] In contrast, in long-term immunosuppressed patients following kidney, or even liver transplantation, the presence of deletion variants was associated with increased risk of developing liver cirrhosis and end-stage liver disease.[180,187] Why this should be the case remains unanswered. It has been suggested that internally deleted core variants may represent immune escape variants, particularly if they are lacking the region containing the main B-cell epitope. It should be pointed out that core internal deletion variants have also been described in the woodchuck animal model.[189,190]

Two further types of genetic variant exist. The first is a 36-bp insertion found around and including the core ATG associated with A1896.[30,142] It was initially seen in an HIV-infected patient and a carrier treated with inter-

feron (IFN). These are likely to be isolates of the newly described genotype G of the virus. Isolates of this genotype have a stop codon at codon positions 2 and 28 of the precore, and a 36 nucleotide insertion at the N-terminal end of core.[191] It has also been observed that this genotype may co-exist with genotype A, which explains the HBeAg positivity in such patients, in spite of the presence of the precore stop codon mutation.[192] However, little is known about the pathogenicity of genotype G, as its detection worldwide remains quite low at present.

The second type of genetic variant is an abnormal spliced mRNA species that may play a role in disease progression.[193,194] This singly spliced 2.2-kb RNA leads to the secretion of defective particles, which are more common in those with chronic hepatitis. After transfection, HBcAg accumulated in cells and increased amounts of HBeAg were secreted.

## Mechanisms of emergence of variants

The major issue in explaining the origin of A1896, the BCP and all other variants that lead to diminished production or absence of HBeAg, is whether such strains have a survival advantage over HBeAg-producing ones. If they do, the implication is that they will eventually become dominant. In addition, if these variants do cause more severe liver disease, then these findings would give cause for concern. T1762/A1764 and/or A1896 strains appear to have become dominant in Mediterranean countries (measured as new presentations to liver clinics) and the Far East, but this could be because these patients present later than those with HBeAg-producing strains, and there has been a general reduction in new HBV cases across the world, as a result of effective vaccination programmes.

There is growing evidence that a vigorous and efficient polyclonal class I restricted CD8+ CTL and class II restricted CD4+ T-helper response to HBV proteins is necessary for recovery from acute symptomatic infection.[51,195,196] A strong and multi-specific response against epitopes from core, polymerase and envelope proteins is critical for a successful outcome during the acute phase of HBV infection and in recovery.[197–201] Such responses persist in recovered individuals, probably as a result of continued CTL activation in the presence of low levels of viraemia, which they continue to keep under control.[202] More recently, it has been shown that HBV replication within hepatocytes can be inhibited by antiviral cytokines without the requirement of hepatocyte lysis.[203,204] The absence of similar responses in patients with chronic hepatitis almost certainly contributes to viral persistence. CTL responses are of low frequency in such patients, they have a narrow repertoire and although insufficient to clear the virus, they contribute to the inflammatory picture seen in liver biopsies.[162,205–207] Failure of antigen presentation to CD4+ helper T cells,[208] failure of such cells to proliferate,[209] immune modulation by secreted proteins,[10,11] viral mutation and integration of viral DNA into the cellular genome[52] may all be contributory factors. The CTL response in chronic carriers seroconverting to anti-HBe naturally or after IFN therapy, has been shown to be similar in strength to that seen during acute hepatitis.[50,210,211] This indicates that immune responses against HBV can recover under the appropriate conditions. The above described findings have in recent years been confirmed and extended using more sensitive assays than chromium release, such as ELISPOT and tetramer technology.[212–216]

It is generally accepted that the natural history of chronic HBV infection goes through at least three phases. These are the immune tolerance and immune clearance phases when the patient is HBeAg-positive, and the low replicative phase following seroconversion to anti-HBe.[40] This latter phase, in view of what has been described above, can in a subgroup of patients be followed by a replicative/reactivation phase, characterized by the emergence of variant viruses.[56] The immunological events that lead to the predominance of the variants and suppression or clearance of the wild-type virus are not clear, but one can speculate on the possible mechanisms that may be involved. The immune clearance phase is characterized by reduced HBeAg and fluctuating but progressively diminishing HBV DNA levels, which are followed by increasing transaminase levels and seroconversion to anti-HBe. It is suggested that HBeAg is the most significant factor in tolerizing the immune system to the presence of high levels of replication during the tolerant phase.[11,217] Moreover, HBeAg has been shown to elicit a Th-2-like immune response[12] while downregulating Th-1 responses by inducing apoptosis of the effector cells.[217] Diminishing levels of HBeAg therefore lead to progressive breakage of tolerance. In addition, the intracellular distribution of HBcAg shifting from nuclear to cytoplasmic, followed by membrane display of core peptides, may result in CTL attack.[218] It has been shown that some variants of the nuclear localization region, found in natural isolates as well as those generated *in vitro*, lead to redistribution to the cytoplasm after expression *in vitro*.[219] Hepatocytes harbouring the wild-type virus come increasingly under attack and are destroyed, and the immune system contains any residual replication. At this time HBV DNA is detectable only by very sensitive techniques, such as real-time PCR. Hepatocytes containing pre-existing variants or variants arising during the non-replicative phase, escape immune recognition.[220–222] Thus, during this phase the number of patients carrying detectable variants increases significantly. Why they do so remains unknown, but one contributing factor may be low level expression of viral proteins, but also accumulation of mutations within critical epitopes that allow

the infected cell to escape lysis by CTLs. It appears that in some patients this may be the case,[162,171,177,223] while in others, mutations do not occur with each flare, at least in HBcAg.[172] Some may have transient variants that may attract immune attention and then be cleared.[171] Whether anti-HBe plays a role in antibody-dependent cell cytotoxicity during this period is not fully known, but is a possibility,[224] as HBeAg can be expressed on the cell surface.[225] These events would undoubtedly favour the elimination of HBeAg-expressing hepatocytes. During this period mixtures of variants and wild-type virus are detectable at very low levels.[23]

In many patients, the low replicative phase may last for years. HBV DNA is absent or barely detectable during this time, histological examination shows minimal changes in the liver and as a result transaminase levels are normal or barely raised. These patients are the asymptomatic or normal carriers.[226,227] However, in some patients the low replicative phase is followed by HBV reactivation, immune-mediated liver cell injury and increases in transaminase levels. Such patients appear to be seen in clinics more frequently in recent years.[228–230] These reactivation episodes may occur at frequent intervals and may be accompanied by increases in IgM anti-HBc levels.[171] During these cycles of reactivation the immune system exercises control of the wild-type virus. It has been shown that antigen-presenting cells infected with HBeAg-expressing constructs but not those infected with HBcAg were able to stimulate HBcAg/HBeAg-specific CD4(+) T-cell clones.[231,232] Therefore, HBcAg-expressing cells may be spared, allowing the dominance of HBeAg-negative variants. By this stage the precore stop codon appears even in strains carrying the BCP double mutation. Moreover, additional mutations may appear in the core region, some of which are in well recognized B- and T-cell epitopes,[171,187,233] or may affect areas that influence intracellular localization such as the carboxy-terminus.[100,50,168] Interestingly, patients who exhibit a more severe form of HBV infection such as FHB[122,150] or who have had liver transplants can have several mutations in the carboxy-terminus of HBcAg. In mice transgenic for HBV replication, CTLs can clear active infection by a post-transcriptional mechanism mediated by IFN-γ and tumour necrosis factor-α (TNF-α).[76] If cytokines prove to exert as strong an antiviral effect in natural HBV infection as is observed in the transgenic mouse model, investigation of this area may prove fruitful in explaining viral persistence and ongoing disease.

## HBeAg-negative variants and treatment

### *Interferon*

Therapeutic interferon (IFN), while inducing an antiviral state, is also believed to amplify immune activity against infected hepatocytes, perhaps by enhancing MHC expression, thus facilitating presentation of peptides to CTL. HBeAg-positive patients who respond to IFN already have evidence of immune activity, as shown by raised transaminases and relatively low levels of HBV DNA. Patients at this stage may have minor populations of HBeAg-negative variants present. One small study showed that those with precore mutants in the HBeAg-positive stage are more likely to respond to IFN,[234] although this was not confirmed in two other studies,[235,236] which showed no precore variant in HBeAg-positive patients before seroconversion, whether they responded to IFN or not. In other studies, however, where HBeAg-positive patients were investigated for both the precore and BCP mutations, such patients responded better to IFN in the presence of these mutations.[127,237,238] Others have found low frequency of mutations in the BCP region in HBeAg-positive patients and of little help in predicting IFN response,[239] while one other study indicated that HBeAg-positive patients responding to IFN had a high number of mutations in the BCP region in contrast to anti-HBe-positive patients who had a low number.[240] Anti-HBe$^-$, HBV DNA-positive Mediterranean patients with chronic hepatitis often respond poorly to IFN therapy[36,241] and if they do respond, they tend to relapse.[242–245] Interestingly, T1856-containing strains may respond poorly to IFN,[246] compared with A1896 or G1896/C1856 strains, but this remains to be confirmed. In keeping with this is the observation that anti-HBe-positive patients with C1858 (which is often associated with T1856) have more inflammation and fibrosis than those with A1896.[46] A wide variety of stop codons, non-functional start codons and amino acid substitutions arise in HBeAg-positive cases who respond to IFN therapy.[247] One study[235] also compared the frequency of selection of mutants after successful IFN therapy with natural seroconversion to anti-HBe. There was a trend towards less frequent and slower emergence of mutants in those who seroconverted during IFN therapy. Sequence variability of HBcAg is greatest in anti-HBe-positive non-responder patients.[235] These substitutions were greatest in the 'promiscuous' Th epitope between amino acids 50 and 69. This concept was supported by the finding that variability in the HLA-A2 CTL epitope was greatest in IFN non-responders.[249] However, no correlation was found by another group in Chinese patients.[169]

### *Lamivudine*

Lamivudine, a nucleoside analogue, has also been used for the treatment of anti-HBe-positive patients with HBV variants carrying the BCP and precore mutations. Such patients respond well whilst on treatment, with 65–96% showing normalization in ALT levels and loss of HBV DNA. However, on stopping therapy the major-

ity relapse and sustained response rates range between 10% and 15%.[245] Treatment for periods longer than 1 year achieves higher rates of sustained response (40%), but the patients run the risk of developing lamivudine-resistant variants. It has been established that lamivudine treatment results in the replacement of the BCP and precore variants by the wild-type virus albeit at low titres, and during prolonged treatment the variants reappear.[249–251] In HBeAg-positive patients carrying genotype C, the presence of BCP/precore mutations was independently associated with HBeAg loss. Moreover, this occurred earlier in the presence of variant isolates than the wild-type virus.[252]

### Precore variants: detection methods

As sequencing can be time-consuming, various approaches have been tried to screen PCR products for A1896. Hybridization with oligoprobes has met with success[253] and has been applied to clinical material to assess the relative importance of mutants to clinical severity.[254] Alternatives are to use a common primer at one end of the DNA of interest and a second that contains either a G or an A at the 3′ end,[70] which has been applied to paraffin-fixed sections, single-labelled nucleotide (G or A) addition to a primer that ends either at 1895 or 1897[255] and digestion of PCR products that have been generated with primers incorporating novel restriction sites.[53] However, none of these are as comprehensive as sequencing. Recently, a line probe assay has been released by Inno-Lipa for the detection of the precore and BCP mutations, as well as HBV genotypes. This has so far proved to be rapid, sensitive and reliable.[256]

## Conclusion

HBV is highly evolved. It has a small and compact genome that makes very efficient use of its nucleotide sequence. Many regions are highly conserved, even between different hepadnaviruses. Yet the potential for significant change is also present. The interaction between HBV and its host is complex. The precore/core gene and BCP variants have been described in detail but why such variants arise is still not clear. Their appearance, however, heralds the beginnings of the seroconversion phase from HBeAg to anti-HBe. Whatever the nature of the variant, the end result is the reduction or abrogation of HBeAg production. The removal of the tolerogenic effect of this soluble serum protein leads to the 'awakening' of the immune response. However, the mechanisms of selection of the variants and clearance, or downturn of wild-type virus production, remain to be defined. Equally undefined are the determinants of active liver disease once the variants are selected. There may be an interplay between host genetic make-up and further virus variation within the core protein, for example, and possibly other viral proteins. These variants are increasingly recognized as independent lineages of HBV, as markers of variation elsewhere in the genome and of clinical relevance. These variants are associated with fulminant hepatitis but host factors are also necessary. Resolution of these issues is still hampered by the absence of a reliable and robust cell culture system. Moreover, the use of cloned material in transfection studies, a lot of the time using described genomic mutations out of the genetic background in which they are found, has caused additional confusion. Undoubtedly, we still have some way to go in identifying those genomic mutations that are important and are clinically relevant.

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# Chapter 16
# Natural history of chronic hepatitis B and hepatocellular carcinoma

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Approximately 350 million people have chronic hepatitis B virus (HBV) infection, which may present as typical hepatitis B e antigen (HBeAg)-positive chronic hepatitis B or as HBeAg-negative infection.

HBV is classified into four serotypes (adr, adw, ayr and ayw) based on antigenic determinants of the surface antigen: prevalence of these subtypes varies in different parts of the world. Based on an intergroup divergence of 8% in the nucleotide sequence, HBV can be classified into seven genotypes, A–G: prevalence of these genotypes again varies in different parts of the world. The most common precore mutation, a G to A substitution at nucleotide 1896, common among HBeAg-negative patients,[1] is found in association with genotypes B, C and D but not genotype A, thus explaining the prevalence of HBeAg-negative chronic hepatitis B in southern Europe and Asia.[2]

Studies of naturally infected humans established the existence of acute and chronic infections with varying degrees of liver damage and the existence of a linkage between HBV infection and hepatocellular carcinoma (HCC).

Further understanding of the natural history of hepatitis B would not have been possible without the contribution of animal models. Chimpanzee inoculation studies demonstrated the infectivity of cloned HBV DNA.[3] In similar experiments, inflammatory changes in the liver, resembling those of viral hepatitis B, were observed in adult rats injected with HBV DNA directly into the liver.[4]

Studies in transgenic mice have led to the hypothesis that HBV may be directly cytotoxic to the liver in the presence of overproduction of HBV envelope polypeptides.[5]

Many intriguing aspects of the natural history of HBV await discovery. One such aspect is the many cases with 'occult' HBV infection that can be detected by studies with the polymerase chain reaction (PCR) technique. There is a low level of HBV replication in a variety of clinical conditions, including in individuals who lack all serological markers of HBV infection. However, it is still unclear whether occult HBV infection causes progressive damage to the liver or is an innocent bystander.[6]

## Natural history of infection

The clinical spectrum of chronic HBV infection ranges from asymptomatic HBsAg carriage with normal hepatic function and histology, to chronic hepatitis with varying degrees of histological severity, to cirrhosis and its complications. HBV infection is a dynamic process characterized by high and low replicative phases controlled by the host immune response. The outcome of HBV infection is largely influenced by the age at which infection is acquired, the integrity of the host's immune response and exposure to environmental co-factors.

### HBeAg-positive patients

See Table 16.1.[7–19] Chronic infection with HBV is more frequent in men than in women, in neonates than in adults (90% versus 5%) and in immunocompromised patients than in immunocompetent ones.[8] The risk of chronicity declines from 60% during the second year of life to 10% by 6 years of age.[7]

HBV infection acquired in the neonatal/perinatal period is characterized by a prolonged immunotolerant phase, normal or near-normal alanine aminotransferase (ALT) levels and a low rate of spontaneous HBeAg clearance.[20] The initial immunotolerant phase with serum HBeAg and high levels of serum HBV DNA is followed by the immunoactive phase, characterized by a decrease in serum HBV DNA and increase of serum ALT levels.[9] Most patients will seroconvert to anti-HBe with remission of liver disease: a significant number of these anti-HBe-positive patients will show hepatitis reactivation; others will proceed to an inactive HBsAg carrier state; a few will have hepatitis resolved with loss of HBsAg and anti-HBs seroconversion. In contrast, patients who acquire HBV during adolescence or adulthood and

**Table 16.1** The natural history of HBeAg infection

| Measure | | Reference |
|---|---|---|
| Chronicity rate | | |
| Neonates | 60% | McMahon *et al.*[7] |
| Adults | 5% | Taylor *et al.*[8] |
| Seroconversion to anti-HBe/year | | |
| Children (China) | 0 | Lok *et al.*[9] |
| Children (Italy) | 16–19% | Bortolotti *et al.*[10] |
| Adults | 16–50% | Fattovich *et al.*,[11] McMahon *et al.*,[12] Yuen *et al.*[13] |
| Reactivation after anti-HBe seroconversion/year | | |
| Children | 0 | Bortolotti *et al.*[10] |
| Adults | 4.4% | Fattovich *et al.*[14] |
| Rate of cirrhosis/year | | |
| Italy | 5.9% | Fattovich *et al.*[15] |
| Taiwan | 2.4% | Liaw *et al.*[16] |
| 5-year survival of patients with cirrhosis | | |
| United States | 66% | Weissberg *et al.*[17] |
| Netherlands | 72% | De Jongh *et al.*[18] |
| Germany | 78% | Niederau *et al.*[19] |

become chronic carriers usually present in the immunoactive phase with active liver disease.

HBeAg seropositive patients with replicating HBV display various degrees of liver damage, from benign forms of chronic lobular hepatitis to more severe forms of active cirrhosis and HCC. Chronic active hepatitis is the result of persistent HBV replication eliciting host immune attacks on infected liver cells, and in most cases it subsides when virus replication is terminated and seroconversion to anti-HBe takes place.[6] For 70 adult Italian patients, the annual rate of HBeAg/anti-HBe seroconversion and disease remission was 16%.[11] Clearance of serum HBeAg in Chinese children is much slower: none of 11 children developed anti-HBe in a 2-year period.[9] Instead, in a 5-year period, 70% of 76 Italian children seroconverted to anti-HBe, cleared HBV DNA, and remained with persistently normal ALT values.[10] In no case was there reactivation of HBV after spontaneous anti-HBe seroconversion, suggesting that these children had become healthy carriers. Termination of HBV replication and clearance of HBeAg and HBV DNA were observed in 45 (51%) of 88 adult Italian patients followed for a mean of 5 years.[14] Although this event was accompanied by clinical, biochemical and histological remission of disease, during a 5-year follow-up 10 (22%) patients had transient spontaneous reactivation of HBV infection and exacerbation of disease. Thus, at variance with infantile HBV infections, seroconversion from HBeAg to anti-HBe during adulthood is not always stable. In two adult patients, HBV reactivation led to deterioration of the underlying liver disease from chronic active hepatitis to active cirrhosis. Similar studies with similar results were carried out in Chinese patients by Tong *et al*.[21] and Lok *et al*.[9] Reactivation of a latent HBV infection was a frequent event in immunocompromised patients infected with human immunodeficiency virus (HIV),[22] homosexuals[23] and patients treated with immunosuppressive agents.[24] In immunocompetent patients, the probability of clearing HBeAg within 5–10 years of diagnosis is about 50%.[10–13]

Seroconversion to anti-HBe is paralleled by exacerbation of hepatitis as a result of immune-mediated liver cell necrosis and progressive clearance of infected hepatocytes and serum HBV DNA ('inactive HBsAg' carriers). After seroconversion to anti-HBe, inactive carriers show long-term non-replicating latent HBV infection as a result of integration of the HBsAg coding for viral DNA into the liver cell genome. The condition of healthy carriers is clinically a long-term benign situation. A prospective cohort study of 92 Italian healthy carriers showed that the prognosis for these subjects was excellent, with a low risk of developing cirrhosis or HCC over 10 years.[25] Similar results were obtained in a prospective study of 317 asymptomatic HBsAg carriers from the Montreal area who were examined after 16 years of follow-up.[26] Three carriers had died of HBV-related cirrhosis, one of alcoholic cirrhosis and nine of causes unrelated to liver disease. No carrier had died of HCC. Determinants of HBeAg seroconversion include female sex, advanced age, ALT levels higher than five times the upper normal limit and severity of histological damage and genotype B of HBV.

### *Severity of the disease*

Persistent HBV replication is instrumental in the progression of the disease to cirrhosis and HCC. The prognosis for infected patients depends on the histological stage of the liver disease. Of 105 Italian patients who were followed prospectively for a mean of 5 years, 34%

of the patients with detectable amounts of serum HBV DNA developed cirrhosis, but only 15% of those without serum HBV DNA detectable by dot-blot technique.[15] In these patients, bridging hepatic necrosis was the histological feature predictive of cirrhosis. In a prospective study of 509 Chinese patients with chronic hepatitis B, cirrhosis developed within 6–64 months after entry in 35 patients, with a calculated annual rate of 2.4%.[16] The incidence of cirrhosis was significantly increased with increasing age at entry, in patients with repeated episodes of hepatitis exacerbation without anti-HBe seroconversion. In an 8-year prospective study in Düsseldorf of 53 patients with chronic infection,[19] 13 developed severe clinical complications without eliminating serum HBeAg. Ascites developed in two patients; jaundice and encephalopathy in one; jaundice, encephalopathy and oesophageal varices in one. Four patients had only oesophageal varices, and bleeding from the varices occurred in three of them during follow-up. Four patients died and one required liver transplantation.

### *Survival*

In 379 patients in the United States who had chronic hepatitis B, the 5-year survival rate was 97% for patients with mild forms of hepatitis, 86% for those with chronic active hepatitis, and only 66% for patients with cirrhosis.[17] In a German study of 53 patients with chronic HBeAg infection, four patients died and one required liver transplantation during a mean follow-up of 38 months. Survival without clinical complications was longer for the patients who spontaneously cleared HBeAg than for those who did not ($p = 0.006$).[19] The overall probability of survival for 43 Dutch patients with compensated cirrhosis was 72% at 5 years.[18] The variables significantly associated with the length of survival were age, serum AST levels, presence of oesophageal varices and the Child-Pugh scores. Multivariate analysis revealed that only age, bilirubin and ascites were independently associated with survival. As expected, the 5-year survival rate of patients with decompensated cirrhosis, determined by the presence of ascites, jaundice, encephalopathy and oesophageal bleeding, was much shorter than that of patients with compensated cirrhosis (14% versus 84%). Both compensated and decompensated HBeAg-positive patients survived for less time than the HBeAg-negative patients with cirrhosis. In a multicentre study, the calculated 10-year survival of 126 European patients was 68%.[27] Clearance of serum HBeAg during follow-up occurred in 64 patients and was more common in the 92 survivors than in the 24 who died (65% versus 17%, $p < 0.001$). Thus, once more, termination of virus replication during follow-up was correlated with better survival.

Despite specific immunoprophylaxis, recurrence of hepatitis B is a well-documented complication of post-transplantation HBV liver disease.[28] HBV almost invariably recurred in patients with serum HBV DNA, with massive virus load and a tendency to hepatitis with progressive fibrosis. The time lag between transplantation and recurrence of hepatitis was 41–307 days, and the short-term mortality rate of the recipients because of liver failure was 27%.[29]

## HBeAg-negative patients

See Table 16.2.[16,18,27,30] Approximately 5% of patients undergoing HBeAg seroconversion may continue to show increase in serum ALT and high levels of HBV DNA. These patients and those undergoing hepatitis reactivation after HBeAg seroconversion constitute the group of patients with HBeAg-negative chronic hepatitis B.

Virus heterogeneity is another important factor in the natural history of HBV infection. Genotypic variants of HBV were originally identified in an interesting group of already anti-HBe-positive patients who had serum HBV DNA measurable by dot-blot hybridization.[31–33] The more common genetic defect was a point mutation in the precore region of the HBV genome, generating an in frame stop codon at nucleotide 1896, which prevented the secretion of the HBeAg product of the nucleocapsid gene.[1] However, there were many other possible sites for mutation of an in frame stop codon.[34] Precore variants were not present as major species in the HBeAg phase of hepatitis, but emerged during or some time after seroconversion to anti-HBe. Immediately after seroconversion, a mixture of strains was present, but with time the wild-type became lost.[35] This selection process occurred in patients with both acute and chronic liver disease.

Chronic hepatitis B with anti-HBe in serum differs from chronic hepatitis B with HBeAg in terms of natural history. Its specific feature is the high frequency of spontaneous remissions followed by hepatitis exacerbations.[36] Latent replicative intermediates of precore variants are present in the livers of the patients during remission phases of the chronic liver disease and may pose problems of differential diagnosis with inactive HBsAg carrier state.[37] However, these apparently inactive reservoirs of mutant HBV strains may predispose to bouts of virus reactivation and worsening of the disease. It is not clear whether precore variants are the cause of

**Table 16.2** The natural history of Anti-HBe infection

| Measure | | Reference |
|---|---|---|
| Rate of cirrhosis/year | 1.3% | Liaw *et al.*[16] |
| Rate of decompensation in patients with cirrhosis/year | 3.7% | Fattovich *et al.*[30] |
| Rate of HCC/year | 1.5% | Fattovich *et al.*[30] |
| 5-year survival | 97% | De Jongh *et al.*[18] |
| 10-year survival | 68% | Realdi *et al.*[27] |

severe liver disease *per se* or whether genetic mutations arise because of a successful response against HBeAg.

### *Severity of the disease*

One intriguing aspect is the varying degree of severity of liver disease observed in these patients, which seems to follow a geographical pattern.[38] In northern Europe and the United States, mutant strains occur rarely in hepatitis B, but were constantly detected in patients with fulminant hepatitis.[39] In Asia, precore variants were never detected in patients with self-limited resolving hepatitis.[40] Interestingly, they have been found in 18 carriers, unrelated to the severity of the underlying disease, including healthy carriers.[34] In 39 Bulgarian patients with chronic hepatitis B, severe liver disease was more common in patients with predominantly precore variants infection and high levels of viraemia.[41] The same was true for 23 Italian patients who were monitored for 8 years. Of the 20 patients with initial diagnoses of chronic hepatitis, seven were found to have cirrhosis at the end of the follow-up period.

In a prospective study of 175 Chinese patients, the calculated annual incidence of cirrhosis was similar to that (2.4%) found in 509 patients with HBeAg infection.[16] Like HBeAg cirrhosis, the incidence of cirrhosis in patients with anti-HBe chronic hepatitis B was correlated closely with the patient's age and frequency of acute exacerbations with HBeAg reactivation.

In a multicentre European study, the 10-year probability of patients with anti-HBe cirrhosis remaining compensated was 63%, indicating that hepatic decompensation usually occurs at a late stage in the clinical course of the disease. [30] During a mean follow-up period of 6 years, HCC developed in 9% of the 349 patients with compensated cirrhosis. By multivariate analysis, age, serum levels of platelets and liver firmness on physical examination were independent predictors of HCC. In our centre, HCC developed in 6 of 57 patients with anti-HBe cirrhosis who were monitored prospectively for 10 years (mean follow-up, 81 months). The yearly incidence of tumour was approximately 1%. During the 10-year follow-up period, eight patients (13%) developed complications other than HCC: four developed jaundice, two developed ascites and two bled from oesophageal varices.

### *Survival*

For 44 patients with compensated cirrhosis, the 5-year survival rate was 97%, compared with 72% for the 54 HBeAg-positive patients.[18] In a multicentre study, the 10-year survival rate of 240 European patients with anti-HBe-positive cirrhosis was 68%.[27] In Milan, the 10-year survival rate of a cohort of 57 patients with anti-HBe-positive cirrhosis and no signs of HCC who were monitored prospectively was 82%. Discrepancies in the survival rates between these studies could be accounted for by different criteria of patient recruitment and follow-up.

## Natural history of HCC

HBV-related HCC is the predominant cause of cancer mortality in Africa and China. Epidemiological and molecular epidemiology studies have disclosed that dietary exposure to aflatoxin is a synergistic risk factor for HBV-related HCC in these geographical areas.[42] There is also evidence that co-infection with hepatitis C virus (HCV) and heavy alcohol consumption increase the risk of developing HCC in HBV hyperendemic areas. In a prospective study of 11 893 men in Taiwan (2359 HBsAg-positive) Yang *et al*.[43] demonstrated 1169 cases of HCC for 100 000 person-years among the HBeAg carriers compared with 324 cases for the HBeAg-negative carriers and 39 cases for the HBsAg-negative subjects. After adjustment for age, sex, hepatitis C, tobacco smoking and alcohol abuse, the relative risk of HCC was 60.2 for HBeAg-positive carriers and 9.6 for HBeAg-negative carriers compared to HBsAg-negative subjects. This study clearly established that in areas hyperendemic for HBV, HCC may develop prior to seroconversion to anti-HBe, probably as a result of continuous or recurrent cycles of liver cell necrosis and proliferation. It is, in fact, well established that the inflamed liver is a mitogenic and mutagenic environment.[44] The same holds true for patients with HBeAg-negative infection who develop HCC. Most information on growth patterns and doubling volume times of HCC and of patients' survival comes from studies of patients with tumours of miscellaneous aetiologies or with predominantly HCV-related disease. With few exceptions, HCC is a slowly developing disease. One such exception is HCC among African black people, which is mostly associated with chronic infection with HBV and exposure to aflatoxin. This tumour, in fact, is quite different clinically and histopathologically from the slow-growing tumours seen in Japan and the Mediterranean basin, which are more often associated with infection with HCV. The most impressive difference noted between HBV-related tumours and HCV-related tumours in both the Western and Eastern hemispheres was that the patients with the former tumours were 10 years younger than those with HCV-related disease.[45,46] Differences in the epidemiology and age of infection with these two viruses could account for the observed discrepancies between HBV- and HCV-related tumours. HCCs developing after HCV infection have been reported to occur in patients with more severe histological features of liver disease than tumours developing after HBV infection.[47,48] In a 10-year prospec-

tive study of 57 patients with compensated HBV cirrhosis at our centre, six developed HCC that was unifocal in three and multifocal in the other three. In 175 Japanese patients who had undergone hepatic resection, HCCs in HCV-positive patients showed a higher incidence of multicentricity than those in HBsAg-positive patients (14% versus 0, $p < 0.05$).[49] In a retrospective clinicopathological study of 113 non-alcoholic Japanese patients with HCC, the HBsAg-negative patients, who were likely to be infected with HCV, more often had expanding-type tumours than the HBsAg-positive patients, who more often had infiltrative-type HCCs.[45] In a study in Japan, the 3-year survival rate for patients with HCV-related tumours was higher than that for patients with HBV-related tumours (68% versus 47%), implying that HBV-related disease was a faster process than the HCV-related cases.[46]

## Summary

The natural history of chronic hepatitis B depends largely on the age at which patients become infected, the integrity of the host's immunity, the interaction with environmental co-factors and the emergence of genetic variants of HBV. Chronic hepatitis caused by HBeAg-secreting strains is more common in men than in women and in neonates than in adults. Seroconversion to anti-HBe occurs less frequently in men and neonates than in women and adults. Hepatitis reactivation after anti-HBe seroconversion never occurs in children. The yearly rate in adults is 4.4%. The yearly incidence of cirrhosis is 2.4–7%, and the 5-year survival rate of patients with cirrhosis is 66–78%. In anti-HBe infection, the yearly rate for cirrhosis is similar to that in HBeAg-positive patients, and the rate of clinical decompensation is 3.7%. Approximately 1.5% of patients with cirrhosis develop HCC every year. The 5-year survival rate of patients with anti-HBe cirrhosis is higher than that of patients with HBeAg cirrhosis (97% versus 66%). The natural history of chronic hepatitis B is accelerated in the immunocompromised patient.

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# Chapter 17
# Hepatocellular carcinoma: molecular aspects in hepatitis B

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Hepatocellular carcinoma (HCC) is among the most common cancers in the world and one of the rare human cancers showing seroepidemiological association with viral infection. The role of hepatitis B virus (HBV) as a major aetiological agent of HCC has been firmly established, and the increased risk of developing HCC has been estimated to be 20–100-fold for chronic HBV carriers as compared with non-infected populations, placing HBV in the first rank among known human carcinogens.[1,2] Apart from epidemiological evidence, another factor linking HBV and liver cancer comes from related animal viruses, which with HBV form the hepadnavirus group and induce acute and chronic infections of the liver and eventually HCC (reviewed by Schödel *et al*.,[3] Buendia[4]). Whether HBV acts through any recognized oncogenic mechanism, either directly or indirectly, is an important unsolved question.

It is generally considered that HBV has no direct oncogenic or cytopathological effect on the infected hepatocyte. Malignant transformation occurs after a long period of chronic liver disease, frequently associated with cirrhosis, suggesting a non-specific mechanism triggered by the host immune response. Chronic inflammation of the liver, continuous cell death and consequent cell proliferation might increase the risk for cancer.[5] In a more specific pathway, persistent production of viral proteins might interfere with endogenous metabolic processes and sensitize liver cells to endogenous or exogenous mutagens. This notion is supported by transgenic mouse models in which unregulated expression of the viral X-protein, a promiscuous transcriptional activator, or that of the large surface protein are associated with hepatocarcinogenesis.[6,7] Alternatively, the virus might play a direct role as an insertional mutagen. Integration of viral DNA into the host genome might cause direct activation of cancer-related genes or secondary chromosomal alterations. Such a role has been suggested by the repeated finding of integrated viral sequences in the cellular DNA of HBV-associated HCC. Indeed, viral integration in nearby potential oncogenes has been described in an increasing number of HCC cases,[8–12] and genetic instability resulting from viral integration events has been documented. Integrated HBV sequences might also alter the host cell growth control in *trans*, through unregulated expression of native or modified viral proteins. All these potential mechanisms might not be mutually exclusive. To date, although information on risk factors causally linked to HCC has accumulated, the role of viral agents and carcinogenic co-factors is only partially elucidated, and no unifying model accounting for the contribution of viral and cellular factors to liver oncogenesis has been established.

In contrast, studies of naturally occurring models for HBV and liver cancer have outlined a predominant role of the *myc* oncogenes in hepatocarcinogenesis induced by rodent hepadnaviruses. Woodchuck hepatitis virus (WHV), a virus closely related to HBV, acts mainly as an insertional mutagen, activating *myc* family genes (c-*myc* and N-*myc*) in >90% of the woodchuck tumours analyzed.[13,14,16,16a] This virus represents the first example of a DNA virus producing insertional events at such a frequency. Another related virus, the ground squirrel hepatitis virus (GSHV), shows weaker oncogenic properties. Integration of GSHV DNA has not been implicated in the tumorigenic process in the natural host, but frequent amplifications of the c-*myc* oncogene have been described in squirrel tumours.[17] More recently, another related hepadnavirus was isolated from arctic ground squirrels in Alaska,[18] but the mechanisms of liver carcinogenesis in infected animals have not been identified so far. In human HCCs, there is no published evidence for a key role of activated *myc* genes, albeit amplification of c-*myc* has been occasionally described, suggesting a different transformation pathway. Whether these striking differences are related to viral determinants or to species-specific factors remains to be determined.

This chapter reviews the different mechanisms that may be implicated in the development of HBV-related HCC, with reference to naturally occurring animal models.

## Epidemiological association between HBV and HCC

Epidemiological research has contributed largely to the understanding of the aetiology of HCC. By the end of the 1970s, it became evident that chronic HBV infection was by far the major risk factor for liver cancer.[2] These conclusions stimulated the search for the molecular mechanisms linking HBV and HCC and pointed out the importance of vaccination against HBV infection as the appropriate strategy to prevent HCC. Other carcinogenic factors, such as exposure to dietary aflatoxins and excessive alcohol intake, have also been implicated in human hepatocarcinogenesis, and epidemiological data support a strong correlation between chronic infection with the human hepatitis C virus (HCV), cirrhosis and HCC.[19,19a]

Primary liver cancer (mostly HCC) ranks among the commonest cancers in many countries. A recent estimate indicates that HCC represents the fifth most common cancer of males, and the eighth most common cancer in females, with a total of 560 000 new cases each year, 83% of which occur in developing countries, and more than one-half in China alone.[21] Moreover, because of its very poor prognosis, HCC represents a leading cause of cancer death worldwide.[22]

Several lines of evidence associate chronic HBV infection with the development of HCC:

**1** The incidence of HCC and the prevalence of HBV serological markers follow the same general geographical pattern of distribution. HCC is common in regions where HBV is endemic, but comes far behind other types of cancer in regions where HBV infection is uncommon.[2,23–25]

**2** Serological evidence of HBV infection is detected in about 70% of HCC patients in Africa and more than 90% in mainland China, as compared with 10–20% of the total population residing in the same areas.[24] In regions of low endemicity such as Western countries, chronic HBV carriers still represent 16–19% of HCC patients.[26,27]

**3** A marked increased risk of HCC has been shown among hepatitis B surface antigen (HBsAg) carriers compared with non-carriers (up to 100 risk factors have been reported in different ethnic or social groups using different methodologies of investigations). [30,31,31a]

A long period of chronic HBV infection generally precedes the onset of liver tumours, but less frequently, HCC may also affect HBsAg-positive children under 12 years of age. In contrast, a large number of HBsAg carriers remain unaware of their carrier status before they die at an old age.[28] In the Far East, early detection of HCC is now frequent in asymptomatic carriers.[32,33] Epidemiological data have shown not only that HBV is causally related to HCC, but also that there is a great variation in HCC incidence among different carrier populations. This variation may be attributed both to differences in the intrinsic properties of HBV infection patterns observed among chronic carriers and to additional genetic and environmental determinants.

Chronic infections resulting from maternal–neonatal transmission present a greater risk of HCC than those acquired as adults, probably reflecting differences in the incubation time (Fig. 17.1). Among HBsAg carriers infected at an early age, an additional HCC risk has been associated with HBeAg carriage,[34] with significant liver damage and high level of anti-HBsAg antibodies in chronic active hepatitis, and with the presence of cirrhosis. A gender discrepancy (males have a twofold to eightfold elevated risk of developing HCC compared with females) and familial tendency (familial clusters of HCC are common in Asia) have also been documented as factors involved in the frequency of tumour development.[33,35]

In addition, inconsistent geographical variations in HCC mortality and HBsAg prevalence have been observed in endemic regions, suggesting that other inde-

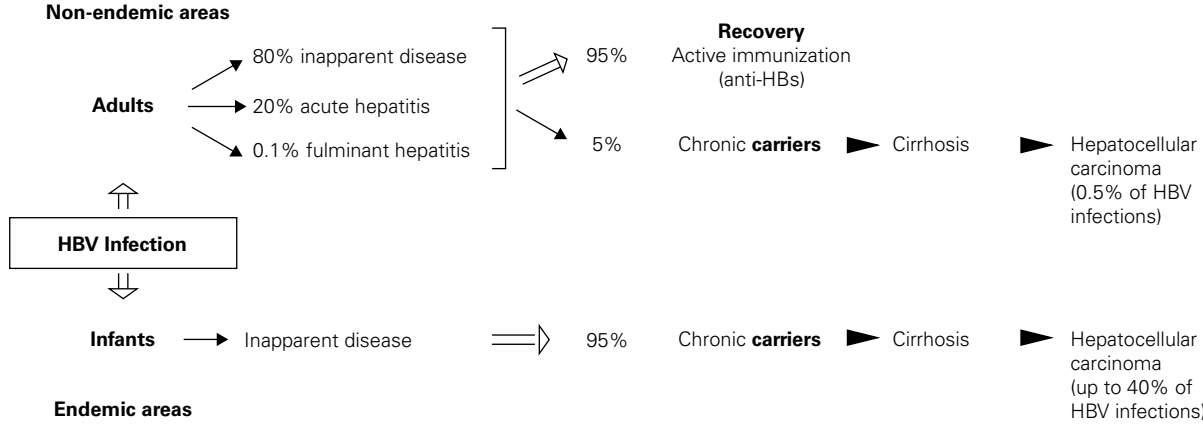


**Figure 17.1** Epidemiological association between HBV and HCC. Description of HBV transmission, progression to chronicity, and HCC incidence in endemic and non-endemic countries.

pendent or cooperative factors might be implicated. In highly endemic regions, particularly in South Africa and in southern provinces of mainland China, an association of dietary aflatoxins, notably aflatoxin B1 (AFB1) and HCC has been recognized in several reports, and synergistic effects of AFB1 and HBV infection have been pointed out.[25,36,37] The carcinogenic potential of aflatoxin $B_1$ in liver cells is well known in many species, and its effects in inducing p53 gene mutations are discussed later. Excessive alcohol intake also increases the risk of HCC in HBV carriers, as well as in cirrhotic men at advanced ages in regions of low prevalence for HBV.[38] However, the co-existence of HBV infection, often undetectable by conventional serological assays, and/or of HCV infection in >90% of alcoholic patients from various countries, has called into question the prevalence of chronic hepatitis induced by alcohol in the absence of viral infection.[39,40] The potential role of cigarette smoking and of long-term use of oral contraceptives is still debated.[38,41] Diabetes mellitus (DM) has been reported to increase the risk of HCC in the presence of other risk factors such as hepatitis C, hepatitis B or alcoholic cirrhosis.[42] In addition, infection with the human hepatitis D virus (HDV), which causes extremely severe hepatic injury and cirrhosis, might be associated with a more rapid onset of liver tumours.[43]

It is important to point out that HBV infection, alone or in association with co-factors, can be implicated only in <20% of HCC cases in low endemic regions such as North America, Europe and Japan. In these countries, the incidence of liver cancer has been continuously increasing during the last decade,[20] but the number of cases related to HBV was in slight decrease. It has been shown that infection with HCV, a human RNA virus related to Flaviviridae and Pestiviridae, plays an increasing part in the development of HCC in these regions, as well as in countries highly endemic for HBV (e.g. China). With the development of HCV markers, epidemiological evidence has been provided of an association between HCV infection, cirrhosis and HCC.[44–46] Liver cirrhosis is generally considered as a preneoplastic condition, but the relationship between cirrhosis and HCC appears to be complex, and the degree of correlation varies with aetiology of cirrhosis. Macronodular cirrhosis precedes or accompanies a majority of HBV- and HCV-associated HCCs (>80% in Asia and 40–60% in Africa) in adults as well as children. In contrast, the risk of HCC has been considered to be lower in HBsAg-negative micronodular cirrhosis observed in alcoholics.[28–30]

## The HBV genome

Since the earliest studies of the genetic organization of HBV and of the viral replication by reverse transcription of an RNA intermediate, there has been constant interest in the unique properties of HBVs. Virological and molecular studies have outlined the structural organization of the HBV genome, its coding potential, the mode of transcription of individual viral genes, and their functional capacities. The main aspects of viral DNA replication and virion assembly within infected hepatocytes have been unravelled (reviewed by Fourel and Tiollais[47]). Less is known about the viral–cellular interactions that control virus attachment, uncoating and entry into susceptible cells, and the cell-surface receptor for HBV has not been identified.

Nucleotide and deduced amino acid sequences of cloned HBV DNA from different virus subtypes (adw, adr, ayw, ayr) have revealed a genome size of 3.2 kb and the presence of four open reading frames (ORFs), localized on one viral strand in the same transcriptional orientation[48–50] (Fig. 17.2). The C and S regions specify structural proteins of the virion core and surface (or envelope). The longest one, P, encodes a polyprotein necessary for viral replication, which contains primase and replicase activities. The smallest, termed X, codes for a transcriptional transactivator. The entire viral genome is coding, and a large portion of the genome harbours two different reading frames. The overlapping of the P gene with the other viral genes is a feature common to

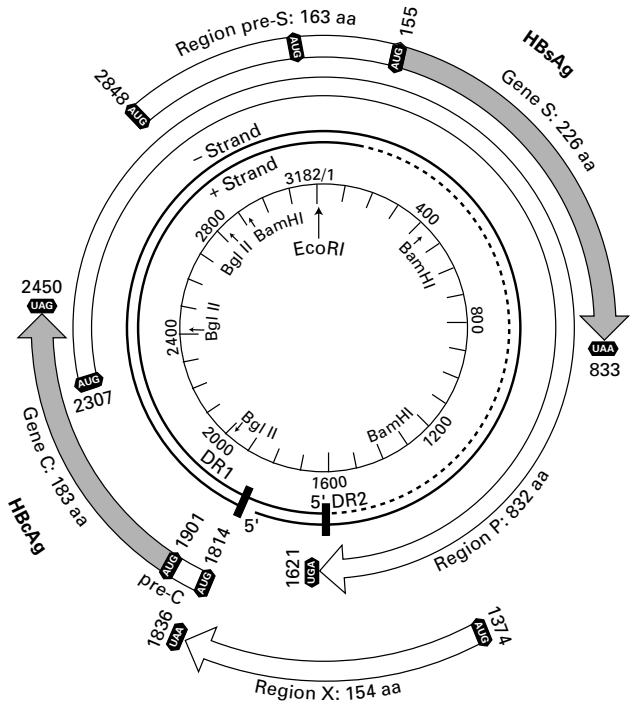


**Figure 17.2** Genetic organization of HBV. The inner cycle shows a restriction map of the *ayw* genome.[48] The partially double-stranded genome is represented as a thick line. Four open reading frames encoding seven peptides are indicated by large arrows. DR1 and DR2 are two directly repeated sequences located at the extremities of the viral DNA strands.

all structures presenting reverse transcriptase activity,[51] but the constant and unusual overlapping of coding and regulatory sequences (promoters, enhancers and termination signal) is more striking. The genomes of different HBV subtypes differ mainly by single nucleotide substitutions or by addition of multiples of three nucleotide blocks, preserving the reading frames and leading primarily to conservative amino acid changes. Genomic variability among viruses of the same subtype has been characterized in different patients and also in the same patient at different times during chronic infection.[52] These genetic variations can be attributed to errors during synthesis of the minus-strand DNA by reverse transcriptase, an enzyme that lacks polymerase-associated proofreading functions. A number of mutations in the pre-S/S region, in the pre-C/C region or in the core gene promoter lead to the outcome of HBV variants with modified immunological and pathological properties.[53–57] The most frequent mutations affecting the precore region inhibit the production of HBeAg and result generally in severe liver injury.[58–60] Defective viral particles carrying a deleted genome have been described,[61–64] showing that HBV shares with other viruses, notably with retroviruses, the property to develop defective variants in the natural host. However, there is no experimental evidence that free defective HBV genomes might present oncogenic properties, or that particular HBV subtypes might be more oncogenic than others.

Comparison of the HBV genome with those of animal hepadnaviruses reveals a basically identical genomic organization and extensive homologies at the nucleotide and amino acid levels. The best-conserved regions are located in the C and S genes and in the viral polymerase. The extent of homology is weaker in the pre-S1 region, which retains the same general conformation and hydrophobicity profile. The rather strict host range of the different hepadnaviruses suggests that the cellular receptor for these viruses, which has not been characterized, might be poorly conserved during evolution. Accordingly, viral sequences implicated in the interaction of the virion with the cellular membrane are located in the amino-terminal part of the pre-S1 domain,[65] a variable region among hepadnaviral genomes. Finally, the internal domain of the X-protein is the less conserved region among mammalian hepadnaviruses. This gene is missing in the avian hepadnaviruses. Two strong homology blocks in the amino- and carboxy-terminal parts of X might correspond to a conservative pressure for functional activity of the X transactivator, and of the viral RNase H encoded by carboxy-terminal P sequences that overlap with the 5′ end of X.[66–68]

The hepadnaviral genome, isolated from infectious extracellular virions, is made of two complementary DNA strands of different length, maintained in a circular configuration by base-pairing at their 5′ extremities.[69] The viral replication pathway, virtually identical for all hepadnaviruses, takes place in the nucleus and cytoplasm of infected cells, and although it can be instructively compared with the retroviral life-cycle, it is entirely extrachromosomal[69–71] (reviewed in Fourel and Tiollais[47]). Hepadnaviruses, like retroviruses and caulimoviruses, use a reverse transcription step during replication. Significant homologies between reverse transcriptases encoded by these viruses have suggested that they display a common evolutionary origin.

In productive hepadnavirus infections, a strict balance in the amount of individual viral gene products is necessary for active viral replication and release of infectious virions, as well as for survival of infected host cells. Despite the small size of the HBV genome and a very compact organization of coding sequences, the expression of the different viral genes is subjected to a complex regulation at various levels, both transcriptionally and post-transcriptionally. Each individual HBV gene is controlled by an independent set of regulatory signals, probably acting in cooperation with HBV elements that coordinate the relative level of viral gene expression. In chronically infected livers and in cell lines supporting active viral replication, two major HBV transcripts of molecular size 3.5 and 2.1 kb are produced from cccDNA template at roughly similar levels[72,73] and minor 3.9-, 2.4- and 0.8-kb transcripts have been described.[75–77] These polyadenylated RNAs direct the synthesis of seven viral proteins: the 3.5-kb species encode the core protein, the e antigen, and the polymerase; the 2.1-kb RNA encodes the middle and major surface glycoproteins, while the 2.4-kb RNA encodes the large surface protein; the viral X transactivator activity is specified by the 3.9- and 0.8-kb transcripts. The transcription patterns of WHV and GSHV in chronically infected livers are strikingly similar, whereas duck hepatitis B virus (DHBV) transcripts differ mainly by higher levels of 2.4kb RNA.[78–80] In the HBV genome, two enhancer elements stimulate transcription from the viral promoters: EN I, positioned about 450 by upstream of the core promoter, and EN II, located in the X gene-coding region.[81,82] Recent studies of the WHV, GSHV and DHBV enhancers have pointed to common liver specificity but significant evolutionary divergence among the hepadnavirus regulatory elements.[83–88]

## Oncogenic properties of viral proteins

It is generally considered that prolonged expression of viral genes may have no direct cytotoxic effects on the infected hepatocytes. Transfection of cultured cells with HBV DNA has not usually been associated with tumorigenic conversion, and most transgenic lines of mice bearing the full-length HBV genome or subgenomic constructs never show any sign of liver cell injury. However, studies, in particular experimental systems, have

indicated that abnormal overexpression of different viral proteins, including the X gene product and the surface proteins in native or modified forms, might play a part in malignant transformation of infected hepatocytes.

## The X transactivator

The smallest HBV ORF was initially designated X, because it was unclear at that time that it might encode a protein produced during HBV infection.[48] Examination of the nucleotide and amino acid sequences of HBx has led to the prediction of a regulatory function for the deduced X polypeptide and has revealed a codon usage similar to that used in eukaryotic cell genes, suggesting that X might have been transduced by the HBV genome.[89] The strong conservation of X sequences among HBV subtypes and the presence of homologous reading frames in the WHV and GSHV genomes suggest that their products may be of importance for viral replication.

Evidence for expression of the HBx gene was first obtained by Moriarty *et al*.[90] and by Kay *et al*.,[91] who reported that the sera of HBV-related HCC patients recognize synthetic peptides based on X sequences. Expression of the X reading frame in prokaryotic and eukaryotic cells, using various vectors, has allowed the identification of a 16.5-kDa polypeptide that reacted with serum samples from a number of HBV-infected individuals.[93–94] Anti-HBx antibodies have been detected in acutely infected patients about 3–4 weeks after the onset of clinical signs,[95] and more frequently in chronic HBsAg carriers showing markers of active viral replication and chronic liver disease.[96–98] The expression of HBx was found to be preferentially maintained in HCCs and the WHV X-protein was detected in HCCs that are permissive for WHV replication.[99,100] The X-protein was found to be localized mainly in the cytoplasm of *in vivo*-infected cells near the plasma membrane and at the nuclear periphery,[95] or at the mitochondrial membrane.[102,103] It has also been detected in the nuclear compartment in transfected cell lines.[104–107] In the absence of a nuclear localization signal in the HBx protein, it has been proposed that nuclear import of HBx might be mediated by its interaction with the NF-κB inhibitor IκBα.[108] Conversely, HBx carries a leucine-rich nuclear export signal (NES) motif, and interacts with Crm1, a nuclear export receptor that binds to Ran GTPase. Cytoplasmic sequestration of Crm1 by HBx has been reported to induce aberrant centriole replication and abnormal mitotic spindles.[109] This process leading to genetic instability of HBV-infected cells might contribute to viral-induced carcinogenesis.

Seemingly contradictory data have recently accumulated on HBx function in natural infections. At least two salient features deserve consideration. On one hand, the requirement for a functional X gene in the establishment of viral infection in woodchucks has been firmly established.[110,111] On the other hand, the finding that the X gene product can activate transcription from a number of HBV and heterologous promoters might be important in defining its role in viral replication and in pathogenesis (reviews[112–114]). The X-protein behaves as a broad-range transactivator. It activates transcription from the viral promoters coupled to HBV enhancers and stimulates the production of viral particles in transient transfection assays.[114a] It is also active on heterologous viral promoters such as the SV40 early promoter, the herpes simplex virus-thymidine kinase (HSV-TK) promoter, and the Rous sarcoma virus (RSV) and human immunodeficiency virus (HIV) long terminal repeats (LTRs), and on cellular promoters including the interferon, HLA-DR, c-*fos* and c-*myc* promoters.[116–119] HBx does not directly bind DNA and may stimulate gene expression by interacting with transcription factors such as CREB, ATF-2, C/EBP and Spl,[120–123] or with elements of the basal transcription machinery such as TBP, TFIIB, TFIIH and the RPB5 subunit of DNA polymerase II.[124–128] It has also been shown that it binds the PHD finger protein HBXAP to co-activate transcription.[129] Other studies support an indirect effect of HBx by activating signalling pathways mediated by the Ras-Raf-MAPK cascade or by protein kinase C, leading to the activation of AP-1 and AP-2.[130–133a] Moreover, HBx has been shown to activate the nuclear translocation of NF-κB and NF-AT through different mechanisms.[103,108,120,134–136] Such functions are important for HBx-mediated regulation of a variety of cellular genes, including oncogenes, growth factors and cytokines, which play important roles in HBV pathogenesis and in tumorigenesis.

More clues on the possible role of HBx in HBV pathogenesis have been provided by studies of HBx interactions with cellular proteins (Fig. 17.3). It has been reported that HBx binds the tumour suppressor protein p53 and inactivates its functions.[137–139] This finding might be correlated with high chromosomal instability of HBV-related tumours. Interaction of HBx with the cellular DNA repair protein UV-damaged DNA-binding protein 1 (DDB1, also called XAP-1), might affect cell ability to repair mutated DNA sequences and allow the accumulation of genetic changes.[15,140–144] The proteosome complex might also be a target of the X-protein.[145] More recently, it has been shown that HBx activation of Src is important for HBV DNA replication and that alteration of cytosolic calcium mediated by HBx activates Pyk2, an Src kinase activator.[146,147] Non-receptor tyrosine kinases of the Src family are known to activate Ras, and activation of calcium-dependent signalling pathway can have pleiotropic effects on cellular functions. Therefore, the ability of HBx to activate calcium-dependent signalling events might account for HBx activity in HBV replication as well as in apoptosis induction and carcinogen-

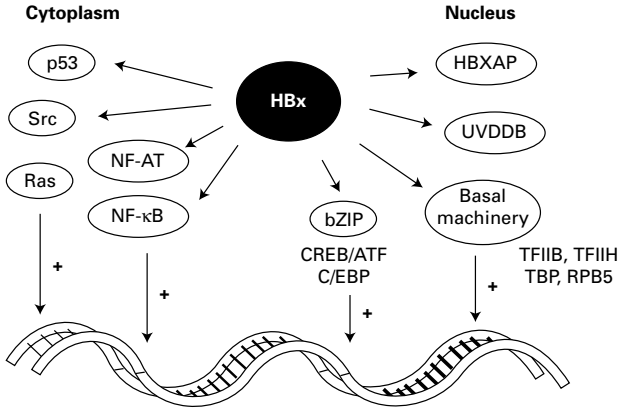


**Figure 17.3** Multiple interactions of HBx with cellular partners from cytoplasmic and nuclear locations. Left: HBx binds and sequesters the tumour-suppressor protein p53 in the cytoplasm, thereby inactivating p53 functions. Activation of Src is mediated by calcium-dependent activation of the Pyk-2 kinase. Activation of the Ras-Raf-MAPK cascade, including the extracellular signal-regulated kinases (ERKs) and jun N-terminal kinases (JNKs) leads to induction of the transcription factors AP-1. HBx induces nuclear translocation of the NF-κB and NF-AT transcription factors by various mechanisms. In the nucleus, HBs binds transcription factors of the bZIP family, including CREB, as well as several components of the basal transcription machinery and the PHD finger protein HBXAP to co-activate transcription. Interaction of HBx with the p127 subunit of the UVDDB complex (DDB1) might play a major role in diverse biological functions of HBx.

esis.[148] In chronic HBV infection, liver cell injury is thought to be mediated mostly by the cellular immune response, but there is increasing evidence that the HBx protein interacts with regulatory pathways controlling apoptosis. However, in this case also, apparently contradictory data have been reported. Arguments favouring pro-apoptotic properties of HBx have been provided in different experimental systems, showing that HBx can directly induce apoptosis through interaction with c-FLIP or by causing loss of mitochondrial membrane potential.[149–152] The hypothesis that HBx might play a role as a 'sensitizer' to other pro-apoptotic stimuli has been notably supported by its ability to increase cell sensitivity to apoptotic death induced by TNF-α, a pro-inflammatory cytokine implicated in chronic liver diseases.[153,154] Importantly, apoptotic functions cannot be dissociated from transactivation activity of HBx and these functions are conserved among mammalian hepadnaviruses.[155] In striking contrast, HBx has been found to inhibit apoptosis induced by p53 or by TGF-β and to promote cell survival through activation of phosphatidylinositol 3-kinase or NF-κB.[156–159] HBx is endowed with pleiotropic functions and is able to bind a variety of cellular partners. It seems therefore probable that HBx's effects on cell viability might be highly dependent on the cellular context. So far, however, the precise mechanisms by which HBx interferes with the control of liver cell proliferation and apoptosis in chronically infected patients and in liver tumours remain largely unknown.

So far, evidence for a direct oncogenic effect of HBx *in vivo* is scarce. It has been shown that high levels of X expression may induce malignant transformation of certain cultured cells, such as the NIH3T3 and REV-2 cell lines, and of immortalized hepatocytes expressing the SV40 large tumour antigen.[105,160,161] In co-transformation assays of rat embryo fibroblasts, HBx has been reported to either cooperate with Ras or to inhibit Ras transformation by inducing apoptosis.[162,163] Studies of transgenic mice carrying the X reading frame controlled by its natural HBV enhancer/promoter sequences or by heterologous liver-specific promoters have given rise to conflicting results. In a transgenic mouse line generated in the outbred CD-1 background, high level expression of HBx in the liver induced the progressive appearance of altered hepatocyte foci, proliferative adenomatous nodules, and malignant carcinoma that killed most male animals before 15 months of age.[7] In contrast, a second mouse lineage with lower HBx expression developed liver tumours at the same rate as normal CD-1 mice,[164] and other HBx transgenic lines generated in different mouse strains developed no obvious hepatic pathology, although the X gene was expressed in liver cells and the HBx protein could be detected in some cases.[165–167] These data suggest that HBx has no acutely transforming activity, but its overexpression in the susceptible CD-1 mouse strain might cooperate with activated cellular oncogene(s) in multi-step hepatic transformation. Arguments favouring this hypothesis were recently provided by the finding that the hepadnavirus X protein sensitizes liver cells to malignant transformation in transgenic mice treated with chemical carcinogens[99,168] and cooperates with c-*myc* in hepatocarcinogenesis.[169] Following AFB1 exposure of HBx transgenic mice, the expression of HBx correlated with an approximately twofold increase in the incidence of G/C to T/A transversion mutations.[170] Other studies have shown that expression of HIF-1alpha and vascular endothelial growth factor was increased in the liver of HBx-transgenic mice, suggesting that the cross-talk between HIF-1alpha and HBx may lead to transcriptional activation of HIF-1alpha target genes, which play a critical role in hepatocarcinogenesis.[171] While some data demonstrated that HBx is able to induce hepatocellular proliferation, other studies by contrast detected increased apoptotic death in HBx transgenic livers.[144,151,172] Moreover, *in vivo* expression of HBx could also exert a paracrine inhibitory effect on liver regeneration, providing an alternative mechanism of HBx-related pathogenesis.[173]

Analysis of integrated viral sequences in tumour DNA has shed new light on one of the mechanisms leading to

persistent expression of HBx in HCC. HBV sequences are frequently interrupted between the viral direct repeats DR1 and DR2 upon integration into host cell DNA, and overproduction of hybrid viral/host transcripts may result from HBV DNA integration in a hepatoma cell line.[174] Consistent with this model, viral/host junctions have been mapped near DR1 in the carboxy-terminal region of X in a majority of human HCCs.[175–178] The presence of viral/host transcripts containing a 3′ truncated version of the X coding region fused with flanking cellular sequences and retaining transactivating capacity was first described in a human HCC.[179] Moreover, enhanced transactivating capacity of the integrated X gene product has been related to the substitution of viral carboxy-terminal residues by cellular amino acids.[180] By contrast, more recent studies have detected truncated and mutated forms of HBx in HCC, which were less effective in transcriptional transactivation, apoptosis induction, and in supporting HBV replication.[177,181–183] However, integrated HBx mutants were also shown to bind to p53 *in vitro* and to retain the ability of wt HBx to block p53-mediated apoptosis *in vivo*, which may provide a selective clonal advantage for preneoplastic or neoplastic hepatocytes. [184] This suggests that the integrated X gene might be essential for maintaining the tumour phenotype that develops at the early stages of carcinogenesis. Collectively, the recent studies on HBx protein function have contributed to better delineate the importance of X gene product as a co-factor in malignant transformation induced by persistent HBV infection.

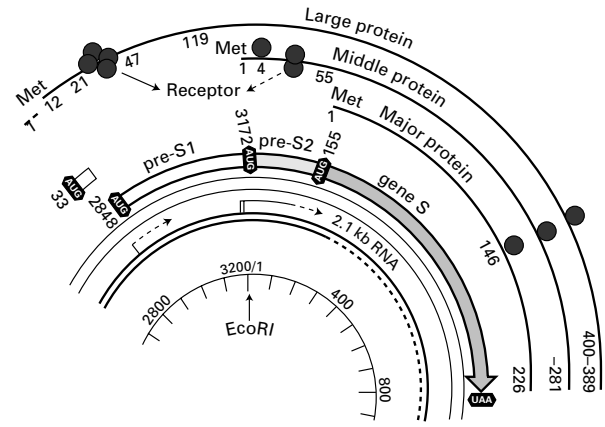


**Figure 17.4** Genetic map of the three HBs proteins (subtype *ayw*). The inner circles represent a part of the HBV genome shown in Fig. 17.2. The transcription start sites are indicated. The outer circles denote the large (LHBs), middle (MHBs), and major or small (SHBs) proteins of the envelope. Numbers refer to the amino acids of the primary translation products. Met, methionine initiator codon. The large black round spots mark the positions of the glycosylated residues, and the black hemispheres correspond to regions probably involved in the binding to the cell membrane.

## Surface glycoproteins

In natural HBV infections, the production of infectious virions and HBsAg particles depends on a tight regulation of the relative levels of the three envelope glycoproteins (Fig. 17.4). Neither liver lesions nor HCCs have been observed in any of the published transgenic lineages that produce the middle and major surface proteins from HBV-derived regulatory sequences.[185–188] When the endogenous pre-S1 promoter is replaced by an exogenous promoter (the metallothionein or albumin promoter), the production of roughly equimolar ratios of large S protein with respect to middle and major S leads to intracellular accumulation of non-secretable filamentous envelope particles within the endoplasmic reticulum of transgenic mouse hepatocytes.[189] This results in histological and ultrastructural features of 'ground-glass' hepatocytes, which have been described in some cases of chronic human liver diseases and are considered to be typical of chronic hepatitis B, ultimately killing the cells.

In pre-S/S transgenic mouse lineages, mild persistent hepatitis was followed by the development of regenerative nodules and eventually HCCs by 12 months of age.[6] At preneoplastic stages, strong hepatocellular proliferation and extensive oxidative DNA damage were noted,[190,191] and the preneoplastic nodules and tumours displayed a marked reduction in transgene expression, suggesting that hepatocytes that express low levels of the large S polypeptide would have a selective survival advantage. Exogenous, chemical co-factors are not required for tumorigenic induction in this model, but exposure of adult transgenic mice to hepatocarcinogens produced more rapid and extensive development of preneoplastic lesions and HCC under conditions that do not alter the liver morphology of non-transgenic controls.[192] These data show that inappropriate expression of the large S protein has the potential to be directly cytotoxic to the hepatocyte and may initiate a cascade of events that ultimately progress to malignant transformation, although the molecular mechanism connecting viral and host factors in this process has not been elucidated.

Studies of integrated HBV sequences in human liver tumours have also suggested a possible role for abnormal expression of rearranged viral S genes in HCC development. Deletion of the carboxy-terminal region of the S gene generates a novel transcriptional transactivation activity.[193] Integrated HBV sequences from a human tumour and a hepatoma-derived cell line, as well as different constructs bearing similarly truncated preS2/S sequences,[194] can stimulate the SV40 promoter in transient transfection assays. Although the mutated S polypeptide is retained in the endoplasmic reticulum and Golgi membranes, transactivation occurs at the transcriptional level. The c-*myc* P2 promoter is also ac-

tivated in *trans*,[180] and more recently, specific activation of c-Raf-1/Erk2 signalling by the truncated pre-S2S protein was observed, resulting in an increased hepatocyte proliferation rate.[195] These findings support the hypothesis that accidental 3′ truncation of integrated pre-S2/S genes could be a causative factor in HBV-associated oncogenesis.

## Viral integrations in HCC

The hepadnavirus replication pathway in infected cells takes place within nuclear and cytoplasmic compartments and does not require a step of viral DNA integration in the host cell genome. However, hepadnaviruses share with other retroelements of common evolutionary origin the ability to integrate their DNA into cellular chromosomes. The molecular events leading to the invasion of cell DNA by hepadnaviral DNA have not been fully elucidated.[101]

The main question is whether viral integrations might play a part in the virally induced transformation process, either by conferring a selective growth advantage on targeted cells, leading to the onset of preneoplastic nodules, or by providing an additional step in tumour progression. Whereas insertional activation of proto-oncogenes has emerged as a common event in WHV-induced woodchuck HCC, a related mechanism seems to be less frequent in human HCCs, in which different activities for integrated HBV sequences have been proposed.

### Integrated HBV sequences

Integrated HBV sequences have been observed in established hepatoma cell lines and in about 80% of human HCCs.[196–198] HBV DNA integrations occur at early stages in natural acute infections and in experimental infections of cultured cells.[115,199,200] As a result of multiple integrations in chronic hepatitis tissues,[201,202] integrated HBV sequences have been detected in most HBV-related HCCs that arise from clonal outgrowth of one or a few transformed liver cells (see Matsubara and Tokino[203]) Single HBV insertions are common in childhood HCCs but are rather uncommon later in life, suggesting that multiple integrations occurring during the course of long-standing HBV infections might accumulate within single cells.[204] Studies of the organization of cloned HBV inserts in liver tissues and HCCs have shown that HBV sequences are fragmented and rearranged and that integration and recombination sites are dispersed over the viral genome, indicating that HBV integration does not occur through a unique mechanism, as in the case of other retroelements and retroviruses. The absence of complete genomes in virtually all HBV inserts, which consist either of linear subgenomic fragments or of rearranged fragments in different orientations, shows that these sequences cannot serve as a template for viral replication. Integrated forms made of a subgenomic fragment, which are frequent in HCC and hepatitis tissues from children[115] are believed to represent primary products of integration. They are of particular interest in the study of the molecular mechanisms responsible for HBV DNA integration.

Highly preferred integration sites have been mapped in the HBV genome within the 'cohesive ends' region, which lies between two 11-bp direct repeats (DR1 and DR2) highly conserved among hepadnaviruses.[175,178,205,206] A narrow region encompassing DR1 has been shown to be particularly prone to recombination.[115,176,207] This region coincides with a short terminal redundancy of the minus-strand DNA, which confers a triple-stranded structure to the circular viral genome. Integration sites are tightly clustered at both the 5′ and 3′ ends of minus-strand DNA, suggesting that replication intermediates and specially relaxed circular DNA might be preferential preintegration substrates.[175,176] Invasion of cellular DNA by single-stranded HBV DNA, using mainly free 3′ ends, might take place through a mechanism of illegitimate recombination, also suggested by frequent patch homology between HBV and cellular sequences at the recombination breakpoints. Although different minor changes in flanking cellular DNA have been associated with viral integration (both microdeletions and short duplications), more precise mechanisms have been proposed. The recombination-proficient region spanning DR1 is located close to a U5-like sequence highly conserved between hepadnaviruses, suggesting that sequences necessary for precise recombination with cellular DNA have been retained from a common ancestor with retroviruses, despite the absence of a gene coding for an integrase in the HBV genome. Mapping of a set of preferred topoisomerase I (topo I) sites near DR1 and DR2 and *in vitro* studies of WHV DNA integration into cloned cellular DNA have sustained the hypothesis that topo I might promote illegitimate recombination of hepadnavirus DNA *in vivo*.[101]

As a consequence of the viral integration process, sequences of the S and X genes and of the enhancer I element are almost systematically present in HBV inserts, whereas those of the C gene are less frequently represented. It has been shown that the pre-S2/S promoter is transcriptionally active in its integrated form in HCCs and that HBsAg may be produced from viral inserts.[208,209] Highly rearranged HBV inserts show virus junctions scattered throughout the viral genome, and, in some of them recombination breakpoints have been mapped in the S coding region.[175] It has been recently shown that truncation of the S gene between residues 77 and 221 confers a transcriptional activation activity to the mutated pre-S2/S products.[194] The shorter pre-S2/S protein lacks carboxy-terminal signals for transloca-

tion through the endoplasmic reticulum membrane and should be retained in the bilayer. Activation of the c-*myc* oncogene promoter, demonstrated in *in vitro* assays, might result from an indirect transacting action of the truncated viral proteins.[193] Whether this or some related mechanism participates in liver cell transformation remains to be determined. Other studies have shown that a significant percentage of viral junctions are localized in the carboxy-terminal part of the viral X gene, predicting a fusion of the X ORF to flanking cellular sequences in a way that might preserve the functional capacity of the X transactivator. Evidence for transcriptional activity at integrated X sequences has been provided in tumours and chronically infected livers[68,179] and might be correlated with the detection of HBxAg in a number of human HCCs. However, there is increasing evidence that integrated, truncated forms of HBx also harbour a number of mis-sense mutations that abrogate the transactivation capacities of HBx.[177,181] Thus, while abnormal expression of integrated and truncated X gene might play a part in HBV-associated oncogenesis, the precise functions of these integrated sequences in *trans* remain to be determined.[210]

## Cellular target sites

Studies of different viral insertions in many human HCCs have revealed that integration can take place at multiple sites on various chromosomes (reviewed by Matsubara and Tokino[203]). These studies failed to demonstrate the presence of a known dominant oncogene or tumour-suppressor gene in the immediate vicinity of any integration site. It has been reasoned that integration of HBV DNA occurs at random in the human genome and that it has no direct mutagenic effect on growth control genes in most cases. It has been proposed that simple repetitive elements are hot spots for HBV insertion in the human genome. Indeed, Alu-type repeats, minisatellite-like, satellite III, or variable number of tandem repeat sequences have frequently been identified near HBV insertion sites, suggesting that chromosomal regions accessible to specific families of mobile repeated sequences are also preferential targets for HBV insertion. However, contrary to a widely held opinion, integration of retroelements and retroviruses might not be entirely random. Genome-wide surveys have shown that integration of retroviruses occur preferentially within transcription units.[211]

Evidence for a direct *cis*-acting promoter insertion mechanism was first provided in two independent HCCs.[8,10] These investigators analyzed early tumours that developed in non-cirrhotic livers and carried a single specific viral integration. In one case, the HBV insertion occurred in an exon of the retinoic acid receptor-β gene (RAR-β) and fused 29 amino-terminal residues of the viral pre-S1 gene to the DNA-binding and hormone-binding domains of RAR-β.[8] Retinoic acid and retinoids are vitamin A-derived substances that have striking effects on differentiation and proliferation in a large variety of systems. Interestingly, another retinoic acid receptor, RAR-α, is implicated in the chromosomal translocation[17,212] found in acute promyelocytic leukemias. In the human HCC, it seems most probable that inappropriate expression of a chimeric HBV/RAR-β protein participated in the tumorigenic process. In a second HCC, HBV DNA integration occurred in an intron of the human cyclin A gene, resulting in a strong expression of hybrid HBV/cyclin transcripts.[10,213] Cyclin A plays a major role in both G2/M and GUS checkpoints of the cell cycle.[214] Recent data from our laboratory, based on the inactivation of cyclin A gene by homologous recombination, have demonstrated that cyclin A is an essential gene for cell cycle progression. The hybrid RNAs code for a 430 amino acid chimeric protein, in which the amino-terminal 152 amino acids of cyclin A are replaced by 150 amino acids from the pre-S2 and S viral proteins, while the carboxy-terminal two-thirds of cyclin A, including the cyclin box, remain intact. Therefore, the cyclin A degradation sequences located in the amino-terminal part of the protein are deleted, and, using an *in vitro* degradation assay with frog oocytes, we have verified that HBV/cyclin A is not degradable.[213] Furthermore, we have obtained *in vitro* results that demonstrate the transforming property of the HBV-cyclin A protein.[215] Several hypotheses can be proposed to account for this effect. The absence of degradation of a molecule retaining the ability to complex to and activate cdk kinases might lead to unregulated and premature DNA synthesis and thus to cell proliferation. It is also quite plausible that the location of the HBV/cyclin A protein is changed, given the membrane location of pre-S2/S molecules in HBV-infected cells, and we have obtained recent evidence that supports an abnormal location of this protein in the endoplasmic reticulum membranes, driven by pre-S2/S sequences. Finally, it is noteworthy that the pre-S2/S viral sequences present in HBV/cyclin A are deleted in their carboxy-terminal part and thus have a structure similar to the deleted pre-S2/S proteins described in the previous section. On the other hand, our group has shown that the expression of cyclin A RNA or protein is an interesting marker of tumour cell proliferation *in vivo*.[216,217]

However, surveys of human liver cancers did not reveal any other case showing rearrangement of a cyclin A or RAR gene. In these two HCC cases, analysis of single HBV insertion sites has allowed the identification of new genes involved in the control of cell growth and differentiation. Both studies have opened the way to novel approaches of the cellular pathways regulating cell division and differentiation.

The notion that HBV might play a significant role as an insertional mutagen in primary liver cancer has been recently strengthened by large-scale analysis of viral insertion sites. We have demonstrated that HBV frequently targets cellular genes involved in cell signalling, and that some of them may represent preferential target sites of the viral integration. By using HBV-Alu PCR, we have isolated, from 22 HCCs, 15 HBV DNA integration sites in which cellular genes targeted by HBV were key regulators of cell proliferation and viability.[11,12,218] We found HBV DNA integrated into the following cellular genes: sarco/endoplasmic reticulum calcium ATPase1 gene (SercA1); thyroid hormone receptor associated protein 150 alpha gene; mini-chromosome maintenance protein (MCM)-related gene; FR7, a new gene expressed in human liver and cancer tissues; nuclear matrix protein p84 gene; neurotropic tyrosine receptor kinase 2 (NTRK2) gene, IL-1R-associated kinase 2 (IRAK2) gene, p42 mitogen-activated protein kinase 1 (p42MAPK1) gene, inositol 1,4,5-triphosphate receptor type 2 (IP3R2) gene, inositol 1,4,5-triphosphate receptor (IP3R) type 1 (IP3R1) gene, alpha 2,3 sialyltransferase (ST3GAL VI or SITA) gene, thyroid hormone uncoupling protein (TRUP) gene, EMX2-like gene and human telomerase reverse transcriptase (hTERT) gene. Overall, we found that both the inositol 1,4,5-triphosphate receptor gene and the telomerase gene were targeted by HBV in two different tumours. Interestingly, several independent groups have found recurrent HBV DNA integrations into the hTERT gene encoding the catalytic subunit of telomerase, which is responsible for elongation of telomeric repeat DNA and maintenance of telomere length and structure.[12,219,220] None of the integrations altered the hTERT coding sequence and all resulted in juxtaposition of viral enhancers near hTERT, with potential activation of hTERT expression. Taken together, these data support the hypothesis that integration of HBV might induce cell immortalization and predisposition to carcinogenesis through activation of hTERT. In conclusion, there is now considerable evidence that the sites of oncogenic viral integrations are non-random, and that genes at the sites of viral integration may provide a growth advantage to a clonal cell population, leading to the accumulatation of additional mutations and to cancer development.

HBV DNA integration may also enhance chromosomal instability. In many tumours, large inverted duplications, deletions, amplifications or chromosomal translocations have been associated with HBV insertions, suggesting that this process may function as a random mutagen, promoting chromosomal defects in hepatocytes.[221–223] In addition, HBV DNA may promote homologous recombination at a distance from the insertion site.[224] However, a role for most of these chromosomal abnormalities has not been assigned as yet, although in a few cases the p53 or Hst-1 loci have been altered as a sequel of HBV integration in the same chromosomal region.[225]

## Insertional activation of *myc* genes in woodchuck HCC

The availability of naturally occurring animal models for HBV-induced liver disease and cancer has been largely exploited for a better understanding of the viral/host interactions. In the WHV/woodchuck model in particular, the natural history of viral infections, the presence and state of viral DNA, and the patterns of viral gene expression have been extensively investigated. Experimental inoculation of newborn woodchucks with infectious virions has given conclusive information on the oncogenic activity of WHV. This virus now appears to be the most potent inducer of liver cancer among the hepadnavirus group.[226] The GSHV and the arctic squirrel hepatitis virus appear to be more weakly oncogenic.[18,227]

Search for transcriptional activation of known proto-oncogenes and for viral insertion sites in woodchuck HCCs has revealed that WHV acts as a potent insertional mutagen, activating *myc* family genes (c-*myc* or N-*myc*) in a large majority of tumour cases.[13,14,16,212] Analysis of the mutated c-*myc* alleles in two individual tumours has shown integration of WHV sequences in the vicinity of the c-*myc* coding domain, either 5′ of the first exon or in the 3′ untranslated region.[13] Deregulated expression of the oncogene driven by its normal promoters resulted from deletion or displacement of c-*myc* regulatory regions known to exert a negative effect on c-*myc* expression, and their replacement by viral sequences encompassing the enhancer elements. Such a mechanism is highly reminiscent of that previously reported for c-*myc* activation in murine T-cell lymphomas induced by murine leukaemia viruses (MuLV). A survey of 56 woodchuck HCCs for c-*myc* rearrangements has shown only a few cases in which WHV DNA was inserted into the c-*myc* locus.[16] Presently, it may be estimated that insertional activation of c-*myc* is involved in about 13% of woodchuck tumours.

The insertional activation of N-*myc* genes was observed more frequently in woodchuck HCCs. In contrast to human and mouse, the woodchuck genome contains two distinct N-*myc* genes: N-*myc* 1, the homologue of known mammalian N-*myc* genes similarly organized into three exons, and N-*myc* 2, a functional processed pseudogene or retroposon, which has retained extensive coding and transforming homology with parental N-*myc*.[212,228] It has been shown that N-*myc*2, like c-*myc*, is a potent inducer of apoptosis in liver cells, and that this effect is counteracted by *Bcl*2 and insulin-like growth factor II (IGF-II). In woodchuck HCCs, N-*myc*2 represents by far the most frequent target for WHV DNA integrations. As shown in Fig. 17.5, in about one-half of cases,

viral inserts were detected either upstream of the gene or in a short sequence of the 3′ untranslated region, also identified as a unique hot spot for retroviral insertions into the murine N-*myc* gene in T-cell lymphomas. Activated expression of the N-*myc*2 retroposed oncogene, frequently correlated with overexpression of C-*fos* and C-*jun*, was observed in a large majority of woodchuck HCCs. Thereafter, integration of WHV DNA in two hot spot regions (*b3n* and *win*) located about 10 and 200 kb away from N-*myc*2 in the same chromosomal region (Xq24) was shown to contribute in N-*myc*2 activation in around one-quarter of woodchuck HCCs.[14,229,230] These loci contain S/MAR motifs that bind specifically nuclear scaffolds, suggesting that WHV integration might deregulate the S/MAR elements and indirectly affect the expression of the upstream N-myc2 gene.[231] Evidence for a direct role of WHV DNA integration into *myc* genes in hepatocyte transformation has been provided by the development of liver tumours in transgenic lines of mice bearing WHV and *myc* sequences from mutated alleles of woodchuck HCCs.[232,233] In these models, expression of the transgenes in liver cells induced primary liver tumours with high incidence.

Collectively, these data indicate that insertional activation of *myc* genes by WHV DNA, from short as well as from long distances, occurs in >90% of woodchuck HCCs (Fig. 17.5). Our findings also suggest that the role of HBV integration should be reconsidered, because a similar mechanism has not been investigated to date in human HCC.

Further evidence that *myc* family genes are predominantly implicated in rodent liver tumours associated with hepadnavirus infection comes from other studies of woodchuck and ground squirrel HCCs. In two independent woodchuck tumours, a genetic rearrangement fusing the coding domain of c-*myc* with the promoter and 5′ translated sequences of a cellular locus termed her has been observed.[234,235] In a study of ground squirrel HCCs, frequent amplifications of c-*myc* were found in tumour cell DNA (11 of 24 cases examined) and associated with enhanced expression of the oncogene.[17] Similarly, c-*myc* amplifications frequently occur in woodchuck HCCs induced by experimental GSHV infection. Integration of GSHV DNA into host cell genome, which occurs only rarely in squirrel tumours, has not been correlated with the observed genetic alterations of c-*myc*. Similar alterations have also been described in rodent liver tumours. Although amplification of c-*myc* has been observed on rare occasions in HBV-positive human liver tumours, there has been no experimental demonstration, until now, that deregulated expression of *myc* genes might be generally associated with HBV-induced tumorigenesis in human livers by any known *cis*- or *trans*-acting mechanism.

The strategy used by WHV in liver cell transformation now appears strikingly similar to that of some non-acute retroviruses, such as Moloney murine leukaemia virus (MoMuLV), which induces disease (usually leukaemias) slowly, emphasizing the described similarities between hepadnaviruses and retroviruses[89] (Fig. 17.5). These conclusions raise two different albeit related questions. What factors drive the oncogenic potential of WHV exclusively toward hepatocytes, as we know that this virus can infect a wide variety of woodchuck tissues, albeit at

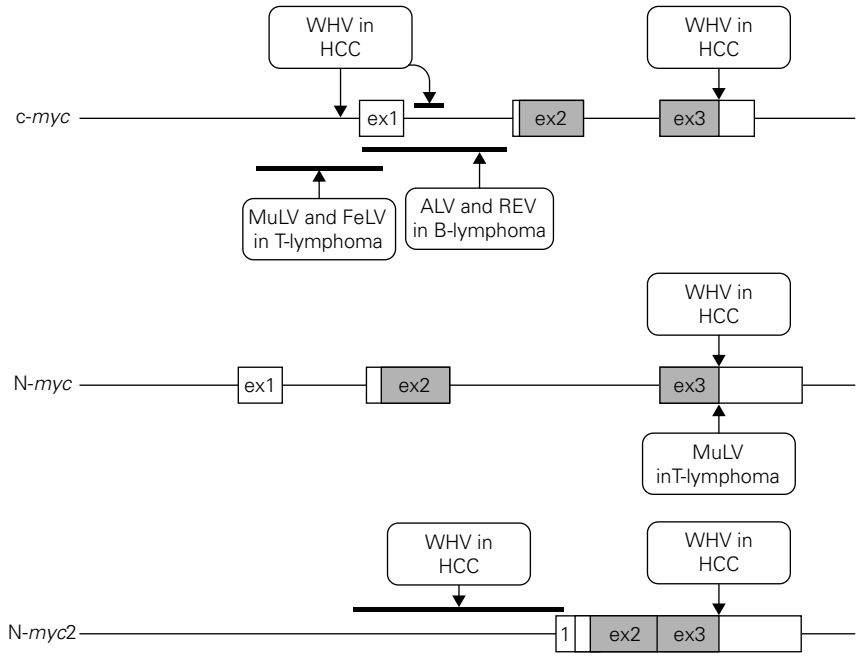


**Figure 17.5** Comparison of hepadnaviral and retroviral insertion sites in the c-*myc* and N-*myc* genes. Preferred target sites for WHV in *myc* family genes, in 56 woodchuck liver tumours are indicated above the corresponding genes, and those of murine and avian retroviruses in B- and T-cell lymphomas are shown under the genes. The intronless N-*myc*2 oncogene, generated by retrotransposition of the parental woodchuck N-*myc*l gene, is specific to the Sciuridae family of rodents. The win locus maps 180–200 kb downstream of N-*myc*2 on the woodchuck X chromosome. Insertional activation of *myc* family genes by WHV DNA was observed in about 90% of the woodchuck HCCs analyzed.

much lower levels? How can the apparent differences in the strategies of the closely related mammalian hepadnaviruses be explained at the molecular level? To address these issues and identify the genomic variations responsible for such discrepancies, the regulatory elements controlling viral gene expression have been compared among mammalian hepatitis B viruses.[83,84,87–88] These studies showed striking evolutionary divergence in the organization of nuclear factor-binding sites between the human and rodent hepadnavirus enhancers. Further *in vivo* studies are required to determine whether these differences play a role in the observed variations in hepadnavirus oncogenicity.

## HCC in HBsAg-negative patients

As stated previously, there are striking geographical variations in the association between chronic infection by HBV and HCC. In Western countries (e.g. northern Europe, United States) and Japan, only 15–20% of the tumours occur in HBsAg-positive patients, and other environmental factors, such as alcohol and infection by HCV, are clearly major risk factors. A number of epidemiological studies previously showed a high prevalence of anti-HBs and anti-HBc antibodies in the group of HBsAg-negative subjects (approximately 40–50% in France), indicating exposure to the virus (Table 17.1).[236–242] These antibodies generally reflect past and resolved HBV infection. However, in HBsAg-negative subjects with HCC, HBV DNA sequences can be detected in the tumours, demonstrating the persistence of the viral infection and suggesting its implication in liver carcinogenesis (Table 17.2).[39,209,237,243–260] With this view, it is indeed important to realize that the improvement in the sensitivity of assays for HBsAg detection, together with the introduction of sensitive tests for HBV DNA identification, has modified the criteria for the diagnosis of HBV infection. Thus, there is a spectrum of chronic HBV infections with a low replication rate, which might also be a risk factor for liver cancer.[39,59,121,261,262] In agreement with this, Shimada and colleagues studied 146 HBsAg negative primary liver tumours and provided evidence that the presence of HBV DNA fragment is a significant independent risk factor for development of HCC.[263]

HBV and HCV can interact with chronic alcohol consumption, and there is circumstantial evidence for a high prevalence of HBV and HCV infections in alcoholics. The increased prevalence of anti-HCV in alcoholics with cirrhosis (approximately 40–50%) as compared with those with minimal liver damage (approximately 20%) suggests that HCV infection might be implicated in the development of the cirrhosis in some of these patients. This observation may also account for the high prevalence of anti-HCV (50%) in alcoholics with HCC.[241,242,264,265] In contrast, there is no evidence for a role of HBV in the development of alcoholic cirrhosis, as the prevalence of anti-HBs and anti-HBc, although higher than in the general population, does not significantly differ whether or not a cirrhosis is diagnosed (approximately 20%). However, there is evidence for

**Table 17.1** Total prevalence of HBV markers in patients with HCC

| Geographical area | HCC (%) | HCC on alcoholic cirrhosis (%) |
|---|---|---|
| Zambia, Taiwan | 100 | NT |
| China | 98 | NT |
| Philippines | 97 | NT |
| Senegal | 96 | NT |
| Uganda | 89 | NT |
| Japan | 81 | NT |
| Greece | 80 | NT |
| USA | 74 | NT |
| Italy (north) | 49 | 81.5 |
| France (south) | 43 | 58 |
| Great Britain | 32 | 27 |

NT, not tested. (Data from refs.[237,239,241,242])

**Table 17.2** HBV DNA in HBsAg-negative patients with HCC: Southern and dot-blot*

| Geographical area | Tumour | Serum | Reference |
|---|---|---|---|
| France | 17(6)/20 | 0/9 | Bréchot *et al.*[39] |
| | NT | 3/54 | Pol *et al.*[243] |
| | 4(4)/21† | NT | Marcellin *et al.*[244] |
| Italy | 0/6 | NT | Pontisso *et al.*[245] |
| | 2/8‡ | NT | Pontisso *et al.*[246] |
| Germany | 0/17 | NT | Walter *et al.*[247] |
| UK | 1(1)/7 | NT | Cobden *et al.*[248] |
| | 0/8 | NT | Dunk *et al.*[249] |
| | 1/6 | NT | White *et al.*[250] |
| US | 0/5 | NT | Fong *et al.*[251] |
| Japan | 2(2)/15 | NT | Hino *et al.*[252] |
| | 4(3)/21 | NT | Hino *et al.*[253] |
| | 5/13 | NT | Koike *et al.*[254] |
| | 0/13 | NT | Horiike *et al.*[255] |
| | ½ | NT | Chen *et al.*[256] |
| China | 1/3 | NT | Zhou *et al.*[209] |
| | 3/3 | NT | Hsu *et al.*[257] |
| Hong Kong | 3/5 | 0/12 | Lok *et al.*[258] |
| Taiwan | 4/21 | NT | Lai *et al.*[259] |
| South Africa | 0/5 | NT | Shafritz *et al.*[260] |
| | 3/8 | NT | Shafritz *et al.*[260] |

NT, not tested.
*Positive cases/tested cases. Numbers in parentheses indicate patients without any serological HBV marker.
†Patients with HCC developing on histologically normal adjacent liver.
‡HCC developing in children.

the role of HBV in the liver cancers occurring in alcoholics, as the prevalence of HBV serological markers is significantly increased in these patients (approximately 50%), and the tumours frequently contain HBV DNA sequences.[236,266–268]

The actual prevalence of these HBV DNA-positive HCCs in HBsAg-negative subjects has been a matter of debate, owing to the presence in the tumours of a low copy number per cell of the viral DNA sequences (estimated at 0.1 to 0.001). Thus, studies performed in different geographical areas showed very different results (Table 17.2), probably as a result of different technical conditions (specificity and sensitivity) and distinct epidemiological situations. The sensitivity of the polymerase chain reaction (PCR) has now allowed confirmation of the previous observations, demonstration of the transmission of HBV particles present in the serum of these HBsAg-negative patients to chimpanzees, and determination of the nucleotide sequences of the HBV genomes. For example, in a study performed in patients from areas of high (South Africa) and low (France, Italy) HBV prevalence, HBV DNA was shown in eight of ten serologically recovered subjects and in six of thirteen patients without any detectable HBV serological marker.[269] Similar results were also recently obtained in Spain, Africa and the United States (Table 17.3).[217,269–275] There is no real correlation between the serological HBV profiles and the presence or absence of HBV DNA detection in serum or tumour. Taken together, they show a persistent HBV infection in a large number of subjects with HBsAg-negative HCCs. Whether these infections are clinically significant or purely 'occult' is an important debate.[276]

Related findings have been reported in the woodchuck and ground squirrel models of HCC. Liver cancer occurs in 17% of serologically recovered woodchucks, compared with a zero rate in uninfected animals.[277] WHV DNA was detected in the tumour tissues of seroconverted woodchucks, with a much lower number of copies per cell (about 0.1–0.3) than in HCCs from WHsAg-positive animals (100–1000 copies per cell). In the ground squirrel model, about 25% of serologically recovered animals develop HCC,[227,262] and integrated GSHV sequences have been characterized in two of five tumours analyzed.[17] In addition, HCC has also been observed in about 20% (six of thirty) of aged, seronegative squirrels. In these tumours, GSHV DNA is undetectable by conventional methods. However, low levels of GSHV DNA have been found in two different cases by using PCR,[17] reinforcing the established association between HCC development in ground squirrels and infection with GSHV.

With regard to the state of HBV DNA, its low copy number per cell has hampered the interpretation of the results of Southern blotting. However, using our PCR test and distinct primers distributed on the S, pre-S/S, C and X HBV genes, we were able to provide further information. For several patients, a positive result was obtained only with some of the HBV primers, a finding consistent with the presence of defective HBV DNA. In other cases, the tumour DNA scored positive with all the HBV primers, consistent with the presence of free or integrated HBV DNA showing no gross rearrangements. Interestingly, defective HBV genomes have been identified more frequently in tumours than in non-tumour tissues. In addition, they also have been shown more frequently in completely seronegative individuals than in anti-HBs- and anti-HBc-positive subjects. This observation probably reflects a technical point, as the presence of defective HBV DNA can be obscured by concomitant complete viral genomes. The PCR profiles obtained with DNA from tumour, non-tumour and serum samples from the same European patients showed marked differences. In the serum and non-tumour samples, amplification was achieved with all the primers tested. In contrast, tumour DNA repeatedly gave negative results with at least one primer. These findings demonstrate that the HBV DNA sequences in the tumour do not derive from contaminating non-tumour cells or serum-derived particles. They are also consistent with the clonal expansion of cells containing defective and integrated HBV DNA.[217] A high rate of mutations has been identified in the X gene in the tumour tissue from such HBsAg-negative HBV DNA-positive patients. This high rate of amino acid changes contrasted with a lower rate of X mutations in the non-tumour section from the same patient. It also contrasted with a lower rate of mutations in the pre-C/C sequence (Poussin *et al*., unpublished observations). These findings might have two important implications. X mutations might be implicated in

**Table 17.3** HBV DNA in HBsAg-negative patients with HCC: PCR*

| Geographical area | Tumour | Serum | Reference |
|---|---|---|---|
| France | 5(2)/10 | NT | Paterlini *et al.*[269] |
| | 5(3)/8 | 11(6)/22 | Paterlini *et al.*[217] |
| Italy | 5(4)/9 | NT | Paterlini *et al.*[269] |
| | 2/3† | NT | Pontisso *et al.*[270] |
| Spain | NT | 12(5)/54 | Ruiz *et al.*[271] |
| Japan | 8/22‡ | NT | Ohkoshi *et al.*[272] |
| South Africa | 6/8 | NT | Paterlini *et al.*[269] |
| Senegal | 18/31 | NT | Coursaget *et al.*[273] |
| Mozambique | 4(1)/11 | NT | Dazza *et al.*[274] |
| USA | 14/38 | 25/105 | Liang *et al.*[275] |

NT, not tested.

*Positive cases/tested cases. Numbers in parentheses indicate patients without any serological HBV marker.

†HCC developing in children.

‡Non-tumorous tissue analyzed only.

the low replication profile of the HBV genome in these tumours. In addition, such mutations might modify the biological properties of X in the tumour, and this possibility is now being tested.

Northern blot analysis has not proven sensitive enough for the detection of HBV RNAs in these tumours. In contrast, cDNA synthesis followed by PCR with primers on the S gene (RT-PCR) revealed HBV RNA sequences in most of HBV DNA-positive tumours from HBsAg-negative patients. Owing to the compact organization of the HBV genome, however, it was not possible to determine precisely which viral transcripts were synthesized.[269] We have investigated the HBV RNAs with primers located on the S, C and X encoding sequences. X, but not C and S, transcripts were identified in tumour tissues from seven of the nine HBsAg-negative HBV DNA-positive patients studied.[216] More recently, we confirmed these observations by detecting the X protein in these tumorous liver cells (Poussin *et al*., unpublished observations). This may provide a clue to the pathogenesis of these tumours in view of the potential transforming properties of the X protein envisaged in HBsAg-positive liver cancers. With this view the issue of the interactions between X and P53 is important. It is indeed tempting to speculate that two types of HCCs might emerge from studies focused on both P53 and X. Some tumours (altogether around 20%) show P53 mutations and thus would not 'need' X expression for P53 inactivation, whereas in the remaining cases, X might functionally inactivate wild-type P53. Preliminary evidence supports this hypothesis,[278] but further confirmation is required.

Taken together, results demonstrate a high rate of persistent HBV infection in patients with HCC negative for serum-HBsAg, many of whom also lack detectable antibodies to the virus. They also show clonal expansion of the tumour cells containing integrated viral DNA and preferential transcription in these cells of HBV RNA sequences encoding the viral X-protein. A direct role of HBV in these liver cancers is further suggested by the detection of HBV DNA in HCCs in HBsAg-negative patients, developing on non-cirrhotic, histologically close-to-normal livers. Thus, cirrhosis cannot solely account for the induction of the cancer in these cases.[279] Although these findings strongly argue for a role for HBV in the development of these tumours, one paradoxical result still remains to be explained: why is the copy number of HBV DNA per cell so low if clonal expansion of infected cells occurs? One may hypothesize that, after triggering the cascade of events that lead to liver cell transformation, the persistence of HBV DNA is no longer required. Furthermore, as discussed in previous sections, there is low or absent HBV replication in the tumour cells and thus no generation of new integrants from free viral genomes. Finally, chromosomal rearrangements might eliminate integrated viral DNA sequences from the tumour clones. This explanation may also account for human and bovine papillomavirus- and some retrovirus-related tumours.[280,281] Interestingly, there is recent evidence in the duck hepatitis model that supports these hypotheses.[282]

## HCV and HCC

HCV is clearly an important aetiological factor of HCC, although the impact of the viral infection varies among geographical areas. Chronic hepatitis plays a major role in the liver carcinogenesis related to this virus. It is presently not clear whether HCV might also exert direct effects in liver cell transformation, although some evidence supports this hypothesis.[284]

HCV RNA sequences can be detected in the tumorous tissues of patients with liver cancer. Sequencing of the hypervariable part of the E2 envelope region has confirmed that HCV RNA sequences persist in the tumour cells and do not merely reflect contamination by non-tumour cells or serum particles. Instead, mutations identified in the HCV RNA sequences from tumour as compared to non-tumour cells reflect infection by an identical HCV isolate, followed by replication at a different rate of the HCV genome when tumour develops.[216,283] In contrast with HBV, HCV genome does not integrate into cellular DNA, and replication is thus necessary to its persistence. Studies based on PCR for detection of negative HCV strand, as well as identification of different HCV proteins into tumour cells, have been consistent with replication of HCV genome. Such studies are complicated by technical pitfalls when searching for HCV RNA-negative strands by PCR[285] and for HCV proteins with the available antibodies. As previously discussed for HBV, a main issue for understanding HCV-related carcinogenesis is whether the virus is only acting by inducing chronic active hepatitis (CAH) and cirrhosis or might also directly modify the cellular phenotype.[286,287] Two factors favour the hypothesis of a direct effect of some HCV isolates. We have reported rare but well-characterized HCCs in livers with minimal histological lesions and that, despite detailed analysis, showed persistence of HCV RNA in the tumours as the only identified risk factor.[288] This study has demonstrated that, in some cases, HCC might be associated with HCV in the absence of CAH. *In vitro*, it has been shown that expression of a region of the NS3 viral protein induces a transformed phenotype in NIH 3T3 cells.[246] Furthermore, it has been demonstrated that the HCV capsid can cooperate with the c-Ha *ras* oncogene to transform rat embryo fibroblasts.[289] More recently, it has been shown that transgenic mice carrying the complete HCV genome, or expressing the HCV core alone, develop liver steatosis and have high rates of HCC.[290,291] The mechanism of core-induced HCC is not well understood, but may

involve interaction with one or more cellular proteins required for the control of cell growth. Moreover, the HCV core protein has been shown recently to induce oxidative stress in transgenic mice.[292]

Altogether, these studies suggest that HCV proteins might exert a direct effect in liver carcinogenesis. The mechanisms involved in this transforming property are not known. However, there are some interesting issues to consider. The viral core, especially carboxy-terminal deleted forms, might modulate expression of cellular genes, such as the *fos*, *myc* and *jun* oncogenes, the retinoblastoma suppressor gene, or the gene encoding for IFN-β.[293,294] The capsid might also interfere with the tumour necrosis factor pathway and thus modulate the sensitivity of the cell to this cytokine.[33] Finally, we have presented evidence for an accumulation of lipid-containing vesicles in the cells upon expression of the capsid, as well as its co-localization with apolipoprotein AII.[295] This is a potentially relevant observation in view of the high prevalence of liver cell steatosis in HCV carriers and the association of circulating HCV particles to lipoproteins. The localization in the cell of the capsid, therefore, is important for understanding the real impact of these findings, and in particular its nuclear localization is important to consider. HCV capsid shows predominant cytoplasmic localization. It has also been suggested that carboxy-terminally deleted forms of the capsid, generated from some isolates, might locate in the nucleus, but this is still a matter of debate.[296] In view of the potential role of HCV capsid in liver cell transformation, it is interesting that point mutations have been identified in HCV core sequences from HCCs developed in HCV carriers in the absence of cirrhosis or severe chronic hepatitis.[297] These mutants co-localize with PKR in Huh7 cells, and this association augments the autophosphorylation of PKR and the phosphorylation of the translation initiation factor eIF2alpha, which are two markers of PKR activity.[297] In addition, HCV genomes potentially encoding for truncated HCV capsids have been identified in other tumours.[298,299] Another protein of HVC, NS5A, also possesses many intriguing properties, including sequestration of p53 in the cytoplasm, downregulation of p21, activation of STAT3, inhibition of TNF-α-mediated apoptosis. It has been shown to alter intracellular calcium levels and induce oxidative stress.[300] Thus, persistent stimulation of cellular stress responses by accumulation of viral proteins within hepatocytes may predispose the cell to genetic alterations and play an important role in hepatitis virus-induced liver diseases and HCC. An important question regarding the role of hepatitis viruses in HCC is whether viral gene products are necessary for the establishment of HCC and whether the cancer, once established, becomes independent of the expression of viral genes.

The impact of HCV genotypes in the risk of developing HCC is an important issue to analyze. A number of studies point to the severity of HCV type 1-associated liver lesions, including HCC. Cross-sectional analyses indeed have shown higher relative prevalence of HCV 1 in patients with cirrhosis than in those with moderate CAH. However, these findings are difficult to interpret because the molecular epidemiology of HCV is presently changing as a result of the introduction, in France and Italy, of other types such as HCV 3 by intravenous drug users. In these conditions, duration of HCV infection differs from one type to another and markedly influences the risk of cirrhosis.[301,302] In prospective studies on the risk of developing HCC in patients with HCV-related cirrhosis, infection by genotype 1 has emerged as an independent risk factor.[303] Along the same line, HCV 1b was the most prevalent type among the patients we reported with HCV-associated HCC in the absence of cirrhosis.[288] Although these examples provide only indirect evidence, they are consistent with a particular profile of HCV 1 infection. *In vitro* studies are now mandatory to compare the different biological properties of various HCV isolates.

## Genetic alterations in HBV-related HCC

Different genetic alterations that cannot be clearly associated with a direct effect of viral infection have been described in human HCCs. These somatic changes include allele losses on several chromosomal regions, mutation and activation of cellular genes showing oncogenic potential, and deletion or mutation of tumour-suppressor genes. Search for activated oncogenes using the NIH3T3 cells in transformation assays has not been conclusive for most HCC DNAs analyzed. DNA analysis revealed a low rate (<10%) of point mutations in H-, K- and N-*ras*,[304] whereas other investigators failed to detect any *ras* mutation in the HCCs analyzed.[305]

Loss of heterozygosity on chromosomes 1p, 4q, 6q, 8p, 9p, 13q, 16p, 16q and 17q occurs frequently in human liver tumours, suggesting that these parts of the human genome contain some genes whose functional loss might be involved in hepatocellular carcinogenesis.[306,307] Essentially similar chromosomal regions were found to harbour changes in DNA copy number by comparative genomic hybridization[308] (reviewed by Buendia[309]). Because the large-scale chromosomal alterations that arise in cancer cells occur infrequently in normal cells, it is probable that control mechanisms that safeguard chromosomal integrity are abrogated in the development of malignancy.[310] Such changes might represent secondary events linked to tumour progression and reflect a general property of transformed cells. Interestingly, it has been found that HBV-related tumours harbour a higher rate of chromosomal abnor-

malities than tumours associated with other aetiological factors.[311,312] A role of HBV DNA integration and of the HBx regulatory protein in promoting genetic instability has been evoked.[109,224]

Allele loss of the short arm of chromosome 17, which includes the p53 gene, has been observed in 30–60% of human HCCs and in hepatoma-derived cell lines (reviewed[313,314]). The wild-type p53 gene negatively regulates cellular growth and, therefore, was designated a tumour-suppressor gene or *anti-oncogene*. Mutant forms of p53 frequently gain a growth stimulatory function. Genetic alterations of the gene generally consist in the deletion of one p53 allele and mutation of the second allele, and represent the most common feature in human neoplasms. Studies of the p53 gene at the DNA, RNA and protein levels have revealed abnormal structure and expression in most established HCC cell lines. A hot spot mutation affecting p53 at codon 249 was originally described in HCCs from regions with high prevalence of HBV infection and high levels of dietary aflatoxins.[315,316] This mutation, mainly a G to T transversion, appears to be specific for the mutagenic action of aflatoxin $B_1$.[317] This particular mutation was not observed in HBV-related HCCs from patients who have not had high exposure to the hepatocarcinogen. P53 codon 249 mutation was detected in apparently normal liver, and its frequency paralleled the level of aflatoxin $B_1$ exposure, suggesting that it occurs early during HCC development.[92,318] Functional characterization of the mutant p53 gene might provide a clue for understanding the oncogenic mechanism triggered by aflatoxin $B_1$. Structural aberrations of the p53 gene (mutations at different codons and loss of one allele) have been observed more frequently in advanced, less differentiated HCCs, often associated with abnormalities of another tumour-suppressor gene, RB.[319] The frequency of p53 mutations has been evaluated in tumours from diverse geographical and ethnic sources and found to be much lower than previously reported. Interestingly, it has been shown that the rate of p53 mutations is significantly higher in HBV-related tumours than in HCV-related and non-viral HCCs.[311] Although HBV DNA insertions in chromosome 17p have been described in some liver tumours, it seems most probable that the genetic alterations observed in a majority of human HCCs are not due to a direct action of the virus. Regional deletions spanning the RB locus on chromosome 13q have also been reported, but a low mutation rate was found in the remaining allele.[319] Candidate tumour suppressor genes in other frequently deleted chromosomal regions include the mannose 6-phosphate/IGF-II receptor (M6P/IGF2R) gene on chromosome 6q,[320] the p16INK4A and p14ARF genes on chromosome 9p[321] and the E-cadherin gene on chromosome 16q.[321a]

In conclusion, recurring deletions spanning many human chromosomes might inactivate a number of tumour suppressors and thus play a key role in hepatocarcinogenesis. Besides genetic alterations, epigenetic factors like methylation-associated gene silencing play an important role in the deregulation of cell cycle control and proliferation. Frequent hypermethylation of CpG islands in the promoters of p16INK4A, E-cadherin and adenomatous polyposis coli (APC) genes has been reported in HCC.[322,323] This epigenetic alteration occurs early during the tumoral process, accumulates step by step during tumour progression, and might be of prognosis value for HCC patients.

Activation of the Wnt/β-catenin pathway has been implicated as an important step in carcinogenesis, through activating mutations in the β-catenin gene, or defects in the adenomatous polyposis coli (APC) or Axin genes.[324] Activation of the Wnt signal induces translocation of β-catenin to the nucleus and its association with the transcription factors Tcf/LEF, leading to transcriptional activation of target genes such as c-myc and cyclin D1.[325] Recently, oncogenic mutations in the β-catenin gene have been evidenced in human and mouse liver tumours.[326,327] β-Catenin mutations were found in around 20% of HCC cases, predominantly in HCV-related tumours and in tumours associated with alcoholic cirrhosis.[311] Additionally, Axin mutations have been detected in 5–10% of liver tumours, but no mutation has been found so far in the APC gene in HCC, contrasting with the predominant role of APC in colorectal cancer. It is noteworthy that p53 mutation has been reported to induce nuclear accumulation of wild-type β-catenin in liver tumours, which might account for the observation that worldwide p53 and β-catenin mutation rates are inversely correlated in HCC.[328] Consistent with this notion, HBV-related tumours exhibit a high rate of p53 mutations, but rare β-catenin mutations, while the inverse situation is found for HCV-related HCCs.[309] Further studies are required to better characterize the molecular mechanisms leading to the appearance of β-catenin mutations and to understand their oncogenic impact. Genome-wide studies of gene expression in HCC and in chronic hepatitis by microarray screening will undoubtedly contribute to a better understanding of the complex mechanisms involved in virally induced liver tumorigenesis.

## Conclusions

Several lines of evidence contribute in linking chronic HBV infection and primary liver cancer. First, a strong epidemiological association has been provided by extensive prospective and retrospective studies in many parts of the world. Second, a network of indirect but convincing arguments has further supported the connection between HBV and HCC, including the existence of related animal viruses that induce HCC in their hosts, the weak

oncogenicity of the viral X transcriptional transactivator, the long-term tumorigenic effect of surface glycoproteins, and the mutagenic action of viral integration into the cell genome, which may, directly or indirectly, contribute to deregulating the normal cell growth control.

The exponential relationship between HCC incidence and age indicates that, as in other human cancers, multiple steps, probably involving independent genetic lesions, are required. In particular, the long latency of HCC development after the initial HBV infection may be interpreted as a sign of an indirect action of the virus. A long-term toxic effect of viral gene products and/or the immune response against infected hepatocytes would trigger continuous necrosis and cell regeneration, which would in turn favour the accumulation of genetic alterations. In this model, productive HBV infections might potentiate the action of exogenous carcinogenic factors, such as aflatoxins and alcohol. It might also be speculated that the latency period depends on the occurrence of a decisive HBV integration event that would promote genetic instability or lead to *cis*- or trans-activation of relevant genes. Investigations of the functional and pathological properties of HBV gene products and of the consequences of HBV integration in the liver DNA suggest that various and probably cooperative mechanisms may operate in the development of liver cancer, and that HBV may share a number of basic strategies with other human oncogenic viruses. The HBV genome encodes at least seven different polypeptides. None of them seems to act as a strong, dominant oncogene, but several lines of evidence indicate that the surface glycoproteins and the viral X transactivator might participate in carcinogenesis in a native or modified state. In this respect, it is noteworthy that in human oncogenic viruses, such as HTLV-1, EBV, and HPV-16 and -18, transforming capacity is associated with the transcriptional transactivation activity of viral gene products. Comparative analyses of the different viral transactivators may help guide future work in this field. Studies of mammalian HBV-related viruses have revealed strikingly different mechanisms and pointed out the importance of the activation of *myc* family genes in rodent hepatocarcinogenesis. However, a role for HBV in activating the c-*myc* oncogene, suggested by *in vitro* assays, has not been established *in vivo*, and recent data support a predominant role of tumour-suppressor genes such as p53 in human HCCs. The identification of the cellular effectors connecting HBV infection and liver cell transformation is the major unsolved component in this question.

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# Chapter 18
# Murine models and hepatitis B virus infection

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## Introduction

Because of the limited host range of the hepatitis B virus (HBV) and the lack of *in vitro* culture systems to propagate HBV, investigators have utilized murine systems as surrogates. Wild-type mice have been used to: (1) assess the comparative immunogenicity of HBV proteins; (2) identify putative T- and B-cell epitopes; (3) examine immunogenetic factors; (4) produce models of infection; (5) examine immune tolerance; and (6) design candidate vaccines. Furthermore, a number of investigators have expressed single HBV proteins, combinations of HBV proteins, the complete HBV genome and T-cell receptors (TCRs) specific for HBV proteins in transgenic (Tg) mice in order to study gene expression, pathogenesis, replication, inhibitors of replication, viral persistence, modulation of immune response and hepatocellular carcinoma (HCC). It was anticipated that expression of viral proteins in inbred mice, which possess well-characterized immune systems, would allow detailed studies of the immune response to viral proteins *in vivo*. Use of liver-specific promoter systems has allowed viral protein expression to be targeted to the physiologically relevant site, the hepatocyte. These Tg systems have yielded a number of interesting insights into the biology, immunology and pathogenic potential of these liver-tropic viruses. This chapter attempts to catalogue and characterize a number of the observations made in murine models that have contributed to our understanding of HBV, an important human pathogen.

## Immunogenicity of HBV proteins studied in mice

Table 18.1 lists a number of important observations first described in mice.[1–47] For example, early studies in the

**Table 18.1** Immunogenicity and function of HBV proteins examined in non-Tg mouse models

| Immunogenicity and function | References |
|---|---|
| HBsAg: | |
| HBsAg is a T-cell-dependent antigen | 1 |
| MHC restriction of HBsAg and pre-S immune responses | 2–4 |
| Pre-S immune response can bypass non-response to HBsAg | 5–7 |
| Pre-S region contains protective epitopes | 8–13 |
| Construction of fully synthetic candidate vaccines | 14–19 |
| HBcAg-specific T cells can 'help' HBsAg-specific B cells | 20 |
| Mouse model of acute infection: hydrodynamic transfection | 21 |
| Identification of T- and B-cell epitopes | 22–28 |
| HBc/HBeAgs: | |
| HBcAg can function as a T-cell-independent antigen | 29 |
| Cross-reactivity between HBc/HBeAgs at the T-cell level | 30–34 |
| HBcAg elicits Th1 cells and HBeAg elicits Th2 cells | 35 |
| B cells can act as primary APCs for HBcAg not HBeAg | 36 |
| HBeAg but not HBcAg can enter the MHC class II endogenous APC pathway | 37 |
| Use of HBcAg as a vaccine carrier platform | 38–43 |
| Identification of T- and B-cell epitopes | 31,32,42,44–47 |

APC, antigen-presenting cell; MHC, major histocompatibility complex; HBsAg, hepatitis B surface antigen, HBcAg, hepatitis B core antigen, HBeAg, hepatitis B 'e' antigen.

murine system indicated marked strain variation in antibody production after immunization with the hepatitis B surface antigen (HBsAg)/P25 (major envelope protein).[2,3] Further studies in mice that were genetically identical except within the major histocompatibility complex (MHC) indicated a strong influence of MHC-linked genes on the immune response to HBsAg and identified high (H-$2^{d/q}$), intermediate (H-$2^{a}$>H-$2^{b}$>H-$2^{k}$), and non-responder (H-$2^{f,s}$) MHC genotypes.[4] When recombinant HBsAg particles containing the larger molecular weight polypeptides became available, studies were extended to examine genetic influences on the immune responses to pre-S sequences. Production of antibodies against the pre-S2 region after immunization with HBsAg/GP33 (middle envelope protein) is also MHC-restricted. However, the hierarchy of response status among strains differs from that of the S region. Additionally, immunization with HBsAg/GP33 can bypass non-response to the S region in HBsAg/P25 non-responder H-$2^{s}$ mice.[5] The immune response to the pre-S1 region of HBsAg/P39 (large envelope protein) is also influenced by H-2 linked genes, but again the hierarchy of response status differs from the responses to the S and pre-S2 regions. HBsAg/P39 immunization elicited anti-pre-S1-specific antibody on all strains and, furthermore, elicited anti-S and anti-pre-S2-specific responses in 'non-responder' H-$2^{f}$ mice.[6] These data indicated that distinct MHC-linked genes influence S-, pre-S2- and pre-S1-specific antibody production *in vivo*.

The ability of HBsAg/GP33 immunization to bypass S region non-responder status in H-$2^{s}$ mice and of HBsAg/P39 immunization to bypass S and pre-S2 region non-responder status in H-$2^{f}$ mice suggested that although these strains lack an S-specific T-cell response, they must possess pre-S-specific T cells that can 'help' B-cell clones specific for S as well as pre-S region epitopes to produce antibody. Studies of HBsAg and pre-S-specific T-cell responses have confirmed that the mechanism responsible for circumvention of non-responsiveness is mediated at the T-cell level. For example, H-$2^{s}$ mice do not possess T cells that can recognize the S region of HBsAg, but T cells of H-$2^{s}$ mice can recognize the pre-S2 region.[7] These studies indicated that the envelope polypeptides of HBV present an array of T-cell determinants to the host's immune system, the specificity of the T-cell recognition process is influenced by MHC-linked genes, and that T-helper cell recognition of one region can bypass non-response to another region. Because most current recombinant HBV vaccines contain HBsAg/P25 but lack pre-S sequences, a number of the results from murine studies have suggested that inclusion of pre-S sequences in future generation vaccines may be beneficial.

Using inbred murine strains investigators have identified a number of T-cell and B-cell recognition sites on HBV proteins.[22–28,31,32,42,44–48] The epitopes identified largely overlap with sites recognized by the human immune response. Use of synthetic epitopes for *in vitro* neutralization experiments,[10] and *in vivo* immunization with pre-S synthetic peptides[8,9,11–13] have shown that pre-S1 and pre-S2-specific antibodies in the absence of S region antibody can protect chimpanzees against experimental HBV challenge. Furthermore, a number of fully synthetic HBV candidate vaccines have been constructed using epitopes defined originally in mice.[14–19] Additionally, the use of synthetic hepatitis B core antigen (HBcAg) derived T-cell epitopes in mice demonstrated that HBcAg-specific CD4+ T cells can help HBsAg-specific B cells produce anti-HBs antibodies through a mechanism termed intermolecular-intrastructural T-cell help.[20] Athymic mice were used to demonstrate that the HBcAg can elicit anti-HBc antibodies in a T-cell-independent manner, whereas hepatits B 'e' antigen (HBeAg) is a T-cell-dependent antigen.[29] Earlier murine studies had demonstrated that HBsAg, although particulate like HBcAg, functioned as a T-cell-dependent antigen.[1]

Murine studies first revealed that HBcAg and HBeAg both can be cross-reactive targets of CD8+ cytotoxic T lymphocytes (CTLs).[30,31,34] HBeAg and the HBcAg also are cross-reactive at the level of CD4+ T-cell recognition.[32,33] However, in a non-infectious system HBcAg tends to elicit a CD4+ T-cell response of the Th1-like type, whereas HBeAg preferentially elicits a Th2-like response but also may activate a Th1-type response depending on the cytokine milieu.[35] For example, *in vivo* treatment with interleukin (IL)-12 can shift an HBeAg-specific Th2-like response to a Th1-like response in an HBeAg-transgenic (Tg) model.[49] Differential activation of Th cell subsets likely is due to the fact that HBcAg-specific B cells can function as the primary antigen-presenting cell (APC) for HBcAg, whereas the primary APCs for HBeAg are macrophages and dendritic cells.[36] It has recently been shown that HBeAg can enter both the MHC class I endogenous pathway for recognition by CD8+ CTLs and the MHC class II endogenous pathway for recognition by CD4+ T cells. HBcAg cannot enter the class II endogenous pathway.[37] Therefore, hepatocytes producing cytosolic HBeAg may be targeted for destruction by CD8+ and/or CD4+ T cells. This observation may help to explain how an HBeAg-negative mutant virus is selected by the immune response. Importantly, murine studies have suggested that secreted HBeAg may have a number of immunoregulatory properties that the HBcAg does not possess, as discussed later (see Table 18.4 below). A number of unique characteristics of the HBcAg including: ability to self-assemble; particulate structure; T-cell independence; ability to elicit intermolecular-intrastructural Th-cell function; ability to activate prima-

ry B-cell APCs, and the ability to accept the insertion of foreign-cell epitopes into the exposed loop region suggested that HBcAg could function as an efficient HBV and non-HBV vaccine carrier platform. A number of murine studies have validated that prediction.[38–43] Lastly, a non-Tg murine model of HBV replication, immunogenicity and viral control has been reported.[21] After hydrodynamic transfection of hepatocytes *in vivo* with a replication-competent, over-length, linear HBV genome, viral antigens and replicative intermediates were synthesized and virus was secreted. Persistence of virus was dependent on the host immune response. This promises to be a useful model system.

## HBV gene expression and viral replication in Tg mouse models

The first studies using Tg mice focused on the expression of the HBV envelope proteins (Table 18.2).[50–70] These early studies demonstrated that the envelope proteins could be expressed in the liver by using liver-specific promoters or the HBV endogenous promoters.[50–57] Using the endogenous HBV promoters resulted in expression in other tissues in addition to the liver. It was also demonstrated that envelope gene expression is developmentally regulated,[56] and is positively regulated by androgens and glucocorticoids.[57] Transgenic expression of the middle and major envelope proteins led to the assembly and secretion of 22-nm spherical particles as occurs during a natural HBV infection.[54,55] Inclusion of the domain encoding the large envelope protein (pre-S1-containing) in the transgene construct resulted in the assembly of long branching filamentous hepatitis B surface antigen (HBsAg) particles.[54] If the large envelope protein was overexpressed in relation to the middle and major proteins by the use of an exogenous promoter (i.e. albumin promoter), the filamentous HBsAg particles became trapped in the endoplasmic reticulum (ER) and were not secreted by the cell. This secretion defect eventually leads to a dramatic expansion of the ER in the hepatocyte and severe liver injury.[55] It has been suggested that such hepatocytes are analogous to 'ground glass' hepatocytes observed in the liver of chronically infected patients.[55] However, the nature of a putative secretion defect in infected hepatocytes is not clear.

**Table 18.2** HBV gene expression and replication in Tg mice

| Gene expression and replication | References |
|---|---|
| HBV gene expression | |
| Envelope proteins | 50–57 |
| Nucleocapsid proteins | |
| Hepatitis B core antigen | 58–61 |
| Hepatitis B 'e' antigen | 60,62,63 |
| X protein | 64–66 |
| HBV replication | 67–69 |
| Infectivity of Tg-derived HBV | 70 |

Both HBV nucleocapsid proteins, the particulate HBcAg and the non-particulate HBeAg, have been expressed in Tg mice (Table 18.2). HBeAg is efficiently secreted into the blood, and HBcAg accumulates in the nucleus of hepatocytes.[58–63] Expression of high levels of HBcAg revealed that intact HBcAg particles could not traverse the nuclear membrane in either direction.[58] The non-structural HBV X-protein has also been expressed in Tg mice.[64–66] The X-protein displays transcriptional transactivation properties, and it has been suggested that expression of this protein in the liver may play a role in the induction of hepatocellular carcinoma (HCC), which is associated with chronic HBV infection. In support of this hypothesis, high level liver-specific expression of the HBV X-protein has led to HCC in Tg mice.[64,65] However, other investigators have not observed the induction of HCC in independently derived X gene Tg mice.[66]

Several laboratories have used constructs containing the entire HBV genome and HBV-derived regulatory sequences to produce Tg mice capable of viral replication (Table 18.2). Interestingly, HBV replication occurred in the kidney as well as the liver, and the supercoiled form of HBV DNA (cccDNA) has not been observed in any lineage of Tg mouse.[67–69] In several Tg lineages, high level replication is sustained in 20–30% of primarily centrilobular hepatocytes, although virtually all hepatocytes express nuclear HBcAg.[69] This suggests that hepatocytes infected in a natural infection may not all be equally permissive for HBV replication. High level replication and HBV gene expression are not associated with liver pathology.[67–69] One group has successfully infected chimpanzees with viral particles derived from Tg mice.[70] This model of HBV replication has provided the opportunity to examine the influence of viral and host factors on HBV replication, pathogenesis and clearance, including the effects of the immune response. For example, a number of factors have been shown to be capable of inhibiting HBV replication in the Tg model (see Table 18.3).[71–90] These and other approaches are discussed in the following sections.

In summary, a number of investigators have expressed the structural proteins of the HBV in Tg mice, and these Tg systems have yielded a number of interesting results, which are detailed below. However, one

**Table 18.3** Inhibition of HBV gene expression and/or replication in Tg mice

| Inhibition | References |
|---|---|
| Direct antiviral effects of HBsAg-specific CTLs | |
| Acute hepatitis | 71,72 |
| Fulminant hepatitis | 73 |
| Chronic hepatitis | 74 |
| Non-cytopathic antiviral effects of cytokines | 75–79 |
| Cellular sources of cytokines include: CTL, NK, NKT and APC | 80–83 |
| HBV gene expression and replication are inhibited by distinct mechanisms | 76,81,84 |
| Post-transcriptional and transcriptional control of HBV gene expression | 76,85–87 |
| The IFN-mediated antiviral response is proteosome-dependent | 88 |
| HBsAg-specific CD4+ cells suppress HBV gene expression | 80,83 |
| Antiviral effects of heterologous hepatic infections | 89,90 |

IFN, interferon.

important conclusion from these studies is that the viral proteins themselves are not directly cytopathic within the liver. The only exception is the overexpression of the HBV large pre-S1-containing protein leading to a secretion defect.[55]

## Tg models of HBV pathogenesis and liver injury

Studies employing the adoptive transfer of CD8+, HBsAg-specific CTLs into HBV envelope-expressing Tg mice have demonstrated that CTLs can induce an acute necroinflammatory liver disease similar to that of natural acute HBV infection (see Table 18.3). The investigators described a three-step process through which the liver disease progresses. The first step involves the attachment of the donor CTLs to HBsAg-positive hepatocytes, which are triggered to undergo apoptosis. Thereafter, between 4 and 12 hours after injection, the CTLs recruit host-derived antigen-non-specific inflammatory cells (i.e. polymorphonuclear cells) that amplify the effects of the CTL (step 2).[71,72] This process results in necroinflammatory foci in which hepatocellular necrosis extends well beyond the location of CTLs, suggesting that most hepatocytes are killed by cells other than the donor CTLs. In these studies, the liver injury in most Tg lineages is transient and is confined to no more than 5% of hepatocytes. However, in recipient Tg mice that overexpress and accumulate HBsAg filaments (see above), the disease process proceeds to step 3, in which approximately half of the mice die of liver failure within 24–72 hours of CTL transfer.[73] The investigators suggest that this process resembles the histopathological features of HBV-induced fulminant hepatitis in humans, characterized by widespread necrosis of HBsAg-laden hepatocytes and diffuse lymphomononuclear inflammatory cell infiltrate and Kupffer cell hyperplasia.[73]

This same group of investigators has developed a model of prolonged chronic immune-mediated (CTL) hepatitis in HBV envelope Tg mice.[74] This was accomplished by transferring HBsAg-specific CTLs into thymectomized, irradiated, bone marrow-reconstituted envelope-Tg recipients. In addition to chronic hepatitis, the Tg recipients eventually (17 months) developed HCC, suggesting that a prolonged inflammatory immune response to an HBV protein can cause liver cancer,[74] consistent with a number of observations in chronically infected humans.[91]

In addition to the direct hepatocyte injury triggered by HBsAg-specific CTLs, a second non-cytolytic mechanism has been described in which the cytokines secreted by the HBsAg-specific CTLs profoundly suppress hepatocellular HBV gene expression and HBV replication in Tg mice.[76,77] The cytokines responsible for these non-cytolytic antiviral effects were CTL-derived interferon (IFN)-γ and CTL-induced tumour necrosis factor (TNF)-α.[76] The antiviral regulatory potential of inflammatory cytokines was confirmed by the fact that administration of recombinant TNF-α,[75] IL-18,[79] IL-2 and to a lesser extent IFN-α and IFN-β,[78] also inhibited HBV gene expression in HBV envelope Tg mice. In addition, direct cytokine-mediated antiviral effects on HBV replication and gene expression have been confirmed *in vitro* in immortalized HBV-Tg mouse hepatocytes.[92,93]

Inhibition of HBV DNA replication and HBV gene expression occur through independent mechanisms that exhibit distinct kinetics and cytokine dependence. The antiviral effects of cytokines in the murine model occur in two phases: (1) HBV DNA replicative intermediates disappear from the liver with no change in viral mRNA; and (2) HBV mRNA is down-regulated at a later time point.[76,81,84] It was demonstrated that HBV replication is inhibited by IFN-α/β at the level of assembly or stability of pregenomic RNA-containing HBV capsids[94] and, further, that this process is proteosome-dependent.[88] In terms of HBV gene expression, the effects of cytokines can be mediated at the post-transcriptional[76,85,86] or the transcriptional level[87] depending on the antiviral stimu-

lus. For example, HBV gene expression was controlled transcriptionally after repeated injections of polyinosinic-polycytidylic acid (poly I/C), an IFN-α/β inducer. Recently, the source of the inflammatory cytokines responsible for inhibition of HBV gene expression has been extended to include CD4+ T-helper (Th)1 cells,[80,83] NKT cells[81] and activated intrahepatic APCs.[82] Furthermore, it was demonstrated that HBsAg-specific CD4+ Th cells could be elicited by DNA immunization in HBsAg-Tg mice.[83]

## Tg models of persistence and/or tolerance

The function of the HBeAg is largely unknown because it is not required for viral assembly, infection or replication.[95–97] However, the HBeAg does appear to play a role in viral persistence. It has been suggested that the HBeAg may promote HBV chronicity by functioning as an immunoregulatory protein.[63,98] Infants born to HBeAg-positive HBV carrier mothers invariably become persistently infected. In contrast, infants born to HBeAg-negative HBV carrier mothers rarely become chronically infected and can often experience fulminant hepatitis.[99] To investigate the role of immunological tolerance mechanisms in chronic infection of the newborn, HBeAg-expressing Tg mice have been generated. HBeAg-Tg mice represent a model system to examine the consequences of *in utero* exposure to HBeAg on HBc/HBeAg-specific immune responses (see Table 18.4).[63,83,98,100-107] Characterization of tolerance in HBeAg-Tg mice and mice rendered neonatally tolerant indicated that T cells but not B cells were tolerized by HBeAg present in the serum at a concentration of 10–100 ng/ml; that T-cell tolerance elicited by HBeAg also extends to HBcAg-specific T cells; that Tg mice produced anti-HBc but not anti-HBe antibodies upon immunization; that the IgG but not the IgM anti-HBc response was diminished in HBeAg-Tg mice; and that the T-cell tolerance induced by a single neonatal exposure to HBeAg was reversible and persisted for 12–16 weeks.[63] Many characteristics of immune tolerance found in HBeAg-Tg mice parallel the long-term immunological status of neonates born to HBeAg-positive HBV carrier mothers, suggesting that the aberrant immunological responses of neonates born to carrier mothers may result from *in utero* exposure to HBeAg, as occurs in the Tg model. In support of the possibility that maternal HBeAg may traverse the placenta, non-Tg littermates born to HBeAg-Tg mothers were tolerant to HBc/HBeAg.[63] Although the ability of HBeAg to cross the murine placenta has been questioned,[108] HBeAg has been detected in the neonatal cord serum of infants born to HBeAg-positive HBV carrier mothers by an increasing number of investigators.[109–112] Furthermore, recent studies in HBeAg-Tg mice, which do not express the HBeAg until several days after birth, indicate that perinatal exposure to HBeAg is sufficient to elicit HBe/HBcAg-specific Th cell tolerance, which may diminish the requirement for HBeAg to cross the placenta in order to have a tolerogenic effect during perinatal infection.[100]

The precore region encoding the HBeAg is also conserved in the avian hepadnaviruses, in which *in utero* tolerance mechanisms are not relevant, suggesting an alternative role for HBeAg. Because adult infection with an HBeAg-negative variant is often associated with a fulminant course, HBeAg may play an immunoregulatory role in adult infection in addition to its effects on neonatal tolerance. As a model of chronic HBeAg exposure and to examine the tolerogenic potential of the HBeAg, HBc/HBeAg-transgenic (Tg) mice crossed with T-cell receptor (TCR)-Tg mice expressing receptors for the HBc/HBeAgs (i.e. TCR/Ag double-Tg pairs) have been produced. In a recent study, serum HBeAg was shown to be tolerogenic for HBc/HBeAg-specific TCR-Tg T cells and three distinct phenotypes of tolerance were observed in double-Tg mice.[100] One phenotype, illustrated by a high avidity (8/12–2) TCR-Tg lineage, was profound tolerance induction by the HBeAg even at very low serum concentrations (10 ng/mL) most likely mediated by clonal deletion and FAS-mediated apoptosis in the periphery. A second phenotype, represented by a low avidity (11/4-12) TCR-Tg

**Table 18.4.** Mouse models of HBV persistence or tolerance

| Model | References |
|---|---|
| Secreted HBeAg may induce T-cell tolerance *in utero* | 63 |
| Exposure to HBeAg perinatally induces T-cell tolerance | 100 |
| T-cell tolerance to the HBeAg is variable and MHC-dependent | 100,101 |
| Secreted HBeAg can delete HBc/HBeAg-specific Th1 cells via Fas | 98,100 |
| 'Split' tolerance between HBeAg and HBcAg | 100,102 |
| A function of HBeAg is to regulate the immune response to HBcAg | 98,102 |
| Use of T-cell receptor (TCR)-Tg mice to study tolerance | 100,102,103 |
| Tolerance to the HBsAg can be broken *in vivo* | 83,104–107 |

lineage, is one of clonal ignorance in which low avidity HBc/HBeAg-specific T cells are neither tolerized nor activated by co-existence with even high concentrations of HBc/HBeAg and remain quiescent *in vivo*. However, 11/4-12 TCR-Tg T cells can be activated by antigen *in vitro* and can mediate liver injury upon adoptive transfer into HBc/HBeAg-Tg recipients.[103] The third tolerance phenotype, illustrated by an intermediate avidity (7/16-5) TCR-Tg lineage, is non-deletional and HBeAg concentration-dependent and is best described as adaptive tolerance or *in vivo* anergy. Exposure of 7/16-5 T cells to high concentrations of serum HBeAg *in vivo* does not result in clonal deletion in the thymus or the periphery, but instead the ability of the T cells to produce cytokines or proliferate to recall antigens *in vitro* and to provide T-cell help for *in vivo* anti-HBe antibody production is severely compromised. Importantly, this form of HBeAg-specific T-cell anergy is reversible in the absence of HBeAg, the tolerogen.

In the context of a chronic HBV infection, a multiplicity of HBeAg-specific T-cell clones are likely to co-exist, as demonstrated in the TCR/Ag double-Tg systems, and the balance between activation or tolerance status among the heterogeneous HBeAg-specific T-cell repertoire may influence degrees of liver injury and viral clearance. High avidity HBeAg-specific T-cell clones are likely to be physically or functionally deleted and not available to participate in antiviral clearance mechanisms after a chronic infection has been established. Intermediate or low avidity HBeAg-specific T-cell clones that are not physically deleted at least have the potential to be activated either through the reversal of adaptive tolerance (anergy) or the primary activation of 'ignorant' HBeAg-specific T cells (Fig. 18.1). Therefore, treatment modalities for chronic HBV infection should be directed at activating these relatively low avidity HBeAg-specific T cells. Given the efficacy of the current antiviral drugs in terms of reducing HBV and antigen load, reversing HBeAg-specific adaptive tolerance by reducing or eliminating circulating levels of the HBeAg may be an achievable goal and may in fact explain viral clearance in a percentage of chronic patients on long-term antiviral therapy.

Recent studies also demonstrated a 'split T-cell tolerance' between HBeAg and HBcAg. Secreted HBeAg appears significantly more efficient at eliciting T-cell tolerance than HBcAg.[100,102] In fact, one TCR × HBc double-Tg pair spontaneously seroconverts to anti-HBc positivity at an early age. However, the presence of serum HBeAg in TCR × HBc × HBe triple-Tg mice prevents anti-HBc seroconversion. Therefore, HBeAg can function as an immunoregulatory protein. HBeAg mediates its immunoregulatory effect by eliciting tolerance in HBc/HBeAg-specific T cells. These results suggest that hepadnaviruses have retained a secretory form of the nucleoprotein because it functions as a T-cell tolerogen and regulates the immune response to the intracellular nucleocapsid. Efficient T-cell tolerance induction by HBeAg is probably related to its monomeric structure and the fact that it is continuously secreted into the serum. Because HBeAg is secreted, it can transit to the thymus and induce central tolerance by deleting HBe/HBcAg-specific Th cells. Secreted HBeAg also may cause deletion of HBeAg/HBcAg-specific Th cells in the periphery by inducing apoptosis primarily in Th1-type cells.[98] Non-deletional mechanisms of tolerance (i.e. clonal anergy, clonal ignorance and Th1/Th2 cross-regulation) also have been described for HBeAg.[100,113] To demonstrate that the immunoregulatory function of the HBeAg is not unique to TCR-Tg murine systems, we recently compared the effects of activating HBc/HBeAg-specific polyclonal T cells in HBeAg-expressing, HBcAg-expressing and dual HBeAg × HBcAg-expressing Tg mice (Fig. 18.2). Injecting the dominant T-cell peptide (p129–140) into HBc-Tg mice elicited efficient anti-HBc seroconversion, whereas HBe-Tg mice were tolerant. Interestingly, anti-HBc seroconversion was significantly inhibited in p129–140-injected, HBc × HBe double-Tg mice (Fig. 18.2a). The HBcAg-specific T-cell responses, measured by IFN-γ production, in the three Tg lineages paralleled *in vivo* antibody production (Fig. 18.2b).

Assuming an immunoregulatory role for HBeAg in natural HBV infection, as suggested by these recent murine studies, one would predict that infection with an HBeAg-negative mutant virus would result in enhanced antiviral immune responses and decreased rates of chronicity. The clinical evidence is largely consistent with this prediction. A number of common mutations in the precore region[114] or in the core promoter region[115] either abrogate or reduce HBeAg synthesis, respectively. Neonates infected with HBeAg-positive wild-type HBV predominantly (90%) become chronically infected, whereas neonates infected with HBeAg-negative mutant viruses experience an acute infection often accompanied by fulminant hepatitis indicative of an enhanced immune response.[99] Similarly, there is a correlation between adult infection with HBeAg-negative mutant viruses and acute fulminant hepatitis.[116,117] Further, emergence of an HBeAg-negative mutant virus during chronic HBV (CHB) infection can lead to exacerbation of liver injury and a poor prognosis, especially if the HBeAg-negative mutant becomes predominant during periods of high viral load.[118,119] However, other reports suggest that emergence of an HBeAg-negative variant during CHB correlates with less liver injury and viral clearance.[120] A recent study by Chu *et al*.[118] may

help resolve this issue. At baseline, most CHB patients possessed mixed viral populations (HBeAg-positive/HBeAg-negative). However, predominance (>50%) of the HBeAg-negative variant before seroconversion hastened the interval to seroconversion, correlated with persistent viraemia and alanine aminotransferase (ALT) level elevations for at least 6 months after seroconversion, and correlated with an increased rate of cirrhosis. This study indicated that the timing of the emergence of an HBeAg-negative variant along the course of the chronic infection is one critical factor in determining the effects of the HBeAg-negative mutation during CHB infection and illustrates the difficulty of interpreting the data from a heterogeneous patient population.

We suggest that the ability of serum HBeAg to function as a T-cell tolerogen and to downregulate the immune response to the HBcAg results in the moderation of HBcAg-specific liver injury during an acute infection and in the promotion and maintenance of viral persistence during a chronic infection. Taken together, the clinical

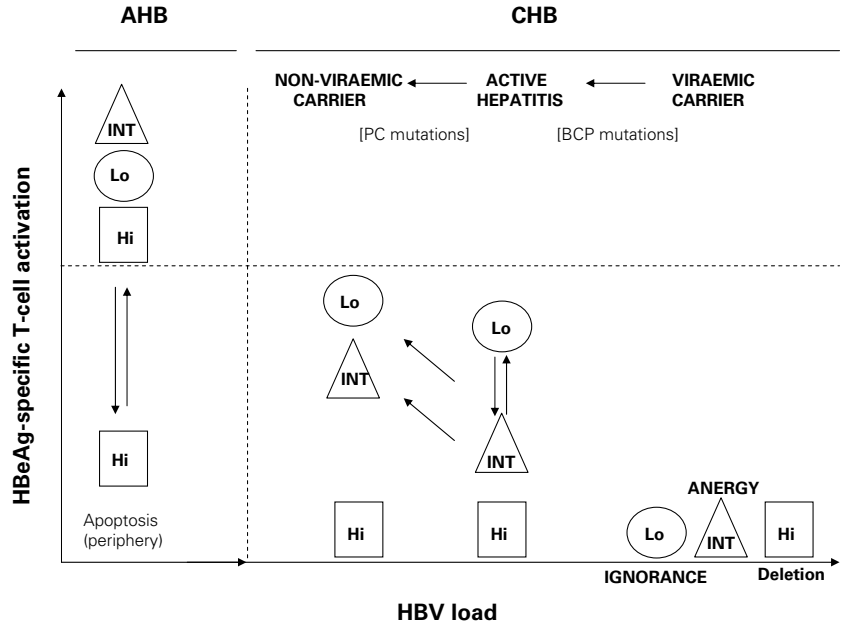


**Figure 18.1** Schematic illustrating the clonality and heterogeneity of HBeAg-specific T-cell tolerance and the possible consequences of the reversal of tolerance during different phases of chronic HBV (CHB) infection. During the so-called 'tolerance phase' or viraemic carrier state the vast majority of HBeAg-specific T-cell clones, including high (□), intermediate (Δ), and low (o) avidity clones, are tolerized by serum HBeAg. The mechanism of T-cell tolerance differs depending on avidity of each clone (see text). The maintenance of the HBeAg-specific T-cell clones in the various tolerance phenotypes (i.e. deleted, anergized or ignorant) depends on the presence of relatively high concentrations of HBeAg and a relatively non-inflammatory hepatic setting as exists in the tolerance phase of a chronic infection or in the neonate at the time of infection. The tolerance phenotypes of low and intermediate T-cell clones, which are NOT physically deleted, can be reversed should the level of the tolerogen (HBeAg) decrease, perhaps due to the emergence of common basal core promoter (BCP) region or precore (PC) mutations, or the hepatic environment becomes more inflammatory. In any event, the activation of previously 'ignorant' low avidity T cells or reversal of anergy in intermediate HBeAg-specific T cells may further reduce HBeAg concentration and/or increase inflammation and shift the balance away from T-cell tolerance and towards HBe/HBcAg-specific T-cell activation. Loss of tolerance in one HBeAg-specific T-cell clone is likely to influence the activation of other T cells via production of inflammatory cytokines, etc. Such a shift in the virus–host relationship may precipitate the so-called 'clearance phase' of chronic HBV infection. During the clearance phase the immune response becomes more active in reducing viral load, in provoking liver injury and in selecting escape mutants. Once a sufficient percentage of the HBeAg-specific repertoire has become activated, HBeAg ceases to be an effective tolerogen and may in fact become a target (i.e. cytosolic HBeAg) of the immune response together with the more immunogenic HBcAg. In this environment, selection of an HBeAg-negative mutant becomes more efficient. The reason the HBeAg-negative virus is not entirely successful in evading immune attack and may even lead to more severe liver injury in some circumstances (see Fig. 18.3) may be due to the loss of the immunoregulatory properties of serum HBeAg, which tend to downregulate the HBcAg-specific response. During an adult, acute HBV (AHB) infection the HBeAg-specific T-cell repertoire is more likely to be activated as opposed to tolerized due to an inflammatory environment in the liver. Even during an acute infection, serum HBeAg may have some regulatory function by eliciting early apoptosis of high avidity HBe/HBcAg-specific T cells in the periphery.

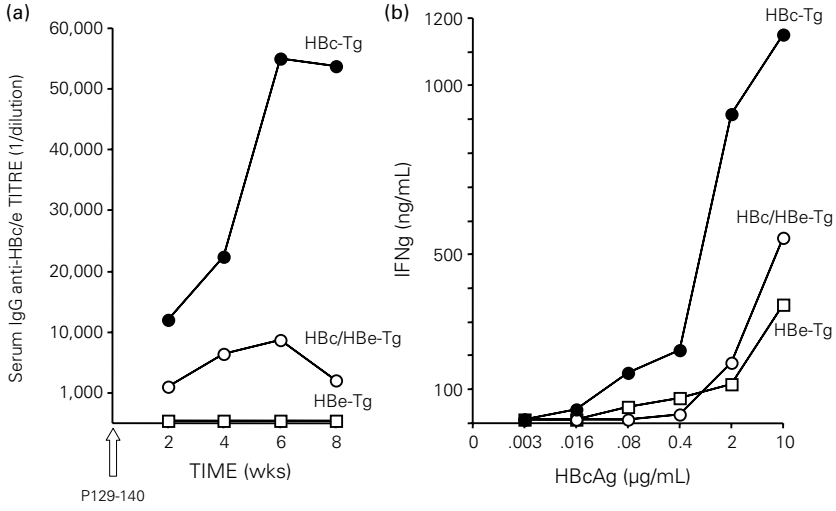


**Figure 18.2** Split T-cell tolerance between the HBeAg and the HBcAg and the immunoregulatory effect of serum HBeAg on the humoral and cellular response to the HBcAg. Groups of B10 HBcAg-Tg (HBc-Tg), HBeAg-Tg (HBe-Tg) and HBcAg × HBeAg double-Tg (HBc/HBe-Tg) mice were injected with the dominant T-cell activating peptide (p129–140), and anti-HBc or anti-HBe seroconversion was monitored by ELISA at the times shown (a). Ten weeks after p129–140 injection, spleen cells were harvested and cultured *in vitro* with varying concentrations of the HBcAg and T-cell activation was determined by IFN-γ production measured in the supernatant by ELISA (b).

observations and murine experimental studies suggest that the HBeAg is important in biasing the virus–host interaction towards chronicity and accomplishes this by downregulating the host T-cell response to the nucleocapsid antigens via a variety of tolerance-inducing mechanisms. The schematic in Fig. 18.3 illustrates the possible important relationship between HBeAg-specific T-cell tolerance, HBV viral load and liver injury. Perinatal or adult primary infection with wild-type (HBeA-positive) HBV results in no or relatively mild liver disease due to the presence of HBeAg-specific T-cell tolerance mechanisms, respectively. During the course of a wild-type chronic HBV (CHB) infection, HBeAg-negative mutants often accumulate with time, reflecting the loss of HBeAg-specific T-cell tolerance as the immune response selects the mutants. Simultaneously, the immune response is reducing viral load, and therefore, only moderate liver injury occurs. It is only when HBeAg-specific T-cell tolerance is lost and the HBeAg-negative mutant predominates in the presence of high viral load that liver injury can become more severe. The most extreme example of high viral load and the complete absence of HBeAg-specific T-cell tolerance is primary infection with an HBeAg-negative mutant virus. Either perinatal or adult infection with an HBeAg-negative virus correlates with a significantly increased risk of severe liver injury.

## Modulation of the immune response to HBV proteins in Tg mice

Tolerance to the HBeAg in Tg mice is MHC-dependent. Tolerance to HBeAg is complete in HBeAg-expressing Tg mice on an H-2$^s$ genetic background, and incomplete on HBeAg-Tg mice on an H-2b background at relatively low concentrations of serum HBeAg (10 ng/mL).[101] At high serum HBeAg concentrations (4–10 μg/mL) even H-2$^b$ mice are largely tolerant.[100] However, a population of functional, 129–140 peptide-specific Th cells co-exists with low levels of circulating HBeAg in

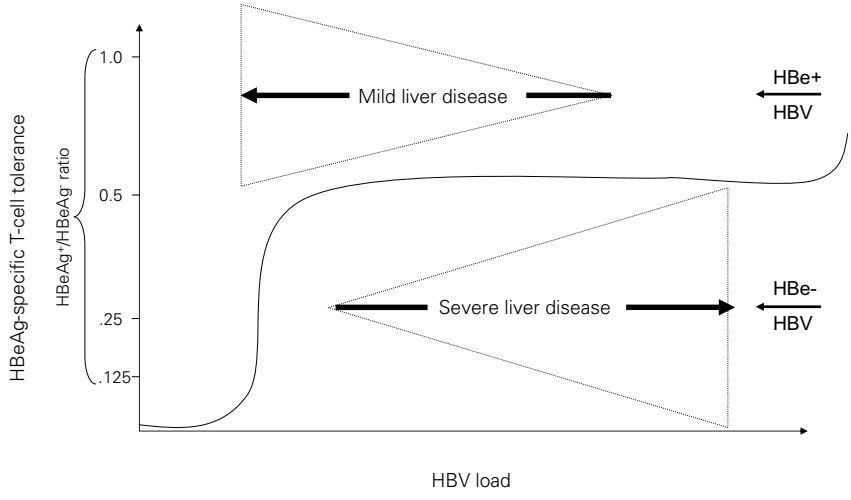


**Figure 18.3** Schematic of a putative relationship between HBeAg-specific T-cell tolerance, viral load and liver injury. The predominance of HBeAg-positive wild-type HBV (above the line) is associated with milder forms of liver injury and liver injury is inversely correlated with viral load. In contrast, predominance of HBeAg-negative mutant viruses (below the line) is associated with more severe forms of liver injury and liver injury is not necessarily inversely correlated with viral load. The triangles represent the degree of liver injury. See text for a discussion.

B10 HBeAg-Tg mice. The Th cells that evade deletion in HBeAg-Tg mice are quiescent unless activated by a dose of the 129–140 peptide as low as 0.6 μg, which induces anti-HBe seroconversion.[101] This model has been useful to test reagents that may modulate anti-HBe seroconversion. For example, soluble CD 152 (cytotoxic T-lymphocyte antigen-4) has been shown to suppress anti-HBe seroconversion in this model.[121] Similarly, IL-12 suppresses anti-HBe 'autoantibody' production and skews the Th cells toward the Th1 subset.[122] Finally, injection of an envelope (pre-S2) T-cell site peptide that binds the same MHC class II molecule as the 129–140 peptide inhibits anti-HBe seroconversion by competitively binding to $IA^b$ and preventing the activation of 129–140 peptide-specific Th cells.[101] This may have important implications during a natural HBV infection because envelope proteins circulate in tremendous excess of the HBeAg. Therefore, 'intraviral' protein competition for MHC binding sites may be a viral strategy to saturate MHC molecules and prevent less abundant, and possibly more relevant, T-cell recognition sites from gaining access to antigen presentation.

HBsAg-expressing Tg mice have also been shown to be tolerant at the T-cell level.[107] Nevertheless, several groups have induced seroconversion to the HBsAg in Tg mice. Immunization of HBsAg-Tg mice with HBsAg in adjuvant induced low level anti-HBs production, approximately 500-fold less than in non-Tg littermates.[105,107] Similarly, multiple immunizations with an HBsAg-vaccinia recombinant virus elicited low level anti-HBs seroconversion.[107] Induction of seroconversion to the HBeAg, the HBcAg or the HBsAg has not resulted in liver injury, indicating that antibodies specific for these viral antigens are not pathogenic. However, activated TCR-Tg HBc/HBeAg-specific T cells adoptively transferred into HBcAg-Tg recipients elicited low level liver injury with an interesting pattern of recurring and remitting ALT elevations.[103] Recently, one group immunized HBsAg-Tg mice with a DNA construct coding for the HBsAg and reported anti-HBs seroconversion, CD4+ Th cell priming and inhibition of HBsAg gene expression, probably due to IFN-γ production.[83] Finally, one group has suggested that the lack of anti-HBs production by HBV chronic carriers and in HBsAg-Tg mice is due to defective function of APCs rather than immune tolerance.[104] It was suggested that circulating HBsAg reduced MHC class II and B 7.2 (CD86) expression on dendritic cells in HBsAg-Tg mice. Treatment with IFN-γ or replacement of Tg dendritic cells with dendritic cells from non-Tg mice allowed Th cells from HBsAg-Tg mice to mediate anti-HBs production *in vitro*.

## Tg mice and hepatocellular carcinoma (HCC)

Several investigators have reported the occurrence of cancerous lesions in the livers of mice transgenic for various HBV proteins (Table 18.5).[54,64–66,74,123] As stated earlier, Tg mice expressing increased amounts of the HBV pre-S1-containing large envelope protein demonstrate an ER storage disorder, leading to massive hepatocyte death, causing a secondary inflammatory and regenerative response that eventually leads to HCC in mice strains that have a low incidence of spontaneous HCC.[123] Although excess pre-S1 protein is unlikely to accumulate in natural infection, a link between chronic liver injury and HCC has been demonstrated in a number of systems, including a recent Tg model of immune-mediated chronic hepatitis by these same investigators.[74] High-level expression of the HBV X-protein can also lead to HCC in Tg mice,[64,65] although other investigators have not observed the induction of HCC in independently derived X gene-Tg mice.[66] This discrepancy may relate to the level of expression or the genetic backgrounds of the mice. The Tg mice that developed HCC were produced on a CD1 background, which displays a high spontaneous rate of HCC.

**Table 18.5** Tg mice as a model for hepatocellular carcinoma

| Model | References |
|---|---|
| Overexpression of the HBV pre-S1 protein | 54,123 |
| High level expression of the HBV X-protein | 64–66 |
| Long-term chronic hepatitis | 74 |

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# Chapter 19
# Pathogenesis of chronic hepatitis B

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## Introduction

The epidemiological studies of hepatitis B virus (HBV) infection produce some remarkable statistics: worldwide 2 billion people have been infected, 350 million are persistently infected and 1 million people a year die as a result of the infection. These statistics generate important questions about the biology of HBV infection which we will address in this chapter. Specifically, those questions are:

**1** Why do some individuals clear the infection whilst others develop persistent infection?

**2** How does the virus cause liver cell damage and fibrosis?

In order to address these questions, it is vital to review the immunological responses to HBV which have been studied in patients with self-limiting or persistent infection and in animal models (see Chapters 12, 13 and 18).

The important question of how the virus causes liver cancer is addressed in Chapter 17.

## Immunological response to HBV

### Innate immunity

The initial line of defence is antigen non-specific and is mediated by the type 1 interferons (IFNs) and cells of the innate immune response, including natural killer (NK) and NK-T cells and macrophages. Type I IFNs are a family of monomeric cytokines with an amino acid similarity of 30–80%, very similar three-dimensional structure (5-α helix-bundle), that use the same receptor (IFNAR) to initiate a signalling response. The genes for type I IFNs do not contain introns and are located on chromosome 9 (9p21.3). They are strongly induced by virus infection, bacterial endotoxin (LPS), double-stranded RNA (dsRNA) and other immunostimulatory molecules, such as interleukin (IL)-1, IL-2 and tumour necrosis factor (TNF)-α. In viral infection IFN induction may be mediated by toll-like receptors (TLRs). TLR3 and TLR7 respond to double-stranded or single-stranded RNA, respectively, whilst TLR9 responds to hypomethylated CpG DNA, features which distinguish viral genomes and replication intermediates from host nucleic acid.[1–3]

Almost all cells have the ability to synthesize type I IFNs, although α-IFNs are mainly produced by lymphoblastoid cells and IFN-β by fibroblasts. Once secreted in response to an infection, after binding of the IFN to the cellular receptor, a signal transduction pathway results in the activation of a large number of cellular genes which inhibit viral replication and enhance the activity of the cells of the innate and adaptive immune responses[4] (Fig. 19.1).

Type 1 IFNs stimulate NK and NK-T cells, which recognize infected cells that are no longer displaying major histocompatibility complex (MHC) class 1 molecules.[5] NK-T cells are a resident population in the liver (see Chapter 2), representing 1% of the hepatic cells in humans, while NK cells are recruited actively from the spleen during the early phase of an infection.[6] NK cells induce apoptosis of virally infected cells and secrete IFN-γ, which suppresses viral replication and activates adaptive immune responses. NK-T cells are also important in regulating cellular immune responses. These components of the host response are therefore likely to be important in determining outcome of HBV infection.

### Adaptive immunity

Dendritic cells (DCs) act as a link between innate and adaptive immunity. Signals delivered by the innate immune system (type 1 IFNs) and contact with NK cells lead to maturation of DCs, which are critical in initiating the adaptive immune response. Virus-specific immunity is induced by DCs presenting viral antigens to T and B lymphocytes in the regional lymph nodes (Fig. 19.2). Thereafter, viral specific T-helper cells are activated and these cells initiate the antiviral immune responses of B lymphocytes and cytotoxic T lymphocytes (CTLs). Early, vigorous and multi-specific T-cell responses are associated with viral clearance, whereas low magnitude

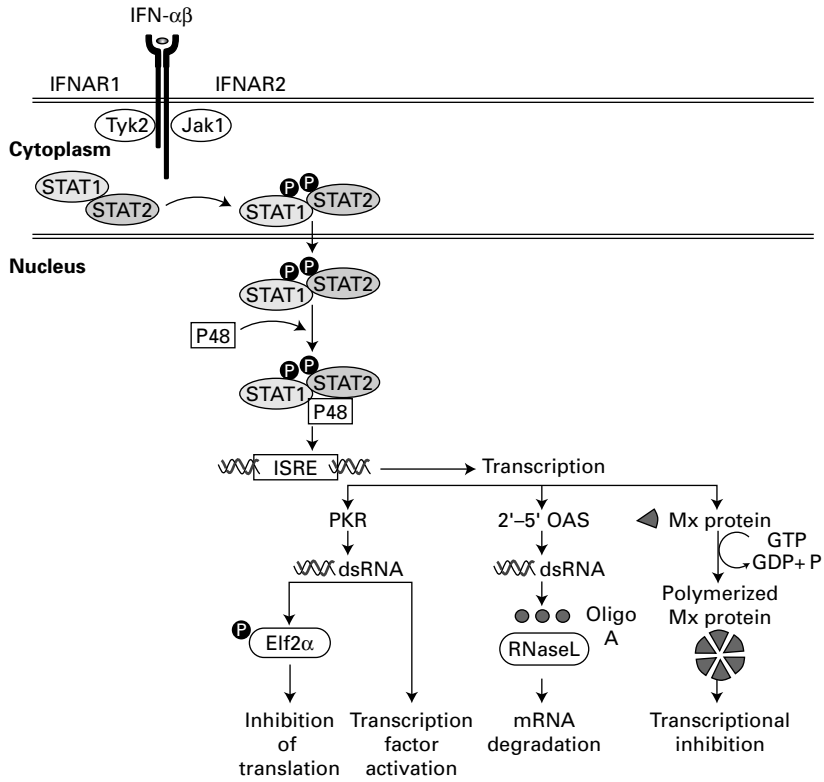


**Figure 19.1** Interferon-α and -β (IFNαβ) interact with the IFN-α receptor which is composed of two subunits (IFNAR1 and IFNAR2). Formation of the IFN–IFN receptor complex leads to autophosphorylation and to phosphorylation of the janus activating kinases (Jak1 and Tyk2). These in turn phosphorylate the signal transduction and activators of transcription molecules (STAT1 and STAT2) which form a complex with P48 (ISGF3), which binds to IFN-sensitive response elements (ISRE) in the regulatory regions of IFN-stimulated genes such as myxovirus resistance gene (MxA), the RNA-dependent protein kinase (PKR) and the 2′5′-oligoadenylate synthetase genes (2′5′-OAS).

oligospecific T-cell responses are associated with viral persistence[7] (Fig. 19.3).

After recognition of viral antigens, T-helper lymphocytes are differentiated to Th1 and Th2 cells according to the type of secreted cytokines. Th1 cells produce antiviral cytokines (IL-2, IFN-γ, TNF-α) and cytokines IL-2, IL-12 and IL-18, which are responsible for effective activation of CTLs. In contrast, Th2 cells secrete IL-4, IL-5 and IL-10, which are involved in activation of B lymphocytes and in production of neutralizing antibodies. It is likely that the failure to generate a strong Th1-lymphocyte response leads to inadequate activation of antigen-specific CTLs. These findings suggest that viral clearance is associated with the early devel-

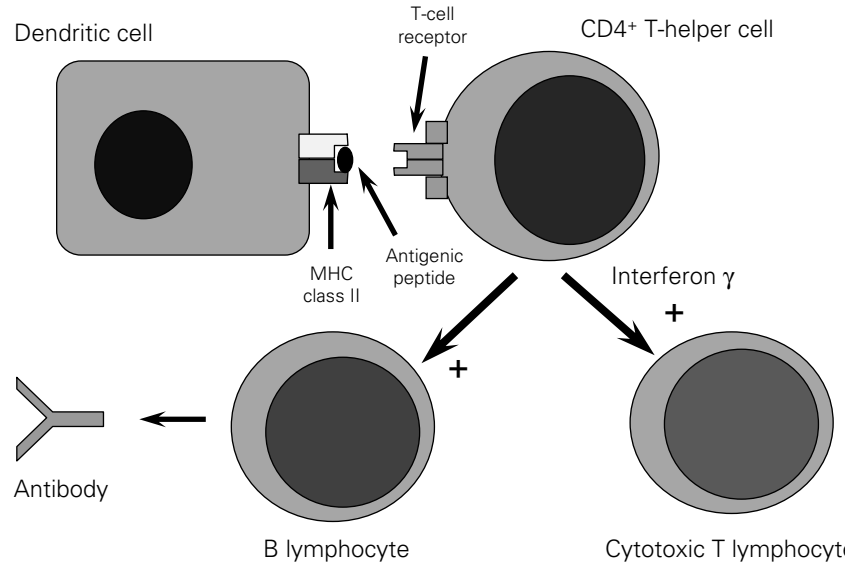


**Figure 19.2** MHC class II products and T-cell responses in acute hepatitis B. During acute hepatitis, the efficiency of antigen presentation is determined by the affinity of the HBV core peptides for the patient's MHC class II proteins. The resulting CD4 response is predominantly Th1 – cells produce IL-2 and IFN-γ – and induces a strong CTL response.

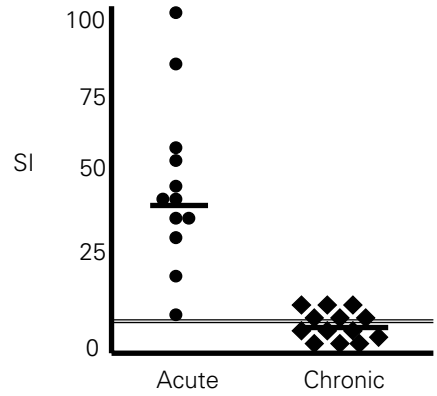


**Figure 19.3** T-cell proliferative responses to the nucleocapsid antigen (HBcAg) of HBV differ in both magnitude and breadth in acute and chronic infection (Tsai *et al.*, 1992).

opment and adequate mounting of the antiviral multispecific immune responses of T-helper and cytotoxic T lymphocytes.

### Cytokine control of viral replication

Type I IFNs induce the expression of more than 100 different genes.[8] Among these IFN-modulated genes are many which control intracellular viral replication. MxA is a GTPase from the dynamin family which, when induced by IFN, forms tubular structures associated with the endoplasmic reticulum.[9] In some viral infections MxA has been shown to associate with nucleocapsids, suggesting that it inhibits virion assembly.[10] The oligoadenylate sythetase gene product OAS is induced by IFN and activated by double-stranded RNA, usually from viral replication intermediates. OAS produces 2′,5′-oligoadenylate which activates the endonuclease RNAseL, thereby inhibiting protein translation. IFN-α suppresses viral replication *in vitro* and also *in vivo* before adaptive immune responses are detectable.

In a transgenic mouse model of HBV, Guidotti and colleagues demonstrated that CTLs controlled viral 'replication' even when there were insufficient CTL numbers to explain the degree of control on the basis of hepatocyte lysis.[11,12] Further experiments demonstrated that control of viral replication was achieved by secretion of TNF-α and IFN-γ. It is therefore likely that control of HBV replication by both the innate and adaptive immune systems is mediated through cytokines in addition to the elimination of infected hepatocytes by NK cells and CTLs.

## Self-limiting infection

The majority of individuals infected with HBV in childhood and adult life develop a clinical or subclinical self-limiting acute hepatitis, with spontaneous clearance of hepatocytes supporting HBV replication or suppression of viral expression, within a few weeks of infection. Some individuals infected with HBV, however, develop chronic infection. The age at which an individual is infected determines the likelihood of the development of chronic infection. Of babies born to mothers with chronic replicative HBV infection (usually, but not invariably, hepatitis B e [HBe] antigen-positive), 98% become infected, and 95% of these will develop persistent infection.[13] However, only 10–15% of children and <5% of adults infected with HBV develop chronic infection.

### Acute HBV infection

The existence of a period of viraemia without liver damage in the prodromal phase of acute infection[14] and of carriers with high level HBV replication without liver damage[15] suggests that HBV itself is not directly cytopathic. Recovery from HBV infection depends on the integrated activities of the patient's innate and adaptive cellular and humoral immune systems and the cytokine network. In acute infection, the production of IFN-α, followed by the induction of the cellular arm of the immune system, results in recognition and destruction of HBV-infected hepatocytes. The humoral (antibody) response to epitopes on the lipid-protein coat that envelops the virus is believed to be responsible for protective immunity against reinfection. Several cell types contribute to the immune response during acute HBV infection (Fig. 19.1).

### Innate response

Cells infected by virus secrete IFN-α, which acts through cell surface receptors to induce a number of antiviral responses. IFN increases the expression of MHC class I molecules, which expose viral antigens to the immune system.[16,17] IFN-α also induces the expression of proteins such as 2–5 A oligoadenylate synthetase and a protein kinase, which inhibit viral replication. The presence of circulating IFN-α at the onset of viraemia may explain the fever and malaise that most patients report 2–3 weeks before they develop dark urine, pale stools and icterus.[16]

### CD4+ T-helper cells (Th)

Soluble and particulate antigens are taken up by antigen-presenting cells (APCs). The antigen is digested into peptide fragments of 10–20 amino acid length, and these bind with varying affinity to MHC class II glycoproteins within the phagosome or endosome. These peptide/MHC complexes are transferred to the cell surface where they may interact with T-cell receptors of Th cells. When this interaction is coupled with the interaction of

co-stimulatory molecules such as B7 and ICAM-1 with their respective ligands, then Th cells will be primed and undergo proliferation. In the absence of co-stimulatory signals Th cells may be rendered anergic.

During acute HBV infection, there is a vigorous MHC class II-restricted Th response to the nucleocapsid antigens of HBV.[18] In contrast, Th responses to the envelope antigen (HBsAg) are virtually undetectable.[19] T-helper responses to the X and polymerase proteins have not been adequately documented for comparison.

T-helper lymphocytes recognize a number of peptide epitopes from the nucleocapsid antigens. The amino acid sequences between positions 1 and 20 and 50 and 69 appear to be recognized by most HBV-infected subjects, irrespective of MHC class II type.[18]

The mechanism by which these T-helper responses induce viral clearance are not adequately documented. However, by analogy to other immunological systems, the likely mechanism can be inferred. CTL responses are dependent on a strong Th response mediated through secretion of cytokines.[20] CTLs are stimulated by the Th1-secreted cytokines, specifically IL-2, IL-12, IL-18 and IFN-γ. CTL responses in acute HBV infection are vigorous and polyclonal and occur in temporal association with the Th responses, whereas in chronic infection CTLs are virtually undetectable.[7,21]

Th responses are also required to support B-lymphocyte production of anti-HBs.[22] As the envelope-specific Th response is poor, even in acute self-limiting infection, it is widely accepted that the nucleocapsid-specific T-helper response supports anti-HBs production. In the mouse, intermolecular help for anti-HBs production has been demonstrated.[22] It is thought that uptake of complete virions by HBsAg-specific B cells leads to expression of nucleocapsid peptides on MHC class II molecules.[23] This leads to further stimulation of nucleocapsid-specific T cells, which support B-cell maturation.

Certain MHC class II alleles (DRB1*1302) are associated with acute infection followed by recovery.[24] The mechanism underlying this association is not clear, but it is likely that the Th response induced by DRB1*1302 is qualitatively or quantitatively superior to that induced by other MHC class II alleles. In support of this, it has been shown that in mice, the MHC class II haplotype determines the Th1/Th2 phenotype of Th cells responding to nucleocapsid antigen stimulation.[25] Furthermore, Diepolder and colleagues demonstrated that Th responses to HBcAg were stronger in patients with HLA-DRB1*1302 that in those who did not possess this allele.[26]

Although it would be expected that a Th1-dominant response would enhance viral clearance (Fig. 19.1), characterization of lymphocytes derived from liver specimens has found predominantly Th1 cells in both acute and chronic HBV infections.[18] This suggests that it is the magnitude and breadth of the Th response rather than the balance between Th1 and Th2 which determines the success of viral clearance. This is supported by the failure of the Th1 stimulant IL-12, to facilitate clearance.[27]

## CTLs and NK cells

Analysis of the inflammatory infiltrate in patients with acute hepatitis demonstrates the presence of natural killer (NK) and cytotoxic T cells.[28,29] Increased NK functional activity has been demonstrated in the blood of patients with early acute HBV infection,[30] and it is probable that this is induced by IFN-α, as has also been demonstrated in the serum of chimpanzees during the prodromal phase of acute HBV infection.[16]

Viral antigens are present on the surface of the hepatocyte,[31–3] and it is probable that these and viral peptides associated with the MHC class I glycoproteins make the cell a target certainly for cytotoxic T-cell lysis[34–37] and possibly for antibody/complement lysis.[38] Uninfected hepatocytes usually express very little MHC class I glycoprotein but in the early stage of acute HBV infection, after the production of IFN-α, MHC expression on hepatocytes increases and coincidentally transaminase levels rise, presumably as a result of hepatocyte lysis.[34]

Investigation of the CTL response to HBV in acute and chronic HBV infection has been inhibited by the inability of HBV to grow in culture. Early experiments using autologous hepatocytes as CTL targets suggested that CTLs were present in both acute and chronic infections, were MHC class I restricted, and were hepatitis B core antigen (HBcAg)-specific.[34,39] However, the specificity of these responses was determined by inhibition of the responses by anti-HBc and anti-HBe polyclonal and monoclonal antibodies. Our current knowledge of MHC-peptide/T-cell receptor (TCR) interactions suggests that it is highly unlikely that an antigen-specific antibody could inhibit CTL responses to the same antigen. Thus, although the observations of the presence of CTLs and the ability of antibodies to HBc and HBe to inhibit CTL activity are reproducible, the mechanism is not understood. The observations are, however, further supported by the observation that infusion of the same monoclonal antibodies into chimpanzees resulted in the animals developing chronic infection after intravenous challenge with a standard innoculum which in control animals, not receiving the monoclonal antibodies, invariably resulted in self-limiting acute infection.[34]

Recently, new techniques have been described with which to investigate the CTL response. Peptide pulsed lymphocyte targets can be used to determine the presence and specificity of CTLs, which are restimulated *in vitro* for about 3 weeks.[40] The major drawback of these experiments is that there is no clear proof that the pep-

tides that elicit a response *in vitro* are the ones expressed in class I clefts *in vivo*. This issue is partially addressed by using target cells that have been transfected with plasmids expressing the HBV antigens.[40] HBV-derived peptides, in these experiments, will be loaded into the MHC class I cleft through the natural mechanism.

Using peptide pulsed targets, Chisari and Ferrari have shown that, in acute HBV infection, the CTL response is polyclonal and multi-specific. In chronic HBV infection, the CTL response is weak and has a limited repertoire.[7,41] In contrast with the findings in the early experiments described previously, relatively more CTL epitopes are found in the HBsAg and polymerase antigens than in HBcAg. The explanation for this phenomenon is unclear, but two possibilities have been discussed. First, exogenous HBsAg, found in great excess during HBV infection, induces MHC class I-restricted CTL responses through an unknown pathway. Second, HBsAg and the HBV polymerase have a large proportion of hydrophobic areas, which may be relatively rich in MHC class I-binding motifs.

HBV is widely recognized as a non-cytopathic infection in which hepatocyte damage occurs as a result of the immune response. Support for this hypothesis is derived from the presence of minimal liver disease in patients with high levels of viral replication and severe liver disease in patients with undetectable viral replication and strong T-cell responses to HBV antigens. Experiments with transgenic mice expressing HBV antigens indicate that neither expression of HBV antigens nor the process of replication itself is directly cytopathic.[42] An exception to this is a strain of mice that overexpress HBsAg. Hepatocytes in this strain are exquisitely sensitive to physiological concentrations of IFN-γ and eventually failure to secrete HBsAg leads to cell necrosis.[43] Adoptive transfer of MHC class I-restricted, HBsAg-specific CTLs into HBsAg transgenic mice induces apoptosis and necrosis with a histological pattern akin to that seen in human acute HBV infection. Increasing the dose of CTL increases the severity of hepatitis until the typical appearance of fulminant HBV infection occurs.[44]

## Humoral immunity

In acute hepatitis, liver damage coincides with the appearance of immunoglobulin M (IgM) anti-HBc, suggesting that the hepatitis associated with HBV infection is caused by immune lysis of infected hepatocytes, an essential part of the recovery process.[14,45] Current dogma suggests that it is the cellular rather than the humoral response that is involved in the mediation of infected hepatocyte lysis. However, the contribution of antibody-mediated lysis has not been ruled out. Compatible with a role for humoral immunity acting through complement activation is the demonstration that C1q and C3 metabolism are increased in acute HBV.[38,46,47]

Virus-neutralizing antibodies are directed to epitopes on the envelope of the virus. This is composed of three polypeptides, each with the same carboxy-terminus. These are designated the large, middle and small envelope proteins and arise from the pre-S and S of HBV. The amino-terminal 120 amino acid region of the large protein, which is not present in the middle and small proteins, is designated pre-S1. This hydrophilic area is myristilated, and recent data indicate that the region amino acids (aa) 21–47 is capable of binding to the membrane of the hepatocyte and is therefore probably involved in virus uptake.[48]

The antibodies found in convalescent serum bind predominantly to the epitopes of the HBs gene-encoded region, the carboxy-terminal region that is present in all the envelope proteins.[49] A hydrophilic region of this polypeptide, aa 124–147, forms two loops via intramolecular disulfide bridges, and these are the binding region for the majority (>80%) of the antigen-binding capacity of convalescent sera.[50] Using monoclonal antibodies binding to these regions, it has been possible to show that antibody to the region aa 124–137, when administered to chimpanzees, will prevent infection.[51] Antibodies to this region, as well as to other epitopes on the S gene-encoded polypeptide, are present in the serum of patients convalescent from HBV infection and in normal subjects immunized with plasma-derived and recombinant DNA-produced HBs vaccine.[21,52]

Although patients recovered from HBV or vaccinated with HBsAg are protected from further infection, there has been considerable debate on the importance of antibodies to the middle and large pre-S2 and pre-S1-bearing polypeptides. During natural infection, antibodies to pre-S1 and pre-S2 appear before antibodies to the HBs region.[50,51] If, as has been postulated, the pre-S1 region is important for binding of HBV to the hepatocyte during infection, antibodies to this region would be virus neutralizing and, therefore, their presence may be important in preventing entry of HBV particles into uninfected hepatocytes.[48] Interference with the production of anti-envelope antibodies thus may be expected to lead to protracted HBV infection, and this is seen in agammaglobulinaemic patients who, when exposed, frequently develop chronic HBV infection.

## Cytokines

In the transgenic mouse models IFN-γ and TNF-α inhibit HBV gene expression. Using a mouse that secreted HBsAg from the liver and that sustained little damage after infusion of HBsAg-specific CTLs, it was shown that secretion of IFN-γ and induction of TNF-α controlled

HBsAg expression in the absence of hepatocyte necrosis.[53] The magnitude of this effect in acute hepatitis B in human subjects remains to be determined.

## Factors leading to HBV persistence

The reasons why certain individuals develop chronic infection, rather than a transient acute infection, may relate to specific viral or host factors.

### Viral factors

The viral and immunological events that occur in the first weeks of HBV infection are probably crucial in determining the outcome of infection. Recent data suggest that the rate of rise of viral replication and of expression of viral antigens is important in determining the induction of innate and adaptive immune responses.[54] It is therefore of interest that the rate of rise of HBV viraemia is very slow. It is not until 6–8 weeks after infection that viraemia is detectable by sensitive polymerase chain reaction (PCR) assays and HBV-specific CD4 helper and CD8 cytotoxic T cells appear.[14,55] This long delay between infection and appearance of the cellular immune response contrasts with the reported kinetics of immune response in other infections (e.g. human immunodeficiency virus, HIV, and cytomegalovirus, CMV).[56,57]

#### *Induction of tolerance*

HBV establishes persistent infection with high-level viraemia which facilitates transmission of the virus to new hosts. Persistence is optimally achieved by inhibiting the initial immunological responses to viral antigens. To this end, the virus inhibits the expression of MyD88, a signal transduction molecule required for viral triggered innate recognition through TLRs.[58] In the absence of the 'danger' signals provided by these receptors, the IFN system is not activated and antigen presentation induces anergy or tolerance to viral antigens rather than effective CTL and T-helper responses.[59] Alternatively, tolerance may be induced by fetal exposure to viral antigens during thymic ontogeny.[29]

HBeAg may play an important role in the high rate of development of chronic hepatitis B infection in children born to mothers who are HBeAg-positive.[29] Almost every child born to an HBeAg-positive mother becomes infected with HBV, and 90% develop a chronic carrier state.[13,15,60] It has been shown that HBeAg crosses the human placenta, and a study (using a transgenic mouse model) has demonstrated that *in utero* exposure to HBeAg leads to a significant period of neonatal T-cell tolerance to HBeAg and HBcAg in non-transgenic offspring of HBeAg-expressing transgenic female mice[61,62] (Chapter 18). This immunological tolerance may play an important role in the development of a chronic carrier state in the neonate.[29]

Thus, it may be postulated that HBeAg in some way may reduce the cytotoxic T-cell response to infected hepatocytes (possibly by inducing immune tolerance to nucleocapsid-derived peptides) and thus reduces the degree of hepatic inflammation, lessening the risk of death of the host and elimination of the virus (both of which are disadvantageous to the virus). A healthy host with persistent infection is more likely to spread the virus to other individuals than a sick host with severe liver disease. When tolerance to the nucleocapsid is lost, as at the time of HBeAg to anti-HBe seroconversion, hepatic inflammation and damage increase until the virus is eliminated.

In addition to secreting soluble nucleocapsid proteins into the serum, the virus also secretes surplus surface (envelope) protein (HBs) into the blood. This excess HBs can be seen in the serum by electron microscopy as 22-nm particles. These particles may confer a biological advantage on the virus, as they will 'divert' antibody to HBs (anti-HBs) away from intact whole virus particles and thus reduce the chances of virus neutralization by these anti-envelope antibodies. Studies have shown that large quantities of murine monoclonal anti-HBs can overcome the antibody-diverting effect of non-infectious HBsAg particles and can shut off HBV replication in agammaglobulinaemic chronic HBV carriers, presumably by stopping infection of regenerating hepatocytes by virus neutralization in the interstitial space or by recruiting additional T-cell help, provided by T cells sensitized to murine globulin epitopes, to viral antigens complexed with heterologous (murine) anti-HBs.[63]

At some stage during the host's life, often during late teenage and early twenties, tolerance to the virus is broken down. The reasons for this are unclear. One explanation may be the random mutation of viral epitopes away from the sequences to which the host has become tolerant, as a result of errors generated by the viral reverse transcriptase. Alternatively co-infection with acute, possibly lytic viruses, may activate danger signals in the vicinity of HBV antigens which may initiate T-cell responses. However, in the face of established peripheral tolerance, T-cell responses are subdued relative to those seen in acute infection. This may explain the relative inefficiency of adaptive immunity in eliminating HBV-infected hepatocytes in chronic HBV infection.

#### *Inhibition of CTL lysis of infected hepatocytes*

The mechanism of replication of HBV – involvement of a reverse transcriptase – leads to the generation of genetic heterogeneity, which creates quasi-species, some of which theoretically may no longer be recognized by Th, CTLs or antibody.[64] Two HLA-A2 patients have been

described in which an HLA-A2 epitope in the HBcAg (aa 18–27) contains amino acid substitutions that do not elicit the usual CTL response.[40,66] Although this confirms the theoretical possibility of CTL escape mutation, it is difficult to weigh up the importance of this finding when the CTL response is documented as multi-specific. Perhaps, only in the context of a weak and limited CTL response, as seen in chronic HBV infection, is this mechanism of any biological consequence. Some of the CTL epitopes that have been documented for HBV arise from highly conserved regions of the virus, which have important roles in viral replication. For instance, the HLA–Aw68/A31-restricted epitope at amino acid positions 141–151 of HBcAg is at a nuclear localization and genomic encapsidation site.[65] Escape mutation in these sites is not a viable mechanism for viral persistence, although there is a theoretical possibility that other amino acid sequences could compete with viral peptides for HLA binding without activation of the T-cell receptor.[66]

### *Collapse of the adaptive immune response*

When resolution of HBV infection is achieved, virus-specific cellular and humoral responses remain detectable for years.[67] In contrast, during chronic infection there is a progressive decline of adaptive immunity with lowering of circulating and intra-hepatic CD4 and CD8 T cells.[68] In addition, the subclass distribution of anti-HBc changes to a predominantly Th2 pattern.[69] Persistently high production of viral antigens can delete or tolerize antigen-specific T cells and B cells.[23,70] The production of the secreted form of nucleocapsid antigen (HBe) induces, in transgenic mice, the preferential production of Th2 cells by deletion of Th1 cells.[71,72] Alteration of the CD8 cells repertoire towards subdominant epitopes has also been observed during the course of the infection. T cells escaping deletion caused by exposure to large quantities of antigen are characterized by an inability to bind antigen-specific tetramers, in spite of being positive for high affinity antigen receptors,[70] indicating that they are functionally deficient in some way.[73]

Furthermore, chronic exposure to HBs has been shown to facilitate T-cell lysis of antigen-specific B cells[23], giving rise to the diminished capacity of patients with persistent HBV infection to produce antibody to HBs antigen after successful therapy of the infection.[23,74]

These changes indicate progressive collapse of the immune response because of continuing exposure to large quantities of soluble HBe and particulate HBs antigen.

### *Emergence of the HBe-negative variant of HBV*

A mutation in the precore region of the nucleocapsid reading frame creates a termination codon that prevents translation of the HBeAg (Chapter 15, Figure 15.1). Virus bearing this mutation appears after HBeAg to anti-HBe seroconversion in many patients around the Mediterranean and in the Far East.[75]

Although seroconversion is usually associated with control of viral replication, in cases where the precore mutant emerges, viral replication continues often with a more aggressive hepatitis. At first sight, therefore, this appears to be a successful escape mutation that emerges under the selection pressure of anti-HBe antibody. However, it has now been established that virus bearing the precore mutation may be found in patients in whom HBV replication has been controlled. DNA sequencing of viral isolates from patients after HBeAg to anti-HBe seroconversion reveals that mutations in antigenic determinants of the core region are associated with persistent viraemia, indicating that the precore mutation alone is probably insufficient for successful evasion of the immune response.

An alternative explanation for the increasing prevalence of the precore mutant virus is that the mutation in the precore stabilizes the structure of a critical stem loop structure in the viral pregenome (Figure 15.2), which acts as a packaging signal and also as the origin of replication.[76] Thus, the base-pair substitution may also give the virus a replication advantage that eventually leads to dominance of this mutant. However, *in vitro* experiments have not convincingly shown any replication advantage of the precore mutant. Interestingly, patients transplanted for HBV, infected with the precore mutant, exhibit re-emergence of the wild-type virus when heavily immunosuppressed for long periods – presumably because of removal of immune selection pressure.[77]

### *HBV integration*

It is now clear that HBV DNA becomes covalently integrated into the hepatocyte genome at some time during the chronic infection.[78,79] The continuous secretion of the viral coat protein (HBsAg) in the absence of active viral replication probably represents a phase of infection in which the viral DNA has become integrated into the host DNA, so that some of the viral genes are transcribed and translated as though they were hepatocyte DNA. This integration event may be involved (late in the infection) in the malignant transformation of hepatocytes.[80] The cells containing integrated sequences must evade the immune elimination processes, and probably do so by not expressing HBc and HBe antigens (the putative targets for CTLs). Because the preferred site for integration on the viral genome is in the promoter region of the HBV core gene, this transcription unit is destroyed during the integration process, and thus no nucleocapsid antigens are produced.[81] HBsAg, however, continues to be expressed in these cells because this gene is intact in the integrated viral sequence. As long as the cell is

not recognized by CTLs as being infected, integration of the virus will result in viral persistence. Whether HBV integrates depends on the duration of infection and is usually found if infection has been present for 2 or more years. Studies of attempts to eliminate HBV infection using IFN therapy have indicated that patients who have had chronic infection for many years, and who have integrated HBV DNA, are much less likely to clear HBsAg than patients with a short period of chronicity and in whom integration is not found.[82] In individuals who have cleared replicating virus (HBV DNA, HBV DNA polymerase and HBsAg all negative) and have no evidence of liver disease, reactivation of infection (presumably from integrated HBV) has sometimes been seen when immunosuppressive therapy or acquired immune dysfunction occurs.[83,84]

## Host factors

### *Genetic susceptibility*

Twin studies from China indicate that the outcome of HBV infection is partly determined by the host genotype.[85]

A number of disease association studies have identified polymorphisms that influence susceptibility to persistent HBV infection. The MHC class II alleles DRB1*1302, DRB1*02, and DRB1*04 are associated with resistance to persistent HBV, whereas the allele DRB1*07 is associated with increased susceptibility to persistent infection.[24,86] In individuals who are heterozygous at the MHC class II loci the repertoire of viral epitopes, which may be presented to Th cells, is increased and as expected, heterozygosity at the MHC class II loci reduces susceptibility to persistent HBV infection.[87]

### *Deficient production of IFN*

IFN-α production during the early phase of acute HBV infection is believed to be important for successful resolution.[16] In acute HBV infection, IFN-α is responsible for the initial increase in MHC class I antigen display on hepatocyte membranes and the activation of the 2′,5′-oligoadenylate synthetase (2,5-OAS), endonuclease and protein kinase systems, which result in an effective antiviral state within the liver. However, in chronic HBV-infected subjects, there is evidence of abnormal activation of hepatocytes by IFN. Levels of 2,5-OAS are only minimally elevated, and MHC class I proteins are present only in very low density on the infected hepatocytes.[32,88] Furthermore, studies of peripheral blood mononuclear cells (PBMCs) taken from patients with acute and chronic HBV infection have revealed that the production of IFN-α is subnormal.[89–92] It is possible that this reduced production of IFN is directly attributable to HBV infection.[93] Suppression of type 1 IFN production in cells expressing the core region of HBV has been shown.[94] However, as reduced IFN production is also seen in lymphocytes taken from patients with resolving acute HBV,[89] it is unlikely that IFN deficiency is the cause of most cases of persistent HBV infection. This is further supported by studies showing that IFN-α therapy results in clearance of HBV infection in only about one-third of treated patients.[95] If persistence of infection was due to deficient IFN production by PBMCs in all cases, then we could expect to see much higher seroconversion rates with IFN-α therapy.

### *Deficient response to IFN*

In patients with moderate to severe chronic hepatitis B, treated with IFN-α, there is a reduced rate of rise of serum beta 2 microglobulin, a component of MHC class 1 glycoprotein, in patients failing to respond to therapy.[96] These abnormal responses to IFN may be acquired as a direct result of infection with HBV. *In vitro* transfection of HBV into cells in tissue culture makes them specifically unresponsive to IFN. They remain susceptible to lysis by Sindbis virus, and MHC class I induction does not occur.[97] MHC class I display is believed to be important for recognition and elimination of HBV-infected hepatocytes by cytotoxic T cells, and the decreased MHC expression, in addition to the reduced 2,5-OAS activation, may contribute to failure to clear the virus.

It is possible that this abnormal cellular response to IFN is directly attributable to the virus itself, but the exact mechanism by which the virus might achieve this is not clear. One hypothesis is that it may be due to the presence of a short nucleotide sequence at the beginning of the core gene of the virus.[98] The nucleotide sequence of this region shows a striking homology to a consensus sequence within the host genome that is upstream from genes induced by IFN in mammalian cells.[99] The IFN-induced secondary messenger binds to this site, and the presence of multiple homologous consensus sequences on episomal or integrated HBV DNA might compete for this messenger.

Another possible mechanism of interference is by the HBV replicative enzyme (HBV DNA polymerase). One study has shown that cells transfected with the HBV DNA polymerase gene produce polymerase protein and become unresponsive to the effects of IFN-α.[100] In this study, transfection with other HBV genes did not affect the cellular response to IFN.

Finally, genome-wide scanning studies in sibling pairs with persistent HBV infection have identified a polymorphic locus on chromosome 21q22.7, which is in linkage disequilibrium with HBV persistence.[101] This

region contains a cluster of cytokine receptor type II genes, including IFN-α receptor chains 1 and 2, and IL-10 receptor B chain.

Transmission disequilibrium studies undertaken on Gambian families identified the causal polymorphism(s) within this region to be in codon 47 of the IL-10 receptor B (IL10RB) gene ($p$ = 0.018) and in codon 8 of the IFN-α receptor 2 (IFNAR2) gene ($p$ = 0.01). When the allele encoding phenylalanine at position 8 of IFNAR2 and the allele encoding lysine at position 47 of IL-10RB are analyzed as a haplotype, the evidence for association is compelling ($p$ = 0.0009).

The IFNAR2-F8S and IL-10RB-K47E variations represent significant changes in the physico-chemical properties of the amino acids at these positions.

Position 8 in IFNAR2 lies within the signal sequence domain of the polypeptide, which is essential for targeting to the secretory pathway of the cell and localization to the cell membrane. Structural modelling of the sequence variants, suggests that the S allele, which is associated with persistent infection, would result in receptor expression at lower density than the F allele, thereby reducing the efficiency of signal transduction and induction of the intracellular antiviral state.

Position 47 in the IL-10RB protein lies in the extracellular domain adjacent to the ligand binding residues. IL-10RB is a promiscuous cytokine receptor. IL-10RB was initially identified as the second subunit of the IL-10 receptor. More recent studies implicate IL-10RB as one of the components of the receptor for novel cytokines IL-28A, IL-28B and IL-29 with antiviral properties which have been termed IFN-λs[102] (Fig. 19.4).[103,104] Consequently, the effects of polymorphism in this receptor will need to be explored in three different systems: IL-10, IL-22 and IL-28/29. It is easy to speculate that changes in the signalling of antiviral functions by IL-28/29 are responsible for the different outcomes in HBV infection but more data are required to support this hypothesis.

### *Antibody to nucleocapsid antigens*

The first host-induced immunological marker of HBV infection is IgM antibody to the core protein (anti-HBc), which appears shortly after the time HBsAg is first detected in the serum.[14,105] Anti-HBc is not a neutralizing antibody, and high titres of anti-HBc in the absence of HBsAg and anti-HBs may indicate the presence of continuing replication of HBV in the liver and infective HBV particles in the serum.[106] Cytotoxic T cells sensitized to nucleocapsid-derived peptides displayed on the hepatocyte membrane recognize and destroy HBV-infected hepatocytes.[34,107,108] However, it has been shown that anti-HBc and anti-HBe block cytotoxic T-cell killing of HBV-infected hepatocytes.[34,108] This blocking effect may be the result of simple steric hindrance if the B- and cytotoxic T-cell epitopes are displayed close to each other on the cell membrane.[34] Thus, circulating anti-HBc, depending on its specificity, may modulate T-cell-mediated lysis of infected cells[109] and, by analogy with chronic measles infection of the central nervous system,[110] may be one mechanism leading to viral persistence. Consistent with this hypothesis is the observation that some adult patients progressing from acute to chronic infection produce anti-HBc before those that clear the virus,[14] and injection of monoclonal anti-HBc and anti-HBe with the virus produces prolonged viraemia.[34]

Of babies born to HBsAg-positive mothers, 90% become infected, and >90% of these develop a chronic infection.[13] It has been speculated that the active transport of maternal IgG anti-HBc via placental Fc receptors contributes to the persistence of HBV-infected cells in the child's liver,[29,34,111,112] which, together with the immatu-

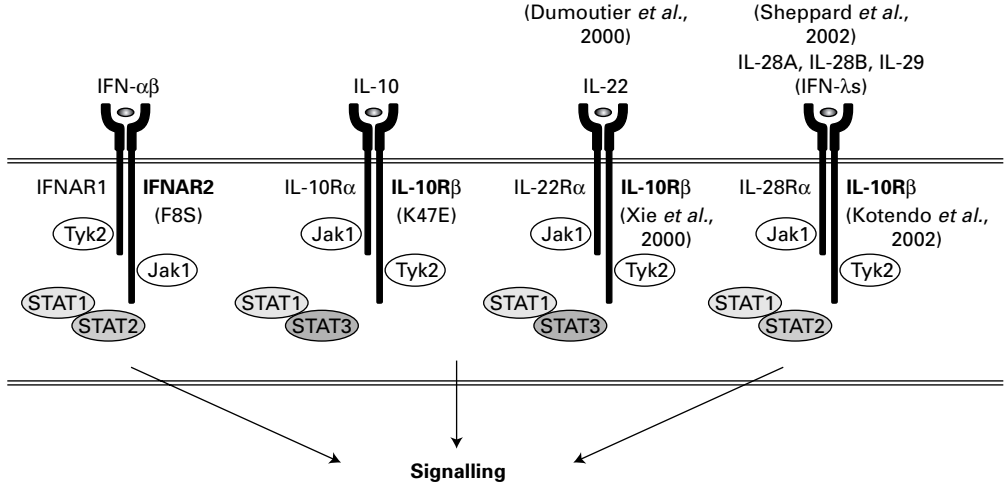


**Figure 19.4** The IL-10 receptor B (IL-10RB) participates in cytokine signalling for IL-10, IL-22 and IFN-λ (IL-28A, IL-28B and IL-29).

rity of the neonatal immune system,[113] results in chronic infection. IgG antibodies have been shown to modulate viral proteins from the membrane of infected cells,[114,115] a process that would prevent recognition by potentially lytic antibodies. Usually, the epitopes recognized by antibodies and cytotoxic T cells are different. In the case of HBV-infected hepatocytes, anti-HBc and anti-HBe will inhibit the lysis of infected hepatocytes by T cells, suggesting that the epitopes recognized by these antibodies and cytotoxic T cells are the same, or displayed near to each other on the cell membrane. Further confirmation of these findings comes from studies showing that murine monoclonal anti-HBc and anti-HBe, when injected into chimpanzees along with an infectious inoculum of HBV, result in a protracted infection lasting 12–18 months.[34]

### *Abnormalities of lymphocyte function*

It is thought that lymphocyte function is broadly deranged in chronic HBV infection,[111,112] and many studies measuring suppressor cell function in both acute and chronic HBV infection have been performed (reviewed by Dienstag[116]). Although the results are inconsistent, it does appear that there is a defect in suppressor cell function in both acute and chronic HBV infection as compared with control patients. Further studies of CD4/CD8 ratios also indicate a reduced ratio in acute and chronic HBV infection.[116] These findings suggest a virus-induced alteration in lymphocyte function in both acutely and chronically infected persons, but do not specifically incline an individual toward HBV persistence.

IL-1 production by monocytes from patients with chronic HBV infection with liver disease is also increased, and this correlates with the severity of fibrosis found within the liver.[117] However, once cirrhosis supervenes, IL-1 production is significantly reduced compared with normal persons,[118] probably because of a serum inhibitor found in these patients with advanced disease. However, this defect is not specific for HBV-induced liver disease. HBV carriers without liver disease have normal interleukin production.

The recent description of CD4+ and CD25+ regulatory T cells which modulate effector CD4 and CD8 cells, and the observation that they are present in chronic viral liver disease, suggests yet another mechanism of viral persistence that requires further investigation.

Defects in B-lymphocyte secretion of antibody in chronic HBV infection have also been described and are believed to be a physiological response of anti-HBs-secreting B lymphocytes to marked excess of circulating HBsAg.[74,119] The importance of these various lymphocyte abnormalities in terms of the likelihood of developing chronic HBV infection is not known.

### *Serum factors*

In addition to the described defect in interleukin production in most patients with severe liver disease,[117,118] studies have suggested that HBV carriers may secrete specific serum factors that predispose to viral persistence. Recent studies have shown that the T cells of the majority of chronic HBV carriers produce a secreted soluble factor (TSF) that selectively suppresses the production of anti-HBs by the B lymphocytes of the patient.[23] Other serum factors that are claimed to adversely alter lymphocyte function have also been described, but these are less specific and are not believed to play a primary role in HBV chronicity.[116]

### *Anti-idiotype response*

One important method of immunoregulatory control is achieved by synthesis of antibodies against the variable region of antibodies. These antibodies, known as anti-idiotypes, can either enhance or, more commonly, limit the immune response. One study looking at anti-idiotype antibodies in HBV infection showed that 70–80% of patients with persistent infection had these.[120] Individuals who subsequently cleared the HBV infection were less likely to have the anti-idiotype antibodies and, if they had the antibodies, they were of a lower titre than patients who did not clear the infection.[120] These data suggest that anti-idiotypic modulation of the immune response against the virus may be important in predicting viral persistence.

### *Gender and hormonal factors*

After exposure in adulthood, the likelihood of remaining chronically infected is greater in males than in females. The reasons for this difference are not known, although it may be speculated that they are hormonal in origin. There is a glucocorticoid responsive element in the genome of HBV,[121] and other studies have indicated that HBsAg gene expression is regulated by sex steroids and glucocorticoids.[122] In addition, HBsAg is cleared more slowly in male inbred mice than in female mice of the same genotype,[123] suggesting that phagocytic cells in males do not 'see' the viral protein as readily as those in females. Males are more likely than females to develop significant chronic liver disease.

## How does the virus cause liver cell damage and fibrosis?

### Lysis of HBV-infected hepatocytes

The existence of a period of viraemia without liver damage in the prodromal phase of acute infection and of

carriers with high-level HBV replication without liver damage suggests that HBV itself is not directly cytopathic.[14,15] Therefore, if it is not the virus itself which causes damage in the liver, then it must be the immune response to the infected hepatocytes. Support for this hypothesis is derived from the presence of minimal liver disease in patients with high levels of viral replication and severe liver disease in patients with undetectable viral replication and strong T-cell responses to HBV antigens. Experiments with transgenic mice expressing HBV antigens also confirm that neither expression of HBV antigens nor the process of replication itself is directly cytopathic.[42] An exception to this is a strain of mice that overexpress HBsAg. Hepatocytes in this strain are exquisitely sensitive to physiological concentrations of IFN-γ and eventually failure to secrete HBsAg leads to cell necrosis.[43]

A major pathway used by CTLs and NK cells to eliminate infected cells is via exocytosis of granule components in the direction of the target cell, delivering a lethal hit of cytolytic molecules. Amongst these, granzyme B and perforin have been shown to induce CTL-mediated target cell DNA fragmentation and apoptosis. Once released from the CTL, granzyme B binds its receptor, the mannose-6-phosphate/insulin-like growth factor II receptor, and is endocytosed but remains arrested in endocytic vesicles until released by perforin. Once in the cytosol, granzyme B targets caspase-3 directly or indirectly through the mitochondria, initiating the caspase cascade to DNA fragmentation and apoptosis. Caspase activity is required for apoptosis to occur. However, in the absence of caspase activity, granzyme B can still initiate mitochondrial events via the cleavage of Bid. Recent work shows that granzyme B-mediated release of apoptotic factors from the mitochondria is essential for the full activation of caspase-3. Thus, granzyme B acts at multiple points to initiate the death of the infected cell.[124] Intrahepatic mRNA of perforin and granzyme B are seen in most patients with hepatitis B and correlate with ALT levels and with HAI scores, indicating that these systems play an important role in liver cell injury in HBV infection.[125]

Recent data raise the likelihood that granzyme M represents a third major and specialized perforin-dependent cell death pathway that plays a significant role in cell death mediated by NK cells.[126] In the presence of perforin, the protease activity of granzyme M rapidly and effectively induces target cell death. In contrast to granzyme B, cell death induced by granzyme M does not feature obvious DNA fragmentation, occurs independently of caspases, caspase activation and perturbation of mitochondria, and is not inhibited by overexpression of Bcl-2.

Hepatocyte apoptosis also occurs in response to ligand activation of various death receptors, such as Fas, TNF-α receptor-1, TNF-related apoptosis-inducing ligand (TRAIL) receptor-1, which are displayed on infected hepatocytes and are activated by the respective ligands, released by HBV-sensitized lymphocytes.[127] The presence of these receptors, which are increased in the presence of HBV replication, suggests that these mechanisms are important in destruction of HBV-infected hepatocytes

Adoptive transfer of MHC class I-restricted, HBsAg-specific CTL into HBsAg transgenic mice induces apoptosis and necrosis with a histological pattern akin to that seen in human acute HBV infection. Increasing the dose of CTL increases the severity of hepatitis until the typical appearance of fulminant HBV infection occurs.[44]

### Hepatic fibrosis

Analysis of CTL populations in the livers of patients with chronic HBV infection reveals further insights into the mechanisms of hepatic damage. In patients with raised transaminases, high levels of viral replication and high levels of inflammatory activity on liver biopsy, the proportion of HBV-specific CTL detectable by tetramer staining is low.[68] In contrast, in patients who are HBeAg-negative with normal transaminases and low levels of viral replication, the proportion of HBV-specific CTLs in the liver infiltrates is high. These data suggest that where there is failure to control viral replication there is recruitment of non-specific inflammatory cells to the liver. These cells may be responsible for 'collateral damage' by secreting inflammatory cytokines and activation of stellate cells.

Liver fibrosis is the result of accumulation of matrix proteins (especially collagen types I and III) in response to liver injury. The process starts with the activation of hepatic stellate cells (HSCs) by a variety of cytokines (TGF-β, PDGF, etc.) produced by HBV-sensitized T lymphocytes and monocytes.[128] These stellate cells differentiate into myofibroblast-like cells which exhibit an increase in their synthesis of matrix proteins such as interstitial collagens. In addition, there is increasing evidence that liver fibrosis is a dynamic process in which altered matrix degradation may also play a major role. Extracellular degradation of matrix proteins is regulated by matrix metalloproteinases (MMPs) – again produced by HSCs – which in turn are regulated by several mechanisms which include apoptosis of the stellate cell, regulation at the level of gene transcription, cleavage of the proenzyme to an active form and specific inhibition of activated forms by tissue inhibitors of metalloproteinases (TIMPs).

## Summary

Acute self-limiting HBV infection usually has little impact on morbidity or mortality, whereas persistent HBV

infection is a major public health problem, particularly in the Far East and in sub-Saharan Africa. Persistent infection is usually accompanied by a state of partial tolerance to viral antigens, which may be the result of both viral and host factors. Total loss of tolerance may lead to viral elimination, but partial loss of tolerance (development of antibody in the absence of an adequate CTL response) may lead to an unstable equilibrium in which prolonged hepatocyte damage leads to fibrosis, emergence of HBV antigenic variants, and ultimately liver failure or hepatocellular carcinoma.

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# Chapter 20
# Treatment of chronic hepatitis B

Patrick Marcellin, Tarik Asselah, Nathalie Boyer

## Introduction

Three agents are currently approved for the treatment of chronic hepatitis B: interferon-alpha (IFN-α), lamivudine and adefovir. Each agent has inherent limitations for use in the treatment of chronic hepatitis B.[1–3] IFN-α is effective in a minority of patients and has frequent side-effects that limit its tolerability.[4,5] The efficacy of lamivudine is limited by the emergence of drug-resistant hepatitis B virus (HBV) mutants, restricting its utility as a long-term therapy for chronic hepatitis B. Adefovir, which has been registered recently, is well tolerated and is associated with a low incidence of resistance, but its antiviral effect is not optimal.

Lamivudine and adefovir have the advantages of oral administration and excellent safety profiles. However, they induce a sustained response after withdrawal of therapy in only a minority of patients, and therefore the treatment needs to be indefinitely administered in the majority of patients. As a result, more effective therapy with more potent drugs used alone or in combination is needed and the treatment of chronic hepatitis B remains an open issue.

After a brief summary of the natural history of chronic hepatitis B to provide an understanding of the indications and the objectives of therapy, this review summarizes first the results obtained with current available treatments, IFN-α, lamivudine and adefovir. The second part of this review summarises the results obtained recently with pegylated IFN (PEG IFN), combination of PEG IFN with lamivudine, combination of lamivudine with adefovir and newer antiviral agents such as entecavir, emtricitabine, telbivudine and clevudine. Phase III studies of entecavir and emtricitabine have been achieved but final results have not yet been fully published. The other drugs are still in phase II trials and therefore the information available is limited (Table 20.1).

**Table 20.1** Nucleoside and nucleotide analogues for the treatment of chronic hepatitis B

| Antiviral agent | Status |
|---|---|
| Lamivudine | Approved |
| Adefovir dipivoxil | Approved |
| Entecavir | Phase III |
| Emtricitabine (FTC) | Phase III |
| Telbuvidine (L-dT) | Phase III |
| Clevudine (L-FMAU) | Phase II |
| L-dC, L-dA | Phase II |

## Natural history of chronic hepatitis B

Chronic hepatitis B is a dynamic process with an early replicative phase and active liver disease and a late low replicative phase with remission of liver disease.[1–3] The natural history of chronic hepatitis B can be schematically divided in three phases (Fig. 20.1).

The first 'immune tolerance' phase is characterized by hepatitis B e antigen (HBeAg) positivity, high HBV replication level (reflected by high levels of serum HBV

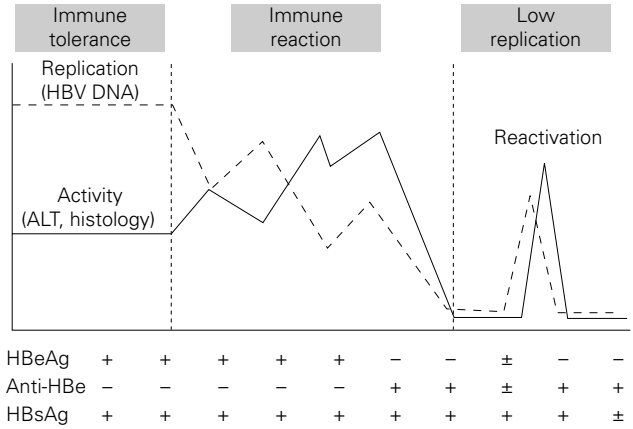


**Figure 20.1** Natural history of chronic hepatitis B. The natural history of chronic hepatitis B can be schematically divided in three phases: a first 'immune tolerance' phase with low activity of the liver disease and high level replication; a second 'immune reaction' phase with moderate to severe activity of the liver disease and low level replication; a third phase with very low level of replication and remission of the liver disease.

DNA), normal or low levels of aminotransferases, mild liver necroinflammation and no or slow progression of fibrosis. During this phase, the rate of spontaneous HBeAg loss is very low. This first phase is more frequent and more prolonged in subjects infected at birth or during childhood.

The second 'immune reaction' phase is characterized by a lower level of replication (as reflected by low levels of serum HBV DNA), increased levels of aminotransferases, moderate or severe liver necroinflammation and rapid progression of fibrosis. During this phase, the rate of HBeAg loss is high. This second phase is more frequent in subjects infected during adulthood.

Seroconversion from HBeAg to antibody to HBeAg (anti-HBe) marks the transition from chronic hepatitis B to the inactive hepatitis B surface antigen (HBsAg) carrier state with low or undectable serum HBV DNA and normal aminotransferases, and confers favourable long-term outcome with very low risk of cirrhosis or hepatocellular carcinoma (HCC) in the majority of patients. HBsAg loss and seroconversion to antibody to HbsAg (anti-HBs) may occur (resolved hepatitis B).

However, a proportion of patients (about 20–30% of cases) continue to have or redevelop high levels of HBV DNA and active hepatitis despite HBeAg seroconversion. These patients usually have HBV variants (with mutations in the precore or the basal core promoter regions) unable to express HBeAg (HBeAg-negative chronic hepatitis B).[6] HBeAg-negative chronic hepatitis B represents a late phase in the natural history of chronic hepatitis B and is associated with a very low rate of spontaneous disease remission. The proportion of patients with this form of chronic hepatitis B has been increasing in the last decades and the majority are in the Mediterranean countries.[7]

## Objectives of treatment

The first objective of the treatment is to decrease HBV replication in order to decrease necroinflammation in the liver and therefore prevent the progression of fibrosis. Thus, the interruption of the fibrogenesis process prevents progression to cirrhosis and its complications, including HCC, and therefore improves survival. The response to treatment can be classified into three phases. The first phase is characterized by the decrease of HBV replication (as reflected by the reduction of serum HBV DNA level). The liver necroinflammation diminishes, fibrosis is stabilized or may even regress but the risk of reactivation persists. If the antiviral effect is sufficient (<100 000 copies of HBV DNA per mL) and is maintained and accompanied by an effective immune response with clearance of infected hepatocytes, HBe seroconversion may occur; then the risk of reactivation is low. If HBV replication is completely interrupted (as reflected by the absence of detectable HBV DNA in the serum by sensitive assays), with stable HBe seroconversion, loss of detectable HBsAg (with or without HBsAg seroconversion) may occur, which is associated with complete disappearance of liver necroinflammation with no risk of reactivation.

With the drugs currently available, two different concepts can be proposed for the treatment of chronic hepatitis B. The first concept is that of sustained response obtained after a limited duration of therapy. The second concept is that of maintained response obtained during administration of therapy. The first strategy is the one usually used with IFN, whose duration of therapy is limited by the poor tolerability (Fig. 20.2). IFN therapy can induce a sustained response after withdrawal of treatment with a not inconsiderable chance of HBsAg loss or seroconversion. However, the limitation of this strategy is that the rate of sustained response is relatively low and sustained response is mainly obtained in patients with good predictors of response (i.e. patients in the immune reaction phase with active liver disease and low level replication). The second strategy is the one generally used with nucleoside or nucleotide analogues, whose withdrawal is generally accompanied by reactivation. Therefore, therapy needs to be administered indefinitely to obtain a maintained response (Fig. 20.3). Maintenance therapy is allowed by the good tolerability of these drugs. However, the limitation is the occurrence during therapy of HBV-resistant mutants associated with breakthroughs. Furthermore, HBsAg loss with or

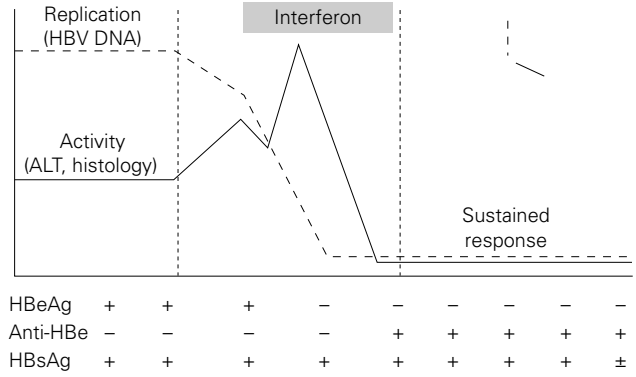


**Figure 20.2** Treatment of chronic hepatitis B with interferon alpha (IFN-α). During administration of IFN-α, there is an early antiviral effect with decrease of serum HBV DNA level, then there is an immune effect with increased cytolysis with elevation of serum alaine aminotransferase (ALT) level. In case of effective antiviral and immune effects, a sustained response is observed after withdrawal of therapy with very low serum HBV DNA level, normal serum ALT level and HBe seroconversion. If HBV replication is completely inhibited with undetectable serum HBV DNA (with a sensitive polymerase chain reaction [PCR] method), HBs seroconversion may occur within the years following treatment.

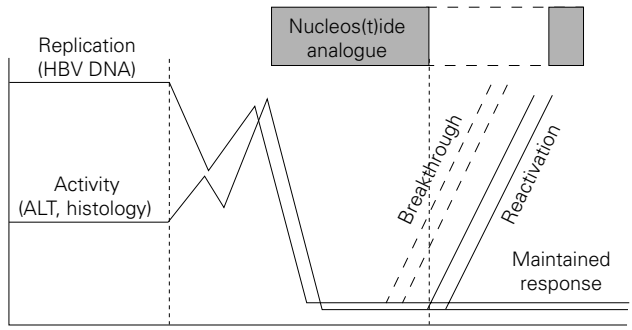


**Figure 20.3** Treatment of chronic hepatitis B with a nucleoside or a nucleotide analogue. During administration of the analogue, there is an early antiviral effect with decrease of serum HBV DNA level. However, there is no immune effect of the drug and therefore there is no increased cytolysis and no elevation of serum ALT level, which remains normal. The response is maintained as long as the drug is administered. However, in case of occurrence of a resistant HBV mutant, a breakthrough is observed with a reincrease of serum HBV DNA level, then a reincrease of serum ALT level. In the absence of an effective spontaneous immune response with HBe seroconversion, withdrawal of therapy is rapidly followed by a reactivation.

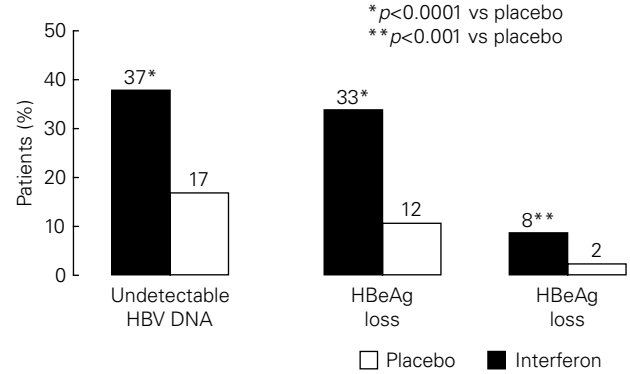


**Figure 20.4** Meta-analysis of interferon-alpha (IFN) trials in HBeAg-positive chronic hepatitis B (from Wong *et al.*, *Ann Intern Med* 1993[5]). In this meta-analysis including 15 randomized controlled trials (published between 1986 and 1992) comparing IFN versus placebo, including overall 837 patients with HBeAg-positive chronic hepatitis B, the superiority of IFN versus placebo was shown for the rates of undetectable serum HBV DNA (hybridization assays), HBeAg loss and HBsAg loss.

without HBs seroconversion is rarely observed with maintenance therapy with nucleoside or nucleotide analogues.

## Current treatments

### IFN-α

IFN-α has been used in the treatment of chronic hepatitis B for many years. IFN-α exerts an antiviral effect on infection with HBV through two mechanisms.[8] First, IFN-α has a direct antiviral effect by inhibiting synthesis of viral DNA and by activating antiviral enzymes. Second, IFN-α exaggerates the cellular immune response against hepatocytes infected with HBV by increasing the expression of class I histocompatibility antigens and by stimulating the activity of helper T lymphocytes and natural killer lymphocytes. Thus, IFN-α induces an early diminution of HBV replication (reflected by a diminution of HBV DNA in serum) and a late (about 2 months later) increase in serum alanine aminotransferase (ALT) levels. Many controlled studies of IFN-α in patients with chronic hepatitis B have been reported. In these studies, with various schedules, mean virological response rate was 37% versus 17%, mean HBeAg loss rate was 33% versus 12% and HBsAg loss rate was 8% versus 2% in the IFN-α-treated groups versus the placebo groups[5] (Fig. 20.4). A dosage of 5–10 MU three times a week for 4–6 months allows a good efficacy with a satisfactory tolerance.[2]

The discrepancies in the results of the different studies could be due, in part, to the different therapeutic schedules, but are mainly due to the populations of patients included in these trials. A certain number of factors are predictive of poor response to IFN-α[9] (Table 20.2). Low serum HBV DNA level and high serum ALT levels are predictors of non-response. Also, infection with HBV at birth or early in the patient's life (as is often the case in countries where HBV infection is hyperendemic, such as south-east Asia) is a factor in poor response to IFN-α.

### Lamivudine

Lamivudine, a nucleoside analogue which directly inhibits HBV DNA polymerase, was first developed as a reverse transcriptase inhibitor for use in HIV infection.[8] It also has activity against HBV at lower concentrations. Lamivudine (2′,3′dideoxythiacytidine) is a minus enantiomer and it is thought that this may help to explain

**Table 20.2** Predictors of non-response to IFN therapy in chronic hepatitis B

| |
|---|
| Asian ethnicity |
| Childhood infection |
| Male sex |
| Immunosuppression due to disease (HIV) or therapy |
| Co-existing HDV infection |
| Disease caused by core promoter or precore mutant virus (HbeAg-negative) |
| Low serum ALT levels |
| High serum HBV DNA levels |
| Mild grade of liver necroinflammation |

the very low rates of side-effects noted with this agent. Randomized controlled trials have shown the efficacy of lamivudine in the treatment of HBeAg-positive and HBeAg-negative chronic hepatitis B.

### *HBeAg-positive chronic hepatitis B*

One randomized placebo-controlled trial[10] showed that almost all patients treated with lamivudine (98%) had a reduction of serum HBV DNA levels. Serum HBV DNA levels became undetectable under lamivudine therapy (<0.7 meq/mL) in 44% versus 16% in the placebo group. At 1 year of treatment, the HBe seroconversion rates were 17% and 6% in the lamivudine and the placebo group, respectively (Fig. 20.5). Normalization of ALT levels was observed in 41% versus 7% in the placebo group. Histological improvement defined by a decrease of at least two points in the Knodell score was observed in 52% versus 23% in those on placebo.

The rates of virological, biochemical and histological response observed in three other randomized controlled trials showed similar results with HBeAg seroconversion rates ranging from 17% to 21%.[10–13]

The tolerability and safety of lamivudine are excellent. The incidence of adverse events was similar to that with placebo. Lamivudine therapy seems to be well tolerated for up to 5 years. However, data reported on long-term therapy with lamivudine are limited to small series of patients.

Pretreatment factors predictive of response are, as for IFN-α, high serum ALT levels and a high degree of histological necroinflammation.[14] Pretreatment ALT level seems to be the most important: response rates are low and similar to untreated patients in patients with levels below twice the upper limit of normal. The rate of HBeAg loss was highest among patients with pretreatment ALT levels greater than five times the upper limit of normal.

After withdrawal of lamivudine therapy, ALT flare-ups have been reported in 41 individuals treated with lamivudine for a minimum of 3 months and followed for at least 6 months after therapy.[15] Marked flares were observed in 17% and occurred 7–44 weeks after treatment. These flares were all associated with rising levels of HBV DNA, unlike the spontaneous flares associated with HBe seroconversion (with falling levels of HBV DNA). Two of these flares were associated with hepatic decompensation.

The major inconvenience of lamivudine is the high rate of occurrence of viral resistance related to mutations in the YMDD motif. Indeed, even if the HBeAg seroconversion rate may be increased by continuing treatment, the frequency of resistance increases with time from 24% at 1 year, to 38% at 2 years, 50% at 3 years and 67% at 4 years.[2,3,16,17]

The most important mutation is a substitution of valine or isoleucine for methionine in the YMDD motif of the HBV polymerase gene (rtM204V/I).[18] In many patients, this is accompanied by a second mutation substituting methionine for leucine in an upstream region (rtL180M). Lamivudine resistance is more likely to occur in patients with high baseline serum HBV DNA levels.

The emergence of lamivudine-resistant mutants is usually associated with a breakthrough with moderate increase of serum HBV DNA and ALT levels, which may remain lower than at baseline for several months. However, severe cases have been reported in patients with cirrhosis. In patients who develop lamivudine-resistant mutant, adefovir is effective and should be initiated rapidly if an increase in ALT is observed, especially in patients with cirrhosis who have a risk of hepatic decompensation. In order to diagnose the emergence of resistance earlier (before the appearance of detectable serum HBV DNA by standard assays and before the increase of ALT), monitoring of serum HBV DNA level by a sensitive assay is useful. Indeed, an increase of serum HBV DNA of more than one log generally reflects the appearance of a resistant mutant and this allows a switch to adefovir several months before the increase of ALT.[18,19]

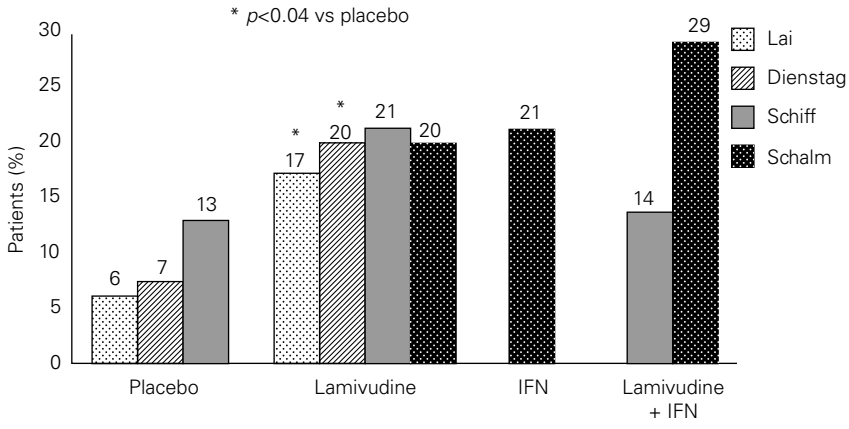


**Figure 20.5** Rates of HBe seroconversion in four randomized controlled trials of lamivudine. In four randomized controlled trials of lamivudine, HBe seroconversion rates ranged from 17% to 21% at 1 year of therapy versus 6% to 13% (spontaneous seroconversion) in the placebo groups. In one study, the rate of HBe seroconversion was comparable to that observed in the IFN group. In two studies, the rates of HBe seroconversion were lower or higher in the IFN plus lamivudine combination groups; however, the differences were not significant.

In case of HBe seroconversion during lamivudine therapy, it is usually recommended that treatment is prolonged for 3–6 additional months to decrease the risk of reactivation.[1,2] In the absence of HBe seroconversion, as withdrawal is almost invariably followed by reactivation, it is usually recommended that the treatment is continued as long as HBV replication is suppressed and serum ALT revels remain normal, until the appearance of viral resistance.

### *HBeAg-negative chronic hepatitis B*

In patients with HBeAg-negative chronic hepatitis B, one randomized controlled study[20] showed an efficacy similar to that observed in patients with HBeAg-positive chronic hepatitis B with the same rate of resistance. HBV DNA was undetectable (non-PCR-based assays) after 12 months of therapy in 90% of patients (70% with PCR-based assays). Serum ALT normalized in 75% of patients. A fall in HBV DNA and normalization of ALT occurred in only 5% of patients on placebo. Histological response was observed in 60% of lamivudine-treated patients. Predictors of response to lamivudine have not been established in this population. In patients with a virological response at the end of a 12-month course of lamivudine, the sustained response rate 6 months post-treatment was <5%.

In studies with prolonged therapy, response rates peak at 12 months and decrease thereafter.[21] At 30 months, rates of virological and biochemical responses were 30% and 60%, respectively.

The rate of resistance observed in patients with HBeAg-negative chronic hepatitis was similar to that observed in HBeAg-positive chronic hepatitis. Lamivudine-resistant mutants appeared in 10–40% of patients after 1 year of therapy, and in 50–60% of those treated continuously for 3 years.[21,22] As in HBeAg-positive patients, the emergence of resistant mutant HBV was accompanied by an increase of HBV DNA and, after a few months, by ALT elevation. Flares occurred in 30% of patients and were symptomatic or severe in some cases. As in HBeAg-positive patients, treatment by adefovir is generally effective in patients who develop lamivudine resistance.

### *Special groups*

In patients with decompensated cirrhosis, lamivudine therapy can induce marked improvement in liver disease, impacting favourably on survival.[23,24] However, the clinical effects are slow and impaired by the risk of emergence of resistance, which can be associated with flares responsible for liver failure and death. Antiviral therapy is required before liver transplantation in patients with detectable HBV DNA to decrease the risk of recurrence of HBV infection after transplantation. In addition, patients in whom lamivudine resistance develops before transplant have a higher risk of recurrent hepatitis B post-transplant. In this clinical situation, adefovir should be initiated rapidly if lamivudine resistance is suspected in order to prevent the flare. This explains why, in this setting, adefovir might be preferred because of the very low risk of resistance.

In case of recurrence of HBV infection after liver transplantation, lamivudine therapy achieves similar results to those in non-transplant patients with respect to inhibition of HBV replication and normalization of ALT. Breakthrough secondary to lamivudine resistance may lead to severe hepatitis flares, rapidly progressive liver failure and graft loss. For these reasons, after transplantation, the optimal prophylactic regimen seems to be the combination of lamivudine with anti-HBs immunoglobulins (HBIg).

In HIV-HBV co-infected patients, from the limited data available lamivudine appears to show similar effects in patients on HAART (highly active antiretroviral therapy) in terms of virological and biochemical response, to those in patients not co-infected with HIV.[25] In 40 HIV co-infected patients, 96% had undetectable serum HBV DNA (<5 pg/mL); serum ALT decreased compared with baseline; HBe seroconversion was observed in 11%. Drug-resistant HBV emerged rapidly during monotherapy with lamivudine: the incidence was 50% and 90% at 2 years and 4 years, respectively.[26] Because of the high rate of resistance to lamivudine and because a large proportion of HIV-HBV infected patients have already received lamivudine for the treatment of HIV and/or HBV, adefovir or tenofovir (which is registered for the treatment of HIV) are the drugs generally used in the treatment of chronic hepatitis B in this population.

## Adefovir dipivoxil

Adefovir dipivoxil has recently been registered for the treatment of chronic hepatitis B. Adefovir dipivoxil is the oral prodrug of adefovir. Adefovir is a nucleotide analogue of adenosine monophosphate. *In vivo*, adefovir dipivoxil is converted to the parent compound, adefovir, and through two phosphorylation reactions to adefovir diphosphate, the active intracellular metabolite that interacts with HBV polymerase. Adefovir diphosphate acts as a competitive inhibitor and chain terminator of viral replication.

Two large randomized controlled trials have demonstrated that adefovir dipivoxil is effective in patients with HBeAg-positive or HBeAg-negative chronic hepatitis B. Also, adefovir dipivoxil effectively suppresses lamivudine-resistant HBV in chronic hepatitis B patients

post-liver transplantation, patients with compensated or decompensated liver disease and patients co-infected with HIV.

### *HBeAg-positive chronic hepatitis B*

A large randomized, placebo-controlled study enrolled 515 patients randomized to receive adefovir dipivoxil 10 mg daily (n = 172), adefovir 30 mg daily (n = 173) or placebo (n = 170) for 48 weeks.[27] There was a rapid decrease in the median serum HBV DNA level in patients treated with adefovir dipivoxil, with statistically significant differences compared with placebo. At week 48, the median change from baseline in serum HBV DNA was –3.5 $\log_{10}$ for adefovir (10 mg) compared with –0.5 $\log_{10}$ copies/mL for placebo. Significantly more patients had undetectable serum HBV DNA levels (<400 copies/mL) (21% vs 0%, $p$ <0.001) (Fig. 20.6).[27] HBeAg seroconversion was observed in 12% of the patients receiving adefovir 10 mg compared with 6% in the placebo group ($p$ <0.05). ALT normalization was achieved in 48% compared with 16% ($p$ <0.001).

A significantly higher proportion of patients receiving adefovir 10 mg showed improvement in liver histology (improvement of at least two points in the Knodell score) at week 48 compared with those receiving placebo (53% versus 25%; $p$ <0.001) (Fig. 20.6). Results of the blinded ranked assessment of baseline and week 48 biopsies demonstrated that patients treated with adefovir 10 mg had better improvement of necroinflammatory activity ($p$ <0.001) and fibrosis ($p$ <0.001). Using the Ishak fibrosis score, fibrosis improved in 34% compared with 19% and fibrosis progressed in 11% compared with 21%.[28]

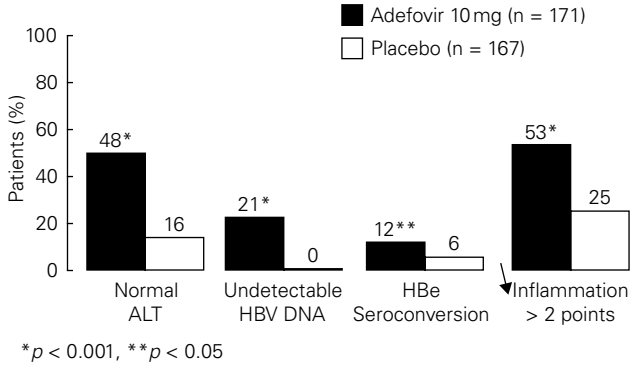


**Figure 20.6** Adefovir dipivoxil in the treatment of HBeAg-positive chronic hepatitis B (from Marcellin *et al.*, *N Engl J Med* 2003[27]). In this randomized controlled trial, at 48 weeks of therapy, the rates of normal ALT, undetectable HBV DNA, HBe seroconversion and histological improvement were higher in the group of patients receiving adefovir, at the dose of 10 mg daily, as compared with the group receiving a placebo.

The tolerability and safety profile of adefovir at the dose of 10 mg was similar to that of the placebo. Adefovir at the dose of 30 mg was associated with an increase in creatinine level in some patients. This increase was moderate, occurred after 24 weeks of treatment and resolved in all cases after withdrawal of the drug. However, this observation chose the 10-mg dose as the best dose with regard to the benefit/risk ratio and the 10-mg dose is the dose registered.

The durability of the response after withdrawal of treatment is now becoming known. Of 60 patients undergoing HBe seroconversion on adefovir, 91% were still HBe antigen-negative after 1 year of follow-up. All the patients that relapsed were genotype C.[29] However, cessation of therapy in some patients without HBe seroconversion was associated with relapse, therefore maintenance therapy is recommended. Preliminary results suggest that the antiviral effect is maintained and the rates of virological response with HBe seroconversion increase with the duration of therapy.[30] However, a longer follow-up is planned (up to 5 years) to answer the question of the durability of the response under treatment and of the possible increased efficacy of long-term treatment.

An extensive genotyping study has been carried out in all patients with detectable HBV DNA (by PCR assay). Systematic sequencing did not show any case of emergence of a mutant HBV resistant to adefovir after 48 weeks of treatment with adefovir.[31] Large-scale studies of cohorts of patients undergoing adefovir therapy are in progress to assess the incidence of resistance in the long term. The explanation for the high threshold for resistance to adefovir dipivoxil may reside in the structural features of adefovir that distinguish it from nucleoside analogues: specifically, its minimal flexible acyclic linker that closely resembles the natural substrate and the fact that it is a nucleotide, containing a phosphorus atom, not a nucleoside.

### *HBeAg-negative chronic hepatitis B*

A large randomized, placebo-controlled study of adefovir dipivoxil has been performed in 185 patients with HBeAg-negative chronic hepatitis B.[32] Patients were randomized at a ratio of 2:1 to receive either adefovir dipivoxil 10 mg (n = 123) or placebo (n = 62) for 48 weeks. Adefovir 10 mg once daily resulted in a significant reduction in serum HBV DNA levels at week 48 compared with placebo, with a median decrease of 3.9 $\log_{10}$ copies/mL versus 1.3 $\log_{10}$ copies/mL ($p$ <0.001). 51% of the patients treated with adefovir dipivoxil had no detectable HBV DNA as measured by PCR compared with none in the placebo group ($p$ <0.001) (Fig. 20.7).[32] ALT normalization was achieved in 72% compared with 29% ($p$ <0.001). Significantly more patients in the adefo-

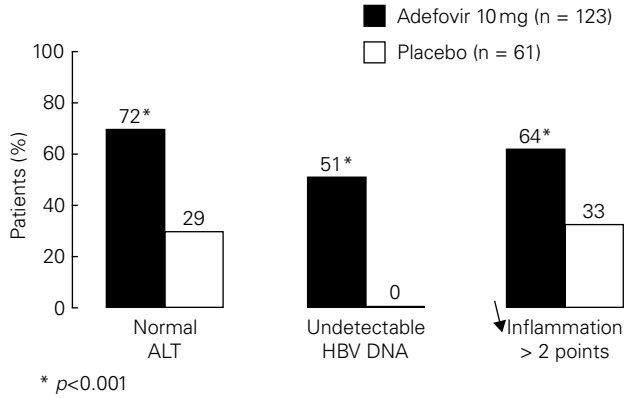


**Figure 20.7** Adefovir dipivoxil in the treatment of HBeAg-negative chronic hepatitis B (from Hadziyannis *et al*., *NEJM* 2003[32]). In this randomized controlled trial, at 48 weeks of therapy, the rates of normal ALT, undetectable HBV DNA and histological improvement were higher in the group of patients receiving adefovir, at the dose of 10 mg daily, as compared with the group receiving a placebo.

vir group had histological improvement from baseline to week 48 compared with the placebo group (64% versus 33%, $p$ <0.001).

At 144 weeks of therapy with 10 mg of adefovir daily, the antiviral effect was maintained with, in a subgroup which had a repeat liver biopsy at 96 weeks, a maintained histological improvement. After 144 weeks of therapy, tolerability was good without significant side-effects and without the occurrence of nephrotoxicity.[33] The incidence of resistance related to the occurrence of specific mutations (rt N236T and rt A181V) was low (3% at 96 weeks and 5.9% at 144 weeks). It seems that the HBV strains resistant to adefovir are sensitive to lamivudine.

### *Special groups*

An open-label efficacy and safety study of adefovir dipivoxil 10 mg has been performed in 324 chronic hepatitis B patients before and after liver transplantation (n = 128 and 196, respectively) with clinical evidence of lamivudine-resistant HBV.[34,35] Patients have received adefovir dipivoxil for varying lengths of time up to a maximum of 72 weeks (before liver transplantation) and 129 weeks (after liver transplantation), respectively. Serum HBV DNA was reduced by approximately 4 $\log_{10}$ copies/mL in both groups, and ALT level normalized in 76% and 49% of patients in the two groups, respectively. Patients also had significant improvement of liver function. Kaplan–Meier survival curve estimates by week 48 were 84% and 93% in the pre- and post-transplantation groups, respectively. These 1-year survival rates are significantly better than survival rates observed in historical groups of patients with no treatment. It is noteworthy that, in a significant number of patients who improved, the liver transplantation was postponed.

Although many of the patients (all of whom had lamivudine-resistant HBV) were medically compromised as a result of advanced liver disease and co-morbidities, there was a low rate of adverse events leading to drug discontinuation in pre- and post-liver transplantation patients (5% and 6%, respectively). A total of 42 deaths (11%) was reported in pre- and post-transplantation patients (24 and 18 patients, respectively). The deaths were considered to be due to complications of progressive liver disease or liver transplantation surgery.

In an open-label pilot study conducted in 35 HIV-HBV co-infected patients with lamivudine-resistant HBV and controlled HIV infection, adefovir therapy, at the dose of 10 mg, induced a 4 log decrease in serum HBV DNA levels at 48 weeks.[36] Two patients underwent HBe seroconversion. A transient increase in serum ALT levels was observed in 15 patients without consequence for liver function. The explanation for this observation is unclear. No HBV DNA breakthrough and no viral resistance was observed through week 48. In addition, no significant changes in either HIV RNA or CD4 cell count were observed.

## New treatments

### Pegylated IFN

More recently, the efficacy of IFN has been improved with the replacement of standard IFN by IFN conjugated with polyethylene glycol (PEG IFN). This new form of IFN reduces elimination of IFN by the kidneys, thus significantly increasing its half-life and resulting in more stable plasma concentrations of IFN. Moreover, pegylation reduces the immunogenicity of the protein (reduction of the production of anti-IFN antibodies). Finally, the number of injections has been reduced from three times to once weekly, thanks to improved pharmacokinetics, which is obviously more comfortable for the patient.

Two PEG IFNs which differ in the quality and quantity of conjugated PEG to IFN have been produced: 12 kD of linear PEG for IFN alpha-2b and 40 kD branched PEG for IFN alpha-2a. In both cases, PEG IFNs have been shown to be twice as effective overall as the corresponding non-pegylated IFNs in chronic hepatitis C.[37,38] Therefore, the efficacy of PEG IFNs has been assessed recently in the treatment of chronic hepatitis B.

A first randomized controlled study of PEG IFN alpha-2a has been performed in patients with HBeAg-positive chronic hepatitis B.[39] Treatment duration and follow-up were each 24 weeks. At the end of follow-up, treatment response defined by loss of HBeAg with serum HBV DNA level <500 000 copies/mL with normal ALT was observed in 19–28% of patients receiving PEG IFN alpha-2a (at a dose of 90 µg, 180 µg or 270 µg per week) versus 12% of patients who received standard IFN al-

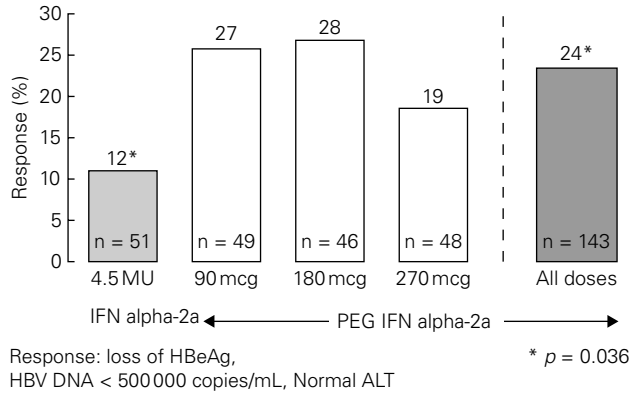


**Figure 20.8** Pegylated interferon (PEG IFN) alpha 2-a in HBeAg-positive chronic hepatitis B (from Cooksley *et al.*, *J Viral Hepat* 2003[39]). In this randomized controlled trial, 24 weeks after therapy, the rates of response were higher in the three groups which received PEG IFN alpha 2a as compared with the group which received conventional IFN alpha 2a; however, the difference was not significant. When the three PEG IFN groups were pooled retrospectively, there was a significant difference as compared with the IFN group (24% versus 12%; p = 0.036).

pha-2a (Fig. 20.8).[39] Side-effects associated with PEG IFN were comparable to those observed with standard IFN. The safety profile of PEG IFN was comparable to that of conventional IFN with the same frequency of adverse events or laboratory abnormalities.

This study does not prove the superiority of PEG IFN alpha-2a as compared with standard IFN alpha-2a, as the dose of IFN alpha-2a used was relatively low (4.5 million units, three times a week) and the differences observed between each of the three PEG IFN alpha-2a treatment groups and the standard IFN alpha-2a group were not significant. However, the overall rate of response in patients who received PEG IFN alpha-2a was higher than that observed in the IFN alpha-2a group. A retrospective analysis showed that the rates of response were higher with the PEG IFN among the most difficult to treat patients (with high HBV DNA level or low ALT levels). Therefore, this study strongly suggests that PEG IFN alpha-2a is more effective than standard IFN alpha-2a for the treatment of chronic hepatitis B.

Three large randomized controlled trials have confirmed the efficacy of PEG IFNs in HBeAg-positive (one trial with PEG IFN alpha-2b and one trial with PEG IFN alpha-2a) and HBeAg-negative chronic hepatitis B (one trial with PEG IFN alpha-2a). These studies, which compared PEG IFN monotherapies to the combination of PEG IFN and lamivudine (three studies) and lamivudine (two studies), are detailed in the next chapter.

## Combination of PEG IFN with lamivudine

Previous studies on the combination of IFN and lamivudine suggested that this combination could be more effective than lamivudine monotherapy.[40] However, the results of different studies were discordant, which could be due to different treatment regimens that may not have been optimal.

### *HBeAg-positive chronic hepatitis*

In a large randomized controlled study, 307 patients with HBeAg-positive chronic hepatitis B were randomized to receive either the combination of PEG IFN alpha-2b 100 µg per week for 32 weeks then 50 µg for 20 weeks and lamivudine 100 mg per day or PEG IFN alpha-2b at the same dose with placebo.[41] At the end of the 26-week post-treatment follow-up, there was no difference in response rates between the two treatment groups: serum HBV DNA was undetectable by PCR (<400 copies per mL) in 7% and 9%; HBeAg loss was observed in 36% and 35%; normal ALT was obtained in 32% and 35% in the PEG IFN monotherapy and the PEG IFN with lamivudine combination therapy groups. Interestingly, a relatively high rate of HBsAg loss was observed (7% in both groups).

This study shows that in patients with HBeAg-positive chronic hepatitis B, 26 weeks after therapy, the combination of PEG IFN alpha-2b with lamivudine (with the simultaneous regimen used) is not superior to PEG IFN alpha-2b used in monotherapy.

Main predictors of response were HBV genotype and pretreatment ALT level. Response was 34% for those with ALT levels under three times the upper limit of normal and 50% for those with ALT levels above five times the upper limit of normal. Response was 60% for genotype A versus 42% for genotype B, 32% for genotype C and 28% for genotype D.

Another large randomized controlled trial of the combination of PEG IFN alpha-2a with or without lamivudine versus lamivudine showed similar results.[42]

Patients were randomized to one of the following treatments: PEG IFN alpha-2a, 180 µg once weekly plus oral placebo once daily for 48 weeks; PEG IFN alpha-2a, 180 µg once weekly plus lamivudine 100 mg once daily for 48 weeks; lamivudine 100 mg once daily for 48 weeks. In total, 814 patients were enrolled in the study. At the end of the 24-week post-treatment follow-up, the two PEG IFN treatment arms (with or without lamivudine) showed the same efficacy, which was superior to that observed in the lamivudine treatment arm. HBe seroconversion was observed in 32%, 27% and 19% of the patients, respectively, and a virological response (serum HBV DNA <100 000 copies per mL by quantitative PCR) in 32%, 34% and 22% of the patients, respectively.

HBsAg loss was observed in patients who received PEG IFN alpha-2a (4% and 3% versus 0% in the lamivudine group).

### *HBeAg-negative chronic hepatitis*

A phase III, partially double-blinded study has evaluated the efficacy and the safety of PEG IFN alpha-2a alone or in combination with lamivudine versus lamivudine in patients with HBeAg-negative chronic hepatitis B.[43]

Patients were randomized to one of the following treatments: PEG IFN alpha-2a, 180 µg once weekly plus oral placebo once daily for 48 weeks; PEG IFN alpha-2a, 180 µg once weekly plus lamivudine 100 mg once daily for 48 weeks; lamivudine 100 mg once daily for 48 weeks. In total, 552 patients were enrolled in the study. At the end of the 24-week post-treatment follow-up, the two PEG IFN treatment arms (with or without lamivudine) showed the same efficacy, which was superior to that observed in the lamivudine treatment arm: a biochemical response (normal ALT) was observed in 59%, 60% and 44% of the patients, respectively, and a virological response (serum HBV DNA <20 000 copies per mL by quantitative PCR) in 43%, 44% and 29% of the patients, respectively (Fig. 20.9). Interestingly, taking into account that HBsAg loss is rarely observed in HBeAg-negative patients, a substantial rate of HBsAg loss was observed in this study in patients who received PEG IFN alpha-2a (4% and 3% versus 0% in the lamivudine group).

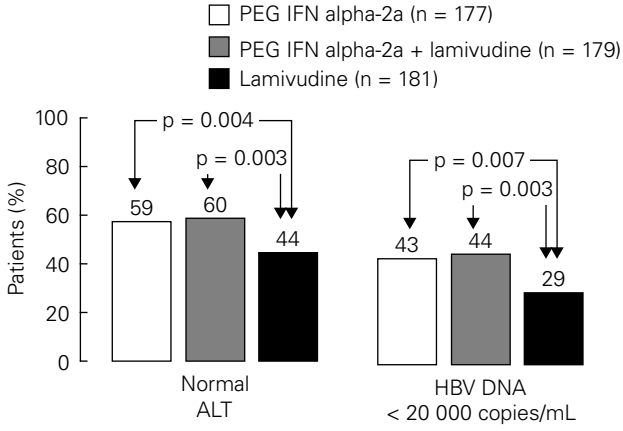


**Figure 20.9** Pegylated interferon (PEG IFN) alpha-2a in HBeAg-negative chronic hepatitis B (from Marcellin *et al.*, AASLD 2003). In this randomized controlled trial, 24 weeks after therapy, the rates of response (normal serum ALT level and serum HBV DNA <20 000 copies per mL) were higher in the two groups which received PEG IFN alpha-2a (with or without lamivudine) as compared with the group which received lamivudine. There was no difference in response rates between the group which received PEG IFN alpha-2a alone and the group which received the PEG IFN alpha-2a plus lamivudine combination.

It is noteworthy that, at the end of the 48-week treatment period, there was a higher incidence of lamivudine resistance in the lamivudine monotherapy group as compared with the PEG IFN alpha-2a plus lamivudine combination group (18% versus <1%), which confirms previous studies suggesting that IFN decreases the risk of lamivudine resistance.[40]

The adverse events associated with PEG IFN alpha-2a therapy were similar to those observed in previous trials in patients with chronic hepatitis C. Interestingly, the frequency of the adverse events was lower than that observed in patients with chronic hepatitis C. In particular, the frequency of depression was much lower: 3–4% as compared with 16–20% in patients with chronic hepatitis C.

This study shows that in patients with HBeAg-negative chronic hepatitis B, (1) the efficacy, as assessed at 24 weeks post-treatment, of PEG IFN alpha-2a monotherapy is superior to lamivudine monotherapy, and (2) the combination of PEG IFN alpha-2a with lamivudine (with the simultaneous regimen used) is not superior to PEG IFN alpha-2a used in monotherapy. However, longer follow-up is needed to assess the durability of response.

## Combination of adefovir with lamivudine

The concept of improving the efficacy by combining two analogues is based on the hypothesis that the combination would maximize the viral suppression and would decrease the occurrence of viral resistance.

One randomized study evaluated the efficacy of the combination of adefovir with lamivudine as compared to lamivudine alone or adefovir alone in 59 patients with HBeAg-positive chronic hepatitis B with lamivudine-resistant HBV.[44] There was no significant difference in median serum HBV DNA reduction (–3.59 and –4.04 log copies/mL), rates of ALT normalization (53% and 47%) and HBeAg loss (three patients in each group) between the adefovir-lamivudine combination group and the adefovir monotherapy group. Notably, serum HBV DNA level remained stable, and there was no significant biochemical or serological change during the study in the patients who remained on lamivudine monotherapy. Therefore, the clinical benefit of continuing lamivudine therapy, once resistance develops, appears to be questionable. However, it seems reasonable, at least in patients with bridging fibrosis or cirrhosis, to continue lamivudine administration after initiation of adefovir since a significant decrease of serum HBV DNA and serum ALT has been obtained.

Another study compared the efficacy of the combination of adefovir with lamivudine versus lamivudine used in monotherapy in 112 treatment-naïve patients (107 HBeAg-positive).[45] There was no significant differ-

ence in median serum HBV DNA reduction (–5.41 and –4.80 log copies/mL), rates of undetectable HBV DNA with PCR (39% and 41%) and HBeAg loss (19% and 20%) between the adefovir-lamivudine combination group and the adefovir monotherapy group. Interestingly, there was a lower incidence of lamivudine resistance in the combination group (2%) than in the lamivudine monotherapy group (20%) ($p$ <0.003).

These two studies do not answer the question of the benefit of the long-term treatment with the combination of adefovir and lamivudine as compared to adefovir monotherapy. Large randomized controlled trials with a long follow-up are needed to address this issue.

## New antivirals

Many new nucleoside analogues are under evaluation for the treatment of chronic hepatitis B. Entecavir, emtricitabine and telbivudine phase III studies have been or will soon be achieved. Other interesting nucleoside analogues like clevudine are still in phase II.

### Entecavir

Entecavir, a cyclopentyl guanosine analogue, is a potent inhibitor of HBV DNA polymerase, inhibiting both the priming and elongation steps of viral DNA replication. Entecavir is phosporylated to its triphosphate, the active compound, by cellular kinases. It is a selective inhibitor of HBV DNA and is less effective against lamivudine-resistant mutants than against wild-type HBV.[46–48]

In a 24-week, double-blind, randomized trial, the safety and efficacy of three different doses of entecavir (0.01 mg/day, 0.1 mg/day or 0.5 mg/day) was compared to lamivudine (100 mg daily); 169 patients were included.[49] Compared with lamivudine, entecavir reduced HBV DNA by an additional 0.97 log at the 0.1-mg dose and 1.28 at the 0.5-mg dose ($p$ <0.0001). At 22 weeks of therapy, in patients treated with entecavir 0.5 mg, 83.7% had an HBV DNA level below the lower limit of detection of the assay (0.7 ME/mL) compared with 57.5% treated with lamivudine ($p$ = 0.008). However, very few patients achieved HBeAg loss (0% and 6% in both groups, respectively. A dose-response relationship was observed.[50] Entecavir was well tolerated at all doses. Most adverse events were mild to moderate and transient with no significant differences observed between the different doses of entecavir and lamivudine. This study indicates that the 0.5-mg dose of entecavir could be the optimal dose.

Entecavir also showed activity in patients with lamivudine-resistant HBV. In one trial including 181 patients with lamivudine-resistant HBV, different doses (0.1, 0.5 and 1 mg daily) of entecavir were tested and compared with lamivudine.[51] At week 24, the percentage of patients with undetectable HBV DNA (<0.7 ME/mL) was 19% with 0.1 mg, 53% with 0.5 mg and 79% with 1 mg of entecavir versus 13% in the group receiving lamivudine ($p$ <0.0001).

Results of phase III studies of entecavir given for 48 weeks as compared with lamivudine in HBeAg-positive and HBeAg-negative patients and in patients with lamivudine-resistant HBV confirmed the efficacy and safety of entecavir. In one study, 709 patients with HBeAg-positive chronic hepatitis were randomized to receive entecavir or lamivudine. Mean HBV DNA level decreases were 7.0 and 5.5 $\log_{10}$ copies/mL ($p$ <0.0001), percentages of patients with undetectable HBV DNA (PCR) were 91% and 65% ($p$ <0.0001) and histological improvement was observed in 72% and 65% ($p$ = 0.008) of patients, respectively.[52]

In another study, 638 patients with HBeAg-negative chronic hepatitis were randomized to receive entecavir or lamivudine. Mean HBV DNA level reductions were 5.2 and 4.7 $\log_{10}$ copies/mL ($p$ <0.0001), percentages of patients with undetectable HBV DNA were 91% and 73% ($p$ <0.0001), histological improvement was observed in 70% and 61% of patients ($p$ = 0.014), respectively.[65] Resistance to entecavir (mutations at positions nt 184, 202 and nt 250) was observed in none of the 432 patients who were not previously treated with lamivudine and in 5.8% of the 172 patients who had previously received lamivudine.[53]

### Emtricitabine

Emtricitabine (FTC) is a cytosine nucleoside analogue with antiviral activity against both HBV and HIV.[46] It differs from lamivudine by a fluorine at the 5-position of the nucleic acid. In a pilot study, 49 patients with HBeAg-positive chronic hepatitis B received five different doses of emtricitabine: 25, 50, 100, 200 or 300 mg daily for 8 weeks. At the end of treatment, serum HBV DNA levels decreased by 2–3 logs in patients receiving the highest doses.

In a randomized, double-blind study, 98 Asian patients (77 HBeAg-positive and 21 HBeAg-negative) were randomized to receive 25, 100 or 200 mg of emtricitabine daily for 48 weeks.[54] At 48 weeks, the median decreases in viral load were 2.6 $\log_{10}$, 3.1 $\log_{10}$ and 2.9 $\log_{10}$ copies/mL, respectively, for the three doses. The proportions of patients with undetectable HBV DNA (<4700 copies per mL) were 38%, 42% and 61%, respectively, for the three doses. HBeAg loss was observed in a high proportion (40%) of the HBeAg-positive patients (ranging from 32% to 50% depending on the dose). Emtricitabine has a comparable efficacy in HBeAg-negative chronic hepatitis B.[55] The results of this study suggest that the optimal dose of emtricitabine is 200 mg once

daily. Genotypic analysis performed at week 48 showed that 12% of patients treated with 100 mg of emtricitabine and 6% of those treated with 200 mg developed drug-resistant HBV.

Phase III clinical trials are under way to determine the long-term safety and efficacy of emtricitabine. However, the role of emtricitabine as a monotherapy may be limited by its structural similarity to lamivudine, with the risk of development of drug resistance.

## Telbivudine

The natural nucleosides in the β-L-configuration (β-L-thymidine [LdT], β-L-2-deoxycytidine [L-dC] and β-L-2-deoxyadenosine [L-dA]) represent a newly discovered class of compounds with potent, selective and specific activity against hepadnavirus.[56] *In vitro* studies have shown that these compounds have marked effects on HBV replication.[46] Telbivudine (LdT) is at the most developed stage of clinical investigation. A phase I study showed a safe preclinical toxicology profile with no mitochondrial toxicity and no mutagenic effect.[57,58]

A phase II study including 104 HBeAg-positive patients compared different therapeutic schedules for 52 weeks: LdT 400 mg daily, LdT 600 mg daily, LdT 400 mg and lamivudine 100 mg daily, LdT 600 mg and lamivudine 100 mg daily or lamivudine 100 mg daily.[59] Median HBV DNA reduction was –6.01, –5.99 and –4.57 in the patients who received telbivudine, the combination of telbivudine and lamivudine, and lamivudine, respectively. Percentages with undetectable HBV DNA by PCR were 61%, 49% and 32%. Percentages of patients with HBeAg loss were 33%, 17% and 28%. YMDD HBV mutants were found in 4.4%, 12.2% and 21.1% of the patients. The safety profile of telbuvidine appeared similar to placebo. Thus, this study confirms the marked antiviral effect of telbivudine with a safe profile. However, there was also a 1-year resistance rate of 4.4%. The combination of telbivudine with lamivudine was not superior to telbivudine alone. On the basis of these data, phase III studies have been initiated.

Another promising β-L-nucleoside compound is val-LdC. It is in phase II testing and preliminary results indicate interesting antiviral activity with a good safety profile. A combination of these two compounds which act at different levels of HBV replication could be of interest.

## Clevudine

Clevudine (L-FMAU;1-[2-fluoro-5methyl-β-L-arabinosyl uracil]) is a pyrimidine analogue with marked *in vitro* activity against HBV but not HIV.[60] The active triphosphate inhibits HBV DNA polymerase but is not an obligate chain terminator. *In vitro* studies suggest that it may also be effective against lamivudine-resistant HBV mutants. In the woodchuck model, a daily dose of 10 mg/kg of clevudine resulted in a 9 $\log_{10}$ decrease in viral load. Interestingly, a delayed reincrease of viral load after cessation of drug administration in a dose-dependent manner was observed.[61] No evidence of drug-related toxicity was observed in treated animals.

An open-label phase I/II, non-randomized, dose escalation study was performed in 35 patients who received clevudine for 28 days at the daily dose of 10 mg (n = 10), 50 mg (n = 10), 100 mg (n = 10) or 200 mg (n = 5) followed by a 20-week post-treatment period.[63] At the end of the dosing period, the median reduction in serum HBV DNA was 2.48 $\log_{10}$, 2.74 $\log_{10}$ and 2.95 $\log_{10}$ in the 10 mg, 50 mg and 100 mg groups, respectively. Interestingly, as in the woodchuck model, there was a slow and delayed reincrease of serum HBV DNA levels with, at the end of the post-treatment follow-up, lower median HBV DNA levels as compared with baseline. Clevudine was well tolerated without serious adverse events. An additional trial with clevudine administered for 12 weeks showed comparable results and confirmed safety.

These preliminary results show that clevudine might be one of the most potent antiviral agents available for the treatment of HBV. Further studies are needed to assess the long-term efficacy and safety of this drug.

## Summary

In recent years, marked progress has been made in the treatment of chronic hepatitis B. The efficacy of lamivudine, the first nucleoside analogue available, is limited by the high incidence of resistance. Adefovir, which was recently approved, has a comparable efficacy with a very low frequency of resistance. However, adefovir needs to be indefinitely administered, as withdrawal of therapy is generally associated with reactivation and sustained response is uncommon.

Preliminary results suggest that PEG IFNs are at least as effective as conventional IFNs. So far, the combination of PEG IFN with lamivudine, used simultaneously, has been disapointing. However, long-term efficacy needs to be assessed and different schedules of combination (for example sequential) need to be evaluated.

A number of nucleoside analogues, with favourable toxicity profiles and a promise of increased effectiveness against HBV, are at various stages of clinical development. Results of phase III trials of entecavir and emtricitabine confirmed their efficacy. However, while entecavir is associated with a low incidence of resistance, emtricitabine is associated with a relatively high incidence of resistance, which limits its use as a monotherapy. The efficacy and safety of new and more potent drugs like telbivudine and clevudine need to be confirmed.

The future of chronic hepatitis B therapy seems to be in the combination of different drugs. Ideally, the optimal drugs to combine would meet the following criteria. They should have different sites of action on HBV DNA replication, a potent antiviral effect, an excellent safety profile and they should induce a sustained response with a limited duration of therapy. Indeed, the concept of combination therapy has been developed recently to increase efficacy and to decrease the occurrence of viral resistance. However, so far few combinations have been evaluated. No combination therapy demonstrated a benefit as compared with monotherapy. More potent drugs and new combinations together with the understanding of the mechanisms of resistance to therapy are important challenges to improve the efficacy of treatment and decrease the global burden related to chronic hepatitis B in the future.

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# Chapter 21
# Management of drug-resistant mutants

Yun-Fan Liaw

## Summary

Lamivudine is a nucleoside analogue with potent inhibitory effects on hepatitis B virus (HBV) replication. Prolonged therapy is required for sustained suppression. However, HBV species with mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) locus of the HBV RNA-dependent DNA polymerase (rt M 204 I/V) conferring resistance to lamivudine may emerge after 9–10 months of therapy with an incidence of 38% and 67% after 2 and 4 years of lamivudine therapy, respectively. During continued lamivudine therapy, patients with rt M 204 I/V HBV usually show serum alanine aminotransferase (ALT) and HBV DNA elevations at lower median levels than their baseline. Marked flare of serum ALT or acute exacerbation may occur as the result of cytotoxic T-lymphocyte (CTL)-mediated immune response directed against the YMDD mutant. Although viral clearance with or without emergence of distinct lamivudine-resistant mutants may occur after such exacerbations, 20% of the exacerbations are complicated with decompensation or even fatality. The exacerbations appear to be more severe than those that occur during the natural course of wild-type HBV chronic infection. The conventional practice of continuing lamivudine therapy therefore seems inappropriate. Studies suggest that there is no benefit in continuing lamivudine therapy in such patients. Limited data show that interferon therapy seems ineffective. Switching to adefovir monotherapy is as effective as adefovir/lamivudine combination therapy, and is safe even in decompensated patients. To develop strategies of enhancing therapeutic response and shortening duration of therapy to avoid drug resistance is more important.

## Introduction

Lamivudine is a cytosine nucleoside analogue with potent inhibitory effects on hepatitis B virus (HBV) polymerase/reverse transcriptase activity.[1] Although it can block new covalently closed circular DNA (cccDNA) synthesis, a residual amount of stable pre-existing cccDNA remains.[2] Therefore, the suppressive effect was usually not sustained after stopping lamivudine therapy[3–6] even after hepatitis B e antigen (HBeAg) had seroconverted to its antibody (anti-HBe) in association with HBV DNA clearance.[6–9] In addition, studies have shown that the HBeAg seroconversion rate in patients treated with lamivudine 100 mg daily for 1 year was <20%.[10,11] These findings suggest that prolonged lamivudine therapy is required for most of the patients. However, HBV with reverse transcriptase mutations conferring drug resistance start to emerge after 6–9 months of lamivudine therapy and create a great challenge for long-term lamivudine therapy. Adefovir dipivoxil (ADV) is another licensed direct antiviral agent with potent suppressive effect on HBV. Resistant mutations (N236T and A181V) may also emerge upon prolonged ADV therapy at a low incidence (<6% up to 3 years) and were responsive to lamivudine. However, its long-term consequences are not clear yet, therefore they will not be covered in this chapter.

## YMDD mutation and its incidence

The lamivudine-resistant HBVs have a characteristic amino acid substitution over the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the RNA-dependent DNA polymerase.[12] According to the new consensus reverse transcriptase domain numbering system,[13] the methionine at position 204 is either replaced by an isoleucine (rt M 204 I) or a valine (rt M 204 V).[14–16] In addition, the rt 204 mutations are frequently accompanied by a leucine-to-methionine substitution at position rt 180 (rt L 180 M).[17,18] While rt M 204 I and rt L 108 M/M 204 V are the two most commonly identified mutants, many other mutants have been reported.[19,20]

Although a mutation in the rt YMDD motif may be detected before therapy,[21] it usually emerges after lamivudine therapy for 6–9 months. One study showed that the genotypic resistance appeared as early as 49 days.[22] The incidence varies from report to report. Based on the results of the largest series, the 1-year incidence was 14% of 285 HBeAg-positive Asian patients,[11] 32% of 44

HBeAg-positive Caucasian patients[10] and 27% of 41 HBeAg-negative Caucasian patients.[23] It was reported that patients with higher serum transaminase, higher HBV DNA or HBV with core promoter mutations select rt YMDD mutations more rapidly.[11,22,24] Data also show that the incidence in Oriental patients is lower than in Caucasians.[10,11] Combined data from four multicentre, controlled phase 3 studies showed that the 1-year incidence was 24% and that sex, baseline body mass index and HBV DNA level were associated with emergence of YMDD mutants.[25] As there is distinct variation in the genotypes of HBV in different geographical regions, it has been questioned whether there is genotype-related incidence of YMDD mutations. One study showed that patients infected with adw subtype HBV had a 20-fold increase in the risk of lamivudine resistance during a mean period of 12 months' therapy as compared with patients with the ayw subtype.[26] A study of longer treatment periods (12–36 months, mean 24 months) showed that the 1-year incidence of YMDD mutation was higher in genotype A (adw) than in genotype D (ady) (43% vs 18%) but the 2-year incidence was similar (53% vs 55%).[27] There was also no significant difference in the cumulative incidence among genotypes A, B and C infection in Japanese patients.[28]

The incidence of rt M 204 I/V increases with increasing duration of lamivudine therapy. In Asian lamivudine studies, it increases from 14% at 1 year to 38%, 55%, 67% and 69%, respectively, at the end of 2, 3, 4 and 5 years.[5,11,29–31] The cumulative rate increases to 56% at 2 years in HBeAg-negative Italian patients,[24] and reaches 91% at 4 years of lamivudine therapy, or 20% per year, in human immunodeficiency virus (HIV)-infected patients with chronic HBV infection.[32] It appears that rt M 204 I/V will ultimately emerge in most, if not all, patients with continuing lamivudine therapy.

## Viral consequences after emergence of YMDD mutations

It has been shown that the replication competency of HBV with rt M 204 I/V is defective.[33] On the other hand, the mutations exert configuration hindrance to the incorporation of lamivudine and therefore confer drug resistance.[34] Upon longer therapy, additional P gene mutations may occur and restore the replication fitness of HBV with rt M 204 I/V.[17,20,24,35] Site-directed mutagenesis experiments have further shown that the defective replication competency of some mutants may be completely restored after addition of lamivudine.[20,36,37] Some P gene mutations may influence or be related to other HBV genes. One study showed reversion of precore stop codon mutation in three HBeAg-negative patients and amino acid substitution in the coding sequence of the overlapping S gene in approximately half of the P gene changes.[24] The functional consequences of such S gene mutations have not been clearly defined and their potential effects are unpredictable but may affect viral envelopment, secretion, antigenicity and infectivity.[38] S gene truncation impairing the secretion of HBsAg has been found in some P gene mutations.[20,36] Of note is that the truncation point in the A529T (rt A 181 T) mutant was located just at the border of the previously defined 'transactivity-on-region'.[20,39] The pre-S2/S mutant with such truncation, as identified in the HBV integrant of a human HCC,[40] is capable of transactivating several promoters including those of oncogenic proteins.[39–41]

## Clinical impact of YMDD mutations during continuing lamivudine therapy

The emergence of rtYMDD mutations is initially silent but is followed by phenotypic resistance or viral breakthrough, after a time lag of 3–4 months.[22] Although the emergence of rtYMDD mutations is followed by viral breakthrough and ALT elevation in >90% of the patients,[25,42,43] it has invariably been reported that patients with YMDD mutations maintain a significantly lower 'median' HBV DNA and ALT level than at baseline.[5,10,11,25,29] However, hepatitis flare with serum ALT over five times the upper limit of normal (ULN) occurred in around 40% of HBeAg-positive patients[22,42] and around 50% of HBeAg-negative patients during continued lamivudine therapy after emergence of rtYMDD mutations (Table 21.1).[22,44–48] These hepatitis flares, usually preceded by a rapid resurgence of HBV DNA,[49] as shown in Fig. 21.1, were considered to be the results of CTL-mediated immune response against rt M 204 I/V.[42] Although such hepatitis flares may be followed by HBeAg seroconversion and/or immune clearance of the mutant virus,[42,43] new and distinct mutant may be selected and elicit another exacerbation and then select another mutant.[20] Whereas patients harbouring HBV with rt M 204 I/V continue to achieve HBeAg seroconversion,[5,29,42,43] the seroconversion rates are much lower than in patients without YMDD mutation.[25] In addition, severe hepatitis with hepatic decompensation or even fatality may occur, particularly in patients with advanced underlying liver disease[42,43,50,51] or in a post-transplantation setting[14,37,52–57] (Table 21.2). Compared with the hepatitis flare or exacerbation occurring in the natural history of untreated chronic HBV infection,[58] hepatitis flares during continued lamivudine therapy in patients with YMDD mutations appear to be more severe in terms of the incidence of hepatic decompensation.[59] The liver damage associated with these hepatitis flares[50] may explain the observation that the hepatitis activity index worsened after initial improvement in patients with rtYMDD mutations.[29,60] One study showed that cirrhotic patients with initial increases in serum albumin dur-

**Table 21.1** Hepatitis flares after emergence of YMDD mutations during continuing lamivudine therapy

| Source | HBeAg | No. | Follow-up (months) | ALT × ULN >5 | ALT × ULN >10 |
|---|---|---|---|---|---|
| Liaw *et al.* 2000[42] | + | 32 | 4–24 | 13 (41%) | 11 (34%) |
| Si Ahmed *et al.* 2000[22] | + | 13 | | 4 (31%)* | |
| | – | 7 | | 3 (43%) | |
| Hadziyannis *et al.* 2000[44] | – | 12 | 3–27 | 6 (50%)* | |
| Santantonio *et al.* 2001[45] | – | 11 | 6 | | 5 (46%) |
| Paganin *et al.* 2001[46] | – | 14 | 9–27 | 5 (36%) | |
| Andreone 2001[47] | – | 14 | 3–31 | 7 (50%) | 3 (21%) |
| Papatheodoridis *et al.* 2002[48] | – | 32 | 12 | 7 (35%)** | |

ULN, upper limit of normal.*4–5 × ULN. ** >8 × ULN. Superscript numbers are reference numbers.

**Table 21.2** Outcomes of post-liver transplantation patients after emergence of YMDD mutations during continuing lamivudine therapy

| Source | No. | YMDD mutation | Onset (months) | Clinical deterioration | HAI ↑ >2 |
|---|---|---|---|---|---|
| Andreone *et al.* 1998[53] | 11 | 3 (27%) | 7–12 | 3 (100%)* | 2/2 |
| Perrillo *et al.* 1999[54] | 52 | 14 (27%) | 8 | 6 (43%) | 3/7 |
| McCaughan *et al.* 1999[55] | 10 | 6 (60%) | 5–20 | 3 (50%)† | |
| Mutimer *et al.* 2000[56] | 10 | 4 (40%) | 9–24 | 2 (50%)† | |
| Ben-Ari *et al.* 2001[57] | 8 | 5 (63%) | 9–20 | 2 (4%)‡ | 4/5 |

HAI, hepatitis activity index. Superscript numbers are reference numbers.
*ALT >10 × ULN. †Hepatic failure, two each died. ‡Hepatic failure, one died.

ing lamivudine therapy developed a highly significant and rapid decline in serum albumin after emergence of rtYMDD mutations.[61,62] It is clear, then, that the possible adverse effects of rtYMDD mutants (Table 21.3) do cause concern and are a challenge, and that the benefits versus risks of prolonged lamivudine therapy must be carefully weighed.

## Better to avoid than to contend with YMDD mutants

Given the problems associated with YMDD mutations, it is better to prevent the occurrence of such a difficult issue. One strategy is not to treat or to postpone the treatment, if the risk is sufficiently low or spontaneous

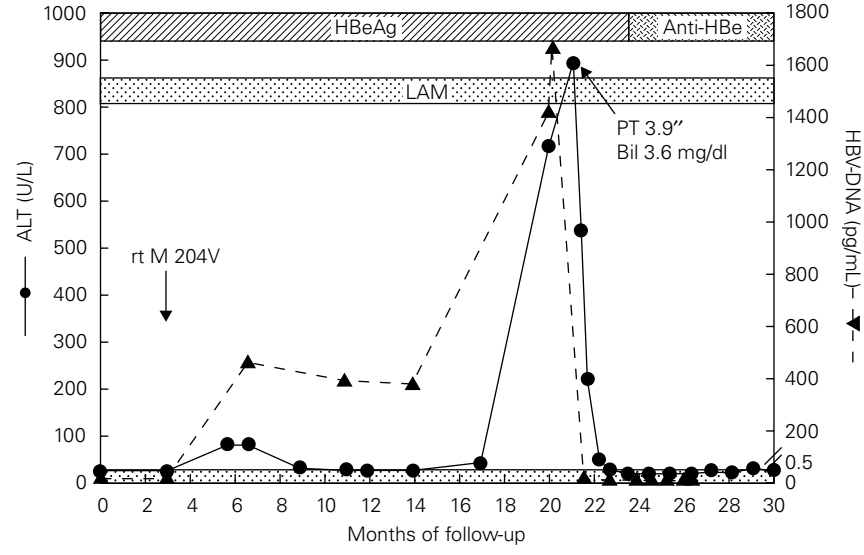


**Figure 21.1** Clinical course of a patient with chronic hepatitis B virus (HBV) infection who developed hepatitis flare after emergence of rt M 204 V during prolonged lamivudine (LAM) 100 mg daily therapy. Note that serum HBV DNA level increased gradually to a high level and was followed by a severe hepatitis flare with high serum alanine aminotransferase (ALT), increased serum bilirubin (Bil) and prothrombin time (PT) prolongation. The hepatitis flare was followed by precipitous decline of serum HBV DNA, subsequent hepatitis B e antigen (HBeAg) seroconversion to its antibody (anti-HBe) and normalization of ALT.

**Table 21.3** Problems of YMDD mutants during continuing lamivudine therapy

| | |
|---|---|
| I Viral | Incidence increases with duration of therapy.<br>Defective replication competency of the mutants restored in the presence of additional compensatory mutations and lamivudine.<br>Viral breakthrough, although 'median' HBV DNA level lower than baseline level. |
| II Clinical | Biochemical breakthrough in >90% of the patients.<br>Hepatitis flares (ALT >5 × ULN) occur in up to 50% of the patients.<br>Severe hepatitis/decompensation may occur, mortality in patients with cirrhosis.<br>Histological benefit diminishes. |

viral clearance is likely to occur. For example, around 70% of hepatitis flares with bridging hepatic necrosis or a serum alpha-fetoprotein level over 100 ng/mL are followed by spontaneous HBeAg/HBV DNA clearance within 3 months.[63] Therefore, a 3-month observation period is acceptable if there is no concern as regards hepatic decompensation.[64,65]

If the patients do need treatment, strategies should be applied to minimize the incidence of YMDD mutation. As was documented with the therapy for HIV infection, combination therapy to completely suppress ongoing replication is required to arrest the emergence of drug resistance. Currently, adefovir dipivoxil (ADV) has been approved and entecavir (ETV), emtricitabine (FTC), clevudine (L-FMAU), telbivudine (LdT) and other new nucleoside analogues, as well as immunotherapeutic approaches, are undergoing appraisal and the preliminary results are encouraging. Combination therapy with some of these agents is a possible strategy to avoid the emergence of YMDD mutants. However, this requires confirmation by controlled studies.

Another strategy is to treat more effectively and thereby shorten the duration of therapy. Studies have shown that patients with high pre-therapy ALT level (>5 × ULN) had a much higher HBeAg seroconversion rate, up to 65% at 1 year[66,67] and 80% at 2 years, [5] suggesting that antiviral agents are more effective in patients who have mounted a stronger endogenous immune response to HBV. With the concern of YMDD mutations, it was recommended to select patients with higher ALT (>5 × ULN) for lamivudine therapy, which should be discontinued as soon as possible after HBeAg seroconversion and/or HBV DNA loss had been documented on two separate occasions at least 1 month apart.[65] Considering the durability of such a response,[6–9] a more recent recommendation is to stop lamivudine therapy after an additional treatment for 6 months after HBeAg seroconversion.[65] This strategy is best demonstrated by a representative case with durable response to a short course of lamivudine therapy (Fig. 21.2).

The strategy for patients with lower ALT level (2–5 × ULN) is to postpone lamivudine treatment until ALT rises over 5 × ULN. Alternatively, to induce ALT flare may be a workable approach, as a pilot study of prednisolone priming has shown an enhanced Th1 response and increased efficacy of a subsequent 9-month period of lamivudine therapy.[68] Enhanced response has also been documented in such patients who received 100 mg of lamivudine daily for 8 weeks followed by combination therapy of 100 mg lamivudine daily with 10 MU IFN-α2b three times weekly for 16 weeks.[69] Studies are required to test whether manipulation of the host immune response against HBV using other immunomodulating agents, such as thymosin $\alpha_1$, before or during lamivu-

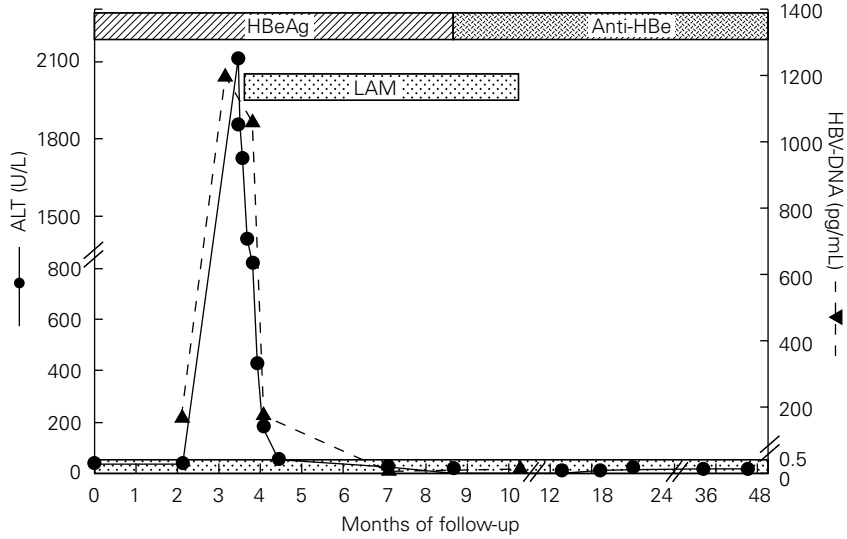


**Figure 21.2** Clinical course of a patient with chronic hepatitis B virus (HBV) infection showing durable response after a short course (6 months) of lamivudine (LAM) therapy. LAM therapy started after serum alanine aminotransferase (ALT) increased over 1500 U/L. Note that serum HBV DNA declined rapidly and was followed by hepatitis B e antigen (HBeAg) seroconversion to its antibody (anti-HBe). The response was sustained for more than 3 years of therapy. No YMDD mutation was detected.

dine therapy, could also enhance efficacy and thereby shorten the duration of therapy.

## Management of patients with YMDD mutations

### Conventional practice: to continue lamivudine therapy

Until effective drug(s) against HBV with rt M 204 I/V are available, patients with YMDD mutations are very difficult to treat. The general recommendation for such patients is to continue lamivudine therapy[64,67] in order to further suppress or to prevent the return of wild-type HBV,[70] which is more replication-competent than HBV with rt M 204 I/V.[33] Another rationale is based on the clinical experience that patients with YMDD mutations maintain lower 'median' serum HBV DNA and ALT levels than their baseline values and continue to achieve HBeAg seroconversion during continuing lamivudine therapy.[5,11,25,29] However, this strategy seems ineffective, and is associated with many problems, as discussed earlier (Table 21.2).

### Discontinue lamivudine therapy

HBV with rt M 204 I/V is not only resistant to lamivudine therapy, experiments have also shown that the defective replication competency of some YMDD mutants was completely restored upon addition of lamivudine.[20,36] This may argue against the practice of continuing lamivudine therapy after emergence of YMDD mutations. Consistent with earlier experimental findings, a recent study on severe recurrent hepatitis B with rt M 204 I/V after liver transplantation also showed enhanced replication of the HBV with rt M 204 I/V *in vitro* in the presence of lamivudine.[37] Similar to the repeatedly reported fatality during continuing lamivudine therapy after emergence of rt M 204 I/V,[43,50–57] two patients in that study died of hepatic failure during continuing lamivudine therapy. In contrast, one patient with hepatic decompensation showed decreasing HBV DNA levels with subsequent clinical improvement and survival after stopping lamivudine therapy. It was therefore concluded that the lamivudine-enhanced replication of HBV with rt M 204 I/V during continuing therapy could be deleterious and that lamivudine therapy should be stopped in the setting of lamivudine-dependent mutants.[37] One Asian study in patients with rt M 204 I/V showed that hepatic flare and decompensation occurred in 67% and 11%, respectively, in patients who continued lamivudine therapy for 12 months as compared with 54% and 7% in those who were followed up for 12 months after stopping lamivudine therapy. In addition, serum HBV DNA increased in 64% and decreased in 36% of the patients who continued lamivudine therapy, but increased in 11% and decreased in 89% of the patients who stopped lamivudine therapy ($p$ <0.001) The study therefore concludes that there is no benefit to continue lamivudine therapy after emergence of rt M 204 I/V.[71] Another Asian study has shown that stopping therapy in this setting is generally safe.[72] All these findings suggest stopping lamivudine therapy even if effective drug(s) for YMDD mutant HBV are not available.

### Switch to drugs able to suppress rt M 204 I/V

Small studies showed that IFN therapy had limited efficacy in treating patients with rt M 204 I/V.[59,73,74] In contrast, studies have shown that ADV and ETV are effective against HBV rt M 204 I/V.[75–78] ADV added to lamivudine in decompensated patients with rt M 204 I/V HBV may suppress HBV DNA level by 2–5 logs and restore their Child Pugh status.[75–77] The clinical course of a representative patient is shown in Fig. 21.3. Switching from lamivudine to ADV monotherapy is as effective as ADV/lamivudine combination therapy in patients with rt M 204 I/V, although hepatitis flare without increase of HBV DNA developed in one-third of the 19 patients after switching to ADV monotherapy.[77] A recent control study in 42 Korean patients with decompensated liver disease[79] and a cohort study in 20 Taiwanese cirrhotic patients with or without decompensation (Liaw *et al*. 2005, unpublished data), however, showed that switching to ADV monotherapy was effective and safe and not associated with significant hepatitis flare. Similarly, switching from lamivudine to ETV monotherapy is safe, has no risk of hepatitis flare and is highly effective.[78] It seems likely that with these drugs, we are able to cope with the problems associated with rt M 204 I/V.

## Conclusions

There is no doubt that chronic HBV infection is difficult to treat, and that even agents with potent suppressive effect on HBV require prolonged therapy to achieve a durable response. Then, drug-resistant mutants will inevitably emerge and create virological and clinical consequences or problems. Although we now have agents able to suppress such mutants, the long-term outcomes are still unknown. To develop strategies for enhancing therapeutic response and shortening the duration of therapy is our ultimate goal to avoid the problems of drug-resistant mutants.[80] For the existing patients suffering from problems with such mutants, it seems more reasonable to switch to a drug that is able to suppress the mutant than to combine with that drug in terms of cost and effectiveness.

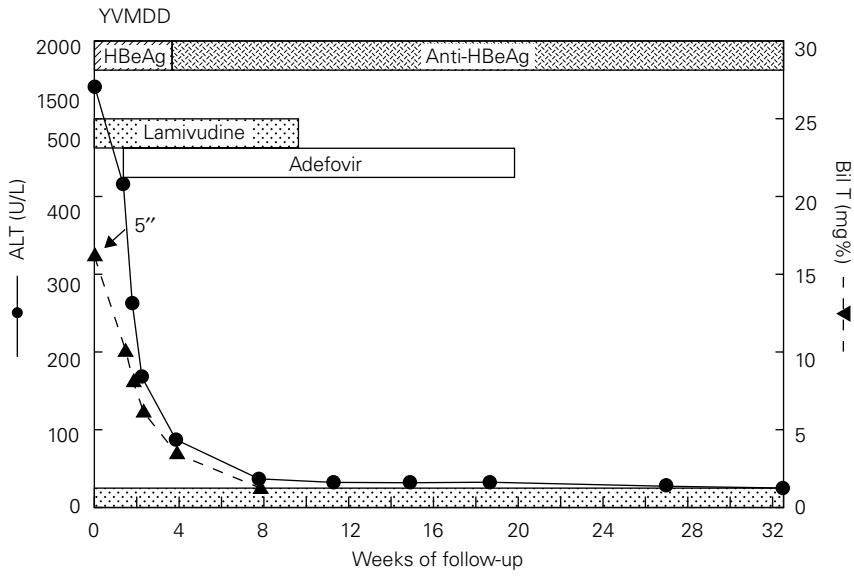


**Figure 21.3** Clinical course of a patient with severe hepatitis flare and decompensation after emergence of YMDD mutants during continued lamivudine (LAM) therapy. After addition of adefovir dipivoxil (ADV), her serum bilirubin (Bil) decreased from 16 mg/dL and alanine aminotransferase (ALT) decreased from 418 U/L to normal levels. Hepatitis B e antigen (HBeAg) seroconversion to its antibody (anti-HBe) also followed. The responses were maintained after stopping LAM therapy and sustained after stopping ADV therapy.

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# Chapter 22
# Liver transplantation in the management of chronic viral hepatitis

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## Introduction

Liver transplantation has revolutionized the care of patients with end-stage liver disease. Indeed, due to significant improvements in immunosuppressive therapy and surgical techniques over the past decades, excellent survival rates and quality of life should now be expected.[1] Among several circumstances that may pose a threat to long-term survival, the greatest is probably the recurrence of the original liver disease.[2–4] This complication may occur for most of the primary causes of liver failure, and more specifically with those of viral origin. While recent advances, predominantly the development of strategies to prevent or effectively treat hepatitis B, have led to substantial improvements in the post-transplantation outcome of hepatitis B candidates,[2] there is a great need for interventions that alter the natural history of recurrent hepatitis C.[3]

This chapter reviews the indications for liver transplantation for viral-related liver diseases, the mechanisms involved in viral reinfection, and the natural history, pathology, methods for prevention and treatment of recurrent viral hepatitis.

## Recurrent hepatitis B virus (HBV) infection

HBV disease, once considered a relative or even absolute contraindication for liver transplantation in many centres, is now considered an excellent indication, accounting for 6–10% of liver transplantations performed annually in the United States and Europe.[1,2] Comparable and even better results than those obtained for other indications can now be obtained, largely due to effective interventions to prevent post-transplantation recurrence – namely high doses of hepatitis B immune globulin (HBIg) in combination with antiviral agents. Historically, rates of HBV recurrence were high (90%) resulting in graft loss in >70% of patients. In fact, the rapid recurrence of hepatitis B with ensuing graft loss was frequent enough for most third party payers to withdraw hepatitis B infection as an acceptable indication for liver transplantation. A number of approaches were developed during the 1990s, and with each intervention, there has been a step-wise reduction in the likelihood of recurrence, down to 5–10% with HBIg plus lamivudine.[2] Currently, the debate has shifted from whether liver transplantation is an option for this patient subgroup to selecting the most cost-effective and applicable strategies to prevent reinfection and treat disease before and after transplantation. Indeed, at present, the main unanswered questions are to define (1) the best long-term prophylactic strategy, with the highest success rate and the lowest cost, and (2) the optimal regimen and timing of pre-transplantation antiviral therapy.

### Indications for liver transplantation

Cirrhosis caused by chronic HBV infection is associated with reduced survival, due to the development of hepatic complications, namely clinical decompensation and hepatocellular carcinoma (HCC). While in the absence of decompensation, the 5-year survival reaches 84%, it drops to 14% once decompensation occurs.[5] The indications and contraindications for transplantation in these patients do not differ from those applied to other forms of liver diseases and typically include liver failure, complications from portal hypertension, or the development of HCC. Criteria for selecting patients with HCC do not differ from those uninfected with co-existent HCC and include a solitary nodule smaller than 5 cm or no more than three nodules, each one smaller than 3 cm in diameter, without macroscopic vascular involvement or extra-hepatic spread.[6]

Studies of the natural history of advanced HBV disease have demonstrated that patients with active viral replication, defined as the presence of detectable HBV DNA using a non-polymerase chain reaction (PCR)-based assay (>$10^5$–$10^6$ copies/mL) or hepatitis B e antigen (HBeAg), are at increased risk of disease progression.[5,7] Suppression of HBV replication in these patients, either spontaneously or with antiviral treatment, measured

by the disappearance of HBV DNA from the serum and seroconversion from HBeAg to anti-Hbe, may result in significant clinical improvement, including improvement in the Child Pugh score of the patient.

## Recurrence of HBV infection

In the absence of preventive measures, most cases of HBV reinfection occur during the first 1–3 years post-transplantation and rarely thereafter.[8] Reinfection is characterized by the reappearance of HBsAg in serum and high levels of HBV DNA.

HBV DNA level before transplantation is the best predictor of the risk of HBV recurrence, with the highest rates reported in HBsAg-positive cirrhotic patients with evidence of active viral replication and the lowest in patients with fulminant hepatitis, HDV co-infection and chronic HBV infection without detectable HBeAg or HBV DNA.[2,8,9] In the absence of effective prophylactic therapies, HBV recurs in 75–90% of patients with a 2-year actuarial risk of 67% in HBV-cirrhotic patients, 32% in cirrhotic patients co-infected with hepatitis D virus (HDV) and 17% in those undergoing transplantation for fulminant hepatitis.[2,8,9] When long-term HBIg was used for HBV prophylaxis, with doses of HBIg used to produce anti-HBs titres higher than 100 UI/L, recurrence was reduced in those without active HBV replication to 17–38% at 2 years but remained at approximately 70–96% for those undergoing transplantation who were HBV DNA-positive (by hybridization methods)[2,8–10] (see below).

## Pathogenesis

Graft reinfection may occur from circulating viral particles in blood immediately post-transplantation or alternatively from virus present in extrahepatic sites, particularly the peripheral blood mononuclear cells (PBMCs). In patients receiving HBIg, circulating Dane particles are presumably neutralized by immune globulins, although the true mechanism by which passive prophylaxis reduces the rate of recurrent HBV infection is unknown. Hypotheses include the binding of antibody to virus in the serum which thereby limits viral entry into the new graft, and the control of horizontal spread of virus within the liver. The specific antibody preparation used for prophylaxis may alter efficacy.[10]

Reinfection in HBIg-treated patients may occur due to one or more of the following causes: (1) viral mutations in the 'a' determinant region; (2) titres of anti-HBs antibody that are too low to provide adequate protection; and/or (3) overproduction of HBV in the extrahepatic sites.[10–15] The 'a' determinant is the region of the surface protein where anti-HBs binds, and mutations in the surface gene encoding this region have been reported in association with monoclonal and with polyclonal HBIg therapy. These mutations, which occur in approximately half of the patients failing HBIg prophylaxis,[12] are associated with detectable HBsAg and HBeAg in serum, increase in HBV DNA levels and disease recurrence, and thus are termed 'escape mutants'. They typically occur after 6 months of treatment. The most common mutation is a substitution of glycine for arginine at amino acid position 145. Discontinuation of HBIg results in reversion of the mutation to the wild-type virus in the majority of patients.[14] In contrast, in the early post-transplant period, failure of HBIg to prevent reinfection is probably due to inadequate post-transplantation anti-HBs titres in the context of high levels of viraemia and/or production of virus from extrahepatic sites. In one study of a patient with HBV reinfection despite anti-HBs Ig prophylaxis, it was shown that the predominant HBV strain after transplantation was the predominant strain present in lymphocytes before transplantation. Interestingly, this strain was different from the predominant strain present in serum and liver, suggesting an immune pressure selection of HBV strain by anti-HBs Ig and demonstrating the role of extrahepatic lymphocytes as a source of HBV reinfection after transplantation.[11]

The mechanisms by which HBV leads to liver injury are incompletely understood. Increased levels of HBV replication are typically observed, probably related to the use of immunosuppressive drugs, particularly corticosteroids. This enhanced replication with excess production of viral proteins in conjunction with the altered host immune responsiveness probably contributes to the pathogenesis of liver damage, particularly to pathogenesis of severe progressive disease of the grafted liver.[16]

## Natural history of HBV recurrence

The natural history of recurrent hepatitis B in the absence of therapeutic intervention is more aggressive than that observed in the immune competent population. Less than 5% of patients developing HBV recurrence maintain a normal graft in the medium to long term.[8,17–19] Typically, patients develop acute hepatitis after detection of HBsAg in serum, with progression to chronic hepatitis and cirrhosis within 2 years of transplantation. One particular entity initially described in these patients is known as fibrosing cholestatic hepatitis, characterized by extensive cytoplasmic HBsAg expression, variable degrees of HBcAg cytoplasmic and nuclear expression.[16] The combined features of high cytoplasm expression of viral antigens and paucity of inflammatory infiltrate suggest a direct cytopathic effect of the virus. The clinical course is rapidly progressive with severe cholestasis, hypoprothrombinaemia, and liver failure within weeks of onset. Patients at risk for this syndrome include those with high levels of viraemia pre-transplantation and

those infected with precore mutants.[20,21] Fortunately, with currently available effective therapies, this syndrome is rarely if ever seen in patients undergoing liver transplantation for HBV disease today.

## Prevention of HBV graft reinfection

### *Hepatitis B immunoglobulins*

In order to prevent reinfection with HBV, lifelong passive immunization with high-dose hepatitis B immunoglobulins is used in most transplant centres and was considered, until very recently, the 'standard of care'.[2,10]

#### *Efficacy*

The administration of HBIg for more than 6 months has been shown to reduce dramatically the rate of HBV recurrence. In a large European multicentre study, the recurrence rate was 75% in patients receiving no or short-term HBIg versus 33% in those receiving long-term HBIg ($p$ <0.001).[9] Long-term administration of HBIg reduced the rate of recurrence in patients with fulminant HBV hepatitis to <10%, in HDV co-infected patients to 10–15%, and in HBV DNA-negative cirrhotic patients to <30%. Yet it did not reduce the rate of recurrence in patients with HBV DNA-positive cirrhosis. A more recent analysis of the long-term results of HBIg administration in 271 patients shows an actuarial rate of HBV recurrence of 26.8%, 34.6%, 40% and 41.6% at 1, 2, 5 and 10 years, respectively.[8] Substantial differences were again observed between patients who were HBV DNA-positive and those who were negative pre-transplantation with 5-year actuarial recurrence rates of 76.7% and 33%, respectively. Interestingly, almost no new cases of recurrence were observed after 5 years.

The adverse prognostic characteristic of active viral replication pre-transplantation may be overcome with more aggressive use of HBIg maintaining titres over 500 IU/L, or by the use of HBIg in combination with antiviral agents.[2,10] With the first alternative, at least during the first 6 months, recurrence in HBV DNA-positive patients may be reduced to approximately 16–35%.[8,22,23] In addition, these aggressive regimens have also been shown to improve outcome in patients re-transplanted for fibrosing cholestatic hepatitis (see above). The second alternative is to use combination prophylactic therapy with HBIg in association with pre- and/or post-transplantation antiviral therapy (see later).

#### *Administration*

Various regimens have been described, with most including the administration of 10 000 IU HBIg intravenously during the anhepatic phase and 10 000 IU HBIg daily for the first week post-transplantation.[2,10] The subsequent dosing is either given on a fixed schedule (generally on a monthly basis)[23] or based on anti-HBs titres (re-administration when antiHBs titre is <100–500 IU/L).[8,22] Individualized immunoprophylaxis based on the administration of variable amounts of HBIg is possibly the best approach. Indeed, there is a great variability in the pharmacokinetic profile of immunoglobulins between patients. In addition, variations are also common in the same individual based on the time since transplantation. The individualized approach is aimed at achieving pre-defined anti-HBs level in the serum known to minimize the risk of recurrence while at the same time reducing the number of infusions and, subsequently, the cost. Based on previous studies, HBIg should be given so as to obtain anti-HBs titres >500 IU/L during the first week after transplantation, >250 IU/L between days 8 and 90 and >100 IU/L thereafter.[2,10]

#### *Safety profile*

HBIg has a very satisfactory safety record with adverse events being typically minor. Mercury poisoning has been reported anecdotally in patients receiving an intramuscular form of the intravenous HBIg.[2,10]

#### *Disadvantages*

Despite the clear efficacy of prophylactic HBIg as a single agent, this therapy has limitations, including the high cost, limited availability, selection of pre-S/S-resistant mutants,[12–15] and overall failure in 20–30% of treated recipients.

A specific limitation is the need for lifelong therapy, given the risk for reinfection associated with discontinuation of this product. Recurrent infection has been documented in patients stopping prophylaxis with HBIg after 1 year. Furthermore, HBV DNA has been detected by highly sensitive molecular techniques in the serum, liver and PBMCs of 50% of HBsAg-negative patients on HBIg ± lamivudine prophylaxis,[23–26] although it is unclear whether these viral sequences represent infectious viral particles capable of reinfecting the graft. Nevertheless, detection of HBV DNA suggests that indefinite treatment is required.

### *Alternatives to HBIg mono-prophylaxis*

In order to overcome the limitations of HBIg, several alternatives are currently being evaluated (Table 22.1)

#### *Pre-transplantation antiviral therapy*

The first alternative is the use of antiviral treatment prior to transplantation to inhibit viral replication.[5,27–36]

**Table 22.1** Strategies in patients undergoing liver transplantation for HBV-related liver disease

| Stage | Goals of therapy | Available drugs | Unsolved questions |
|---|---|---|---|
| Pre-transplantation therapy | Inhibition of viral replication:<br>Inclusion in the waiting list<br>Reduction of the risk of recurrence<br>Stabilization of liver function | Interferon (?)<br>Lamivudine<br>Adefovir | Time of initiation of lamivudine<br>Lamivudine vs adefovir vs combination therapy |
| Post-transplantation pre-emptive therapy | Prevention of recurrence | HBIg<br>Adefovir (?)<br>HBIg + lamivudine<br>HBIg + adefovir | Long-term prophylaxis<br>Lamivudine vs adefovir |
| Treatment of established disease | Histological improvement<br>Inhibition of viral replication | Lamivudine<br>Adefovir<br>Entecavir | Combination therapy vs monotherapy |

HBIg, hepatitis B immune globulins.

Interferon (IFN) is not recommended in patients with decompensated HBV cirrhosis, due to the low tolerability, the risk of triggering life-threatening complications such as bacterial infections and/or the risk of precipitating flares of underlying liver disease leading to worsening liver failure and even death. In contrast, nucleoside analogues are very well tolerated, are orally administered, and have a potent antiviral effect, inducing rapid suppression of HBV DNA in serum. Recent experience with these drugs in cirrhotic patients awaiting liver transplantation is encouraging.[28–36] Most of the experience published to date is with lamivudine, with data on >500 cirrhotic patients treated with this drug while on the waiting list for liver transplantation. With lamivudine, HBV replication is suppressed below the level of detection of standard assays within 2–3 months of initiating therapy, allowing liver transplantation to be performed in conditions of low risk of HBV recurrence. In most published studies, pre-transplantation lamivudine results in loss of HBV DNA by molecular hybridization in 90% (range 62.5–100%) of patients with detectable serum HBV DNA prior to beginning nucleoside therapy, including those infected with the e-minus strain.[28–36] However, viraemia will recur in >80% of patients following treatment discontinuation, and thus treatment should be administered indefinitely in these patients with advanced HBV cirrhosis. Unfortunately, prolonged therapy is associated with development of mutations in the YMDD motif of the HBV DNA polymerase gene. Moreover, development of resistance has been associated with progression of liver disease, and even fatal flares of HBV disease.[28–36]

Suppression of HBV replication has resulted in improvement in hepatic synthetic function as well as in Child Pugh score, in some cases delaying or even obviating the need for liver transplantation.[28–34,36] In these cases, clinical and biochemical improvement, defined by normalization of serum aminotransferases, decrease in serum bilirubin, increase in albumin levels and a >2–3 point improvement in the Child Pugh scores occur gradually, typically after 6–9 months of therapy.[35,37] Unfortunately, not all decompensated cirrhotic patients with active viral replication who are treated with lamivudine will benefit from this drug. Indeed, progression of the disease and even death may occur, particularly in the subset of patients with severely advanced liver failure with increased serum bilirubin and creatinine levels and elevated Child Pugh and model for end-stage liver disease (MELD) scores.[37,38] As deaths tend to occur early after the initiation of therapy, generally within the first 6 months, patients with the above characteristics, who most likely have presented late in their disease course, should be prioritized for urgent liver transplantation, irrespective of the antiviral response to lamivudine.

The major downside of lamivudine is the emergence of 'drug resistance' with HBV DNA reappearance, and generally, modest increase in serum aminotransferase levels. This complication, which is strongly linked to the duration of therapy, develops in 0–27% of patients, with the risk increasing substantially after 6 months of treatment.[28–38] Although the clinical course after the development of drug resistance is often benign,[39] fatal flares of hepatitis and rapidly worsening liver failure have been reported.[40–42] In addition, development of resistance while on the waiting list may lead to reluctance to transplant an HBV DNA-positive patient known to have lamivudine-associated HBV mutants.[43–45] Indeed, it has been recently shown that the risk of postoperative HBV recurrence may be relatively high in these patients, despite the use of high doses of HBIg in combination with lamivudine in the post-transplantation period.[44,45] Hence, for the last few years, the dilemma has been to decide the optimal timing of initiation of lamivudine in potential liver transplant candidates. Against the concept of delaying lamivudine therapy has been the observation that some patients with decompensated cirrhosis have improvement in the hepatic synthetic function with treatment-related viral suppression. In contrast,

against the concept of early antiviral therapy is the fear of emergence of lamivudine resistance with prolonged therapy and the potential for worsening liver disease and increased risk of HBV recurrence. One approach to minimizing the risk of progressive liver disease resulting from viral resistance has been to give other oral agents that carry a lower risk of resistance.

Adefovir dipivoxil, a prodrug of adefovir, is a phosphonate nucleotide analogue of adenosine monophosphate, which appears to be, at least in part, the solution to this problem. The major advantage of this recently approved oral antiviral agent is the potential to treat effectively HBV infection in patients who have previously developed lamivudine resistance. This drug has demonstrated activity *in vitro* and *in vivo* against HBV strains resistant to lamivudine. Recent data from 128 patients with decompensated cirrhosis treated with adefovir at doses of 10 mg daily demonstrated that this drug is safe and effective in treating lamivudine-associated breakthrough, significantly suppressing serum HBV DNA levels. As with lamivudine, normalization of ALT is achieved in most treated patients (approximately 60%), with improvement in serum bilirubin, albumin and prothrombin time.[46] The one concern with this drug is the potential for renal toxicity, which may be of particular importance in patients with hepatic decompensation. The dose of adefovir should be adjusted based on creatinine clearance and careful monitoring of renal function is warranted. As elevations of serum creatinine are mostly observed in patients with pre-existing renal insufficiency, dose reductions of adefovir have been recommended for patients with creatinine clearances of <50 mL/min.

In summary, adefovir dipivoxil appears to be an excellent rescue therapy for patients with advanced decompensated HBV-related cirrhosis who develop lamivudine resistence while awaiting liver transplantation. While polymerase gene mutations associated with long-term adefovir have been recently reported, their incidence appears to be extremely low (2% after 2 years of continuous use), and other nucleoside analogues that are in various phases of development offer hope as rescue therapy for viral resistance.[47] The observation that lamivudine-resistant variants are sensisitive to adefovir dipivoxil, and conversely that adefovir-resistant variants are sensitive to lamivudine provides scientific rationale for combination therapy with both of these agents in treating patients with advanced liver disease awaiting transplantation.

Several questions remain unanswered at present. For instance, the best post-transplantation prophylaxis in patients with lamivudine-resistant mutants is not known. Theoretically, a triple therapy with lamivudine, adefovir dipivoxil and high dose HBIg may be the best approach, but data are lacking. In addition, it is unclear whether there is a need to continue with lamivudine once adefovir is started. Finally, what is the appropriate management for patients with improved hepatic function in response to antiviral therapy while awaiting transplantation? Should these patients be removed from the waiting list? Should an HCC surveillance programme be adapted to these patients? Is the risk of HCC development the same as it is for HBV carriers or is it higher? Will the risk of adefovir resistance increase significantly with prolonged therapy? And finally, will combination therapy with two or more oral agents (such as lamivudine plus adefovir) reduce the rate of emergence of resistant HBV variants, which poses a risk of hepatic decompensation for these patients with advanced liver disease?

### *Post-transplantation antiviral therapy*

Once liver transplantation has been performed, there are several alternatives to long-term HBIg. The first is to continue therapy with lamivudine as a single agent that was begun prior to transplantation. Although this approach is initially effective and patient compliance is good given the few side-effects of this drug,[25,34,48,49] therapy is limited by the emergence of HBV mutants with prolonged treatment.[25,34,48,49] The rate of recurrence due to the emergence of escape mutations, mainly in patients with a high level of viral replication before starting lamivudine, may reach as much as 40% and 60% at 1 and 3 years, respectively. It is presently unknown whether these results may be improved when combining lamivudine with other oral antiviral agents, such as adefovir.

### *Combination therapy*

As treatment failures occur with both HBIg and with lamivudine given as single agents, the second alternative is the use of the two in combination. The advantages over a single agent are the following: (1) possibility of administering lower doses of HBIg (400–2000 IU), which results in significant cost reduction; (2) potential reduction of development of resistant mutants, which is a frequent event when either drug is given as a single agent; and (3) synergistic effect with lower rates of treatment failures. The potential mechanisms for the observed synergistic protection of these two agents against graft reinfection are not completely known but include (1) reduction of lamivudine resistance due to reduction of circulating virions by HBIg, and (2) reduction of surface gene mutations due to the decrease in viral load induced by lamivudine. In a preliminary report, lamivudine in combination with high doses of HBIg was shown to be safe and highly effective.[50] In this study, 13 patients were treated prophylactically with lamivudine (150 mg daily) and high-dose HBIg starting with 10 000 IU during the anhepatic phase. No treatment failures were observed

after 1 year of treatment and 1-year patient survival reached 92%.[50] Combination prophylaxis with low doses of intramuscular HBIg and lamivudine has also been shown to be highly effective (Table 2.2) with recurrence rates of <10% in most studies.[24,34,44,51–55] Higher rates of recurrence are typically found in patients who have developed lamivudine resistance prior to transplantation.[44] This strategy is becoming the standard of care in most transplant programmes. However, the best protocol is still unknown as doses, routes, type and lengths of administration vary substantially from centre to centre. Moreover, the amount of HBIg infused ranges from 2800 to 80 000 IU during the first week, and from 7600 to 200 000 IU during the first year. Some centres continue to use the intravenous (i.v.) route while others switch to the intramuscular (i.m.) injection. Fixed i.m. or i.v. HBIg doses are utilized in some centres, while others prefer to individualize this therapy to achieve predefined serum anti-HBs concentrations. Finally, some centres continue HBIg indefinitely, while others stop HBIg at different time intervals from transplantation. Future studies are needed to assess these aspects, in particular the benefits of fixed versus individualized therapy and the need for indefinite versus limited durations of HBIg therapy.

### *Long-term prophylactic therapy*

At present, the main unanswered question relates to long-term prophylaxis. In patients receiving combination therapy, there is a need to define the optimal duration of HBIg. Two different approaches have been investigated. The first is *active HBsAg vaccination* with the aim of inducing active immunity against HBV without the need for additional antiviral treatment. Results from preliminary studies are disparate,[56–59] with good results being achieved in some studies, and poor response to HBV vaccination observed in others. Reasons for these apparently discrepant results likely include differences in study populations, differences in methodologies and differences in definitions of seroprotection. In the first study by Sanchez-Fueyo *et al*.,[56] 17 patients at low risk for HBV recurrence (non-replicative before transplantation, HBIg for at least 18 months without evidence of allograft HBV infection, and on low immunosuppressive doses) were vaccinated against HBV at a median of 30 months post-transplantation. The maintenance dose of HBIg prior to vaccination was approximately 2000 IU given i.m. every 1–2 months so as to maintain anti-HBs titres >100 IU/L. Then 1–4 weeks after the last dose of HBIg, three i.m. doses of 40 μg of recombinant HBsAg were given at 0, 1 and 6 months. Seroconversion to anti-HBs (antiHBs titres >10 IU/L) occurred in 82% of the patients (six after the first three doses, eight after a second course of three additional doses). A recently updated report showed that among 22 vaccinated transplant recipients, the percentage of seroconversion decreased to 64%.[57] In contrast to this initial study, a second study by Angelico *et al*. did not confirm these findings and the rate of seroprotection (defined as anti-HBs titres >10 IU/L) was only 23%.[58] The main differences between these two studies include the differences in the study population (all patients in the Italian study underwent transplantation for cirrhosis) and the use of lamivudine following HBIg discontinuation (in the Italian study). More recently, a third group has reported the results of HBV vaccination using a more immunogenic vaccine.[59] In that study, 10 patients received repeated doses of recombinant HBV vaccine (20 μg) in combination with a new adjuvant, monophosphoryl lipid A. Vaccination was performed at least 2 days prior to HBIg administration and titres of anti-HBs concentrations were determined before HBIg injections. HBIg was then discontinued whenever levels were >500 IU/L. Five of the 10 patients developed anti-HBs levels ranging from 721 to 45 800 IU/L, thus allowing HBIg withdrawal. After 1 year of follow-up, all responders had serum anti-HBs concentrations >900 IU/L. Larger

**Table 22.2** Combined prophylaxis with hepatitis B immune globulins at low doses and lamivudine in the prevention of recurrent hepatitis B

| Author, year (ref. no.) | Patient no. | HBV DNA-positive pre-LT | Pre-LT lamivudine (%) | Follow-up (months) | HBV recurrence (%) |
|---|---|---|---|---|---|
| Yao *et al*. 1999 (51) | 10 | 2 | 100 | 16 | 10 |
| Yoshida *et al*. 1999 (52) | 6 | 4 | 100 | 18 | 0 |
| McGaughan *et al*. 1999 (55) | 9 | 8 | NA | | |
| Han *et al*. 2001 (54) | 59 | NA | 41 | 15 | 0 |
| Angus *et al*. 2000 (53) | 32 | 16 | 100 | 18 | 3 |
| Rosenau *et al*. 2001 (44) | 21 | 13 | 90 | 20 | 11* |
| Marzano *et al*. 2001 (24) | 25 | 25 | 100 | 30 | 4 |
| Seehofer *et al*. 2001 (45) | 17 | 17 | 100 | 25 | 18* |

LT, liver transplantation; NA, not available.

*The patients with recurrent hepatitis B despite prophylaxis had lamivudine resistance prior to transplantation.

trials using different vaccination protocols over a longer observation period are needed to determine the real efficacy of this approach, particularly in low-risk patients without active viral replication pre-transplantation. Once established in this group, the applicability of this approach will have to be tested in patients at high risk for treatment failure. Other issues will need to be addressed, including the optimal vaccine doses, the timing of booster injections, as well as the potential benefit of using more immunogenic vaccines. Finally, the scientific basis for this approach may be questioned, as these patients with previous chronic HBV cirrhosis have already been exposed to high levels of naturally occurring HBsAg and have failed to develop protective immunity to HBV. It is therefore unclear why vaccination of these immunocompromised individuals would be likely to now result in immune protection.

A less risky alternative may be to *substitute HBIg with lamivudine in the long term*. Successful results using this alternative have already been reported by some authors in low-risk patients without markers of viral replication prior to transplantation.[25,60,61] More data are needed to define the optimal timing for HBIg withdrawal and the potential applicability in high-risk individuals.

In summary, an individualized multi-step prophylactic regimen may be used to prevent HBV recurrence. In patients at low-risk for recurrence, i.e. those undergoing transplantation for HBV HDV cirrhosis, fulminant hepatitis B and HBV cirrhosis with undetectable HBV DNA by PCR assays, there may be no need for pre-transplantation antiviral therapy. In contrast, for those with detectable HBV DNA prior to transplantation, suppression with oral antiviral appears to be appropriate. Following transplantation, three alternatives may be proposed: (1) indefinite high-dose HBIg starting with i.v. HBIg 10 000 UI in the anhepatic phase and daily during the first week, followed by the administration of variable amounts of HBIg aimed at achieving anti-HBs titres >100–150 UI/L; (2) indefinite low-dose HBIg in association with lamivudine (100 mg/day) using the same dose of HBIg during the first week followed by i.m. monthly administration of low-dose HBIg (2000–4000 UI/L); (3) high-dose HBIg in association with lamivudine (100 mg/day) during the first 6–12 months, followed by lamivudine alone for up to 2–3 years, and subsequently active HBV vaccination. In those who develop protective immunity, lamivudine may be stopped, and booster doses may be administered to maintain anti-HBs levels (at least, >100 UI/L). If there is no response to vaccine, the prophylaxis should continue with lamivudine. In high-risk patients, i.e. those with detectable HBV DNA by hybridization assay and/or HBeAg, pre-transplantation antiviral therapy with lamivudine is required for at least 1 month. Following transplantation, two alternatives may be proposed: (1) indefinite high-dose HBIg in association with lamivudine (100 mg/day) starting with i.v. HBIg 10 000 UI in the anhepatic phase and daily during the first week, followed by the administration of variable amounts of HBIg aimed at achieving anti-HBs titres >500 UI/L during the first 3 months, and 100–150 IU/L subsequently; (2) high-dose HBIg in association with lamivudine (100 mg/day) during the first 12–24 months, followed by lamivudine alone for up to 2–3 years, and subsequently active HBV vaccination. HBIg discontinuation should only be proposed for patients with undetectable serum HBV DNA by PCR assay.

## Treatment of HBV disease of the graft

There are three categories of patients who are potential candidates for HBV therapy after liver transplantation: (1) those who have undergone liver transplantation in the pre-HBIg and/or lamivudine era; (2) those who have undergone liver transplantation in the post-HBIg/lamivudine era and who have broken through treatment; (3) those with apparent '*de novo*' acquisition of HBV. Selection of therapy likely depends on the category to which the patient belongs. For example, treatment options for patients in the first category include lamivudine, entecavir or adefovir. These patients may have developed 'vaccine escape mutants', but they are likely to be sensitive to all three antiviral agents. The proportion of patients falling into the second category today is small, yet if these patients have 'broken through' both HBIg and lamivudine, they are likely to have developed resistance and as such may be appropriate candidates for antiviral agents such as adefovir or entecavir that have activity against resistance variants. Finally, patients that fall into the third category with '*de novo*' infection, are likely to be infected with virus that is sensitive to all currently available agents.[27,62]

Lamivudine is the most widely used nucleoside analogue. In most studies, liver transplant recipients with documented HBV recurrence (elevated serum ALT levels, and detectable HBsAg and HBV DNA) have been treated with lamivudine 100 mg daily (adjusted for renal function) with good tolerance and rapid loss of HBV DNA in serum.[27,62–66] Good biochemical and virological responses have been achieved not only in patients with chronic hepatitis B following transplantation but also in the setting of acute hepatitis B of the graft and in the most severe cases of fibrosing cholestatic hepatitis. Histological improvements in the inflammatory grade are also achieved with therapy. In a multicentre study based on 52 patients with detectable DNA after liver transplantation, lamivudine therapy for 1 year resulted in 60% loss of HBV DNA in serum and 31% 'e' seroconversion.[63] Other studies have confirmed these results, showing HBV DNA loss in 68–100% of patients treated for periods of 12–36 months.[27,64–66] The downside of this agent is

the need for continuous treatment, as relapse is the rule once the drug is discontinued. Prolonged therapy is, in turn, associated with the development of breakthrough due to the emergence of HBV escape mutants, which has been shown to reach 50% with long-term therapy.[62–68]

Famciclovir (500 mg three times daily with dosing adjusted to renal function) has also been used to treat liver transplant recipients with HBV recurrence.[64,67] Although initially good results were obtained with this drug both in terms of HBV DNA reduction and transaminase normalization, viral clearance was observed in a lower percentage of patients than has been seen for lamivudine. In addition, famciclovir-resistant virus may not be sensitive to lamivudine, a situation recently described in several patients.[42]

### *Emergence of nucleoside analogue resistance*

Monotherapy with both lamivudine and famciclovir has resulted in the emergence of HBV variants that are resistant to these compounds. This resistance generally occurs after prolonged therapy (>6 months).[27,62–68] In both instances, the appearance of resistant mutants is associated with a rise in serum HBV DNA and ALT levels, indicating a breakthrough in therapy. Molecular analysis has shown mutations in the gene encoding the viral DNA polymerase. Because of the overlapping nature of the HBV open reading frames (ORFs), nucleotide changes in the polymerase may result in amino acid changes not only in the polymerase protein but also in the surface protein, which could in turn theoretically alter binding of HBIg at the time of transplantation.[42]

When the antiviral drugs are stopped, the wild-type variant re-emerges as the dominant viral population, but retreatment is again associated with the development of resistant mutants at an accelerated rate. Drug-resistant mutants are not consistently associated with hepatic disease progression. *In vitro* studies have shown that the M 204 V/I mutants have decreased replication fitness compared with wild-type HBV.[39] However, in some cases, severe and fatal hepatitis B infection can occur during lamivudine therapy and may be associated with certain HBV mutants selected during sequential nucleoside and HBIg treatment.[42] It has been suggested that these changes may represent compensatory mutations that restore the replication fitness of HBV. The lamivudine-enhanced replication shown by these mutants suggests that continuation of therapy with lamivudine could be deleterious in some patients. From a clinical point of view, patients who are maintained on lamivudine therapy after the emergence of lamivudine resistance should be closely monitored to detect flares of the underlying disease. Fortunately, the availability of new hepatitis B antivirals such as adefovir has resulted in viral suppression of lamivudine-resistant variants.[46] Thus far, resistance to adefovir is unusual (<2% after 2 years). It is not yet known whether patients who are treated for lamivudine resistance with adefovir post-transplantation need to continue on lamivudine, but given the potential for progressive liver disease with overt recurrence, it seems prudent to continue both drugs for some period after the institution of adefovir.

For patients who have become HBsAg-positive after liver transplantation despite therapy in the pre- and/or peri-transplantation period, possible interventions include adefovir or entecavir. Experience with adefovir (at a dose of 10 mg per day) has shown that this drug is viral suppressive but that treatment may be limited by toxicities, possibly associated with impaired renal function.[46] Experience with entecavir is limited. As this nucleoside is devoid of renal toxicity, it may play an important role in the future management of these recipients.

## Prevention and treatment of *de novo* HBV infection

The prevalence of *de novo* HBV hepatitis ranges from 2% to 8%, and is generally related to transmission from an HBsAg-negative, anti-HBc-positive donor to an uninfected recipient. The most significant factor associated with transmission is the serologic status of the recipient, so that the risk is almost null in patients who are anti-HBs-positive, minor (≅ 10%) in those who are anti-HBs-negative but anti-HBc-positive, and high (≅ 50–70%) in those without markers of previous exposure to HBV.[69,70] Although there have been reports of severe progression, the natural history of *de novo* hepatitis B is generally more benign than that described for recurrent hepatitis B.

In order to avoid *de novo* HBV infection, two complementary approaches may be undertaken. (1) HBV vaccination prior to liver transplantation of all anti-HBs-negative candidates. Accelerated vaccination regimen with double doses (40 μg) has been adopted at 0, 1 and 2 months with a follow-up vaccine at 6 months. Unfortunately, as with other immunosuppressed populations, the results of vaccination in these patients have been disappointing with response rates that barely reach 40%.[71] A second course of vaccination may slightly increase these results. (2) Anti-HBc determination of the donor with use of organs from anti-HBc-positive donors only in recipients already infected with HBV. In order to obtain a maximum benefit from these organs while at the same time reducing the risk of HBV transmission, these organs may be used in special circumstances in recipients not infected with HBV. The following criteria should then be applied.[72] As the risk of HBV transmission is low if the recipient is anti-HBs- and anti-HBcore-positive, selection of an anti-HBs-positive recipient may be sufficient without other interventions. However, there is concern

that vaccine-induced immunity may not be as effective as innate immunity in preventing acquisition of HBV from an infected donor. Livers from anti-HBc-positive donors can be directed next to recipients with isolated anti-HBc, although a low risk of HBV transmission appears to exist if no specific HBV prophylactic measures are taken. While initiating prophylaxis with HBIg and/or lamivudine to all patients will prevent transmission, this strategy has the potential for treating a high proportion of recipients who would have never developed infection. When no individuals with the above criteria exist in the waiting list, anti-HBc-positive donors can be offered to naïve recipients with critical clinical needs or with HCC although, in such cases, HBV prophylaxis with either lamivudine or HBIg or a combination of both agents is advisable given the high likelihood of HBV acquisition.

Treatment of *de novo* hepatitis B is similar to that described for recurrent hepatitis B. If started at early time-points, the results are generally better than those obtained with recurrent hepatitis B. Unfortunately, resistant mutants develop at the same rate, and adefovir or entecavir may prove to be preferred therapy for the primary management of this disease.

### Re-transplantation

The initial results on re-transplantation for patients with graft failure due to recurrent hepatitis B were discouraging due to high rates of HBV reinfection and even more aggressive disease in the second graft. Improved outcomes have been achieved with specific interventions, mainly with the use of aggressive immunoprophylaxis to prevent HBV reinfection in combination with lamivudine that is typically started prior to re-transplantation in order to suppress viral replication.[73–75] Fortunately, re-transplantation for recurrent HBV disease is rare, given the effective measures described above that prevent recurrent infection in the vast majority of those with pre-transplantation infection. If it is needed, three measures should be followed in order to improve the outcome: (1) avoid late re-transplantations when the hepatic failure is too advanced and renal insufficiency has developed; (2) use antiviral therapy to clear the virus prior to retransplantation; and (3) choose an aggressive prophylactic regimen to prevent reinfection.

### Living-related liver transplantation (LRLT)

There are no data regarding HBV recurrence following LRLT. Given the availability of excellent therapies to prevent and treat recurrence, it is likely that the results obtained with this technique will be similar to those obtained with cadaveric organs. In addition, adequate timing of pre-transplantation antiviral therapy is a potential advantage, as transplantation can be performed when HBV DNA has been cleared in serum but before the development of resistant mutants.[76]

### Transplantation in patients co-infected with HDV

Historically, patients with HBV and HDV infection were considered to be better candidates for liver transplantation than those with HBV infection alone, because of the lower HBV DNA levels and consequent lower risk of recurrent infection in the former than in the latter group.[9] Delta infection is an uncommon indication for liver transplantation. In the absence of HBIg, both HBV and delta virus can infect the graft, but HDV is not pathogenic until HBV replication also occurs. Interestingly though, HDV is detected early post-transplantation either in serum (HDV RNA) or liver (HDV RNA and/or delta antigen) in 80% of HBsAg-negative liver transplant recipients.[77,78] With prolonged follow-up and absence of HBV recurrence, these markers of HDV infection disappear. If HBV recurs, HDV is also detected leading to chronic hepatitis. HDV-HBV-related hepatitis is generally less severe than in patients with HBV alone. HBIg prophylaxis is effective at preventing recurrence in the majority of those with pre-transplantation co-infection.[77,78] The additional benefit of pre-transplantation treatment with lamivudine is largely unstudied but is commonly administered in clinical practice. Thus, current recommendations would be to pre-empt HBV and HDV infection of the graft as is undertaken for the prevention of recurrent HBV infection described above.

## Recurrent hepatitis C virus (HCV) infection

Once considered an excellent indication for liver transplantation, enthusiasm for liver transplantation in patients with end-stage HCV cirrhosis is waning, due to the growing evidence that recurrent HCV disease is an important cause of morbidity and even mortality in the post-transplantation period.[79] In contrast to HBV disease, there are no effective interventions to prevent post-transplantation HCV recurrence.[80]

HCV-related liver disease with or without alcohol and with or without HCC now represents the major indication for liver transplantation in the United States and Europe.[1,79] Historically, rates of HCV recurrence were modest (70%) and typically resulted in benign liver injury with no effect on survival. In contrast, more recent natural history studies based on strict histological follow-up, updated virological techniques and prolonged follow-up, have demonstrated that viral recurrence is universal, histological recurrence is very frequent, and advanced graft HCV disease occurs in a substantial

number of patients, leading to an adverse effect on graft and patient survival.[79,81,82] Due to the magnitude of the problem, with a preponderance of HCV as the primary indication for liver transplantation, the worsening patient and graft survival in HCV recipients,[81,83,84] and the growing demand for re-transplantation in recipients with recurrent HCV,[85] a Consensus Conference was recently held to overview the state of the art concerning liver transplantation and HCV disease. The conference participants examined the definition of recurrent HCV, the natural history of HCV after liver transplantation, the potential clinical predictors of adverse outcomes, treatment and management strategies, the contribution of different immunosuppressive regimens to outcome, and the role of re-transplantation for recurrent HCV in the face of an overall donor shortage.[86]

## Indications for liver transplantation

Cirrhosis due to chronic hepatitis C infection is associated with reduced survival, due to the development of hepatic complications, namely clinical decompensation and HCC. In the absence of decompensation, the 10-year survival approximates 80%, but survival decreases markedly once hepatic decompensation occurs (approximately 50% at 5 years).[87–89] The cumulative risk of clinical complications is 30% over a 10-year period. The risk of clinical decompensation unrelated to HCC has been estimated to be 15–20% after 4 years of follow-up, while the risk of developing HCC is 6–24% over the same time. Given the potential for an aggressive form of recurrent hepatitis C, timing of liver transplantation is a major challenge in transplant centres. Liver transplantation should only be offered when the risk-benefit balance clearly favours this option and not merely because the patient meets minimal listing criteria for transplantation. Thus, as the outcome of compensated HCV-cirrhotic patients is sufficiently good, liver transplantation should be reserved ideally for those with progressive liver disease that cannot be managed medically. Thus, while indications and contraindications for transplantation in these patients do not differ from those applied to other forms of liver diseases, decompensated patients should be aggressively managed with traditional medical therapy in an effort to avoid the premature transplantation of some of these patients. This is also true in the new era of living donor liver transplantation.

Criteria for selecting patients with HCC do not differ from those with HCC who are not HCV-infected.[89] However, because recurrent HCV will likely complicate the post-transplantation management of these patients, aggressive medical and surgical management of HCC including chemoembolization and hepatic resection should be undertaken before consideration of liver transplantation. Thus, resection may be considered the treatment of choice, particularly in those with compensated cirrhosis without relevant portal hypertension, with liver transplantation remaining as 'adjuvant' therapy.[89,90] The addition of antiviral therapy in these patients could potentially lead to viral eradication and improvement of hepatic fibrosis,[91] eliminating the future need for transplantation.

## Recurrence of HCV infection

While recurrence of HCV infection is based on virological parameters, the recurrence of HCV disease requires protocol and/or clinically indicated liver biopsies that

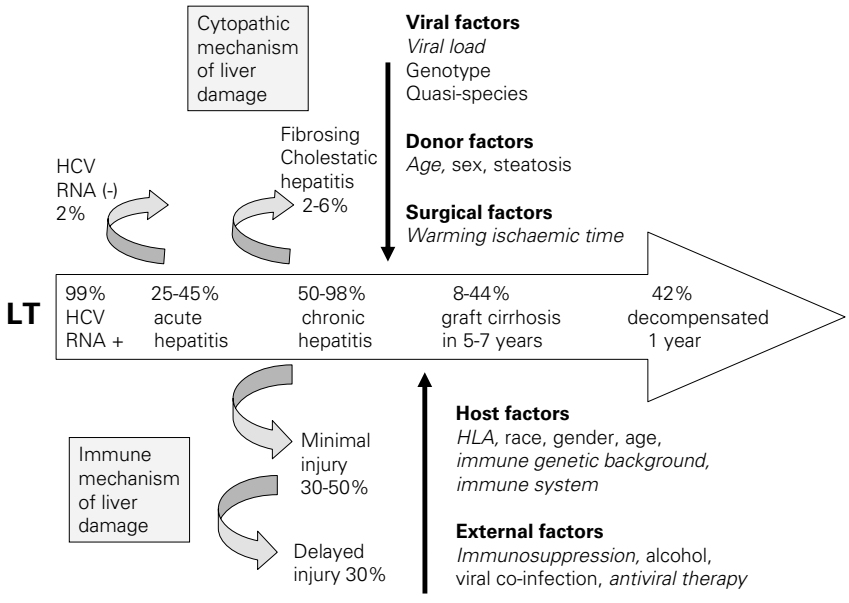


**Figure 22.1** Natural history of recurrent hepatitis C: mechanisms of liver lesion and factors that determine outcome. LT, liver transplantation.

report both grade and stage of disease. Indeed, recurrence of HCV infection is defined as the presence of virus in serum and liver. It occurs nearly universally following transplantation[92] and is characterized by a steep rise in levels of HCV RNA, that typically are 10–20-fold higher than before transplantation.[93] Recurrence of HCV disease, in contrast, occurs at various time points and exhibits a wide spectrum of histological findings from trivial inflammation in the absence of fibrosis to severe and progressive liver disease[94] (Fig. 22.1)

## Pathogenesis

A rapid and sharp decline in viral load occurs immediately after removal of the infected liver.[95] Following reperfusion, HCV RNA levels typically decrease further at a rate that exceeds the decrease observed during the previous anhepatic phase. HCV binding to and/or uptake by hepatocytes may contribute to this early post-transplant decrease in viraemia. Following this initial decline, HCV RNA levels either increase exponentially reaching pre-transplantation levels as soon as day 4, or continue a decline in the first post-transplant week to increase thereafter, peaking by the fourth postoperative month.[95] These differences in kinetics appear to be related to the use of corticosteroids, so that HCV RNA levels increase rapidly in patients receiving corticosteroids as part of the immunosuppressive regime, while they continue to decrease in those not receiving this drug. At 1 year post-transplantation, the levels are 10–20-fold higher than pre-transplantation.[93]

The mechanisms by which HCV leads to liver injury are incompletely understood. Several lines of indirect evidence support a role for the cellular immune response in shaping outcome following transplantation. It is likely that the increased levels of HCV replication typically observed – in conjunction with the altered host immune responsiveness – contribute to the pathogenesis of liver damage, particularly to the severe course of disease of the grafted liver. In addition, there is a spectrum of severity of recurrent HCV disease that may differ in pathogenesis and clearly differs in outcome.[94] Recurrent chronic hepatitis C disease, which can lead to cirrhosis, should be distinguished from recurrent cholestatic HCV disease. In the former, it is possible that the relative antiviral control by the immune response prevents cytopathic injury while perpetuating chronic liver injury.[96] In contrast, a direct cytopathic mechanism of liver injury appears to predominate in patients with severe cholestatic hepatitis.[97]

## Natural history of HCV recurrence

Early short-term survival is comparable to that obtained with other aetiologies. However, emerging data are demonstrating that the long-term outcome is not as benign as previously thought.[79,81–83]

Acute lobular hepatitis develops in approximately two-thirds of the patients within the first 6 months post-transplantation. By the fifth postoperative year, >80% of recipients will develop chronic recurrent HCV disease, characterized histologically by portal and periportal inflammation with or without the presence of portal and/or periportal fibrosis. Lobular hepatitis can also be part of the pathological picture.[98–102]

The natural history of this chronic hepatitis C is more aggressive than that observed in the immunocompetent population. Indeed, in the non-transplantation setting, it is estimated that approximately 20% of the infected patients will develop cirrhosis after a mean duration of infection of 30 years. In the transplant setting, this course is accelerated, and progression to cirrhosis occurs in a percentage of recipients that ranges, depending on the series, from 6% to 23% after a median of 3–4 years since transplantation.[81,83,84,98–104] The development of cirrhosis is associated with reduced graft and patient survival.[94] A recent study has estimated that the median time to develop cirrhosis after transplantation is approximately 9–12 years,[105] a duration significantly shorter than that described in the immunocompetent population. The progressive nature of liver disease in the transplant setting is also evident once the cirrhosis is established, with a higher likelihood of developing clinical decompensation and death compared with that observed in non-transplant patients with cirrhosis.[106] The 1-year actuarial risk of decompensation in patients with post-transplantation HCV cirrhosis is 42%, a percentage that is higher than the 28% at 10 years observed in HCV-infected non-transplant patients with cirrhosis.

In addition, in 5–10%, an accelerated course of liver injury leading to rapid development of liver failure has been observed,[107] reminiscent of that previously described in HBV-infected recipients with fibrosing cholestatic hepatitis. Recurrent cholestatic HCV disease can occur as the initial manifestation of recurrent HCV disease or can emerge in the setting of chronic HCV disease. The definition of fibrosing cholestatic hepatitis includes all of the following criteria: 1 month post-transplant, serum bilirubin >100 μmol/L, serum alkaline phosphatase and GGT more than five times the upper limits of normal, characteristic histology of central ballooning (not necrosis or fallout), a paucity of inflammation ± cholangiolar proliferation without bile duct loss, very high serum HCV RNA levels and absence of surgical biliary complications or hepatic artery thrombosis.[86]

Delayed hepatitis C-related severe liver damage may occur in recipients following initial benign recurrence. This acceleration in disease progression develops in

one-third of patients who have relatively slow fibrosis progression in the first 3–5 years of post-transplantation disease.[108]

Emerging data suggest that the rate of fibrosis progression, and thus the rate of development of graft cirrhosis due to recurrent hepatitis C, is accelerated in patients transplanted in recent years.[81,83,84,105]

Based on all these data, it is not surprising that in several centres a reduction in patient and graft survival among HCV-infected recipients has been observed, with a 5-year survival significantly lower among HCV-infected recipients than in those not infected with HCV (60–70% vs 76–77%).[81,82] In addition, the worse histological outcome seen in recent years is beginning to translate in some centres into a reduced survival among those transplanted recently.[81]

## Histological changes

Liver function tests are not correlated with either viraemia or with histological disease severity,[98–101,109,110] and protocol liver biopsies are generally needed to identify progression to severe forms of chronic hepatitis.

Early histological findings may be helpful in selecting the patients at high risk of disease progression.[98–101,109–113] In particular, the degree of activity and fibrosis staging observed in the first-year liver biopsy is associated with the subsequent risk of developing cirrhosis with only 3–10% of those with mild hepatitis developing cirrhosis compared with 30–60% in those with moderate to severe activity in their first-year liver biopsy.[98,99] Furthermore, not only the severity of initial histological findings, but also some specific histological features such as significant steatosis, ballooning degeneration, cholestasis and confluent necrosis observed on early biopsies may be helpful in predicting progression of disease.[98–101,111–113] Finally, the presence of some degree of fibrosis at baseline (i.e. F = 1 in the first-year liver biopsies) appears to predict the delayed onset of severe liver damage.[108]

## Pre- and post-liver transplant risk factors impacting on HCV recurrence

While some patients have an accelerated course to cirrhosis, there are others, approximately one-third, who remain stable with normal or near-normal histology during 5–10 years of follow-up. Identifying patients at risk of accelerated allograft loss is paramount to the decision about the timing of antiviral agents and/or need for re-transplantation. Factors which have been described as predictors of disease severity/progression and/or survival include those related to the host (demographics, genetic background, immune status, co-morbidities, hepatic function at transplantation), those related the virus (genotype, viral replication, viral quasi-species), those that are donor-related (age, degree of hepatic steatosis, weight, living vs cadaveric) and those related to the environment (surgical-related factors, immunosuppression, alcohol).[79,86]

### *The immune status*

Several indirect findings point towards the deleterious effect of the immunosuppressed status, a variable intrinsically related to the transplant setting (Table 22.3). It has even been suggested that the recent worsening in disease progression may be related to over-immunosuppression following the introduction of more potent immunosuppressive agents.[81,83,105] However, the true adverse effect of more potent immunosuppression on disease outcomes, immunosuppressive agents such as mycophenolate mofetil, lymphocyte-targeting immunoglobulins (e.g. daclizumab and basiliximab), as well as the newer agents with dose-dependent antiproliferative properties,[114–121] needs to be defined.

**Table 22.3** Indirect findings supporting the role of immunosuppression in the natural history of recurrent hepatitis C

| | |
|---|---|
| The course of hepatitis C is accelerated in liver transplant recipients as compared with that observed in immunocompetent patients: | Higher rate of fibrosis progression – 0.3/year (0.004–2.19/year) vs 0.2/year (0.09–0.8/year) – and higher risk of developing severe hepatitis<br>Shorter duration to cirrhosis from time of infection (median of 9–12 years vs 20–40 years)<br>Higher risk of clinical decompensation once HCV-related cirrhosis is established (50% in 1 year vs 20–25% in 10 years) |
| The course of hepatitis C is worse in recent years when significant changes have mainly occurred in the immunosuppressive therapy: | Use of newer and more potent first-line immunosuppressive drugs<br>Earlier and more abrupt discontinuation post-transplantation of second-line immunosuppressive drugs |
| The course of recurrent hepatitis C is worse in other immunosuppressed populations such as HIV-co-infected patients or those with hypogammaglobulinaemia | |

There is already ample evidence that severity of recurrence of HCV is linked in part to the degree of immunosuppression, with most studies showing a positive correlation between a high rate of cirrhosis development and potent immune suppression (high number of boluses of methyl-prednisolone, use of anti-lymphocytic preparations, high total cumulative doses of steroids).[122–125]

### *Immunogenetic background*

There is now strong evidence for the important role of genetics in host immune responses to infectious pathogens and in susceptibility to infection as well as in outcomes after exposure to microbial agents. Both the major histocompatibility complex (MHC) genes and non-MHC genes are increasingly identified as candidate genes with significant importance in outcome of infectious diseases. In HCV, specific HLA class II alleles, such as HLA B14 and HLA DRB1*04 have emerged as possible modulators of disease severity. Regulatory mechanisms that control cytokine production, including tumour necrosis factor (TNF)-α, operate in part at the gene level. It has been shown that cytokine genes are polymorphic at specific sites. These mutations may influence the production of cytokines encoded by the polymorphic gene. In one study, Rosen *et al*. evaluated the significance of polymorphisms at positions –238 and –308 of the TNF-α gene promoter (mutations associated with susceptibility to active hepatitis after HCV exposure in immunocompetent patients) in liver donors on the severity/outcome of recurrent HCV. They found that time to recurrence was shorter and the hepatic activity index greater in those who received an organ from donors with TNF-α –308 allele.[126] These data suggest that a specific donor TNF-α genotype, known to be associated with high TNF-α production, may contribute to the accelerated graft injury observed in liver transplant recipients with HCV infection.

Furthermore, some but not all authors have suggested that while HLA-B sharing between the donor and the recipient reduces the incidence of acute cellular rejection, it also promotes the recurrence of viral hepatitis.[127,128] Discrepancies may be explained by differences between studies in HLA typing methods, selection and ethnic backgrounds of patients as well as differences in immunosuppressive regimens used.

### *Donor-related variables*

The *age of the donor* was recently found to be independently associated with disease severity, disease progression and survival.[81,83,84,129] In one series, only 14% of the recipients who received an organ from a donor younger than 30 developed recurrent HCV-related cirrhosis. In contrast, 45% and 52% of those receiving the organ from donors aged 31–59 or older than 59 developed graft cirrhosis, respectively ($p$ <0.0001).[81] The changing organ donor profile with increasing age of the donors in recent years may explain the observed worsening in outcomes over the same time period. This observation has important implications for donor liver allocation, in that older donors might be more appropriate for HCV-negative recipients in whom the adverse effects of donor age appear to be less deleterious. In addition, the effect of donor age correlates with data from the immunocompetent population where age at the time of infection is an important and powerful determinant of fibrosis development. Age-related changes in liver response may be the key factor that determines the increased susceptibility of the older liver to HCV-related fibrosis.

The *anti-HCV status of the donor* may also influence the post-transplantation outcome. Superinfection has been described in this setting,[130] but it appears that histological outcome and survival do not differ from those observed in patients receiving organs from anti-HCV-negative donors.[131–133]

### *Viral-related variables*

*HCV RNA levels* prior to and/or soon after transplantation may predict survival and the rate/severity of hepatitis C of the graft.[105,134] These findings parallel those previously described for hepatitis B. In one study, patients with higher pre-transplantation HCV RNA levels (>log 1 mEq/mL) experienced mortality and graft loss rates 30% more frequently than those with lower levels.[134]

Studies evaluating the relationship between severity of liver disease post-transplantation and *HCV genotypes* are conflicting.[135–137] Some but not all studies have implicated genotype 1b in a more aggressive post-transplantation disease than non-1b genotype. It may possible that different strains belonging to genotype 1b may be implicated.[138,139]

*HCV heterogeneity* may be implicated in the pathogenesis of progressive HCV disease. However, results from the few studies published to date are inconclusive and somewhat discrepant,[94,140,141] and may be related to the small number of patients included, the different methodologies applied to assess HCV heterogeneity, the type of end-points chosen, the region of the genome evaluated, and the definition of disease severity.

### *Other host variables*

*Race* was recently found to influence outcome in patients with HCV recurrence, with non-Caucasians doing worse than Caucasians.[105,134]

In some studies, female *gender* has been associated with a severe course of recurrent hepatitis C and subse-

quent low survival.[82,83] Reasons that might explain this association are lacking.

In one study, a lack of association was observed between the rate of *fibrosis progression prior to transplantation* and that observed after transplantation,[105] suggesting that variables present at the time of transplantation and those related to post-transplantation management are more important in influencing disease progression than genetic or viral variables unique to the individual. The interpretation of these results is complicated, however, by difficulties in measuring accurately the duration of infection prior to liver transplantation.

In some studies, the presence of *HCC* was found to be a significant predictor of reduced patient and graft survival.[103] This may be explained by differences in patient and tumour selection criteria.[142]

Prolonged *re-warming time* during allograft implantation has been associated with severe recurrent disease.[143] If these data are confirmed, special emphasis should be placed on techniques to minimize re-warming time, such as vena cava preservation.

*Timing of recurrence* appears also to be related to the outcome, with early recurrence within the first 6 months associated with worse prognosis.[100]

Patients who develop *cytomegalovirus* (CMV) viraemia may be at increased risk of severe HCV recurrence.[84,144] The reasons for this association are unknown but probably relate to the degree of immune deficiency, the release of TNF by CMV and/or the existence of cross-reactive immunological responses.

Co-infection with other hepatotropic viruses such as *HBV* may influence histologic disease severity but results are conflicting. While no effect was found in an initial study,[145] co-infected patients appeared to have milder histological course than patients infected only with HCV.[103] Although viral interactions could explain this phenomenon, the passive transmission of antibodies against HCV in co-infected patients receiving HBIg during the pre-HCV era is a more likely explanation.[146]

In contrast, co-infection with other viruses such as *hepatitis G virus* (HGV) does not seem to influence the post-transplantation course of HCV disease.[147]

In summary, it is likely that the interaction between all these variables accounts for the differences in outcomes observed between different centres with cumulative rates of developing HCV-related graft cirrhosis that range from 10% to 50%.

## Prevention of HCV graft reinfection

Given the increasing number of patients progressing to HCV-related graft cirrhosis and the shortage of organ donors, development of strategies to improve the outcome of these recipients is mandatory. These strategies include an adequate timing of liver transplantation in patients with HCV-related end-stage liver disease (see above), antiviral therapy, and the use of specific immunosuppression regimens that may be less deleterious to HCV-infected patients than others.

### *Hepatitis C immunoglobulins (HCIg)*

In contrast to HBV, there is currently no available intervention to prevent universally HCV recurrence. In one study, polyclonal immunoglobulins containing anti-HCV were shown to decrease the incidence of recurrent HCV viraemia measured 1 year post-transplantation.[146] However, given the humoral immune failure in providing adequate and long-lasting neutralizing immunity against HCV, one would predict that additional approaches will be necessary. Indeed, a recent randomized controlled study of hepatitis C immunoglobulin found no benefit in terms of post-transplant clinical reinfection rate or HCV RNA levels.[148] Whether this negative study is a function of the dose of HCIg used, the timing of administration or the type of preparation used (non-neutralizing antibodies) is unknown. It is also possible if not likely, that diverse *quasi-species* nature of HCV makes this virus inherently more difficult to neutralize than the more stable HBV virus populations present in any individual.

### *Pre-transplantation antiviral therapy*

In theory, the rationale for treating patients with decompensated HCV-related cirrhosis is the same as for hepatitis B, i.e. (1) to slow down clinical disease progression and improve the hepatic synthetic function, and in doing so, to reverse the complications of liver disease and obviate/delay the need for liver transplantation; and (2) to achieve an improvement in post-transplantation outcome by clearing the virus prior to transplantation. While the first aim has become a reachable goal in patients with HBV-related decompensated cirrhosis, it remains to be proven in patients infected with HCV, at least with current antiviral agents. In contrast, clearance of HCV prior to transplantation may prevent viral recurrence, and reduction of HCV RNA levels could potentially improve post-transplantation disease progression. Patients waiting for liver transplantation due to HCV-related liver disease typically include two types of patients, those with compensated cirrhosis and HCC and those with decompensated cirrhosis. While in the former group it is likely that a complete course of antiviral therapy may be achieved with currently available drugs, it is less so in those with advanced hepatic insufficiency.

#### *Treatment of compensated cirrhosis (for HCC)*

Therapy of chronic hepatitis C has improved in recent

years with the addition of ribavirin and development of pegylated forms of IFN (PEG-IFNs). Sustained virological responses are achieved in 54–56% of patients compared with 44–47% of those treated with standard IFN and ribavirin. Response is strongly related to the infecting genotype, so that it may reach 78% in those infected with non-1 genotypes compared with 51% in those infected with genotype 1. Other factors that have been shown to influence virological response include viral load, body mass index, race and the degree of liver fibrosis. Although tolerance is adequate, the response rate to these therapies appears to be lower in patients with compensated cirrhosis or transition to cirrhosis than in patients with less advanced liver disease (43–50% vs 57–65% in non-cirrhotic patients).[149] In addition, histological analysis has shown that 49% of cirrhotic patients with a sustained virological response have an improvement in the fibrosis score, suggesting that advanced fibrosis can be reversed with therapy. It remains to be shown whether improvements in fibrosis can be reliably achieved in patients without a virological response who are treated with long-term, maintenance IFN therapy. Tolerability and side-effects are similar to those observed with standard IFN, with the exception of cytopenias, particularly neutropenia, that are more frequently seen with the PEG-IFNs. These side-effects may become a limitation when treating cirrhotic patients with marginal counts. The use of growth factors (i.e. erythropoietin and neutrophil stimulating factor) may be helpful in some cases and avoid the reduction and/or discontinuation of antiviral drugs. However, the potential benefit of these compounds needs to be tested in prospective randomized trials.

Thus, in summary, combination therapy with PEG-IFN and ribavirin should be recommended in patients with cirrhosis provided that no contraindications are present.[80] For patients infected with genotype 1, the optimal length of therapy and dose of ribavirin are probably 48 weeks and 1000–1200 mg. In contrast, for those infected with genotypes 2 and 3, 24 weeks and 800 mg of ribavirin are probably sufficient.

*Treatment of patients with decompensated cirrhosis (Table 22.4)*

The data available on treatment outcomes of antiviral therapy in patients with decompensated liver disease are limited to small uncontrolled case series.[150–152] However, it is clear from these studies that antiviral therapy should be administered with extreme caution in this population, as treatment may increase the risk of infectious complications and increase the likelihood of hepatic decompensation.

In two studies, therapy was started with low doses of IFN ± ribavirin, and doses were increased slowly as tolerated.[10,151] In contrast, complete doses of both drugs were used in a third study by Forns and colleagues.[152] These studies emphasize the advantages and disadvantages of this approach.

In the multicentre study by Crippin and colleagues,[150] HCV-infected patients at or near the top of their respective waiting lists were randomly assigned to one of three treatment arms, two involving therapy with IFN in monotherapy, and one in combination with ribavirin. Less than half the patients screened met entry criteria, with thrombocytopenia and leukopenia being the most common reasons for exclusion. Eventually, only 15 patients from five large transplant centres were treated. Nine patients received IFN monotherapy while six received combination therapy with ribavirin (400 mg twice daily). While on treatment, loss of detectable HCV RNA was seen in 33%. Unfortunately, recurrence of infection was not prevented in the single patient who underwent liver transplantation at the time when he was HCV RNA-negative (although he was negative by the relatively insensitive bDNA assay). In addition, a significant number of adverse effects occurred (n = 23), many of which were considered severe. While thrombocytopenia was the most frequent adverse event, infection was the most severe one. These side-effects, particularly life-threatening infections, ultimately led to the early termination of the study. There are several interesting conclusions from this study. (1) A large proportion of

**Table 22.4** Therapy of patients with HCV-related decompensated cirrhosis

| Author, year (number of patients)[ref] | Eligibility | Genotype 1 | Child A | VR* | Adverse events | Treatment DC | Prevention of recurrence of those HCV RNA-negative at transplantation |
|---|---|---|---|---|---|---|---|
| Crippin *et al.* 2002 (n = 15)[150] | 47% | 67% | 0% | 33% | 87% | 100% | No (n = 1) |
| Everson *et al.* 2000 (n = 91)[151] | NA | 77% | 50% | 38% | ND | 28% | 100% (n = 8) |
| Forns *et al.* 2003 (n = 30)[152] | 62% | 83% | 50% | 30% | 63% | 20 | 67% (6/9) |

DC, discontinuation; VR, virological response; NA, not available; ND, not done.
*HCV RNA clearance while on therapy.

patients awaiting transplantation will not benefit from this approach due to the presence of contraindications, particularly thrombocytopenia and neutropenia. (2) Awareness of the potential complications should be kept in mind when treating the small proportion of patients who meet initiation criteria.

The second reported study of treating patients awaiting liver transplantation comes from the University of Colorado in the United States. At the time of writing data have only been presented in abstract form.[151] In this study, Everson and colleagues treated 101 patients, 70% of whom were infected with HCV genotype 1, and approximately 50% of whom were Child's class A cirrhotics (i.e. they were relatively well compensated). Thus, most of the patients had a low Child Pugh score of <B7–8. Patients were treated with low doses of IFN (1.5 mU three times per week) and ribavirin (600 mg daily), with slow increases in dose of both drugs every 2 weeks as tolerated. Growth factors were administered as needed. Preliminary results of treating 91 patients were recently reported. On treatment, virological responses occurred in 38% and sustained virological response in 22% of patients. Sustained responses were more common in patients infected with genotypes other than 1. Interestingly, recurrent infection which was observed in all patients with detectable HCV RNA at the time of transplantation was prevented in the eight patients who were HCV RNA-negative at the time of transplantation. Although overall rates of severe adverse events were not reported, 28% of the patients had treatment discontinued because of the development of side-effects, and serious complications occurred in 8% of the patients. Results from this study are more optimistic than those of Crippin and colleagues. The main difference between the two studies lies in the severity of liver disease at initiation of therapy and the regimen used. While in the study by Crippin and colleagues, the mean Child Pugh score was 12, patients in the Everson study had significantly less advanced disease. In addition, lower doses were used at initiation in the latter study.

In the study by Forns and colleagues,[152] patients on the waiting list for liver transplantation were considered for antiviral therapy if the expected time on the waiting list was shorter than 4 months, if there was not evidence of renal failure and if patients met minimal blood count criteria. Of 50 patients evaluated, only 62% met entry criteria. The majority of these patients were infected with HCV genotype 1. At the time of inclusion, half of the patients were Child Pugh A while the other half were Child B/C. The regimen used consisted of IFN-α-2b 3 MU/daily and ribavirin 800 mg/day. A virological response was achieved in 30% of patients. Pretreatment viral load was significantly lower in responders than in non-responders ($3 \times 10^5$ vs $6.5 \times 10^5$ IU/mL). Of the nine patients who were HCV RNA-negative at the time of transplantation, six remained free of infection after a median follow-up of 46 weeks. Although side-effects and dose reductions were frequent, particularly due to cytopenias, no patients died while on therapy. However, treatment had to be discontinued in six patients due to thrombocytopenia (n = 4) and sepsis (n = 2). An interesting finding of this study was that of the relationship between viral load at 4 weeks of initiation of therapy and virological response. Indeed, the positive and negative predictive values of an early decrease in viral load (≥2 logs) were 82% and 100%, respectively.

## Post-transplantation antiviral therapy

Post-transplantation therapy may be started early following surgery or alternatively, at a later stage, when the disease is established. The major goal of pre-emptive post-transplantation therapy is to prevent reinfection of the graft, and in doing so, to reduce the rate and/or severity of recurrent disease. In contrast, the major goal of treatment of established disease is to eradicate viraemia, and in doing so, improve the histology.[80]

### *Post-transplantation pre-emptive therapy*

IFN alone or in combination with ribavirin has been attempted within the first 2 weeks after liver transplantation to prevent disease recurrence, or at least diminish the risk of aggressive histological progression. Two small but randomized trials have evaluated the role of IFN in this setting without observing an effect on survival.[153,154] In the first study, histological disease recurrence was observed less frequently in IFN-treated patients (eight of 30 evaluable at 1 year) than in those who were not treated (22 of 41; $p = 0.01$).[153] Interestingly, neither the rate of persistence of HCV RNA nor the level of HCV RNA was affected by treatment, suggesting that IFN may have beneficial effects other than decreasing viral replication. In a smaller controlled trial (n = 24), neither the incidence of histological recurrence nor its severity differed between groups.[154] However, IFN treatment delayed the development of HCV hepatitis, which occurred at a median of 408 days after transplantation in the treated group compared with 193 days in the untreated group. In a third placebo-controlled trial of pre-emptive therapy with IFN plus ribavirin, HCV RNA loss was observed in 3/21 (14%) of the treated group versus none of the placebo group. However, tolerability of therapy was problematic, with profound anaemias observed, requiring transfusion in many patients.[155] One small, uncontrolled series has evaluated the effect of pre-emptive combination therapy. In this case series, 36 recipients were treated with IFN-α2b and ribavirin starting the third post-transplant week and continued for 1 year.[156] After a median follow-up of 52 months, the actu-

arial 5-year survival was excellent (87.5%). At 36 months post-discontinuation of therapy, a sustained virological and biochemical response was achieved in 12 patients (33%), 20% in those infected with HCV genotype 1 and 100% in those with genotype 2. Liver biopsies were normal in these patients. In contrast, progression to severe hepatitis C was observed in four of the non-responders (11% of the overall series). Common side-effects included haemolytic anaemia and asthenia, which were well controlled with dose reduction. Well-designed controlled randomized studies are needed to confirm these findings.

Another recent study only published in the form of an abstract has emphasized the low applicability of this approach, as a substantial proportion of patients do not qualify to receive either IFN or ribavirin due to the presence of co-morbidities in the early post-transplant period, particularly leukopenia and thrombocytopenia.[157] In addition, the efficacy is low in those infected with HCV genotype 1, the group who constitute the vast majority of patients undergoing transplantation for HCV-related cirrhosis.

### *Treatment of patients with recurrent hepatitis C*

Monotherapy has resulted in extremely disappointing results with biochemical response achieved in a minority of IFN- or ribavirin-treated patients but in general, without virological and/or histological response.[80] Although combination therapy probably yields the greatest potential benefit, results are far from being acceptable. Studies on therapy of recurrent hepatitis C are scarce, non-randomized and generally based on small sample sizes.[158–168] The sustained virological response achieved in these studies ranges from 9% to 33%. In addition, both dose adjustments and drug discontinuations, mainly due to ribavirin toxicity, are frequent (30–40%). Although less common, severe adverse effects (particularly hepatic decompensation) may occur in treated patients (5% in previous studies). In a recent study, 6 vs 12 months of combination therapy were compared in 57 transplant patients. A sustained virological response was achieved in 6 of 27 patients treated for 6 months (22%) and in 5 of 30 patients treated for 12 months (17%) ($p = 0.4$). In addition, the response was found to be superior in those infected with HCV genotypes non-1 than in those infected with HCV genotype 1, after both 6 and 12 months of therapy (43% vs 15% and 43% vs 9%, respectively), thus suggesting that 6 months of therapy may be sufficient for recurrent hepatitis C even for genotype 1 cases.[167] However, the numbers of patients studied are too small to draw definitive conclusions. It appears from these preliminary findings that response to standard IFN and ribavirin is generally lower following liver transplantation than in immunocompetent patients. Reasons that may explain this low response rate probably include all of the following: (1) high levels of viraemia, (2) high frequency of HCV genotype 1 in these patients, (3) the low tolerability of IFN and ribavirin leading to frequent dose reductions, (4) lower responses to HCV therapy in immunocompromised patients, suggesting that an intact immune response facilitates treatment-associated viral clearance. In addition, the efficacy of therapy appears to be reduced in recipients with advanced recurrent hepatitis. Reversal of fibrosing cholestatic hepatitis is highly unusual. Many patients have renal insufficiency secondary to immunosuppressive agents, and because of impaired renal clearance of ribavirin, drug-associated haemolysis can be profound. Thus, if ribavirin is initiated as part of combination therapy, the dose should be 600–800 mg, depending on renal function and presence of anaemia. In small studies, early initiation of erythropoietin has been reported to increase the tolerability of ribavirin. In the early studies using IFN in monotherapy, there was concern regarding the induction of allograft rejection due to the potential for upregulating the expression of HLA class I and II. In the majority of studies, the bulk of evidence using combination therapy suggests that there is no increase in frequency or severity of rejection.

Data on post-treatment histology are generally lacking. In a recent randomized study, there were no major differences in the activity and fibrosis stage before treatment and at the end of follow-up.[168] In contrast, improvement in histology was observed in other small non-randomized studies. It is possible that an effect on histology will be seen with a longer follow-up.

There are still many aspects that need to be addressed, such as the optimal dose and duration of therapy, whether ribavirin maintenance is needed following IFN discontinuation, and/or the potential benefit of using growth factors.

In summary, each of the strategies has advantages and disadvantages. Prophylactic therapy while the patient is awaiting transplantation is the best theoretical approach. It is, however, limited by the low tolerability of antiviral agents in patients with advanced liver disease. In addition, it is unlikely that this approach will have a major impact on post-transplantation recurrence of HCV, as only a relatively small proportion of patients may qualify for therapy and the minority achieve viral clearance. In addition, treatment with IFN may theoretically result in the selection of more virulent or resistant viral strains, that ultimately lead to a more aggressive course of the disease or to a post-transplantation situation in which the limited antiviral agents that might have been available are no longer effective.

Pre-emptive post-transplantation therapy is another attractive approach. However, there are many unanswered questions remaining with this strategy. It is not

known whether this approach reduces the rate of recurrent hepatitis C or if it merely delays recurrence. In addition, if this strategy is to be used, it is not known whether all patients should be treated or if treatment should be focused on those at high risk of severe progression. Unfortunately, to date, no single factor or combination of factors is capable of accurately differentiating which patients will ultimately progress to cirrhosis from those with a more benign prognosis. Finally, tolerability of therapy is a major problem when HCV antiviral agents are administered early in the post-transplantation period, and adverse events are common and significant.

With the available drugs, treatment of the established disease is probably the most cost-effective option.[169] Although limited by a relatively low efficacy, tolerance appears to be better than when these drugs are given pre-emptively. Treatment should be offered preferentially to patients who develop progressive liver disease. In that sense, protocol liver biopsies may identify early histological changes that herald an aggressive course, and in such cases, therapy should be initiated at earlier stages when a response appears to occur more frequently.

## Alternative approaches

As the efficacy of antiviral therapy is limited in HCV-infected recipients, optimal management of long-term immunosuppression may be important in improving long-term outcomes. As excess immunosuppression has a deleterious effect on outcome of HCV infection, steroid doses should be minimized, anti-lymphocyte globulin avoided whenever possible, and other immunosuppressive agents should be kept to minimum levels. This is a trend already followed in many transplant centres, but its efficacy has yet to be proven. In fact, recent preliminary data suggest that a rapid steroid tapering may adversely impact HCV disease progression,[121] and it may be possible that the 'acceleration' in disease progression observed in recent years is in fact related to the recent changes in maintenance immunosuppression.[119] Potential hypotheses include the development of subclinical chronic rejection as a consequence of under-immunosuppression or liver damage due to the partial reconstitution of the immune system.

The choice of the appropriate immune suppression is currently under evaluation. Indeed, the effect of different immunosuppressive drugs on viral load and disease progression are unclear to date. Significant difficulties in assessing these associations have arisen recently as newer and more potent immunosuppressive agents are being used to prevent rejection of the graft. As a consequence, there has been an increase in drug combinations of varying potency. This, together with changes in immunosuppressive regimens in individual patients over time, limits our ability to assess accurately the role played by the immunosuppression in the progression of HCV-related liver disease. Our limited understanding as to how to optimize immune suppression underscores the importance of prospective studies comparing different types of immunosuppression-based regimens in HCV-infected recipients. These studies should focus not only on the induction phase but also on the maintenance phase, particularly the way in which steroids are tapered over time.

As a general rule, the optimal strategy should be to achieve a balance between prevention of acute and chronic rejection while minimizing the adverse effects of immunosuppression on recurrent hepatitis C. Based on the available information, the following recommendations were made at the recent Consensus Conference:[86] (1) induction immunosuppression should be performed with two drugs or reduced doses of one or more agents when using triple-drug therapy, and (2) when rejection is diagnosed histologically, the first approach should be to increase the dose(s) of the agents used for maintenance immunosuppression while avoiding bolus corticosteroids or T-cell depleting agents.

## Re-transplantation

Re-transplantation is the last option for patients with failing grafts due to recurrent disease. The number of patients infected with HCV in need of a second transplantation is expected to increase as primary transplant recipients survive long enough to develop graft failure from recurrent disease.[85,170] With the prospect of this increase, it has become imperative to determine whether all or indeed any patients with graft failure due to recurrent HCV disease are candidates for further transplantation. To date, there has been a certain reluctance to accept these patients for re-transplantation, particularly for those patients who have developed recurrent disease leading to graft failure over a short period of time. This fear with re-transplantation is related to four major concerns: (1) early reports suggesting a worse outcome following re-transplantation in HCV-infected recipients than in those uninfected;[170–173] (2) uncertainty regarding the natural history of recurrent hepatitis C in the second graft; (3) advanced age of the recipient, as most had their first transplant in their late fifties or early sixties; and (4) increased organ shortage.

More recent data have reported improved outcome, particularly when re-transplantation is performed before development of infectious and renal complications.[174] Given the poor natural history of compensated graft cirrhosis,[106] if re-transplantation is considered, it should ideally be performed before decompensation.[175] In that sense, serial liver biopsies may help in the diagnosis of clinically compensated HCV-related graft cirrhosis, fa-

cilitating an early referral for liver re-transplantation, at a stage where the probabilities of a favourable outcome are higher. However, under the current MELD organ allocation system,[176] patients have no realistic hope of receiving an organ until they have developed advanced graft failure with significant coagulopathy and frequent renal insufficiency. As a result, patients with hepatitis C-related graft failure will only receive an organ at a point when the chances of survival with re-transplantation are low. In order to improve the outcome, and in a similar way to what has been done with HCC, policies regarding the allocation of organs would have to be revised to offer re-transplantation to patients with recurrent hepatitis C for whom there is a reasonable expectation of long-term survival.

One aspect that remains debatable is whether the severity of recurrent HCV disease in the second graft is related to that observed in the first graft. In a recent case series based on annual protocol biopsies, HCV-related disease severity following re-transplantation was found to be related to that observed following the first transplant.[177] This study suggests that if re-transplantation is to be considered in these patients, it should only be done in the setting of some therapeutic strategy. Unfortunately, the frequent co-existence of co-morbidities and development of side-effects limit the potential utility of antiviral therapy in re-transplanted patients.[177]

Additional important aspects to be considered and that were recommended at the Consensus Conference[86] are: (1) the presence of cholestatic HCV disease should preclude re-transplantation other than in exceptional circumstances; (2) additional transplantation beyond the second graft for recurrent hepatitis should be discouraged; (3) there are minimal data concerning the role of re-transplantation in living donor recipients with recurrent HCV disease and no recommendations could be made.

In summary, as for HBV, three measures should be followed in order to improve the outcome: (1) avoid late re-transplantations when the hepatic failure is too advanced and renal insufficiency has developed, (2) use antiviral therapy at an early stage following re-transplantation, and (3) choose a mild anti-rejection regime so that infections and severe HCV recurrence may be decreased.

### Live donor liver transplantation for HCV-infected patients

Despite all the measures to improve the outcome of HCV-infected patients, the increased organ shortage has led to a dramatic increase in the number of patients on the waiting list and in those dying while waiting. The implementation of live donor liver transplantation (LDLT) was believed to be a potential solution to this problem.[178] The main unanswered question relates to the results obtained with this new technique. Are they the same as with standard liver transplantation in patients infected with HCV? To date, the question remains unanswered as results between centres are controversial.[179,180] While some studies have suggested that HCV recurs earlier and is associated with more severe hepatitis, other studies have not confirmed these data. Most of the evidence though, points towards a worse outcome than when using cadaveric donors. Recent unpublished data from the United Network for Organ Sharing (UNOS) show that the relative risk of graft loss and death are significantly higher with LDLT than with cadaveric liver transplantation (2.23 and 1.88, respectively).[179] In addition, and in a similar way to what has been described with standard transplantation, results in the LDLT group, although not reaching statistical significance, also appear to be worse in the HCV-positive group than in the HCV-negative group. However, the number of HCV-infected LDLT recipients to date is very small, the post-transplant follow-up interval is limited and the reports to date lack protocol liver biopsies. As a result, the data regarding the impact of LDLT on severity of HCV recurrence were deemed inconclusive by the Consensus group.[86]

## Conclusions

The management of HBV in the liver transplant setting has substantially improved in recent years,[8,181] and currently, the likelihood of serious disease due to HBV reinfection is unlikely. Indeed, with available combination therapy, survival is excellent, even in those with active pre-transplantation viral replication, and risk of recurrent disease of the allograft is low. The challenge now remains to identify the most cost-effective strategy to prevent recurrence. Benefits of treatment with nucleoside analogues prior to transplantation must be weighed against potential risks associated with the development of drug-resistant mutants, which in turn may impair the ability of HBIg to prevent HBV recurrence. Prolonged HBIg therapy is limited by costs and side-effects. If oral antiviral drugs are to be used in combination with HBIg, the type of antiviral agent and dose and duration of HBIg treatment remain uncertain.

In contrast to HBV, results in HCV-infected patients have not improved with time. HCV-related end-stage liver disease represents the predominant cause of transplantation in most transplant centres. Despite improvements in antiviral and immunosuppressive therapies in recent years, worsening of patient and graft survival has been observed in HCV recipients, with a resultant growth in demand for re-transplantation. As the severity of recurrence of HCV is linked to the degree of immunosuppression, better utilization of immunosuppressive drugs should lead to an improvement in outcome. In

contrast, if additional studies confirm that more severe recurrence of HCV results from the use of older or living donors, patient and graft survival may decline sharply as mean donor age increases inexorably and the frequency of LDLT increases substantially. Treatment of established recurrent hepatitis C is the only alternative currently available. There is a great need for improved antiviral drugs for use in this setting.

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# Chapter 23
# Prevention

Jane N Zuckerman

## Introduction

Hepatitis B, which is a major public health problem throughout the world, is preventable by immunization. First-generation vaccines were prepared from the 22-nm hepatitis B surface antigen (HBsAg) particles purified and inactivated from plasma donations from chronic asymptomatic (healthy) carriers of hepatitis B virus. These preparations were safe and immunogenic but have been superseded largely by recombinant DNA vaccines produced by expression of HBsAg in yeast cells. The expression plasmid contains only the 3′ portion of the viral surface open reading frame (ORF) and only the major surface protein, without pre-S epitopes, is produced. Vaccines containing pre-S1 and pre-S2 as well as the major surface protein, so-called third-generation hepatitis B vaccines, have also been produced.

Before the availability of hepatitis B vaccines, passive immunization with hepatitis B immunoglobulin (HBIg) was used for prophylaxis under certain defined conditions.

## Passive immunization

HBIg is prepared specifically from pools of plasma with high titres of anti-HBs and may confer temporary passive immunity under certain defined conditions. The major indication for the administration of HBIg is a single acute exposure to HBV, such as occurs when blood containing HBsAg is inoculated, ingested or splashed onto mucous membranes and the conjunctiva. The optimal dose has not been established but doses in the range of 250–600 have been used effectively. HBIg should be administered as early as possible after exposure and preferably within 48 hours, usually 3 mL (containing 200 IU of ant-HBs per mL) in adults. It should not be administered 7 days or more after exposure. It is generally recommended that two doses of HBIg should be given 30 days apart.

Results following the use of HBIg for prophylaxis in babies born to carrier mothers indicate encouraging prevention of infection in the newborn if the immunoglobulin is given immediately, and certainly within 12 hours of birth. The dose of HBIg recommended in the newborn is 1–2 ml (200 IU of anti-HBs per mL). The risk of the baby developing the persistent carrier state is reduced by up to 70%. Combined passive and active immunization indicate an efficacy approaching 90%.

## Active immunization

See Figs 23.1 and 23.2. The discovery of variation in the epitopes presented on the surface of the virions and subviral particles identified several subtypes of HBV which differ in their geographical distribution. All isolates of the virus share a common epitope, a, which is a domain of the major surface protein that is believed to protrude as a double loop from the surface of the particle. Two other pairs of mutually exclusive antigenic determinants, d or y and w or r, are also present on the major surface protein. These variations have been correlated with single nucleotide changes in the surface ORF which lead to variation in single amino acids in the protein. Four principal subtypes of HBV are recognized: adw, adr, ayw and ayr. Subtype adw predominates in northern Europe, the Americas and Australasia and is also found in Africa and Asia. Subtype ayw is found in the Mediterranean region, eastern Europe, northern and western Africa, the Near East and the Indian subcontinent. In the Far East, adr predominates, but the rarer ayr occasionally may be found in Japan and Papua New Guinea. Based on the analysis of subdeterminants, nine subtypes of the virus have been proposed.

Hepatitis B virus (HBV) is now classified into seven genotypes, A–G, by sequence divergence >8% of the entire genome.[1,2] An eighth genotype, designated H, has been reported from Central America,[3] but has not been fully characterized yet. Although both genotypes and subtypes segregate geographically, the same subtype can be represented by several genotypes.

The major response of recipients of hepatitis B vaccines is to the common group determinant (epitope) a with consequent protection against all subtypes of the virus.

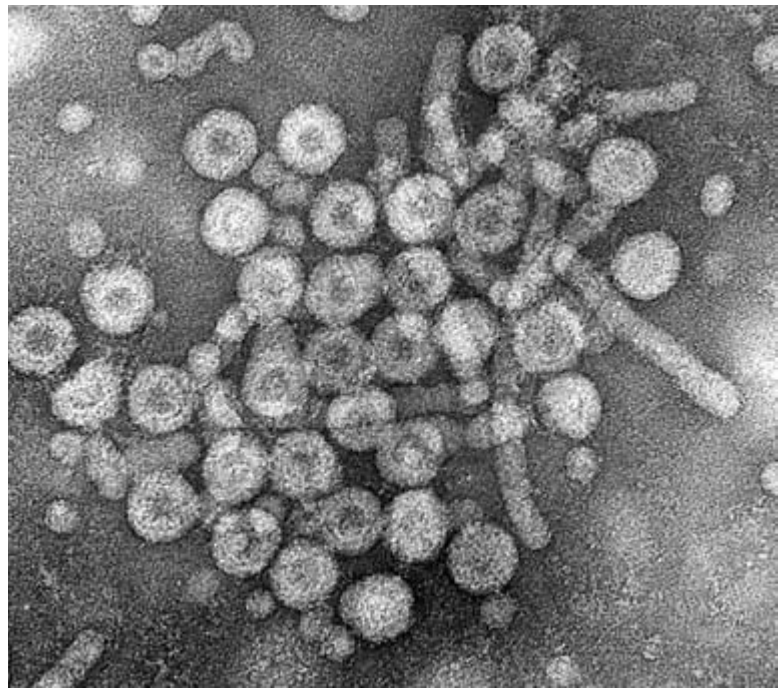

**Figure 23.1** Electron micrograph of plasma from a carrier of hepatitis B virus showing the 42-nm double spherical particles of the complete virus, tubular forms and small spherical particles measuring 22 nm in diameter (×200 000).

Immunization against hepatitis B is now recognized as a high priority in preventive medicine in all countries and strategies for immunization are being revised. Universal vaccination of infants and adolescents is recommended as a possible strategy to control the transmission of this infection. More than 150 countries now offer hepatitis B vaccine to all children, including the United States, Canada, Italy, France and most Western European countries.[4]

However, immunization against hepatitis B is at present recommended in a number of countries with a low prevalence of hepatitis B, mainly the UK and Scandinavian countries, only to groups which are at an increased risk of acquiring this infection. These groups include individuals requiring repeated transfusions of blood or blood products, prolonged inpatient treatment, patients who require frequent tissue penetration or need repeated circulatory access, patients with natural or acquired immune deficiency and patients with malignant diseases. Viral hepatitis is an occupational hazard among health-care personnel and the staff of institutions for people with mental disabilities and in some semi-closed institutions. High rates of infection with HBV occur in narcotic drug addicts and intravenous drug abusers, sexually active male homosexuals and prostitutes. Individuals working in high endemic areas are, however, at an increased risk of infection and should be immunized.

Young infants, children and susceptible persons (including travellers) living in certain tropical and subtropical areas where socio-economic conditions are poor and the prevalence of hepatitis B is high should also be immunized. It should be noted that in about 30% of patients with hepatitis B the mode of infection is not known and this is, therefore, a powerful argument for universal immunization.

## Site of injection for vaccination and antibody response

Hepatitis B vaccination should be given in the upper arm or the anterolateral aspect of the thigh and not in the buttock. There are over 100 reports of unexpectedly low antibody seroconversion rates after hepatitis B vaccine using injection into the buttock. In one centre in the United States a low antibody response was noted in 54% of healthy adult health-care personnel. Many studies have since shown that the antibody response rate was significantly higher in centres using deltoid injection than in centres using the buttock. On the basis of antibody tests after vaccination, the Advisory Committee on Immunization Practices of the Centres for Disease Control, USA recommended that the arm be used as the site for hepatitis B vaccination in adults, as has the Department of Health in the UK (reviewed in Zuckerman *et al*.[5]).

Apart from the site of injection, there are several other factors which are associated with a poor or no antibody response to currently licensed vaccines. Indeed, all studies of antibody response to plasma-derived hepatitis B vaccines and hepatitis B vaccines prepared by recombinant DNA technology have shown that between 5%

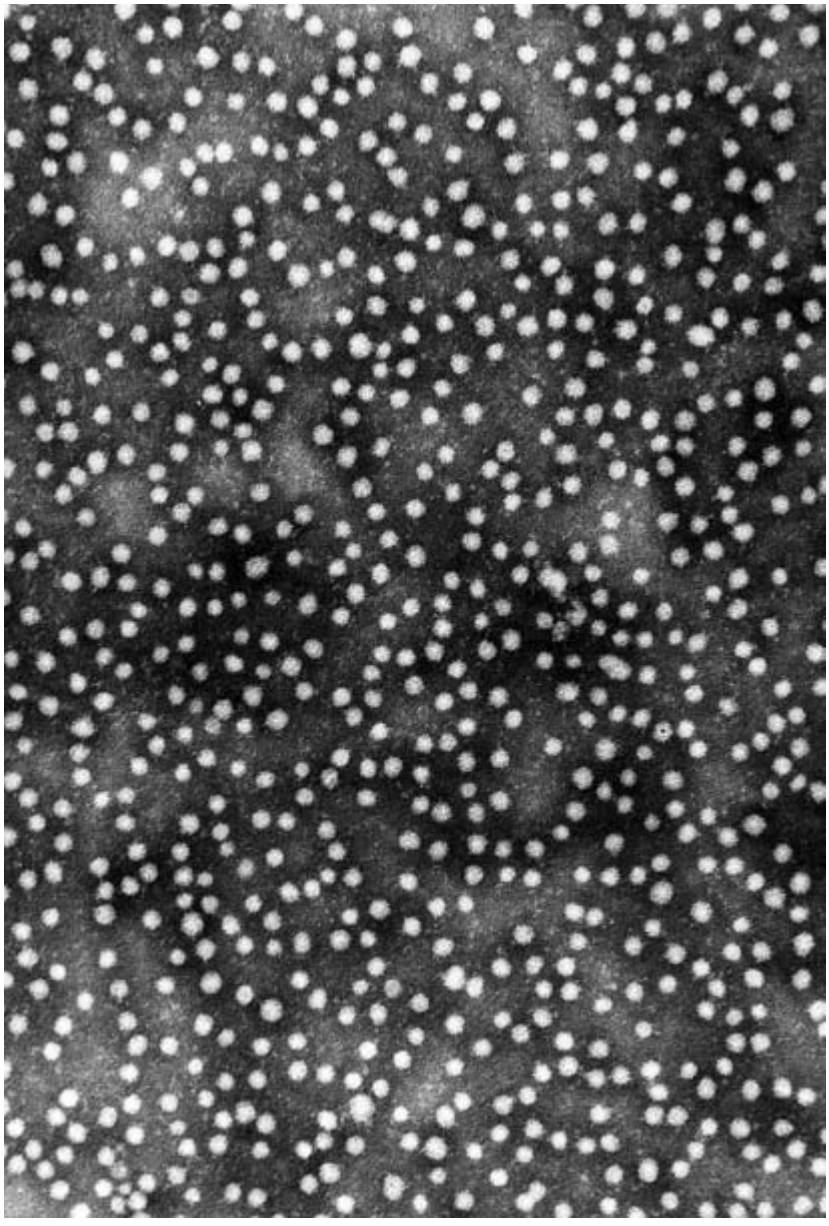

**Figure 23.2** Electron micrograph of plasma-derived hepatitis B vaccine showing the presence of purified 20–22-nm spherical particles of the excess protein coat (surface antigen, HBsAg) of the virus (×180 000).

and 10% or more of healthy immunocompetent subjects do not mount an antibody response (anti-HBs) to the surface antigen component (HBsAg) present in these preparations (non-responders), or that they respond poorly (hypo-responders). The exact proportion depends partly on the definition of non-responsiveness, generally less that 10 IU/L or 100 IU/L, respectively, against an international antibody standard (reviewed in Zuckerman *et al*.[5]).

## The kinetics of anti-HBs response to immunization and booster doses

The titre of vaccine-induced hepatitis B surface antibody (anti-HBs) declines, often rapidly, during the months and years following a complete course of primary immunization. The highest ant-HBs titres are generally observed 1 month after booster vaccination followed by a rapid decline during the next 12 months and thereafter more slowly. Mathematical models have been designed and an equation was derived consisting of several exponential terms with different half-life periods. It is considered by some researchers that the decline of anti-HBs concentration in an immunized subject can be predicted accurately by such antibody kinetics with preliminary recommendations on whether or not booster vaccination is necessary (see review by Zuckerman[6]).

If the minimum protection level is accepted at 10 IU/L, consideration should be given to the diversity of the individual immune response and the decrease in levels

of ant-HBs as well as to possible errors in quantitative ant-HBs determinations. It would then be reasonable to define a level of >10 IU/L and <100 IU/L as an indication for booster immunization. It has been demonstrated that a booster inoculation results in a rapid increase in anti-HBs titres within 4 days. However, it should be noted that even this time delay might permit infection of hepatocytes.

Several options can therefore be considered for maintaining protective immunity against hepatitis B infection.

- Relying upon immunological memory to protect against clinical infection and its complications, a view which is supported by *in vitro* studies showing immunological memory for HBsAg in B cells derived from vaccinated subjects who have lost their anti-HBs, but not in B cells from non-responders, and by post-vaccination surveys.
- Providing booster vaccination to all vaccinated subjects at regular intervals without determination of anti-HBs. This option is not supported by a number of investigators because non-responders must be detected.
- While an anti-HBs titre of about 10 IU/L may be protective, this level is not ideal from a laboratory point of view, as many serum samples may give non-specific reactions at this antibody level.
- Testing anti-HBs levels 1 month after the first booster and administering the next booster before the minimum protective level is reached.

No empirical data are available for the anti-HBs titre required for protection against particular routes of infection or the size of the infectious inoculum. The minimum protective level has been set at 10 IU/L against an international standard. However, the international standard is a preparation of immunoglobulin prepared from pooled plasma of individuals recovered from infection rather than immunized subjects and the antibody avidity is likely to be different. Furthermore, studies carried out in the 1980s indicated asymptomatic infection after immunization in subjects and health-care workers who had antibody titres below 50 IU/L.

There are studies showing that hepatitis B vaccines provide a high degree of protection against clinical symptomatic disease in immunocompetent persons despite declining levels of anti-HBs (reviewed by Zuckerman and Zuckerman[7]). These studies encouraged the Immunization Practices Advisory Committee of the United States, the National Advisory Committee on Immunization of Canada and the European Consensus Group[8] to recommend that routine booster immunization against hepatitis B is not required. Caution, however, dictates that those at high risk of exposure, such as cardiothoracic surgeons and gynaecologists, would be prudent to maintain a titre of 100 IU/L of anti-HBs by booster inoculations, more so in the absence of an appropriate international antibody reference preparation. Breakthrough infections have been reported and, whereas long-term follow-up of children and adults indicate that protection is attained for at least 9 years after immunization against chronic hepatitis B infection, even though anti-HBs levels may have become low or decline below detectable levels (reviewed by the European Consensus Group[9]), brief periods of viraemia may not have been detected because of infrequent testing. Longer follow-up studies of immunized subjects are required to guide policy, as is well illustrated by a study carried out in Gambian children.[10] It was found, by a cross-sectional study in the Gambia, that the efficacy of hepatitis B vaccination against chronic carriage of HBV 14 years after immunization was 94%, and the efficacy against infection was 80% and lower (65%) in those vaccinated at the age of 15–19 years. Further and longer follow-up studies of immunized subjects are therefore required to guide policy.

## Strategies for immunization against hepatitis B

The World Health Organization (WHO) has set a target for global control of hepatitis B, and recommended that all countries integrate hepatitis B vaccine into their national immunization programmes by 1997. Over 150 countries have introduced such programmes.

A number of strategic options are outlined below, including a proposal for immediate implementation of universal antenatal screening and immunization of infants born to carrier mothers as a minimum policy initiative.

There is strong support for the introduction of universal antenatal screening to identify hepatitis B carrier mothers and the vaccination of their babies. It is important that any other strategies do not interfere with the delivery of vaccine to this group. Immunization of this group will have the greatest impact in reducing the number of new hepatitis B carriers. For children outside this group, it is difficult to estimate the lifetime risk of acquiring a hepatitis infection, and four main approaches should be considered.

- To continue vaccination of 'high-risk' babies as defined above
- Universal infant immunization
- Universal adolescent immunization
- Vaccinate everybody.

### Vaccination of adolescents

This approach delivers immunization at a time close to the time when 'risk behaviour' would expose ado-

lescents to infection. Vaccination could be delivered as part of a wider package on health education in general, to include sex education, risk of AIDS, dangers of drug abuse, smoking, benefits of a healthy diet and lifestyle.

The problems with this approach are as follows.

- Persuading parents to accept vaccination of the children against sexually transmitted disease, a problem they may not wish to address at the time.
- Ensuring that a full course of three doses is given.
- There would be difficulty evaluating and monitoring vaccine cover. The systems for monitoring uptake of vaccine in this age group may not operate efficiently.

### Vaccination of infants

The advantages of this approach are as follows.

- It is known that vaccination can be delivered to babies.
- Parents would accept vaccination against hepatitis B along with other childhood vaccinations without reference to sexual behaviour.

The disadvantages of this approach are:

- Whether immunity would remain until exposure occurred in later life. This was thought to become less of a problem as more people were vaccinated as the chance of exposure to infection was reduced.
- That the introduction of further childhood vaccination would reduce the uptake of other childhood vaccinations. This problem would be avoided if hepatitis B could be delivered in a combined vaccine containing diptheria tetanus and pertussis (DPT), and such preparations are under evaluation.

Vaccination of infants is preferable to vaccination of adolescents as there are sufficient mechanisms to ensure, monitor and evaluate cover. A booster dose could be given in early adolescence combined with a health education package. A rolling programme could be introduced, giving priority to urban areas.

## Non-response to hepatitis B vaccines

All studies of the antibody response to currently licensed plasma-derived hepatitis B vaccines and hepatitis B vaccines prepared by recombinant DNA technology have shown that between 5% and 10% or more of healthy immunocompetent subjects do not mount an antibody response (anti-HBs) to the surface antigen component (HBsAg) present in these preparations (non-responders) or that they respond poorly (hypo-responders). The exact proportion depends partly on the definition of non-responsiveness or hypo-responsiveness, generally <10 IU/L or 100 IU/L of anti-HBs, respectively, against an international antibody standard (reviewed by Zuckerman[6]).

Non-responders remain susceptible to infection with HBV. While several factors are known to affect adversely the antibody response to HBsAg including the site and route of injection, gender, advancing age, body mass (overweight), immunosuppression and immunodeficiency, and mechanisms underlying non-responsiveness to the S component of HBsAg in humans remain largely unexplained, evidence is accumulating that there is an association between different HLA-DR alleles and specific low responsiveness in different ethnic populations. Considerable experimental evidence is available to suggest that the ability to produce antibody in response to specific protein antigens is controlled by dominant autosomal class II genes of the major histocompatibility complex (MHC) in the murine model.[11,12] Much effort has been devoted to overcoming class II-linked non-responsiveness to current hepatitis B vaccines.[13–16]

There is evidence that the pre-S1 and pre-S2 domains have an important immunogenic role in augmenting anti-HBs responses, preventing the attachment of the virus to hepatocytes and eliciting antibodies which are effective in viral clearance, stimulating cellular immune responses, and circumventing genetic non-responsiveness to the S antigen.[8,15] Thus, a number of studies indicated that the inclusion of pre-S components in recombinant or future synthetic vaccines should be developed. For example, the pre-S2 region is more immunogenic at the T- and B-cell levels than the S region in the mouse model,[15,17] as is the case with pre-S1 in the mouse[16] and in man and circumvents S region non-responsiveness at the level of antibody production.

Indeed, it was demonstrated in the murine model that the independence of MHC-linked gene regulation of immune responses to pre-S1, pre-S2 and S regions of hepatitis B surface antigen would assure fewer genetic non-responders to a vaccine containing all three antigenic regions.[16] Studies conducted in humans with experimental recombinant hepatitis B vaccines containing all three S components of the viral envelope polypeptides demonstrated the enhanced immunogenicity of such preparations when compared with conventional yeast-derived vaccines (as shown by Pay *et al*.[18] and others), although several earlier studies with vaccines containing the S, pre-S1 and pre-S2 components revealed significant differences from preparations containing only the S antigen.[19,20]

These observations led to the development of a new triple antigen hepatitis B vaccine, a third-generation recombinant DNA vaccine containing pre-S1, pre-S2 and S antigenic components of hepatitis B virus surface antigen of both subtypes adw and ayw. All three antigenic components are glycosylated, closely mimicking the surface protein of the virus itself, produced in a continuous mammalian cell line, the mouse c127 clonal cell line,

after transfection of the cells with recombinant HBsAg DNA.

Animal studies showed that the vaccine was well tolerated and a viral challenge study in chimpanzees demonstrated protective efficacy.

This vaccine was evaluated for reactogenicity in a number of clinical trials (reviewed by Zuckerman and Zuckerman[21]). The major conclusions from these studies were that the vaccine was safe and immunogenic and overcame the responsiveness to the single S antigen vaccines used widely in some 70% of non-responders, and that even a single dose of 20 μg of the triple antigen provided significant seroprotection levels of antibody. However, the anticipated high costs of the triple antigen vaccine will limit the use of the triple antigen vaccine initially to the following groups.

- Vaccination of non-responders to the current single antigen(s) vaccines, who are at risk of exposure to HBV infection.
- Subjects with inadequate humoral immune response to single antigen hepatitis B vaccines, e.g. those over the age of 40 years, males, obese individuals, smokers and other hypo-responders.
- Persons who require protection rapidly, e.g. healthcare employment involving potential exposure to parental procedures involving blood-to-blood contact (current schedules of immunization with single antigen hepatitis B vaccines involve three doses at 0, 1 and 6 months).

Studies are required to determine the efficacy of the triple antigen in patients who are immunocompromised and also to determine whether the inclusion of pre-S1 and pre-S2 antigenic components in this new vaccine will protect against the emergence of HBsAg mutants (see below.)

### Attempts to overcome non-responsiveness by the use of immunomodulators

Attempts have been made to enhance the anti-HBs response following immunization, particularly in patients treated by maintenance haemodialysis, but often with conflicting results or in limited studies, which have not been confirmed, including: α-interferon (IFN-α), interleukin (IL)-2 and thymopentin and other substances such as experimental oral adjuvants in mice and oestrogen.

## Hepatitis B surface antigen variants and immunization

There is evidence that amino acid substitutions within the *a* determinant of the surface antigen can allow replication of HBV in vaccinated persons, as antibodies induced by current vaccine do not recognize critical changes in the surface antigen domain. The emergence of variants of HBV, possibly due to selection pressure associated with extensive immunization in an endemic area, was suggested by the findings of HBV infection in individuals immunized successfully in Italy. These studies were extended subsequently by the finding of non-complexed HBsAg and anti-HBs and other markers of hepatitis B infection in 32 of 44 vaccinated subjects, and sequence analysis from one of these cases revealed a mutation in the nucleotide encoding that a determinant, the consequence of which was a substitution from glycine to arginine at amino acid position 145.[22]

Various mutations and variants of HBsAg have since been reported from many countries, including Italy, the UK, Holland, Germany, the USA, Brazil, Singapore, Taiwan, China, Japan, Thailand, India, and West and South Africa.[23,24] However, the most frequent and stable mutation was reported in the G145R variant. A large study in Singapore of 345 infants born to carrier mothers with HBsAg and HBeAg, who received HBIg at birth and plasma-derived hepatitis B vaccine within 24 hours of birth and then 1 month and 2 months later, revealed 41 breakthrough infections with HBV despite the presence of anti-HBs. There was no evidence of infection among 670 immunized children born to carrier mothers with HBsAg and anti-HBe, nor in any of 107 immunized infants born to mothers without HBsAg. The most frequent variant was a virus with the G145R mutation in the *a* determinant.[25] Another study in the USA of serum samples collected between 1981 and 1993 showed that 94 (8.6%) of 1092 infants born to carrier mothers became HBsAg-positive despite post-exposure prophylaxis with hepatitis B immunoglobulin and hepatitis B vaccine. Following amplification of HBV DNA, 22 children were found to have mutations of the surface antigen, most being in amino acids 142–145; five had a mixture of wild-type HBV and variants and 17 had only the 145 variant.[26]

In a more recent study in the USA[27] direct sequencing of amplified or cloned polymerase chain reaction (PCR) products, solid-phase detection of sequence-specific PCR products (SP-PCR) and limiting dilution cloning PCR (LDC-PCR) were compared to determine their sensitivity in detecting differing concentration of HBsAg variants in the same population of the infants studied in the 1981–1993 post-exposure prophylaxis of hepatitis B in infants born to carrier mothers. LDC-PCR had the greatest sensitivity and could detect HBsAg variants at a concentration of 0.1% of the total viral population. NBsAg variants were detected in 47 of 93 (51%) of infants with chronic HBV infection acquired after post-exposure prophylaxis, and more than half of the variants were detected only by the most sensitive methods. The G145R variant (glycine to arginine at aa 145) was identified most frequently.

A report from Taiwan[28] noted the increase in the prevalence of mutants of the a determinant of HBV over a period of 10 years in immunized children, from 8 of 103

(7.8%) in 1984 to 10 of 51 (19.6%) in 1989, and 9 of 32 (28.1%) in 1994. This is of particular concern. The prevalence of HBsAg mutants among those fully immunized was higher than among those not vaccinated (12/33 vs 15/153, $p = 0.0003$). In the 27 children with detectable mutants, the mean age of those vaccinated was lower than of those not vaccinated, and mutation occurred in a region with greatest hydrophilicity of the surface antigen (amino acids 140–149) and more frequently among those vaccinated than among those not vaccinated. More mutations to the neutralizing epitopes were found in the 1994 survey in Taiwan.

Another important aspect is the evidence that HBsAg mutants may not be detected by all of the blood donor screening tests and by existing diagnostic reagents. Such variants may therefore enter the blood supply or spread by other means. This is emphasized by the finding in Singapore, between 1990 and 1992, of 0.8% of carriers of HBV variants in a random population survey of 2001 people.[25,29] These findings add to the concern expressed in a study of mathematical models of HBV vaccination, which predict, on the assumption of no cross-immunity against the variant by current vaccines, that the variant will not become dominant over the wild-type virus for at least 50 years – but the G145R mutant may emerge as the common HBV in 100 (or more) years' time.[30]

It is important, therefore, to institute epidemiological monitoring of HBV surface mutants employing test reagents which have been validated for detection of the predominant mutations, and consideration should be given to incorporating into current hepatitis B vaccines antigenic components that will confer protection against infection by the predominant mutant(s).[31]

The current position can be summarized as follows.

- Variants of hepatitis B virus surface antigen proteins were identified over a decade ago and may have a potential impact on immunization against this important infection and on public health.[21]
- The G145R mutant is replication-competent and is stable. It appears to be the most common variant and may persist in the host for at least 14 years.
- There is evidence that sera of 10% (up to 40% in high-risk groups) of individuals with antibodies to hepatitis B core antigen (anti-HBc) as the only marker of HBV infection may contain HBV DNA. At least some of the chronic low level carriers of HBV, where surface antigen is not detected and anti-HBc is the only serological marker of HBV infection, are infected with surface mutants. Further studies are required.
- Epidemiological monitoring of HBV surface mutants is essential, employing test reagents that have been validated for detection of the predominant mutations.
- Urgent consideration should be given to the introduction of routine screening for hepatitis B by nucleic acid-based technology of blood donors and tissue and organ donors for transplantation.
- Consideration should be given to incorporation into the current hepatitis B vaccines of additional antigenic components that will confer protection against infection by the predominant *a* determinant mutation(s), if dictated by epidemiological findings.

Considerable progress has been made in the prevention of HBV infection by active immunization since the vaccine was first licensed in 1982. The global eradication of hepatitis B is attainable.

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# Section V
# Hepatitis C Virus

# Chapter 24
# Structure and molecular virology

Michael J McGarvey, Michael Houghton

## The relationship between hepatitis C virus and the Flaviviridae family

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus of the genus Hepacivirus in the family Flaviviridae. The HCV genome is comprised of a positive-stranded RNA molecule of about 9500 nucleotides (nt)[1] containing a single long translational open reading frame (ORF) that encodes a large polypeptide of approximately 3000 amino acids (aa), beginning with the first in-frame methionine codon[2–4] bounded by 5′ and 3′ untranslated regions (UTRs) of approximately 341 and 230 nucleotides (Fig. 24.1). The 5′ UTR of the HCV genome has substantial primary sequence identity with the corresponding region of pestivirus genomes,[3,5,6] and a region of the encoded polypeptide exhibits significant sequence identity to nucleoside triphosphate (NTP)-binding helicases encoded by the pestiviruses and to a lesser extent, the flaviviruses.[3,7,8] Protease and replicase sequence motifs, conserved among the pestiviruses and flaviviruses are also present within the HCV-encoded polyprotein, which along with the more extensively conserved helicase sequence are all similarly colinear among the three types of viral polyproteins.[3] Although these are the only regions of HCV exhibiting significant primary sequence identity with pestiviruses and flaviviruses, the hydropathicity of the HCV-encoded polypeptide is remarkably similar to that of the flaviviruses and, to a lesser extent, to that of the pestiviruses, thus indicating similarities in their basic structures and functions.[3] Nucleotide and protein sequence analysis of GBV-A, GBV-B and GBV-C[9,10] show that these three viruses are more closely related to each other and HCV than to the other members of the Flaviviridae family. HCV, together with GBV-A, B and C form a new genus, Hepacivirus in the flavivirus family (Fig. 24.1)[11,12] of related viruses within the larger genus of *Flaviviridae*.[10] HCV causes chronic infection in >80% of cases,[13,14] which then predisposes patients to the sequential development of cirrhosis and primary hepatocellular carcinoma (HCC).[15]

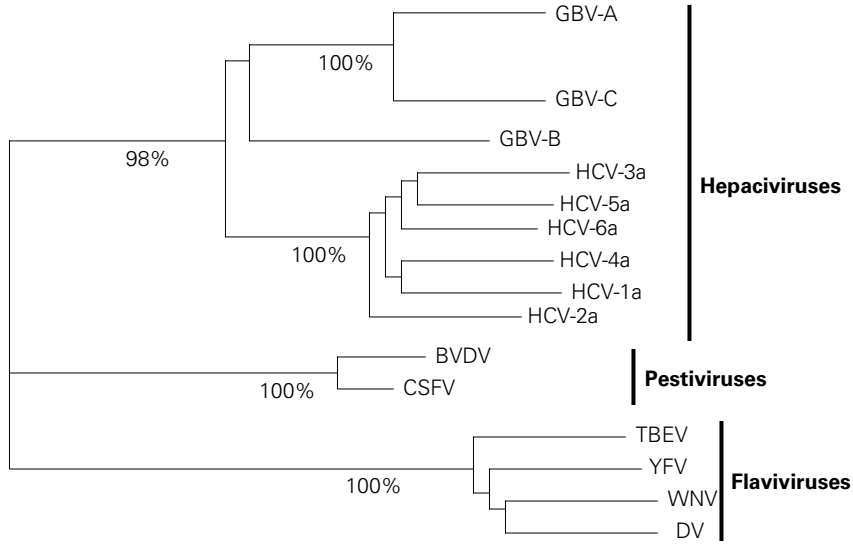


**Figure 24.1** Phylogenetic relationship between HCV and other members of the Flaviviridae. This figure shows a comparison of the predicted polymerase regions of HCV-1a, -2a, -3a, -4a, -5a and -6a, GBV-A, GBV-B, GVB-C, classical swine fever virus (CSFV), bovine viral diarrhoea virus (BVDV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV), West Nile virus (WNV) and dengue virus (DV). (Adapted from Simmonds.[11,12])

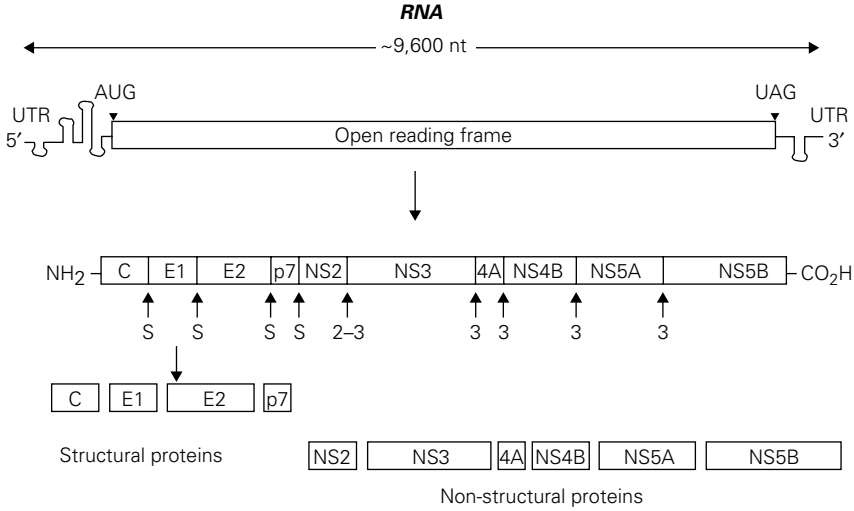


**Figure 24.2** Schematic organization of the HCV genome and the encoded proteins. The viral RNA consists of a single open reading frame (ORF), encoding about 3011 amino acids, bounded by 5′ and 3′ untranslated regions (UTRs). The polyprotein is cleaved post-translationally with signal peptidases (S) and the virus encoded NS2-3 (2-3) and NS3-4 (3) proteases to generate the mature structural and non-structural proteins.

## Structure of the virus

### The HCV virion

Analysis of the structure of HCV particles has been hampered by the low titre of virus in infectious sera and the difficulties of replicating the virus in culture systems. It is likely that HCV is an enveloped icosahedral virus, like other members of the Flaviviridae.[16,17] However, detailed structural analysis of HCV virions is still lacking. Particles with a diameter of 45–65 nm have been observed by electron microscopy in human plasma[18] and in chimpanzee and human liver chronically infected with HCV.[19,20] Non-enveloped nucleocapsids have also been detected in the plasma of infected chimpanzees.[21] HCV-like particles (HCV-LPs) have been detected in experimentally infected[19] or transfected cell lines.[22] HCV-LPs of 40–60 nm have also been produced by the expression of HCV structural proteins using: baculovirus vectors in insect cells;[23] Semliki Forest virus replicons[24] and VSV (vesicular stomatitis virus) vectors[25] in BHK 21 cells; and yeast expression vectors.[26] There is evidence that HCV particles are present in the circulation as immune complexes[27] or in association with serum lipoproteins.[28–31]

### Structural proteins

A large ORF extends throughout most of the HCV RNA genomic sequence and encodes a polypeptide of between 3010 and 3033 amino acids, depending on the source of the viral isolate.[2–3,32–36] As in the case for pestiviral and flaviviruses, the large HCV ORF encodes a polyprotein precursor that is processed co- and post-translationally to yield a variety of structural (virion) and non-structural (NS) proteins (Fig. 24.2 and Table 24.1). Structural proteins are processed from the N-terminal region of the HCV polyprotein precursor, beginning with an RNA-binding nucleocapsid polypeptide of basic charge (C; ca. 23 kDa) followed by two glycoproteins – E1 (33–35 kDa) and E2 (68–72 kDa) (Fig. 24.2). These proteins have been identified by *in vitro* translation of HCV RNA transcribed *in vitro* from cDNA clones[37,38] and also from mammalian cells[39–42] and insect cells[41] transfected with HCV cDNA. Additional proteins derived from core, 21 kDa and 17 kDa and a 7 kDa protein (p7) which lies between E2 and NS2 have also been found. The processing of the putative region of the HCV polyprotein is similar to that of flaviviruses and pestiviruses in being mediated at least in part by the host signal peptidases.[43–48]

### Core protein

The expression of the HCV structural protein coding region, by cell-free translation in the presence of microsomal membranes, or in tissue culture cells, produces a 23-kDa (previously identified as 21–22-kDa) core protein of 191 amino acids. This indicates that the cleavage of the core protein from the polyprotein is dependent on signal peptidases in the endoplasmic reticulum (ER).[27,49,50] HCV core protein has been identified in the sera of HCV-infected humans as a 21-kDa protein.[51] The core protein binds to membranes *in vitro* and in transfected cells, and has been shown to be associated with the cytoplasmic side of the ER. E1 was precipitated by anti-core antibodies in the presence of core protein, showing that core interacts with E1. In contrast, the E2 protein is not co-precipitated with anti-core antibodies in co-expression experiments. Following signalase cleavage, the E1 signal sequence may act, at least in part, to retain the upstream C protein in the ER in an analogous manner to that of the flaviviruses.[44–46] Little E1 is exposed on the cytosolic side of the ER, therefore the interaction between core and E1 probably takes place in the ER membrane

**Table 24.1** HCV proteins

| Protein | Molecular weight (kDa) | Main functions* |
|---|---|---|
| C | 21–23 | Nucleocapsid, |
| | | RNA binding |
| | 17 | F/AFRP protein? |
| E1 | 33–35 | Envelope protein |
| E2 | 70–72 | Envelope protein |
| p7 | 7 | Ion channel? |
| NS2 | 21–23 | NS2-3 protease component |
| NS3 | 70 | NS3/4 protease (protease domain) |
| | | NS2-3 protease (protease domain?) |
| | | NTPase |
| | | RNA helicase |
| | | DNA helicase? |
| | | RNA binding |
| NS4A | 8 | NS3/4 protease – co-factor |
| NS4B | 27 | Induction of ER 'membranous web' |
| | | NS5A phosphorylation |
| NS5A | 56–58 | Inhibition of IFN-α? |
| | | Inhibition of apoptosis? |
| NS5B | 65–68 | RNA-dependent RNA polymerase |

*See text for a discussion of the functions of the HCV proteins.

and deletion studies indicate that the C-terminal regions of both proteins are important for their interaction.[52]

The amino-terminal hydrophilic portion of the core protein contains a homotypic domain which allows the p21 core protein to interact with itself.[53] In contrast, core that was expressed as p23 did not interact with itself, indicating that the C-terminal domain may inhibit multimerization.[53] Both membrane-bound and membrane-free core exist in dimeric and multimeric forms. However, the processes involved in the assembly of the HCV capsid are not yet clear. Core proteins, which had the hydrophobic C-terminal domain deleted, also translocated to the nucleus. One of the three clusters of basic amino acids in the core protein, PRRGPR (aa 38–43) is similar to the nuclear localization signal found in human papilloma and human herpes viruses.[54] Intact core may therefore remain in the cytoplasm by being anchored to ER membranes and, if cleaved, it translocates to the nucleus. HCV core protein expressed *in vitro* has been shown to form nucleocapsid-like particles (NLPs).[55] The 75 N-terminal residues have been shown to be sufficient to assemble as NLPs *in vitro*.[56] NLPs have been detected in the nucleus when core was expressed in yeast[57] and in HCV-infected human liver.[58] Expression of core using a Semliki Forest virus replicon shows that core amino acid D111 is essential for the formation of NLPs.[58a]

Core protein has been shown to interact with lipid droplets via its central domain. Core proteins lacking part of the central domain (residues 125–144) were degraded by the proteosome.[59] Similar sequences in the core protein of GBV-B and in plant oleosin protein were shown to facilitate the association of these proteins with lipid droplets. It was shown that the HCV lipid binding domain could substitute for the equivalent domain in oleosin, allowing the hybrid protein to bind to lipid droplets.[60] After cleavage of the signal sequence between core and E1, the intramembrane cleaving protease, signal peptide peptidase (SPP), cleaves the signal sequence releasing core which can then associate with lipid droplets.[61] Core can be phosphorylated by both protein kinase A (PKA) and protein kinase C (PKC).[62] Serine residues ser-99 and ser-116 are the major phosphorylation sites for PKA and PKC, respectively, and the phosphorylation of ser-116 by both PKA and PKC may enhance the nuclear localization of core.[63] Ran/Gsp1p (a small GTPase) is required for the nuclear localization of core protein. However, the reduction in the nuclear localization of transcription factors, which normally use Ran/Gsp1p, in cells that express core suggests that core nuclear localization may disturb the transport of cellular proteins into the nucleus.[64] The proteosome activator PA28γ was shown to bind to core and the deletion of the PA28γ binding region from core led to its export from the nucleus to the cytoplasm.[65] HCV core can regulate the activity of cellular genes, including c-myc and c-fos, the long terminal repeats of Rous sarcoma virus and HIV-1 and SV40 early promoters.[66] Core activation of transcription from the interleukin-2 (IL-2) promoters via nuclear factor of activated T cells (NFAT), suggests that core expression may have consequences for IL-2-mediated T-cell development.[67]

Different reports have presented evidence that core can stimulate or inhibit apoptosis. Core can bind to the lymphotoxin β receptor (LTβR) and activate NF-κB ac-

tivity by increasing the degradation of Ikappa B kinase (IKKβ).[68,69] Consequently both Fas and tumour necrosis factor-α-(TNF-α)-mediated apoptosis are inhibited. Expression of core in HepG2 and HeLa cells activated the NF-κB signalling pathway in a dose-dependent manner. The use of dominant negative forms of TNF receptor-associated factor (TRAF2) showed significant blockage of the activation of this pathway by core. This suggests that core protein mimics pro-inflammatory cytokine activation of NF-κB through TRAF2.[70,71] Cells expressing HCV core were also shown to undergo apoptosis in response to stimulation with anti-Fas monoclonal antibodies[72] and core protein induced cell death via TNF signalling pathways[73] primarily by facilitating Fas-associated death domain (FADD) recruitment to TNFR1.[74] The expression of core was also shown to downregulate the expression of pRb and correspondingly upregulate the expression of E2F-1, which may sensitize cells to apoptosis.[75] Aggregation of Fas receptors by cytoplasmic core was shown to facilitate apoptosis in Jurkat cells.[76] By contrast, core expression has also been reported to inhibit TNF-α-induced apoptosis[77] and increase the level of bcl-x1 mRNA, which may inhibit apoptosis.[78] Core has also been implicated in cell transformation. Core has been shown to bind to p53 and enhance the expression of p21 (waf1/Cip1) indicating that this binding may lead to p53 activation.[79,80] Core inactivates a bZIP nuclear transcription protein, LZIP, by sequestering it in the cytoplasm and subsequently potentiating cellular transformation in NIH 3T3 cells.[81] Core expression is also able to immortalize primary human hepatocytes.[82] An increase in p53 and a reduction in c-myc were observed in core immortalized hepatocytes treated with an antisense core gene leading to cell death via apoptosis. This indicates that core is responsible for the regulation of the immortalized phenotype.[83] The expression of HCV core protein inhibits the replication of HBV.[84] The interaction of HCV core with the HBV X-protein suppresses HBV gene expression and the interaction of HCV core with HBV polymerase inhibited the formation of HBV virions.[85]

Members of the DEAD box family of RNA helicases, CAP-Rf,[86] DDX3[87] and DBX,[88] were shown to bind to core by the use of the yeast two-hybrid system, suggesting the possibility of core inhibiting host cell translation or other functions that involve RNA metabolism.

Recent evidence indicates that there may be a novel HCV protein produced by a –2/+1 ribosomal frameshift in the core reading frame and that this could give rise to the previously observed p17.[89] Peptides corresponding to this F (frameshift) protein or ARFP (alternative reading frame protein) have been shown to react with serum antibodies from chronically infected HCV patients, which suggests that p17 is produced during infection.[89–91] Cell-based expression systems have also shown that this protein is present in the cytoplasm, particularly in the perinuclear region.[92] The function of this protein is not clear.

## Envelope proteins

HCV has two envelope glycoproteins, E1 and E2, of molecular weights 33–35 kDa and 70–72 kDa. E1 and E2 are essential components of the HCV virion envelope and they are necessary for virion entry into host cells by direct receptor binding and possibly for membrane fusion. Internal signal sequences upstream of the E1 and E2 proteins direct the polyprotein precursor to the endoplasmic reticulum (ER), where they are translocated into the lumen, and after signal sequence cleavage[37,38,93] they remain anchored inside the lumen.[37,42] E1 and E2 are integral membrane proteins and are heavily glycosylated in the ER lumen with high-mannose carbohydrate chains, which contribute approximately one-half the mass of each of these glycoproteins.[37,38,42,93] The C-terminal region of E1 domain is very hydrophobic and terminates with the hydrophobic E2 signal sequence, which is cleaved by signal peptidase upon translocation into the ER lumen. Thus, the E2 signal sequence may serve, at least in part, as the E1 anchor in a manner reminiscent of the flaviviral envelope proteins.[44–46] Similarly, the signal peptidase cleavage between core and E1 results in the attachment of the hydrophobic E1 signal sequence to the C-terminus of the core protein.[37,38] Both E1 and E2 contain internal transmembrane domains (TMDs). Expression of E1 or E2 alone showed that they localized to the ER in a similar way to the E1E2 complex, and chimeric proteins which were constructed with E1 and E2 TMDs, e.g. with the ectodomains of CD4 or CD8, were also shown to localize to the ER. These TMDs are sufficient to localize E1 and E2 to the ER, to serve as membrane anchors and are involved in heterodimerization of E1 and E2.[94,95] The anchor domains of E1 and E2 are similar in structure to those of other members of the family *Flaviviridae*. They comprise two short stretches of hydrophobic residues separated by a short region which contains charged amino acids. Replacement of these charged amino acids in the HCV envelope glycoproteins led to alterations in the functions performed by the TMDs, i.e. protein anchoring, ER localization and assembly of glycoproteins, indicating that these functions are tightly linked together.[96] E1 and E2 proteins can be co-precipitated by antibodies against E1 or E2 when they are expressed together, indicating that these two proteins are associated in HCV-infected cells. However, little evidence for intermolecular disulfide bonding was observed between E1 and E2.[97,98] Furthermore, E1 in which cysteine residues have been replaced by other amino acids still forms aggregates with E2 by non-covalent (native) interactions. Analysis of the regions of E2 responsible for interaction with E1 shows that the TMD is critical for native com-

plex formation.[99] Before signal sequence cleavage the TMDs of E1 and E2 form a hairpin-like structure. After signal sequence cleavage of E1 and E2 in the ER, there is a dynamic change in the orientation of the C-terminal of these TMDs from luminal to cytosolic. Thus, by reorientating their C-terminals towards the cytosol, signal sequences at the C-terminal of E1 and E2 contribute to new functions, i.e. protein anchoring, E1E2 heterodimerization and ER retention.[100]

Immunostaining and electron microscopy showed that the E1 and E2 were predominantly located in the ER rather than at the cell surface, suggesting that there may be mechanisms for retention of these proteins in this compartment and that they are not translocated beyond the cis-Golgi.[101] Analysis of E2 expression by immunofluorescence and nocodazole treatment indicates that E2 does not cycle through the Golgi but is instead retained by the ER. Further evidence for the retention of HCV E2 by the ER is indicated by the absence of modifications to E2 glycans by Golgi enzymes. In addition, recombinant CD4, which contains the TMD of E2, is also retained in the ER without cycling through the Golgi.[102] E1 and E2-NS2 are rapidly released from the polyprotein by host cell signal peptidases and autocatalytic cleavage at the NS2/NS3 site. These primary cleavage products are core glycosylated and appear heterogeneous. In addition, the complex glycans that are usually acquired in the trans-Golgi do not appear in the E1 or E2.[103] The fact that the HCV glycoproteins do not appear to migrate further than the cis-Golgi may indicate that, like other Flaviviridae, the HCV buds from the endoplasmic reticulum and is released from cells via the endocytosis pathway. The absence of HCV glycoproteins in the plasma membrane should also reduce host immune responses and help to maintain chronic infections. However, immunofluorescence showed that 93% of chronically infected HCV patients had antibodies to E2 expressed in a Chinese hamster ovary cell line.[104] Most of these antibodies were against the hypervariable region 1 (HVR-1) of E2, which spans aa 384–414. Co-expression of the ER chaperones calnexin, calreticulin and BiP shows that they all interact with E1E2 heterodimers. Tunicamycin treatment indicates that these interactions are due to N-linked oligosaccharides on the HCV proteins interacting with the chaperones. Calnexin appears to play a role in the productive folding of the HCV glycoproteins, whereas calreticulin and BiP are involved in non-productive folding pathways.[105,106] Calnexin may therefore help to retain the E1E2 glycoproteins in the endoplasmic reticulum and thus prevent their migration to the plasma membrane and facilitate their interaction with the nucleocapsid.[103,107]

VSV pseudotyped with E2 showed that the HVR-1 binds to cell surface proteoglycans.[108] E2 glycoprotein was shown to bind to cell surface heparin sulfate proteoglycans (HSPG) and liver-derived highly sulfated heparin sulfate specifically inhibited cellular binding and entry of pseudotyped virions in a dose-dependent manner. Binding of E2 to HSPG may be the first step in the interaction between HCV and the cell surface, resulting in receptor-mediated entry and initiation of infection.[109] Analysis of antibodies to E1, E2 and HVR-1 in chronically infected chimpanzees indicates that there are low levels of detectable antibodies to E1 (22%) and E2 (15%) and HVR-1. Changes in the sequence of HVR-1 were analyzed by passaging HCV-infected human sera in chimpanzees. Subsequent analysis of the sequence complexity in the chimpanzees showed that there was much lower sequence diversity generated in chimpanzees than in humans and that very little change in sequence was shown during serial passage in chimpanzees. This indicates that the appearance of non-synonymous mutations is due to immune selection pressure rather than being an incidental result of HCV replication.[110,111] Sequence analysis of 460 HVR-1 clones, isolated at different times from six HCV-infected humans receiving interferon-α (IFN) therapy indicated that despite strong amino acid variability, the conformation of HVR-1 was highly conserved, with an overall basic stretch of amino acids located at specific sequence positions. This suggests that HVR-1 may be involved in interaction with negatively charged molecules, e.g. lipids, proteins or glycosaminoglycans (GAGs), and that the interaction with GAGs may have a role in host cell recognition and attachment.[112]

E2 protein binds to CD81, a tetraspanin which is present on the surface of B lymphocytes and hepatocytes. The binding region of E2 was mapped to the extracellular loop on CD81. Recombinant CD81 molecules which contain this loop were able to bind HCV and anti-HCV antibodies inhibited virus binding to CD81.[113] A truncated soluble form of E2 was shown to bind to CD81 but not other members of the tetraspanin family, e.g. CD9, CD63 and CD151. Binding of CD81 by soluble E2 induced the aggregation of lymphocytes and inhibited B-cell proliferation[114] and blocked natural killer (NK) cell activation, cytokine production and cytotoxic granular release and proliferation.[115,116] This suggests that E2 inhibition of cells of the immune system may be an evasion strategy by HCV to establish chronic infection. However, the interaction of E2 with CD81 showed that only 30% of CD81 molecules had become internalized after 12 hours, indicating that CD81 has a poor capacity to mediate virus entry[117] and the expression of human CD81 in transgenic mice did not confer susceptibility to HCV infection on them.[118] This indicates that CD81 is not the main/only receptor for HCV.

Infectious HCV pseudotyped retroviruses which displayed functional E1E2 glycoprotein complexes were shown to enter cells via a pH-dependent endocytosis

pathway. Expression of CD81 in non-permissive CD81-negative hepatocarcinoma cells restored susceptibility to the pseudotyped retrovirus infection. However, receptor competition assays show that scavenger receptor class B type 1 (SR-B1) is also required for the infection of CD81-expressing cells. Competition with soluble E2 could also prevent infection with the pseudotyped retrovirus. This suggests that CD81 and SR-B1 may both be important for the entry of HCV into hepatocytes.[119] E2 binds to dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and liver endothelial L-SIGN via high-mannose glycans. Competition with mannan and carbohydrate recognition domain-specific antibodies interferes with this binding.[120,121] Soluble E2 and pseudotyped retroviruses that express both E1 and E2 bound efficiently to DC-SIGN and DC-SIGNR expressed on cell lines and primary human endothelial cells. However, no binding to other C-type lectins was observed. The interaction of E2 with DC-SIGN and related lectins may help to deliver HCV to the liver and possibly modulate the activity of dendritic cells.[122] The possible role of E1 and/or E2 in membrane fusion is unclear. However, computational analysis suggests that HCV E1 and pestivirus E2 glycoproteins are truncated class II fusion proteins.[123]

E2 contains a sequence identical to the phosphorylation sites of IFN-induced protein kinase R (PKR) and eIF2-α, a target for PKR. E2 expression blocked PKR kinase activity and its subsequent inhibitory effects on protein synthesis and cell growth.[124] E2 also binds to and inhibits PKR-like ER resident kinase (PERK) in a similar manner to its inhibition of PKR. By relieving the inhibition of translation caused by these kinases, E2 expression may also reduce ER stress, possibly promoting the persistence of HCV infection.[125]

### p7

Between E2 and NS2 there is an additional small polypeptide, p7, which is a 63 aa integral membrane polypeptide and comprises two transmembrane domains connected by a cytoplasmic loop and intraluminal amino- and carboxyl-terminal tails.[126,127] It is mainly located in intracellular membranes, but it is not yet clear whether these are primarily associated with the endoplasmic reticulum[126] or the mitochondria.[128] Studies *in vitro* have shown that p7 acts as a calcium ion channel[129,130] and can be inhibited by amantidine. HCV p7 has also been shown to be able to substitute for the influenza ion channel in a cell-based assay.[129] p7 appears to be essential for infection, as deletion or substitution mutations in the p7 region of an infectious HCV cDNA clone were not viable in chimpanzee infection studies.[127] Studies on genotype 1a and 2a chimeras of p7 also indicate that the intraluminal tails may interact with other HCV components in a genotype-specific manner.

## Replication of the virus

### Non-structural proteins

The organization of the non-structural (NS) protein-coding region of HCV (Fig 24.2) resembles those of flaviviruses and pestiviruses. Small amino acid sequence motifs conserved among proteases, helicases and replicases are colinear in all three types of viral-encoded polyproteins[3,8] and, despite the absence of extensive, overall primary sequence homologies in this region, the hydropathicity profiles of all three viral NS regions are similar, particularly for HCV and the flaviviruses.[3]

### NS3/NS4A protease

The NS3 protease domain contains a serine protease catalytic triad, his 1083, asp 1107 and ser 1165[131–133] (polyprotein numbering) and cleaves the HCV polyprotein at the junctions between NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. Protein sequence analysis localized the N-terminal residues (P1′) of NS4A (aa 1658), NS4B (aa 1712), NS5A (1773) and NS5B (2421)[131] and showed that small uncharged residues, e.g. ser or ala, appear to be preferred at P1′ and polar residues cys or thr are found at position P1 with a gly or asp found at position P5.[131,134,135]

NS4A was also shown to be important for NS3-mediated cleavage, particularly at the NS4B/NS5A site.[19,136–143] However, cleavage at the other non-structural protein junctions was variable in efficiency, with cleavage at the NS5A/NS5B junction being most efficient.[144–146] The N-terminal 181 residues of NS3 were found to be sufficient for the protease activity and it can cleave all of the non-structural protein sites in trans except the NS3/NS4A site.

Using a COS cell transfection system, it was shown that NS4A stabilizes NS3 and helps to localize it in the ER membrane.[147] The interaction of NS3 and NS4A requires the N-terminal 22 amino acids of the NS3 protein.[148] This was further demonstrated when a fusion protein, which contained the N-terminal 22 amino acids of NS3 fused to DHFR, also formed a stable complex with NS4A.[149] Co-expression and immunoprecipitation experiments showed that interactions between the NS4A and NS3 proteins could not be disrupted by non-ionic detergent treatment, showing that the interaction was relatively strong. Further studies showed that the central region of NS4A is important for the NS4A-dependent activation of NS3. Hydrophobic residues, in particular ile 29 and ile 25, were shown to be important for the NS4A activity. Peptides covering regions 21–34 of NS4A were able to substitute for intact NS4A in trans cleavage reactions, and mutants which destabilize the NS3–NS4A interaction abolish the NS4A-mediated stimulation of NS3.[150] Analysis of more than 250 HCV genomes showed that the threonine residue at aa 631 (T631) is strictly con-

served in the P1 position at the NS3–NS4A autoproteolysis junction. Wild-type T631 NS3-NS4A constructs require the central co-factor domain of NS4A to be present (aa 21–34). By contrast, T631C mutant protein underwent self-cleavage in the absence of the co-factor. Replicons derived from T631 mutants showed a reduced level of colony formation. T631 may slow processing at the NS4A cleavage site by ensuring proper interaction with the NS4A co-factor prior to cleavage with the polyprotein and prevent subsequent product inhibition by the NS3 C-terminus.[151]

Kinetic cleavage analysis of P6-P4′ peptides corresponding to all the intermolecular cleavage sites of the non-structural region of the HCV polyprotein had the following order of cleavage: NS5A/5B > NS4A/4B >> NS4B/5B. This indicates that the primary structure of the cleavage site is an important determinant for the efficiency with which each site is cleaved.[152] Purified NS3 protease domain (aa 1027–1281) efficiently cleaves a 17 mer peptide corresponding to the NS5A/5B junction. The addition of NS4A peptide amino acids 1673–1692 increases the Kcat from 2 to 15.8 minutes$^{-1}$ and decreases the Km from 250 to 99 μM.[153] Product inhibition of NS3 protease was observed with peptides corresponding to the NS4A/NS4B, NS4B/NS5A and NS5A/NS5B cleavage sites. No inhibition was observed with peptides corresponding to the NS2/NS3 cleavage site.[154] NMR spectroscopy data show that inhibitor peptides based on N-terminal cleavage products of NS3 peptide substrates bind to the protease in a well-defined and extended conformation,[155] and they appear to induce stabilization of the enzyme by causing tightening of the interdomain interaction in NS3[156] by an induced fit mechanism.[157]

Alignments of the NS3 protease of HCV, and GBV-A, B and C hepatitis viruses[9,10] with the 2A proteases of picornaviruses, showed the conservation of zinc binding domains in all of these proteases. Cys 97, cys 99, cys 145 and his 149 are likely to represent the zinc binding motif in the NS3 protease. Changes in the nuclear magnetoc resonance (NMR) signals of his 149 between pH 7 and 5 suggest that there may be structural changes at the metal binding site switching from a 'closed' to an 'open' conformation.[158] No similar zinc binding domain was found in NS3 of other members of the Flaviviridae family.[159] The presence of a zinc atom within this binding site appears to be necessary for structural integrity and activity of the enzyme, which is similar to the chymotrypsin-like 2A cysteine proteases of picornaviruses, indicating that NS3 is a member of a novel class of zinc binding chymotrypsin-like proteases.[159,160]

X-ray crystallography at 2.2–2.7 Å resolution was carried out with recombinant NS3 protease domains corresponding to the HCV BK[161,162] and H[163] strains. The catalytic triad of his 57, asp 81 and ser 139 of HCV is highly conserved and the protease domain folds in a chymotrypsin-like protease fashion, which includes two beta barrel domains and four short alpha helices with the catalytic site located in the crevice between the beta barrel domains. The substrate binding pocket is shallow, non-polar, with few features and is formed by the side chains of invariant residues phe 154, ala 157 and leu 135 and has a similar structure to the predicted S1 pocket.[135] The first 30 amino acids of the N-terminus of the NS3 protease extend away from the protein and contain β-strands that interact with neighbouring molecules. The interaction of NS3 with NS4A[161] leads to the ordering of the N-terminal 28 residues of the protease into a beta strand and an alpha helix and also causes local rearrangements which are important for the production of a catalytically favourable conformation of the active site.[162,163] A comparison of NS3 protease and NS3 protease–NS4A complex by NMR also indicates that there are large structural rearrangements of the strand loop regions formed by residues V51-D81 as a result of the NS4A binding.[164]

There have been many attempts to develop small molecule inhibitors against the NS3 protease, e.g. a hexapeptide inhibitor of NS3 protease was shown to have similar inhibitory properties to IFN-α in the treatment of replicon cells (see section on Replication models for HCV below). The half-life of HCV RNA was shown to be about 11 hours with this protease inhibitor and 14 hours with IFN-α treatment.[165] BILN 2061 is a small molecule that inhibits NS3 protease. It was shown to inhibit NS3 both *in vitro* and *in vivo*. Patients treated with BILN 2061 for 2 days had a large decrease in HCV RNA plasma levels.[166] However, the development of resistance variants to protease inhibitors can be problematic, e.g. replicon cell lines could be selected so that they became resistant to the presence of these inhibitors and sequence analysis revealed that point mutations in the protease were responsible for this resistance.[167] A comparison of two protease inhibitors, VX950 and BILN 2061, showed that point mutations led to resistance to both of these protease inhibitors. However, the major BILN 2061 resistance mutations, at asp168, are fully susceptible to VX950, whereas mutants resistant to VX950 at ala 156 remain sensitive to BILN 2061.[168] A number of approaches have also been used to try to develop novel inhibitors of NS3 proteases. A variable domain antibody fragment derived from a phage display library was a potent inhibitor of NS3 protease,[169,170] and the characterization of a mini-antibody inhibitor of NS3 protease led to the development of a cyclic peptide which inhibits NS3 protease.[171] Recently, a novel set of NS3 protease inhibitors was developed based on *Drosophila* serpin SP6 reactive site loop. These were shown to bind to NS3 via the induced fit behaviour of the catalytic site and inhibit NS3 protease.[172]

An NS3–NS4 complex was isolated from transfected COS cells and the effect of polynucleotides on the protease, and the NTPase and helicase (see below) was examined. The addition of poly(U) increased the activity of the protease fivefold, showing that there is interaction between the protease and NTPase/helicase domains, and that this interaction is likely to be important during virus infection. The apparent enhancement of the protease activated by poly(U) may also help to localize the processing of the NS5A/5B to the 3′-UTR where replicase assembly and minus-strand RNA synthesis are initiated.[173]

## NS3 NTPase/helicase

The C-terminal 442 aa of the HCV NS3 protein contains a DECH amino acid sequence motif,[3,174] which is characteristic of the DExH subfamily, of DEAD-box superfamily-2 NTPase/RNA helicases.[175,176] HCV NS3 NTPase activity can be stimulated by polynucleotides in a similar fashion to those of pestiviruses and flaviviruses. Comparisons of the binding of recombinant NS3 proteins showed that the helicase domain had to be present for poly(U) to bind to NS3. Recombinant NS3 helicase domains display RNA unwinding activity in the presence of divalent cations, $Mn^{2+}$ or $Mg^{2+}$ and adenosine triphosphate (ATP) in strand displacement assays. NS3 helicase can unwind RNA strands from substrates which contain 5′ and 3′ single-stranded regions (5′/3′) or substrates containing only 3′ single-stranded regions (3′/3′) but not substrates which contain 5′, single-stranded regions only (5′/5′) or on substrates lacking single-stranded region.[177] This indicates that the HCV helicase, like most RNA helicases, is unidirectional, in a 3′ to 5′ direction.[178] HCV NS3 helicase was also shown to efficiently unwind DNA/DNA and DNA/RNA substrates. The significance of DNA helicase activity is not clear, but it is possible that it can affect the unwinding of cellular nucleic acid, and this in turn could have some role in viral pathogenesis or persistence.[179] Full-length NS3, in an NS3–NS4A complex isolated from transfected COS cells, had a pH optimum of 7.5–8.5 for NTPase activity, compared with the previously observed optimum of pH 6.5 for the NTPase/helicase domain expressed on its own. The maximum activity of the NTPase was obtained when poly(U) was present. The concentration of poly(U) required for stimulation of the NTPase in the NS3–NS4A complex was up to 1000 times less than that required for recombinant proteins that contain only the NS3 NTPase/helicase domain. This indicates that the protease domain is important for efficient activity of the NTPase, indicating that there is interaction between the protease and helicase domains, and suggests that this interaction is likely to be important during virus replication.[180]

A 2.1 Å resolution of the structure of the HCV NS3 RNA helicase domain suggests that initial recognition involves interaction of the 3′ end of single-stranded substrate RNA with a conserved arginine-rich region on the RNA binding domain of NS3.[181] NS3 interacts efficiently and specifically with the 3′ ends of both positive- and negative-stranded RNA. Interaction with the 3′ negative-strand RNA appears to involve binding to a stem-loop structure (nucleotides 5–20 from the 3′ end). Deletion of this structure almost totally impaired NS3 binding. Binding to the 3′ positive-strand RNA involves the entire 3′ region.[182] In addition, RNA aptamers that were selected by their ability to bind to and inhibit HCV NS3 helicase were shown to have similar structures to the 3′ UTR of HCV genomic RNA.[183]

Mutational analysis of conserved regions in the helicase and NTPase domains of NS3 has shown that these residues are crucial for both NTPase and helicase activity,[184,185] indicating that these two activities are coupled. Mutational analysis also showed that some mutants that had lost NTPase activity, K1236A (in the GxGK motif) and D1316A (in the DExH motif) could still bind RNA efficiently. However, R1490A (in the QRxGRxGR motif) was defective in NTPase and could no longer bind dsRNA. This suggests that the conserved motifs cooperatively constitute a larger fuctional domain rather than acting as several independent domains.[186]

NS3 RNA helicase undergoes conformational changes, on binding of NTPs or ssRNA and after NTP hydrolysis, which drives helicase translocation along a nucleic acid. Binding of an NTP reduces the affinity of NS3 for ss nucleic acid but increases its affinity for ds nucleic acid. Therefore, the binding energy of NTP is used to bring NS3 out of a state in which it is tightly bound to ss nucleic acid, to facilitate translocation along the unwound duplex nucleic acid, whereas NTP hydrolysis and product release promotes tight binding to the ss nucleic acid.[187,188] Kinetic analysis suggests that NS3 helicase may function as a dimer,[189] and one study indicates that NS3 complexed with NS4A has a higher RNA helicase activity than NS3 alone.[179] HCV NS3 appears to be post-translationally methylated at R1488 in QRRGRTGR1493G motif IV. However, although methylation of specific arginine residues has been shown for a number of RNA binding proteins, the significance of this methylation for NS3 is unclear.[190] Adaptive mutations in the NS3 helicase (residue 470) have been shown to transform inactive replicon constructs into fully active RNA replicons. The mutations R470M for HCV BK and P470L for HCV 77 gave optimal activity.[191]

Monoclonal antibodies[192,193] and recombinant antibody fragments[194] specific for HCV NS3 protein abolished the helicase activity and an scFv, expressed in HCV-infected human hepatocytes by using adenovirus delivery of the

scFv coding sequence, resulted in a reduction in HCV genomic RNA.[195]

## NS3 interactions with the cell

NIH3T3 cells transfected with cDNA corresponding to the NS3 protease domain became transformed and these transformed cells were able to form tumours when injected into nude mice.[196] NIH3T3 cells which expressed the N-terminal region of NS3 were also shown to be resistant to actinomycin D-induced apoptosis. Induction of p53 during actinomycin D treatment was observed to be weaker in these cells than in untransformed cells. This suggests that the NS3 N-terminal domain has a suppressing effect on apoptosis by decreasing the amount of p53.[197] The N-terminus of NS3 (aa1055–1200) was shown to interact with the C-terminus of p53 (aa 301–306) and form a complex.[198] Full-length NS3 and a C-terminal truncated NS3 region (aa 1027–1459) were co-expressed in cells with p53. In the absence of expressed p53, full-length NS3 was found to be localized in the cytoplasm and truncated NS3 in the nucleus. In the presence of co-expressed p53, both proteins were co-localized in the nucleus. This indicates that p53 enhances the nuclear localization of NS3.[199] Staining of hepatocytes from HCV-infected patients with anti-NS3 antisera shows that while NS3 is mainly present in the cytoplasm, in a minority of infected cells NS3 was also present in the nucleus.[200] NS3 can bind Sm-D1, a small nuclear ribonucleoprotein (snRNP) complex component, associated with autoimmune disease and in cells overexpressing Sm-D1 there is an increase in the level NS3 in the nucleus.[201]

Examination of the NS3 aa sequence indicated a region of 14 amino acids (aa 1487–1500) which is similar to the inhibitory site of the heat-stable inhibitor of cAMP-dependent protein kinase (PKA). Synthetic peptides which corresponded to this region and a recombinant NS3 protein have been reported to be competitive inhibitors of PKA, and NS3 expression was shown to inhibit the translocation of the C subunit of PKA into the nucleus after stimulation with forskolin and reduce the level of histone phosphorylation.[202] The intracellular presence of HCV NS3 could block the normal function of PKA C subunits by directly binding to them.[203] However, one report suggests that the inhibition of PKA by NS3 is due to ATP hydrolysis.[204] NS3 has also been shown to bind to PKC and histones.[205] The significance of NS3 binding to host cell proteins is as yet unclear. Some reports have provided evidence for the internal cleavage of NS3 into two proteins of 49 kDa and 23 kDa when the NS3 region alone or the entire HCV ORF was expressed in mammalian or insect cells. Site-directed mutagenesis indicates that a cleavage position is within a highly conserved domain of the RNA helicase domain.[206,207] HCV NS3/4A serine protease was shown to block the phosphorylation and effector action of IFN regulatory factor 3 (IRF-3), which is a key molecule in the antiviral signalling pathway. The abolition of protease activity by mutation or protease inhibitor restored IRF-3 activity after challenge with another virus, showing that the effector of IRF-3 blockage was attributable to the protease activity of NS3.[208]

## NS5B

The NS5B protein coding sequence has primary sequence motifs, in particular 'GDD', which are conserved among all single-chain viral RNA-dependent RNA polymerases (RdRps)[3,7] and NS5B has been shown to have RdRp activity.[209] X-ray crystallography shows that most of the NS5B protein (the first 530 aa) forms a catalytic domain with 'fingers' and 'thumb' subdomains encircling the enzyme active site, in the 'palm' subdomain, typical of single chain polymerases.[210] X-ray crystallography also indicates that the fingers domain has a long binding groove which guides template RNA to the catalytic site[211] and an NTP binding region close to the active site.[212] The initiation of *in vitro* synthesis of RNA, using a template corresponding to the HCV 3' X region by NS5B, indicates that it is localized to the pyrimidine-rich region of stem I[213] and that *de novo* synthesis favours initiation from the +1 position to maintain the integrity of the template.[214,215] The C-terminal 21 amino acids of NS5B form an alpha-helical transmembrane domain that anchors it to the cytosolic face of the ER.[216] Mutational analysis showed that replicons lacking this transmembrane domain were unable to replicate, and the treatment of replicon cell lines with a synthetic peptide corresponding to the NS5B C-terminal domain prevented the membrane association of NS5B and reduced the level of replicon RNA, indicating that the association of NS5B with ER membranes facilitates RNA synthesis.[217]

Two non-nucleoside analogue inhibitors were shown to bind to the base of the thumb subdomain near its interface with the C-terminal extension of NS5B, suggesting that they interfere with a conformational change essential for the activity of the polymerase.[218] X-ray crystallographic analysis of the C-terminal region (aa 545–564), upstream from the membrane anchor, shows that it forms a hydrophobic pocket in the putative RNA binding cleft. However, deletion of this region enhanced the rate of RNA synthesis by up to 50-fold, indicating that it may have an important role in regulating RNA synthesis.[219] Two residues (glu 18 and his 502) that lie outside the catalytic site are critical for RdRp and are also necessary for the oligomerization of NS5B[220] which occurs in the presence of template RNA.[212,221] A ubiquitin-like protein, hPLIC1, appears to directly interact with NS5B, suggesting that it may be a regulator of HCV RNA replication.[222] Yeast two-hybrid system analysis has shown

that alpha actinin binds to NS5B, and this has been confirmed in RNA replicon cell lines. The use of RNA interference to ablate the synthesis of alpha actinin has also shown that it may be important for RNA replication.[223] The development of RdRp inhibitors is an important approach to the development of antivirals against HCV. Binding studies have shown that a heterocyclic agent ('compound 4') inhibitor of the polymerase, binds to NS5B but does not inhibit the binding of RNA, suggesting that it inhibits the initiation of RNA synthesis.[224] The addition of 2′ modified nucleoside analogues to RNA replicon cell lines inhibited HCV RNA replication. The analogues were incorporated into RNA but prevented the addition of the next nucleotide.[225]

## NS5A

NS5A occurs as a 56-kDa protein and as a 58-kDa hyperphosphorylated form of the 56-kDa protein.[226] The production of p58 is enhanced by the presence of NS4A[226] or NS4B,[227] which may modulate the activity of a cellular protein kinase. Peptide analysis of phosphorylated NS5A identified ser 2321 as the major phosphorylation site. The regions flanking ser 2321 are proline-rich, suggesting that a proline-directed protein kinase may be responsible for the majority of NS5A phosphorylation.[228] NS5A can be hyperphosphorylated when it forms part of a continuous NS3-NS5B polyprotein but not when the non-structural proteins are expressed separately in trans.[227] Deletion analysis has shown that the N-terminal 30 aa of NS5A form an amphipathic alpha helix which functions as a membrane anchor[229] and is conserved across different HCV isolates.[230] NS4A co-localizes with ApoA1 in the Golgi and with lipid droplets in the cytoplasm as well as the perinuclear membrane.[231] NS5A has been implicated as having a role in inhibiting host cell responses to IFN.[232] A region of genotype 1b NS5A termed the IFN sensitivity determining region (ISDR) together with an additional 26 aa carboxyl to the ISDR are required for NS5A to bind to the protein kinase R (PKR – which is induced by IFN-α) dimerization domain. This has the effect of disrupting kinase dimerization, thereby inhibiting its ability to phosphorylate eIF2-α and to shut off translation.[233] However, the role of NS5A in the inhibition of IFN-induced PKR remains controversial. For example, an analysis of mutations of NS5A in HCV-infected patients treated with INF-α did not show a statistically significant correlation of changes to the ISDR with the patients' responses to IFN-α therapy.[234] A cleaved form of NS5A that had lost both the N- and C-terminal portions (aa 155–389) was localized to the nucleus and showed transcriptional activity when fused to a Gal4A DNA binding domain, suggesting a role for NS5A in transcriptional regulation.[235] Binding studies and confocal microscopy indicate that NS5A can bind to p53[236] and suppress p53-mediated transcriptional transactivation and apoptosis.[237]

NS5A inhibits growth factor receptor bound protein 2 (Grb2) by the binding of NS5A polyproline motifs to Grb2 Scr homology 3 (SH3) domains[238] and inhibiting Grb2-mediated mitogenic signalling.[239] It also acts at the same time on the phosphatidylinositol 3-kinase–AKT protein kinase B (PI3K-AKT) cell survival pathway by interacting with the p85 subunit of PI3K. This increases the level of phosphorylation and activity of AKT, which in turn provides protection against the induction of apoptosis in cell lines that express NS5A.[240] This may be important in HCV pathogenesis and viral persistence.[239] NS5A inhibited the mitogenic and stress-activated transcription factor activating protein-1 (AP1) by binding via polyproline motifs, thereby perturbing mitogenic signalling pathways in HCV-infected hepatocytes.[241] NS5A also binds to the SH3 domains of a number of other members of the Src family of tyrosine kinases, e.g. Hck, Lck, Lyn and Fyn.[242] However, the significance of these interactions is not clear. NS5A blocked TNF-α-mediated apoptosis[243] but not Fas-induced apoptosis in cultured cells.[244] Similar results were obtained in NS5A transgenic mice, which were protected against hepatic apoptosis after injection by TNF-α but not when hepatic apoptosis was induced by injection with anti-Fas antibodies.[245] The binding of NS5A to TNF-α receptor associated death domain protein (TRADD) in these transgenic mice suggests that the protection against TNF-α may be by the inhibition of TRADD-mediated NF-κB activation.[245] However, this conflicts with other reports which show that expression of NS5A appears to activate NF-κB by tyrosine phosphorylation of IκB-α[246] and the translocation of NF-κB to the nucleus.[247]

## NS2

NS2 is a 21–23-kDa protein[248,249] forming part of the NS2-3 protease which is specific for the NS2/NS3 cleavage site.[249,250] Cleavage of the N-terminus of NS2 from the C-terminus of p7 is mediated by host signalase enzymes. Single amino acid changes of the NS2/NS3 cleavage site had little effect, and only mutations which altered the conformation of the region P5 to P3′, e.g. pro at P1 or P1′, had a major inhibitory effect on cleavage.[251] NS2 is a transmembrane protein in the ER,[252] and its association with the membrane may be required to assist in the proper folding of the nascent precursor for processing at the NS2/NS3 junction. Unlike most mammalian membrane or secretory proteins, the NS2 protein is translocated post-translationally, suggesting that changes occur in the folding of the protein, from an active autoprotease to a form that allows unfolding and translocation across the ER.[252] There are two internal signal sequences at aa 839–883 and aa 928–960 which target NS2 to the ER

where it appears to have multiple transmembrane domains, and it may have both the amino- and carboxyl-termini located in the lumen of the ER.[253] Different analyses of recombinant NS2-3 proteins indicate that it may be either a zinc-dependent enzyme, with a minimal protease catalytic region spanning aa 904–1206 that contains a zinc ion,[159,254] or alternatively, that it may be a cysteine protease with Cys993 and His 952 as a catalytic dyad.[255,256] NS2 may also have direct effects on host cells. It has been reported that NS2 binds to the death-inducing domain of the pro-apoptotic factor CIDE-B and inhibits the CIDE-B-induced release of cytochrome C from mitochondria and the subsequent caspase-mediated cell death mechanism.[257] In addition, the amino-terminal portion of NS2 has been shown to inhibit the expression of reporter genes controlled by liver and non-liver-specific promoter and enhancer elements, which indicates that it might have a role in altering host gene expression in chronic HCV infections.[258]

## NS4B

Confocal microscopy and proteinase protection assays indicate that NS4B is a cytoplasmically orientated integral membrane protein which is localized to the ER.[259] However, more recent data show that NS4B is a transmembrane protein with the carboxyl-terminus in the cytoplasm and the amino-terminus in the ER lumen.[260] The function of NS4B is not yet clear, however, the expression of NS4B can induce the formation of a 'membranous web' derived from the ER with which all of the HCV proteins[261] and genomic RNA are associated.[262] It is likely that this membranous web is the site of RNA replication in HCV-infected cells, as is the case for other *Flaviviridae*. There is also evidence that NS4B inhibits the traffic of proteins between the ER and the Golgi in infected cells,[263] that it inhibits protein synthesis[264,265] and that it modulates the hyperphosphorylation of NS5A.[227]

## The 5′ terminal UTR

The 5′ untranslated region (5′ UTR) of 341 nt length precedes the initiator methionine codon of the large ORF encoding the polyprotein precursor.[2–4,6,33–36] This is the only region of the HCV genome that shows substantial nucleotide sequence identity with other known viral genomes, exhibiting approximately 50% identity with the 5′ leaders of animal pestiviral RNA genomes[3,5,6] and up to 60% identity with the GBV-B 5′ UTR.[266] The 5′ UTR contains four domains (I–IV) which have extensive stem-loop structures (Fig. 24.3).[267,268] Domain IV extends beyond the 3′ UTR and contains the start codon of the polyprotein with a 3′ boundary between nucleotides +12 and +30,[269–271] and together with domains II and III forms the HCV internal ribosome entry site (IRES), which controls HCV protein translation. The HCV IRES differs, therefore, from the picornavirus model in having the authentic initiation codon located between upstream and downstream elements of the IRES.[270] Chimeric viruses in which the poliovirus IRES[272] or the BVDV 5′ UTR[273] was replaced by HCV 5′ UTR constructs have also confirmed that the HCV IRES functions *in vivo*, and that it will only function when the sequences downstream from the initiation codon are included. Electron microscopy of the IRES indicates that domains II, III and IV all form distinct regions within the molecule with a possible flexible hinge region between domains II and III. Pyrimidine tract binding (PTB) protein was also show to bind to domain III.[274]

The maintainance of the domain II stem-loop structure is necessary for the activity of the HCV IRES. However, this structure is relatively tolerant of sequence changes as long as the stem-loop is preserved.[268] NMR studies show that the free form of domain II is similar in shape to the 40S bound form.[275] Mutants that stabilize stem-loop IV reduce the translational efficiency of the IRES. Long-range interactions between nucleotides 24–38 and 428–442 in the stem-loop IV also reduce IRES-mediated translational efficiency.[276] Stem-loop IV may therefore regulate the translational efficiency of HCV, by interacting with viral proteins as part of a translational feedback mechanism similar to the translational control mechanisms found in bacteriophages. This would require stem-loop IV to melt before 40S ribosomal subunits could bind to the AUG and begin the process of translation.[277] In binding studies it was shown that core protein preferentially binds to oligonucleotides corresponding to the loop IIId domain of the 5′ UTR,[278] suggesting a possible site of interaction between the nucleocapsid and the genome. The La antigen has been shown to bind to the initiator AUG codon and to be a requirement for the initiation of translation, suggesting that La has a role in the selection of the AUG during the initiation of HCV polyprotein translation.[279]

40S ribosomal subunits form a stable pre-initiation complex with HCV IRES RNA without the requirement for initiation factors eIF3, eIF4A, eIF4B and eIF4F, which are required for cap mediated initiation in eukaryotes.[280] Chemical and enzymatic analysis of the structure of the IRES under physiological salt conditions shows that it folds into a unique three-dimensional structure in the absence of the translational apparatus and additional co-factors.[281] Analysis of the interaction of 40S ribosomal subunits with the HCV IRES by cryo-electron microscopy shows that domain IIId/e/f binds directly to 40S subunits; domain IIIb does not bind to to 40S subunits but is positioned such that it can bind eIF3; and domain II may be positioned in the E site of the 40S subunit such that it can facilitate the interaction of the 60S subunit with the 40S and initiate translation from the coding

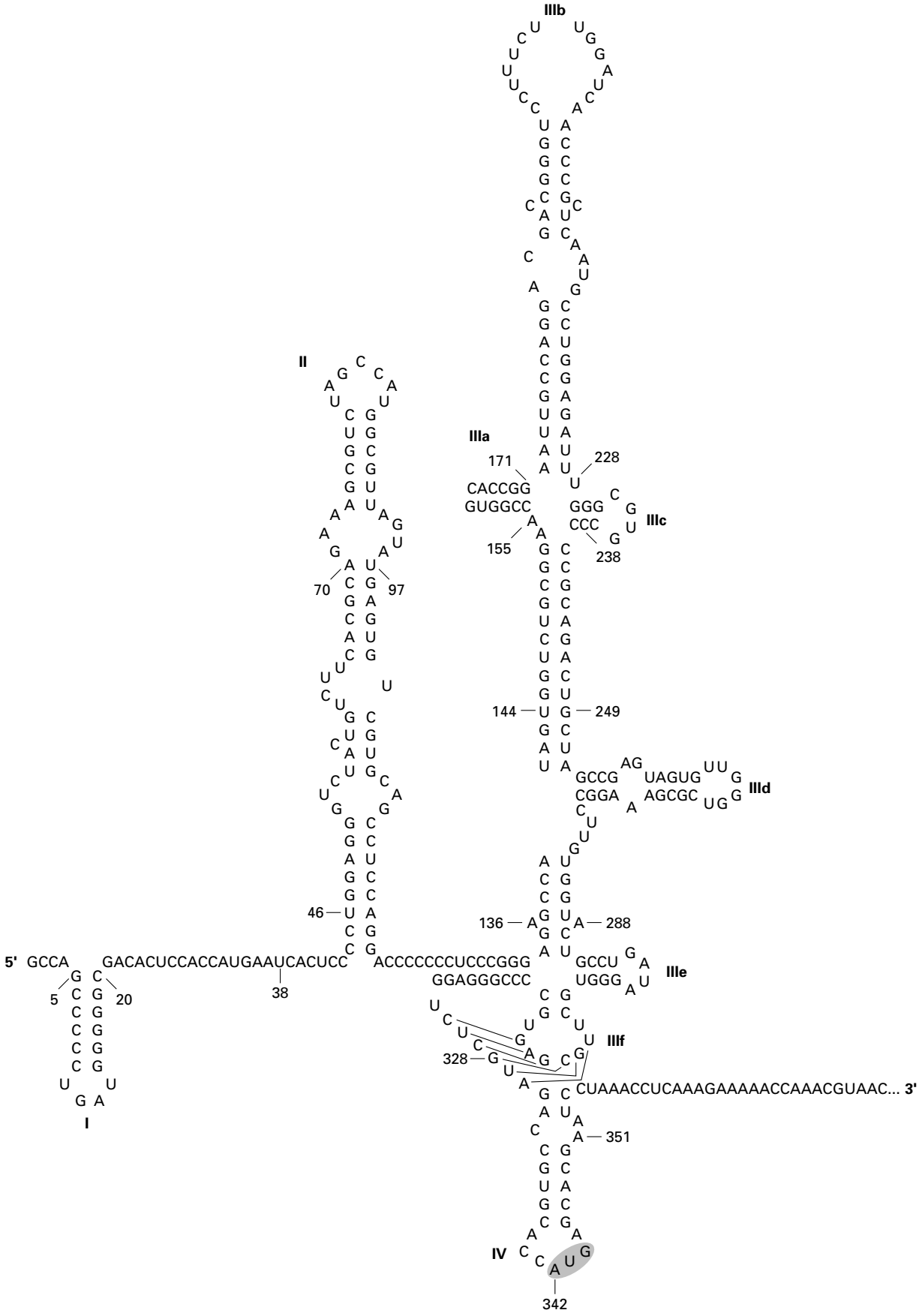


**Figure 24.3** Proposed secondary structure of the HCV RNA 5′ UTR. I–IV indicates the stem-loop domains. AUG (shaded) is the start codon of the polyprotein (after Honda *et al*.[268]). See text for a detailed discussion of the structure and function of the 5′ UTR.

sequence of the HCV genomic RNA.[282] Chemical and enzymatic modification analysis shows that domain IIIb and possibly domain IIIa binds to eIF3, and that domains IIIa/c/e/f all bind the 40S subunit with additional contacts between the 40S subunit and domains II and IV.[283] Cross-linking studies suggest that ribosomal proteins mediate the interaction of the 40S subunit with the IRES. In a UV cross-linking study ribosomal protein S5 could be linked to the IRES.[284] However, 4-thiouridine-mediated cross-linking linked several ribosomal proteins to the IRES, though not S5.[285]

Cultured lymphoblastoid cells inoculated with H77 serum led to the appearance of three substitutions, G107A, C204A and G243A, in the 5′ UTR,[286] all of which showed 2–2.5-fold greater translational activity in human lymphoblastoid cell lines compared with monocyte, granulocyte or hepatocyte cell lines or cell-free translation. The different translational efficiencies of quasi-species variants in different cell types from the same patient could help to explain why they are selected for in these different cells.[287,288] In RNA replicons, the first 125 nucleotides of the 5′ UTR are sufficient for RNA replication, but at a much lower level than when the intact 5′ UTR is present. Deletion of the 40 nucleotide sequence upstream of the IRES abolishes RNA replication, showing that sequences required for RNA replication also lie outside the IRES.[289] 5′ UTR sequences that had mutations in the first 43 nucleotides, and which also had compensatory mutations which preserved the stem-loop structure, were still functional in RNA replication.[290]

## The 3′ terminal UTR

The 3′ terminal untranslated region (3′ UTR) is located downstream of the stop codon terminating the large ORF encoding the viral polyprotein. The 3′ UTR comprises three structurally distinct RNA domains. From 5′ to 3′ these are: (1) an upstream variable region (VR) of about 40 nucleotides which varies considerably between different genotypes, (2) a long poly(U)-poly(U/UC) tract and (3) a 98 nucleotide (3′X) sequence which forms three stem-loop structures (SL1, SL2 and SL3) and terminates in a U residue and which is highly conserved between all HCV genotypes.[291–295] The 98 nucleotide 3′X sequence appears to be the authentic 3′ end of the genome and has been found in genomic RNA in serum and its complement has been found in anti-genomic RNA extracted from HCV-infected liver. Comparisons between the HCV 3′ UTR and those of flaviviruses and pestiviruses show little sequence homology. However, the 3′ UTRs of HCV and the GBV-B[9,292] appear to be similarly organized. The function of the 3′ UTR is not yet clear. It may play an important role in minus- and plus-strand RNA synthesis, in the packaging of viral RNAs or even in the regulation of protein translation. The highly conserved 3′X sequence may interact with viral or cellular proteins or RNA elements to initiate these processes. Mutational analysis and UV cross-linking showed that PTB binds to the pyrimidine-rich region of the 3′ UTR[296] and possibly to 3′X stem-loops 2 and 3.[295] A 35-kDa protein corresponding to heterogeneous nuclear ribonuclearprotein C (hnRNPC) was also shown to bind to this region.[296] In one study, the presence of a 3′X region which is capable of binding PTB has been shown to enhance the efficiency of translation from the HCV IRES three- to fivefold.[297] However, other studies have reported little effect of the 3′ UTR on IRES-mediated translation.[298,299] Infectivity studies in chimpanzees show that the 3′X sequence[300,301] and the poly(U/UC) sequences are essential. However, levels of virus replication similar to wild-type HCV were observed with a genome that had a 24 nucleotide deletion in the variable region.[300] RNA replicons that had the variable region deleted were able to replicate but at a much lower level. However, deletion of either the poly(U/UC) or the 3′X were not viable.[302,303] Full-length NS3 and the helicase domain of NS3 both bind specifically to the 3′ UTR, possibly as part of a replication complex. These proteins also bind to the 3′ UTR of negative-strand HCV RNA.[182]

## Replication models for HCV

PBMCs (peripheral blood mononuclear cells) from healthy donors were experimentally infected with HCV-positive sera, and using reverse transcriptase polymerase chain reaction (RT-PCR) it was reported that HCV negative-strand RNA was present in cells but not in cell culture supernatant.[304] However, highly stringent strand-specific RT-PCR, using a thermostable reverse transcriptase (rTth), detected negative-strand RNA in liver but not in PBMCs or other extrahepatic sites in HCV-infected chimpanzees and humans, indicating that HCV does replicate in liver but not in non-hepatic cells.[305] Similarly, there is uncertainty about many of the reports of HCV replicating in cell culture systems, e.g. HPBMa102-2, a human lymphocytic cell line,[286,306] Daudi cells, a human B-cell line[286] and the human T-cell leukaemia virus type 1-infected cell clone MT-2C,[307] as this very stringent rTth RT-PCR procedure for the detection of HCV negative-strand RNA was not used.

Primary chimpanzee hepatocytes were maintained in culture and it was shown that they could be infected with HCV *in vitro*.[308] Subsequently, RNA transcripts from full-length clones of HCV genotype 1a were found to be infectious after direct intrahepatic inoculation of chimpanzees.[309–312] An analysis of the sequences of HCV following the infection of chimpanzees by transcribed RNA from infectious cDNA clones indicates that the establishment of persistent infections is not due to changes in the HVR-1 region.[313] Serum from a chimpanzee infect-

ed after transfection with RNA from an infectious HCV cDNA clone was shown to be infectious when injected into a second naïve chimpanzee.[314] Similarly, virions derived from HepG2 cells transfected with full-length HCV RNA were also shown to be infectious in chimpanzees.[315]

The difficulties with chimpanzee-based models for HCV replication models mean that there is considerable interest in the development of alternative small animal models. Athymic nude mice which were implanted with human HCC from HCV-infected patients showed that HCV RNA was detectable in tumour cells as well as mouse liver and serum up to 42 days after the implant.[316] SCID mice carrying a plasminogen activator transgene (Alb-uPA) were engrafted with human hepatocytes which resulted in chimeric mouse-human livers. Following the inoculation of these mice with HCV-infected human sera, negative-strand HCV RNA and proteins could be detected in the chimeric liver nodules. Virus from these mice was shown to be capable of being serially passaged through three subsequent generations of mice with chimeric livers.[317] HCV-infected mice with chimeric human livers were infected with an adenovirus vector containing the gene for the BH3 interacting domain death agonist (BID), which had been modified to contain an NS3 cleavage site and consequently could be activated by the NS3-NS4A protease, thus triggering apoptosis. Mice with chimeric livers exhibited HCV-dependent apoptosis and showed a considerable decline in serum HCV titres after infection with the BID-expressing adenovirus.[318] A transgenic mouse which expresses the human poliovirus (PV) receptor was infected by chimeric PV/HCV in which the PV IRES was replaced by the HCV IRES. Chimeric PV/HCV virus replicates well in the liver but not in the brain, in contrast to control PV virus, which replicates in both of these tissues. This suggests that the tissue tropism of HCV may be partly determined by the activity of the IRES.[319] One report also suggests that tree shrews (*Tupia belangeri chinensis*) can be infected with HCV.[320]

Given its similarities to HCV, GBV-B may provide a surrogate model for HCV replication. Intrahepatic transfection of transcripts from a full-length GBV-B cDNA clone has also resulted in the development of severe hepatitis in tamarins. High viral titres were detected in these animals, indicating that GBV-B is a true hepatitis virus[321,322] and virions derived from the transfected animals could be transmitted to naïve tamarins.[322] Hepatocytes obtained from GBV-B-infected tamarins secreted virions and had high levels of cell associate RNA for up to 42 days in culture. NS3 protein could also be detected by immunofluorescence.[323] Infection of a tamarin has also resulted in a chronic infection.[324] GBV-B has been transmitted in marmosets and has also been shown to infect isolated marmoset primary hepatocytes.[325]

Subgenomic replicating HCV RNAs (RNA replicons) have become important cell-based models for HCV RNA replication. These were developed from full-length HCV genomes in which the sequence coding for core to p7 or from core to NS2 was replaced by a gene cassette which consisted of neomycin phosphotransferase (i.e. a 'neo' selectable marker and a downstream encephalocardiomyocarditis virus [EMCV] IRES). In this construct the neogene is translated under the control of the HCV IRES and the HCV NS proteins are translated under the control of the EMCV IRES. Huh7 cells transfected with RNA replicons are grown in culture medium which contains G418, which only allows the growth of cells lines that contain the replicating HCV RNA replicons.[326,327] It was originally thought that only Huh7 cells were able to allow the propagation of HCV RNA replicons, possibly indicating a requirement for essential human liver-specific 'factors'. However, more recently, subgenomic replicons have been shown to replicate in mouse hepatoma and non-hepatic human epithelial cells. This indicates that translation and RNA-directed RNA replication of HCV RNA do not depend on hepatocyte- or human-specific factors.[328,329] Fractionation and EM of NS proteins from RNA replicon cell lines showed that they were all associated with the ER. Morphological alterations of the ER in these cell lines, e.g. convoluted cisternae and paracrystalline structures, resemble alterations previously observed in liver biopsies of HCV-infected individuals and in flavivirus-infected cells (i.e. the membranous web).[330] Ribonuclear complexes (RCs) purified from these cell lines were shown to synthesize HCV replicon RNA *in vitro*. Replicative forms (RFs) and replicative intermediates (RIs) were synthesized from endogenous RNA templates and were shown to contain both minus- and plus-strand RNAs.[331] Pulse chase analysis showed that incorporation into ssRNA was chased into dsRNA, indicating that newly synthesized RNA could serve as a template for further rounds of RNA synthesis.[332] The activity of HCV synthesis was analyzed in digitonin-permeabilized replicon cells. It was shown that replication complexes were protected from access from nuclease and protease by membrane compartmentalization. It was also shown that only a small part of the NS proteins were resistant to protease action, suggesting that the majority of NS proteins do not form part of the compartmentalized replication complexes in replicon cells.[333]

## Heterogeneity of the HCV genome

There is substantial sequence variation in the HCV genome resulting from the lack of proofreading of NS5B RdRp and the subsequent accumulation of errors in replicating HCV genomic RNA. This means that, even in a single infected individual, the HCV genome does not exist as a homogeneous species. Rather, it exists as

a quasi-species of closely related but nevertheless heterogeneous genomes.[334] In addition, the process of host selection and adaptation of a rapidly mutating genome has led to the evolution of many distinct HCV genotypes. A large number of studies have been conducted on the phylogeny of HCV. Analyses of the 5′ terminal RNA sequence upstream of the large ORF indicated the existence of three main HCV types – 1, 2 and 3.[335] When the more variable NS3 and NS5 sequences of the ORF itself were included, HCV types 1 and 2 could be divided into subtypes a and b.[335] Comparison of the sequences of NS5 from around the world showed evidence of six major genotypes. These could be redivided into 11 different populations or subtypes.[336] In a different study, comparison of the E1 gene in 51 isolates from around the world showed that they could be divided into at least 12 genotypes.[337] To standardize the various systems that had developed in different laboratories, a new system for the nomenclature of HCV genotypes was proposed. Sequence comparisons were made between the NS5 region of HCV variants from a worldwide panel. The sequence similarities between members of different genotypes were 55–72%. Similarities between those within related subgroups ranged from 75% to 86% and individual isolates from each of the clusters showed 88% sequence similarity.[338,339] Six genotypes or clades (1, 2, 3, 4, 5, 6) with a variable number of subtypes (designated a, b, … etc.) for each of these genotypes were suggested, and this has now become the standard system for the nomenclature of HCV genotypes.[339] The core and E1 regions show similar ranges of sequence similarity, corresponding to those previously described for NS5. This indicates that there is little evidence for variants which are due to recombination between more than one genotype.[336] Sequence and genotyping studies indicate that the quite closely related subtypes, HCV-1a and HCV-1b, predominate in the USA and Japan, respectively, although less common types also occur in both countries.[335,340–348] In Europe, genotypes 1a, 1b, 2 and 3 and have been commonly observed.[334, 335,345–352] Type 4 HCV appears to predominate in Egypt and Zaire,[336] 1b, 2b and 2a in the Far East, 5a is the main genotype in South Africa, but was rarely seen elsewhere in the world.[358] While the average rate of change of the HCV genome within a single persistently infected individual has been estimated to be $1–2 \times 10^{-3}$ nt changes per site per year,[353–355] there is a much higher rate of change at the 5′ end of the E2 coding sequence.[353–356] This E2 hypervariable region (HVR-1) is the most variable region of the HCV polyprotein and is different in virtually every isolate studied so far.[350,357] The emergence of HVR-1 variants suggests that escape mutants in the HVR-1 region may play an important role in the development of chronicity.[358,359] Heteroduplex gel shift analysis was used to compare the quasi-species nature of sera from different patients. Analysis of the HVR-1 sequence obtained by PCR amplification showed that there were two kinds of quasi-species, simple quasi-species that had a homogenoeous gel shift profile and complex quasi-species that gave rise to multiple bands on gel shift analysis.[360] However, despite the high level of variability of the HVR-1, there are strong constraints which maintain similar physicochemical characteristics of HVR-1 from different genotypes.[112] The lack of patient response to IFN therapy has also been correlated with heterogeneity of the E2 HVR region,[361] which is consistent with the hypothesis that persistence of HCV is attributable to E2 HVR-1 escape mutants.[358] The HCV populations in chronically infected patients were shown to be composed of quasi-species of heterogeneous viruses with variations in the E2 HVR region. During IFN treatment some quasi-species disappeared and at the end of treatment reappeared, and a number of quasi-species which had originally been present as a minor fraction were selected.[362,363] Major changes in HVR-1 were also observed during treatment with IFN-α.[364] This indicates that there are differences in the sensitivity of different variants to IFN.

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# Chapter 25
# Epidemiology

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The hepatitis C virus (HCV) is one of the leading known causes of chronic liver disease. It is responsible for more than 50% of liver transplantation among adults in Western countries.[1] The prevalence of HCV infection averages around 3% of the world population, resulting in about 210 million HCV-infected persons (Table 25.1), nearly five times more than human immunodeficiency virus (HIV)-infected individuals. The main route of HCV transmission in the past was blood transfusion. The virus has been spread since the early 1960s, and incidence increased until the early 1980s, then levelled off and declined sharply in the 1990s after cloning of HCV in 1989 and subsequent development of sensitive antibody tests that allowed the universal screening of blood donors.[2] With the current safety of the blood supply, nosocomial transmission has become the predominant mode of health-care-associated spread of HCV.

Although universal screening of blood donors in developed countries and improvements in infection control measures following Centers for Disease Control and Prevention (CDC) guidelines have significantly decreased exposure to the virus,[3] which in the younger generations is confined to high-risk groups such as drug addicts,[4] the large reservoir of chronically infected individuals, the high evolutionary potential of the virus,[5–7] the lack of routine screening of donated blood in countries with developing or transitional economies, the use of traditional medicine and tattooing in some cultures, and the global expansion of the human population with increasing fluxes of immigration from endemic areas to less prevalent regions strongly support the hypothesis that HCV is still spreading throughout the world. In western countries, although the incidence of HCV infection may be decreasing, the prevalence of liver disease caused by HCV is on the rise.

## Transmission mechanisms and groups at risk for HCV infection

The routes of HCV transmission include **percutaneous** exposure to infected products (red blood transfusion, injection of blood derivatives, sharing needles and equipment), **nosocomial** (patient-to-patient during haemodialysis or other situations, patient to health-care worker by accidental needle-stick, health-care worker to patient after surgery), **non-apparent percutaneous modes** (tattooing, circumcision, ear and body piercing using inadequately sterilized equipment), **preventive health campaigns** (vaccination, microtransfusions in underweight newborns,[8] therapy for schistosomiasis under suboptimal hygienic conditions), **sexual** (in case of vaginal mucosa damage, multiple sexual partners and commercial sex workers) and **vertical transmission** (infants born to high viraemic HCV-positive mothers and

**Table 25.1** Estimated prevalence and number infected according to world population (2003)

| http://www.census.gov/cgi-bin/ipc/idbagg | Population 2003 | Prevalence of HCV (%)* | Infected population 2003 |
|---|---|---|---|
| Africa | 857 087 413 | 5.17 | 44 311 419.25 |
| Asia | 3 816 573 388 | 3.55 | 135 488 355.3 |
| America | 867 610 839 | 1.93 | 16 744 889.19 |
| Europe | 728 996 759 | 1.75 | 12 757 443.28 |
| Oceania | 31 919 758 | 1.88 | 600 091.4504 |
| Total (2003) | 6 302 188 157 | 2.856 | 209 902 198.5 |

*Averaging data from Table 25.4 of prevalence among the general population.

co-infected with HIV). HCV is not spread by breastfeeding, sneezing, hugging, coughing, food or water, sharing eating utensils, or casual contact.[9]

The predominant modes of transmission have changed over time and differ between and even within countries. Before implementation of blood testing for HCV, blood transfusion and intravenous drug use were the predominant modes of transmission in Eastern[10] and Western hemispheres.[11,12] Groups dependent on human blood products (haemophiliacs, haemodialyzed and polytransfused patients) have the highest prevalences. Nowadays, in economically developed countries, new HCV infections are mainly due to drug use, while in developing countries with high prevalence of HCV with all age groups affected, unsafe injections, folk and traditional medical procedures and contaminated medical equipment appear to be the major risk factors for acquiring HCV infection. Besides these major routes of HCV transmission, there is still a percentage of anti-HCV-positive patients (30–70% depending on the area), with undefined epidemiological risk factor. These patients probably acquired HCV by one of the rare and low prevalence but potential routes of transmission such as inapparent parenteral or permucosal exposures to HCV (e.g. surgical and dental procedures, tattooing, acupuncture, intranasal cocaine, perinatal, sexual, accidental needlestick, and household transmission).

## Parenteral transmission

The parenteral route of HCV transmission is responsible for one-third to two-thirds of hepatitis C cases and constitutes the most commonly recognized and best characterized transmission mechanism of HCV. Anti-HCV testing has largely confirmed that HCV is responsible for the vast majority of hepatitis cases in which transfusion of blood or blood components or obvious percutaneous exposure to blood is involved.[13] The incidence of HCV in some risk groups directly depends on the baseline prevalence of HCV in the general population, so that recipients of blood products obtained from a low prevalence area have a low incidence of infection.

### *Percutaneous transmission*

#### *Transfusion recipients*

Before the implementation of mandatory anti-HCV screening in 1990, there was a wide range of transfusion-associated hepatitis C (TAH-C) incidences in different geographic areas, ranging from 0.5% in England,[14] 1.1% in Australia,[15] 3–4% in the United States,[16] 7.7% in Japan,[17] 11% in Spain,[18] 12.5% in Taiwan,[19] to 13% in Greece.[20] As expected, patients requiring multiple transfusions have a high prevalence of HCV infection. Among more than 1000 transfusion-dependent Italian thalassaemic patients, 80% had confirmed anti-HCV,[21] as well as 47% of Egyptian thalassaemic children and 75% of multitransfused patients in long-term remission from leukaemia with evidence of liver disease.[22,23]

Screening of blood donors for anti-HCV drastically reduced the risk of HCV transmission to very low levels that were associated with the window period. The recent implementation of investigational NAT (nucleic acid technology) in small pools of blood donations has improved the safety of the blood supply by reducing the window period and this has practically eradicated TAH-C, so that transfusion of screened blood should no longer be considered a primary risk factor for HCV infection (Table 25.2). The current risk of a blood recipient becoming infected with HCV is extremely low (1:1 935 900 in the United States[24]) and is predicted to be reduced to 1:425 714 in Spain,[25] 1:747 058 in Italy[26] and 1:8 300 000 in France.[27]

**Table 25.2** Estimated current risk of transfusion-transmitted hepatitis C virus infection associated with the use of routine antibody or antibody plus nucleic acid technology testing, as compared to those for hepatitis B virus (HBV), human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV)

| Viral agent | Incidence (/100 000 person-years) | Window period (days) | Risk per unit | | | |
|---|---|---|---|---|---|---|
| | | | United States | France | Italy | Spain |
| HBV | 1.267 | 45 | 1:640 000 | 1:470 000 | | 1:74 000 |
| HCV (Aby) | 1.889 | 70 | 1:276 000 | 1:860 000 | 1:127 000 | 1:149 000 |
| HCV (+NAT) | 1.889 | 10 | 1:1 935 900 | 1:8 300 000* | 1:747 058* | 1:425 714* |
| HIV (Aby+Ag) | 1.554 | 16 | 1:1 468 000 | 1:1 370 000 | 1:435 000 | 1:513 000 |
| HIV (+NAT) | 1.554 | 11 | 1:2 135 000 | 1:2 700 000* | 1:870 000* | 1:1 026 000* |
| HTLV | 0.239 | 51 | 1:2 993 000 | Nil | | |

Aby, antibody test only; +NAT, antibody plus nucleic acid technology, i.e. TMA (transcription-mediated amplification) or PCR (polymerase chain reaction); Aby+Ag, antibody plus p24 antigen.

*Predicted residual risk after implementation of NAT in 2001.

*Plasma product recipients*

The prevalence of HCV among haemophiliacs correlates with the amount and type of product transfused. Most of all haemophiliacs exposed to untreated commercial clotting factor concentrates have evidence of HCV infection,[28–32] whereas among those treated with cryoprecipitates, the rate was 66%.[31] In contrast, haemophiliacs who have exclusively received appropriately inactivated coagulation components or single-donor cryoprecipitate are generally anti-HCV-negative.[29–33] Screening of plasma pools used to manufacture concentrates should eliminate the risk of infection.

Transmission of HCV by transfusion of a platelet concentrate made from an anti-HCV and HCV-PCR-negative blood donation means that not only the routine screening of plasma donors used to manufacture concentrates but a careful selection, quarantine of plasma and virus inactivation of plasma and its derivatives, remain as necessary as they were before the nucleic acid amplification test came into use.[34]

Intravenous immune globulin (IVIG) has excellent safety records. However, two outbreaks of HCV infection have been described in women given intramuscular[35] or intravenous[36] contaminated anti-D immunoglobulins in the late 1970s and also two major and three minor outbreaks of hepatitis C in agammaglobulinaemic patients treated with different products manufactured by the Cohn fractionation method from unscreened donor plasma have been described.[37] Ironically, in 1993 a major outbreak of hepatitis C in agammaglobulinaemic patients was caused by a commercial immune globulin (Gammagard) prepared from enzyme immunoassay (EIA)-2 screened plasma. But this was not the last case, and in 1996, there was an outbreak of HCV infection in the United States linked with the administration of Gammagard.[38] Addition of an inactivation step to plasma screening and recombinant clotting factor use have solved the problem, and no further cases have been detected with the new preparations.[39]

*Haemodialysis patients*

Among patients on maintenance haemodialysis, prevalence of HCV infection averages 20%, although there are wide geographical variations (Fig. 25.1) ranging from <5% in northern Europe[40] to 30–50% in Japan,[41–43] Poland,[44] 30.25% Saudi Arabia,[45,46] Bosnia-Herzegovina 43.51%,[47] Brazil 39%,[48] Taiwan,[49,50] and Egypt.[51] Prevalence between 5% and 30%, has been reported from the United States,[52,53] 23.3% in New York (USA),[54] India,[55,56] Hong Kong,[57] Western Europe,[3,58–61] Thailand[62] and 5% Kenya.[63] The high prevalence of HCV infection in haemodialysis patients has been attributed not only to the frequency of blood transfusion among these patients, but also to increasing years on dialysis, suggesting that HCV may be transmitted among patients in the dialysis unit, probably as a result of poor infection control practices.[3,40,64,65] Comparison of HCV spread between two dialysis units in southern Sweden revealed that in one unit, there was no evidence of spread within the unit, and that the prevalence of HCV was dependent on the status of the patients entering for treatment. In the other unit, 36% of patients were infected during a 3-year period, including patients who had not received blood transfusions.[66] Outbreaks of HCV infection in dialysis units have also been attributed to poor infection control practices.[64] Finally, molecular epidemiology analysis of haemodialysis patients treated in an Italian unit with a high prevalence of HCV infection strongly suggested nosocomial spread of HCV genotype 4 within the unit.[67] In contrast, no seroconversions were found in a study in which anti-HCV-positive and anti-HCV-negative patients shared the same machines in a haemodialysis unit when universal precautions were rigorously applied.[68]

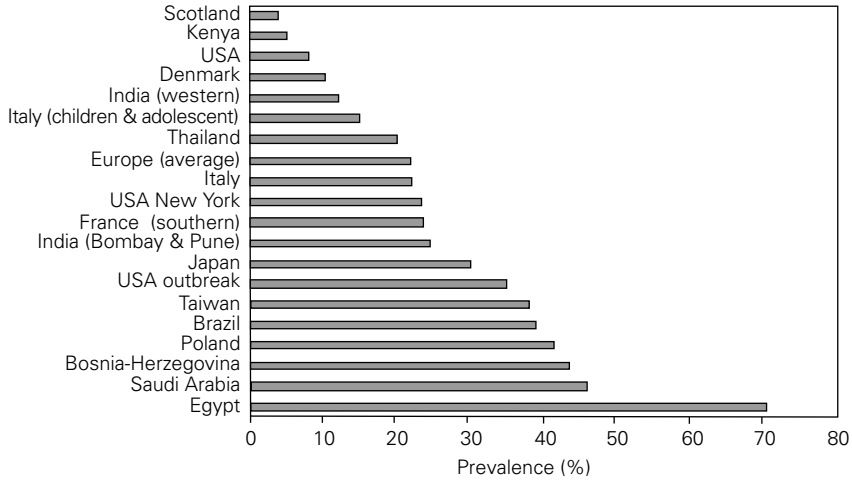


**Figure 25.1** Worldwide prevalence of HCV infection among haemodialysis patients

*Organ transplantation*

Organ transplant recipients are at high risk of acquiring HCV infection.[69] Infection in this setting can occur as a result of recurrence of HCV infection already present before transplantation, transfusion-associated transmission during transplantation, or the presence of HCV infection in the organ donor.[70,71] Antibody tests may underestimate the incidence of transmission and the prevalence of HCV infection among immunosuppressed organ recipients.[70] Hence, HCV RNA testing may be required to detect those patients who lose or do not develop HCV antibodies.[72–74]

The risk of transfusion-associated infection has dramatically decreased with the increased safety of the blood supply. In one study conducted between 1987 and 1991,[75] 14% of patients undergoing allogenic bone marrow transplantation developed HCV infection, but this rate fell to 1.6% after implementation of blood donor screening for anti-HCV.

The risk of transmission from an anti-HCV-positive organ donor to a seronegative recipient is very high. In some studies, 90–100% of recipients of kidneys, liver or heart from HCV-infected donors acquired HCV infection after transplantation.[69,70] In other studies,[76,77] infection rates have been reported to be much lower (approximately 50–60%). It is unknown whether differences in procurement or organ preservation techniques might account for these different transmission rates. Recurrence of HCV infection in the grafted organ after liver transplantation is the rule.[78–83]

### *Nosocomial transmission*

Previous hospitalization is an epidemiological risk factor in patients with HCV infection.[4,84] Because the prevalence of HCV infection among hospitalized patients is rather high (between 2% and 20% depending on the patient setting),[11] nosocomial transmission is likely if disinfection procedures are inadequate and contaminated equipment is shared between patients. In fact, nosocomial transmission may account for a substantial proportion of HCV infection among patients lacking a history of transfusion or other obvious parenteral exposure to HCV (see below). Patient-to-patient transmission has been implicated in outbreaks of HCV infection in a haematology ward[85] and in a paediatric oncology unit. Surgeon-to-patient transmission of HCV during cardiac surgery has been documented.[86] Transmission from a patient to an anaesthesiology assistant and from this to five patients has been reported recently.[87] The risk of transmission from an HCV-infected orthopaedic surgeon to patients is 0.48% (1/207),[88] but only 0.04% (1/2286) from gynaecologist to patient by caesarean.[89] It is likely that with the dramatic decrease of transfusion-associated hepatitis C, nosocomial transmission, albeit infrequent, will become the predominant mode of health-care-associated spread of HCV.

### *Intravenous drug use*

Intravenous drug addiction carries an extremely high risk of HCV infection. The prevalence of HCV among groups of intravenous drug users (IDUs) in all European Union countries ranged from 30% to 98%, with an incidence rate of 6.2–39.2 per 100 person-years.[90,91] Study data from the CDC showed that from 1986 to 1988, drug abuse was responsible for 42% of cases of acute hepatitis C acquired in the community,[92] and global studies have found anti-HCV in 70–90% of IDUs.[93] HCV infection occurs quickly after initiation of injecting illicit drugs, with higher risk for those injecting heroin than amphetamines. Infection during the first year of intravenous habit has been also reported in a study among prison inmates.[94] Recently, it has been reported that the shared use of drug injection paraphernalia other than syringes such as 'cookers' or cotton filters may be an important cause of HCV transmission between IDUs.[95]

## Non-apparent parenteral transmission

### *Tattooing*

Tattoos have been involved in HCV transmission.[96,97] In a Taiwanese study, 12.6% of 87 tattooed healthy young men without other risk factors were found to be anti-HCV-positive as compared with 2.4% of 126 matched control subjects. Tattooing has been epidemiologically linked to HCV infection in Australia.[98] Not exceptionally, tattoos are surrogate indicators of unconfessed intravenous drug abuse.

### *Acupuncture*

Acupuncture can be a potential risk factor when improperly sterilized needles are used by an inexperienced acupuncturist. Acupuncture has been associated with an increased risk of HCV infection among Korean adults with chronic liver disease.[99]

### *Health-care workers*

Transmission of HCV from infected patients to health-care workers has been documented,[100] and molecular evolutionary analysis has confirmed this mode of transmission.[101] Hollow-bore needle-stick exposures are the main cause of HCV transmission from patient to health-care worker.[39] In this sense, no seroconversions were detected after 105 accidental injuries caused by suture needles or sharp objects as compared with 1.2% after 331

needle-stick injuries involving hollow-bore needles.[102] Prospective studies have shown that the average risk of infection after a needle-stick injury involving HCV-positive blood may be as high as 3% with a range between 0.013% and 10% in different studies.[89,102–107] On the other hand, mucous membrane or skin contamination have not been associated with an increased risk of HCV infection.

Reported prevalence among general health-care workers in the UK has ranged between 0.3% and 0.7%,[108] 0.8% in Germany,[109] 1.8% in Spain,[110] 1.97–2% in Italy[105,111] and 2.7% in Hungary.[112] Hence, the prevalence of HCV infection among health-care workers appears to correlate with that of the general population or the specific high-risk group they serve, as well as the risk of accidental percutaneous injuries associated with a specific type of care.

## Non-parenteral transmission

### *Maternal–infant transmission*

Vertical (mother-to-infant) transmission of HCV is uncommon. The prevalence of HCV-infected pregnant women is 0.1–2.4%, which is no different to that in the general age-matched population, although this value is higher in countries such as Cameroon (5.5–6.0%) and in DR Congo (4.3%) in which prevalence in blood donors is higher. From 60% to 70% of HCV-infected pregnant women have active infection with viraemia. Transmission of HCV occurs only when serum HCV RNA is above $10^6$ copies per mL. The global rate of vertical transmission is 4–7% per pregnancy. Co-infection with HIV increases the rate of transmission four- to fivefold. When or how mother-to-infant transmission of HCV occurs remains unknown, and there is the possibility of *in utero* infection and/or at the time of delivery. However, currently available data do not allow the recommendation of routine use of caesarean section for women with chronic hepatitis C unless there is HIV co-infection.[113] Breastfeeding carries no further risk of transmission.[73,113,114,115]

### *Sexual and household transmission*

Sexual transmission of HCV is still a controversial issue. However, evidence from studies of regular partners of HCV-infected patients[116] and direct evidence from particular cases[117,118] has shown that there is a small but definite risk of sexual transmission of HCV, and sexual transmission of HCV from a chronic patient to his sex partner after removal of an intrauterine device has recently been reported.[119] Sexual transmission of HCV has been shown to be extremely uncommon and it has been related to duration of the relationship, which implies that HCV transmission may be more likely to occur if the exposure is repetitive and extends over time. A possible reason for this low risk could be the presence of HCV RNA in semen at a very low level (<200 copies/mL).[120,121] It has been reported that the annual risk of inter-spousal transmission of HCV infection is extremely low (0.23% per year).[122] Damage of the vaginal mucosal might favour transmission, so in this case the use of barrier precautions is recommended despite the fact that the use of latex condoms is not currently recomended for stable monogamous sexual partners. Sexual partners of chronically infected individuals should not be accepted for blood donation. In this sense, it has been reported that lymphocytes from around 25% of seronegative spouses from HCV-infected patients proliferate in the presence of at least one HCV antigen.[123] This has raised the question as to how many spouses, despite being infected by a very small inoculum of virus, have been able to induce an effective lymphocyte immune response that generates a perdurable memory without any other sign of previous infection (anti-HCV- and HCV RNA-negative).

The pooled prevalence of anti-HCV among siblings and household contacts of patients with chronic liver disease was 4% compared with 0% among contacts of anti-HCV-negative controls. The pooled prevalence of anti-HCV among offspring of Japanese HCV-infected chronic liver disease patients was 17% compared with 10.4% of controls.[116] Hence, evidence exists that familiar, non-sexual and sexual transmission of HCV does occur.

### *Non-injecting drug users*

The prevalence of HCV among non-injecting drug users is higher than in the general population (4.7% in New York, USA). Recently, it has been suggested that sniffing or snorting heroin in combination with cocaine may increase the risk of HCV infection among non-injecting drug users.[124]

### *Sporadic hepatitis*

Acute hepatitis C with no apparent risk factor continues to occur. The mechanism of transmission of sporadic hepatitis C cases is possibly a combination of intravenous drug use, which is not revealed in the history, non-apparent or covert, nosocomial, percutaneous exposure, non-percutaneous mechanisms including sexual transmission, and perhaps as yet unidentified modes of virus dissemination. Recently, cocaine snorting has been suggested as a significant risk factor for HCV infection when it involves sharing of blood-contaminated straws.[125]

## Prevalence of HCV infection among blood donors and the general population

Global prevalence of HCV infection in blood donors is summarized in Plate 25.1 (found between p.786–7) and Table 25.3,[11,46,62,84,93,126–192] while prevalence in the general population is shown in Plate 25.2 (found between p.786–7) and Table 25.4. [28,43,126–128,130,135,138,155,161,167,174,178,184,190,193–269]

Low and moderate prevalence (0.3–2.5%) are predominant in the world map of blood donors cohort (Plate 25.1). Very low prevalence (<0.3%) has been reported in some areas of northern and central Europe, parts of the United States, Jamaica in Central America, New Zealand in Oceania, some countries in southern Africa such as Zambia, Zimbabwe and South Africa, and also in Asian countries such as Yemen, Iran and parts of Japan. High prevalence (2.5–5%) has been reported in some areas of Brazil (Rio de Janeiro), some countries in Central Africa such as Guinea, Togo, Chad, Rwanda, Burundi, parts of Nigeria, and in Asia in studies reported from the city of Jeddah in Saudi Arabia and in Harbin (China). Very high prevalence (5–10%) has been found particularly in countries in Africa, such as Nigeria, Cameroon, CAR and Tanzania, but also in some areas such as north-eastern Thailand, in the city of Beijing (China), Soqotran Island in Yemen and also in the Latina province in Italy. Extremely high prevalence (>10%) has been reported in Egypt (13.6–24.8%), Jakarta in Indonesia (24.3%), in the city of Ho Chi Minh in Vietnam (20.6%) and in Nigeria (12.3%), due to specific human interventions such as the parenteral therapy for schistosomiasis in Egypt,[270] or the poor sanitary conditions in Ho Chi Minh during and after the war, or the association between dental care and recombinant immunoblot assay (RIBA) positivity in Latina province in Italy.

**Table 25.3** Estimated HCV prevalence among blood donors (BD)

| Continent | Geographical localization | City, region | Sample size | Prevalence (%) | Population cohort | Ref. no. |
|---|---|---|---|---|---|---|
| Africa | Benin | Cotonou | 582 | 1.4 | Volunteer | 126 |
| Africa | Benin | | 931 | 1.7 | Volunteer | 127 |
| Africa | Burundi | | 340 | 4.9 | Volunteer | 127 |
| Africa | Cameroon | Manyemen | | 6.4 | Volunteer | 128 |
| Africa | Cameroon | | 117 | 8.7 | Volunteer | 127 |
| Africa | CAR | | 163 | 6.1 | Volunteer | 127 |
| Africa | Chad | | 290 | 4.8 | Volunteer | 127 |
| Africa | Egypt | 26 governorates | 2644 | 24.8 | Volunteer | 129 |
| Africa | Egypt | El Cairo | 163 | 13.6 | Volunteer | 130 |
| Africa | Ethiopia | | 500 | 1.4 | Volunteer | 131 |
| Africa | Ghana | | 3264 | 0.5 | Volunteer | 127 |
| Africa | Guinea | | 228 | 4.4 | Volunteer | 127 |
| Africa | Kenya | | 780 | 0.9 | Volunteer | 132 |
| Africa | Malawi | | 140 | 0.7 | Volunteer | 127 |
| Africa | Mauritania | | 349 | 1.1 | Volunteer | 127 |
| Africa | Mozambique | | 194 | 2.1 | Volunteer | 127 |
| Africa | Niger | | 1685 | 1.3 | Volunteer | 127 |
| Africa | Nigeria | | 304 | 3.6 | Volunteer | 127 |
| Africa | Nigeria | | 260 | 12.3 | Volunteer | 133 |
| Africa | Rwanda | | 482 | 2.7 | Volunteer | 127 |
| Africa | Somalia | | 157 | 0.6 | Volunteer | 127 |
| Africa | South Africa | | 66 531 | 0.1 | volunteer | 127 |
| Africa | South Africa | Western province | 66 314 | 0.41 | Volunteer | 134 |
| Africa | Tanzania | | 100 | 8.0 | Volunteer | 127 |
| Africa | Togo | | 241 | 3.3 | Volunteer | 127 |
| Africa | Zambia | Lusaka | 240 | 0.0 | Volunteer | 135 |
| Africa | Zimbabwe | | 437 | 0.2 | Volunteer | 127 |
| America | Brazil | Rio de Janeiro | 4762 | 2.89 | Volunteer | 136 |
| America | Cuba | Havana city | 461 | 1.5 | Randomly selected | 137 |
| America | Jamaica | | | 0.3–0.4 | Volunteer | 138 |
| America | USA | Indianapolis | 149 756 | 0.17 | Volunteer | 139 |
| America | USA | | 862 398 | 0.36 | Consecutive BD | 140 |
| America | USA | | | 0.2–0.8 | Volunteer | 11,93,141 |
| Asia | Bangladesh | | 83 | 0.0 | Volunteer | 142 |
| Asia | Bangladesh | | 163 | 1.2 | Paid donors | 142 |
| Asia | China | Beijing | 1909 | 0.3 | Volunteer | 143 |

(*Continued*.)

**Table 25.3** (*Continued*.)

| Continent | Geographical localization | City, region | Sample size | Prevalence (%) | Population cohort | Ref. no. |
|---|---|---|---|---|---|---|
| Asia | China | Wuhan | 503 | 1.2 | Volunteer | 144 |
| Asia | China | Harbin | 132 | 2.3 | Volunteer | 145 |
| Asia | China | Beijing | 1017 | 5.7 | Volunteer | 143 |
| Asia | India | New Delhi | 15 922 | 1.85 | Volunteer | 146 |
| Asia | Indonesia | 21/27 provinces | 7572 | 2.1 | Volunteer | 147 |
| Asia | Indonesia | Surabaya | 2233 | 2.3 | Volunteer | 148 |
| Asia | Indonesia | Ujung Pandag | 196 | 3.1 | All | 149 |
| Asia | Indonesia | Jakarta | 243 | 24.3 | Volunteer | 150 |
| Asia | Iran | North Iran-Rasht | 5976 | 0.033 | Volunteer | 151 |
| Asia | Israel | | 136 977 | 0.6 | Volunteer | 152 |
| Asia | Japan | | 114 266 | 0.2 | All | 153 |
| Asia | Japan | Osaka | 448 020 | 0.3 | Volunteer | 154 |
| Asia | Japan | Matsumoto | 34 989 | 0.56 | Volunteer | 155 |
| Asia | Japan | 11 cities | 16 500 | 2.2 | All | 156 |
| Asia | Japan | | 7479 | 0.6 | Volunteer | 142 |
| Asia | Kong Kong | | 5000 | 0.4–3.8 | Volunteer | 157 |
| Asia | Korea | Seoul | 150 | 1.3 | Volunteer | 158 |
| Asia | Lebanon | | 8700 | 0.6 | Volunteer | 159 |
| Asia | Pakistan | | 16 705 | 1.2 | Volunteer | 160 |
| Asia | Philippines | | 392 | 2.2 | Volunteer | 161 |
| Asia | Qatar | Qatari nationals | | 0.4–2.8 | Volunteer | 162 |
| Asia | Saudi Arabia | Riyadh | 4818 | 1.5 | Volunteer | 163 |
| Asia | Saudi Arabia | Dammam | 8934 | 1.65 | Volunteer | 46 |
| Asia | Saudi Arabia | Jeddah | 744 | 3.2 | Overall | 164 |
| Asia | Singapore | | 65 208 | 0.37 | All | 165 |
| Asia | Syria | | 2100 | 1.0 | Volunteer | 166 |
| Asia | Taiwan | | 500 | 0.8 | Paid donors | 167 |
| Asia | Taiwan | | 420 | 0.95 | Volunteer | 168 |
| Asia | Thailand | | 961 | 0.8 | Volunteer | 62 |
| Asia | Thailand | | 66 340 | 0.98 | Volunteer | 169 |
| Asia | Thailand | | | 1.0 | Volunteer | 170 |
| Asia | Thailand | North-eastern | 3255 | 5.6 | Volunteer | 171 |
| Asia | Vietnam | Hanoi | 499 | 0.8 | Volunteer | 172 |
| Asia | Vietnam | Ho Chi Minh | 491 | 20.6 | Volunteer | 172 |
| Asia | Yemen | Aden | 494 | 0.6 | Volunteer | 173 |
| Asia | Yemen | Sana'a | 493 | 0.2 | Volunteer | 173 |
| Asia | Yemen | Soqotra Island | 99 | 5.1 | Volunteer | 173 |
| Asia | Yemen | Taiz | 294 | 1.0 | Only males | 174 |
| Europe | Germany | | 428 896 | 0.005 | Volunteer | 175 |
| Europe | Ireland | | 14 917 | 0.3 | Volunteer | 176 |
| Europe | Italy | | 16 515 | 0.03 | Volunteer | 177 |
| Europe | Italy | | | 0.2 | Volunteer | 178 |
| Europe | Italy | Sardinia | 1690 | 0.9 | Volunteer | 179 |
| Europe | Italy | Latina province | 20 741 | 5.5 | 1996–2000 | 180 |
| Europe | Italy | Latina province | 5978 | 8.5 | 1995 | 180 |
| Europe | Northern Ireland | | 231 321 | 0.01 | All | 181 |
| Europe | Norway | | 16 756 | 0.3 | Volunteer | 182 |
| Europe | Romania | | 87 894 | 0.3–1.5 | 1999 | 183 |
| Europe | Russia | Moscow | 2217 | 0.91 | | 184 |
| Europe | Russia | Dagesthan | 10 682 | 0.93 | Volunteer | 185 |
| Europe | Russia | Dagesthan | 267 | 7.5 | Paid donors | 185 |
| Europe | Scotland | | 180 658 | 0.088 | All | 186 |
| Europe | Spain | | 30 231 | 1.2 | Volunteer | 84 |
| Europe | Turkey | Ankara | | 1.9 | Volunteer | 187 |
| Europe | UK | | 287 332 | 0.35 | Volunteer | 188 |
| Europe | Ukraine | Sumy | 681 | 2.3 | Volunteer | 189 |
| Oceania | Australia | | Review | 1.0 | Volunteer | 190 |
| Oceania | Australia | | 167 511 | 0.25 | Volunteer | 191 |
| Oceania | New Zealand | | | 0.1–0.34 | Volunteer | 192 |

**Table 25.4** Estimated HCV prevalence among the general population

| Continent | Geographical localization | City, region | Sample size | Prevalence (%) | Population cohort | Citation |
|---|---|---|---|---|---|---|
| Africa | Benin | | 1110 | 1.6 | gp | 127 |
| Africa | Burkina Faso | Bobo Dioulaso | 965 | 4.9 | gp | 126 |
| Africa | Burundi | | 1184 | 11.3 | gp | 127 |
| Africa | Cameroon | Yaounde | 1494 | 1.9 | Pregnant rural | 193 |
| Africa | Cameroon | Yaounde | | 5.5 | Pregnant urban | 128 |
| Africa | Cameroon | Manyemen | | 6.0 | Pregnant rural | 128 |
| Africa | Cameroon | | 807 | 12.5 | Southern provinces | 194 |
| Africa | Cameroon | | 6015 | 13.8 | gp | 127 |
| Africa | CAR | | 709 | 2.4 | gp | 127 |
| Africa | Côte d'Ivoire | | 429 | 3.3 | gp | 127 |
| Africa | Chad | | 290 | 4.8 | gp | 127 |
| Africa | DR Congo | Kinshasa | 1092 | 4.3 | Pregnant women | 195 |
| Africa | DR Congo | | 2572 | 5.5 | gp | 127 |
| Africa | DR Congo | | 173 | 6.4 | gp | 196 |
| Africa | Egypt | upper Egypt | 6012 | 8.7 | gp | 197 |
| Africa | Egypt | | 155 | 51.0 | Non-random selected residents | 130 |
| Africa | Equatorial Guinea | | 2042 | 1.7 | gp | 127 |
| Africa | Eritrea | | 323 | 1.9 | gp | 127 |
| Africa | Ethiopia | | 2080 | 1.9 | gp | 127 |
| Africa | Gabon | Eastern | 1172 | 6.5 | gp | 198 |
| Africa | Gabon | | 1597 | 9.2 | gp | 127 |
| Africa | Gabon | | 109 | 24.0 | gp | 199 |
| Africa | Gambia | | 212 | 2.4 | gp | 127 |
| Africa | Ghana | | 5033 | 1.7 | gp | 127 |
| Africa | Ghana | Ashanti-Akim North | 803 | 5.4 | Children & adolescent | 200 |
| Africa | Guinea | Villages forest region | 459 | 1.1 | gp | 126 |
| Africa | Guinea | | 2050 | 5.5 | gp | 127 |
| Africa | Kenya | | 1567 | 0.9 | gp | 127 |
| Africa | Libya | | 266 | 7.9 | Healthy people | 201 |
| Africa | Madagascar | | 1564 | 2.1 | gp | 127 |
| Africa | Madagascar | | 643 | 3.3 | | 202 |
| Africa | Malawi | | 140 | 0.7 | gp | 127 |
| Africa | Mauritania | | 349 | 1.1 | gp | 127 |
| Africa | Mozambique | | 536 | 2.8 | gp | 127 |
| Africa | Niger | | 2327 | 1.8 | gp | 127 |
| Africa | Nigeria | | 669 | 2.1 | gp | 127 |
| Africa | Rwanda | | 610 | 4.1 | gp | 127 |
| Africa | Senegal | | 352 | 2.2 | gp | 127 |
| Africa | Somalia | | 2203 | 1.5 | gp | 127 |
| Africa | South Africa | | 68 931 | 0.1 | gp | 127 |
| Africa | Sudan | | 865 | 2.8 | gp | 127 |
| Africa | Sudan | Juba | 666 | 3.0 | gp | 203 |
| Africa | Swaziland | | 194 | 1.5 | gp | 127 |
| Africa | Tanzania | | 2188 | 3.2 | gp | 127 |
| Africa | Togo | | 478 | 3.9 | gp | 127 |
| Africa | Tunisia | | 33 363 | 0.4 | Healthy people | 204 |
| Africa | Uganda | | 881 | 6.6 | gp | 127 |
| Africa | Zambia | | 583 | 0.2 | gp | 127 |
| Africa | Zambia | | 495 | 0.6 | gp | 135 |
| Africa | Zimbabwe | | 579 | 2.0 | gp | 127 |
| America | Brazil | | 2.91 | 3.0 | gp | 205 |
| America | Canada | | 433 | 1.2 | Urban population | 206 |
| America | Jamaica | | 513 | 0.0 | Women reproductive age | 138 |
| America | Mexico | Mexico City | 1100 | 0.7 | gp | 207 |
| America | Nicaragua | Leon | 399 | 0.0 | Cross-sectional | 208 |
| America | Peru | jungle region | 2111 | 0.0 | gp | 209 |
| America | USA | Boston | 869 | 0.1 | Adolescent | 210 |

(*Continued*.)

**Table 25.4** (*Continued*.)

| Continent | Geographical localization | City, region | Sample size | Prevalence (%) | Population cohort | Citation |
|---|---|---|---|---|---|---|
| America | USA | Sonoma | 1235 | 0.3 | Residents | 211 |
| America | USA | | 10 000 | 0.48 | Military personnel | 212 |
| America | USA | San Francisco | 1354 | 0.5 | Asian-American | 213 |
| America | USA | | 21 241 | 1.8 | gp | 214 |
| America | USA | San Diego | 3367 | 2.5 | gp | 215 |
| America | Venezuela | Yukpa Amerindians | 293 | 0.0 | gp | 216 |
| Asia | China | Beijing | 438 | 2.1 | Normal subjects | 217 |
| Asia | Hong Kong | | 910 | 0.5 | gp | 218 |
| Asia | India | Bombay & Pune | 830 | 0.0012 | gp | 219 |
| Asia | India | West Bengal | 3579 | 0.31 | <10 years | 220 |
| Asia | India | West Bengal | 3579 | 0.87 | gp | 220 |
| Asia | India | West Bengal | 3579 | 1.85 | >60 years | 220 |
| Asia | India | Madhya Pradesh | 91 | 5.5 | | 221 |
| Asia | Israel | | | 0.44 | gp | 222 |
| Asia | Japan | | 1442 | 0.0 | Schoolchildren | 155 |
| Asia | Japan | Okinawa | 1295 | 0.4 | gp | 43 |
| Asia | Japan | | 16 714 | 0.98 | Pregnant women | 223 |
| Asia | Japan | Osaka | 298 | 2.0 | gp | 224 |
| Asia | Japan | Fukuoka | 2237 | 3.3 | gp | 43 |
| Asia | Japan | Shizuoka | 971 | 5.1–49 | Epidemic town | 225 |
| Asia | Jordan | Amman | 578 | 0.65 | Hospital population | 226 |
| Asia | Korea | | 4917 | 1.4 | Adults >20 years | 227 |
| Asia | Pakistan | Hafizabad | 309 | 6.5 | gp | 228 |
| Asia | Philippines | Davao city | 123 | 1.6 | Physician staff | 161 |
| Asia | Saudi Arabia | Riyadh | 4496 | 0.9 | Children (1–10 years) | 229 |
| Asia | Saudi Arabia | Gizan Area | 1482 | 1.8 | Healthy subjects | 230 |
| Asia | Saudi Arabia | Al Baha | 380 | 3.6 | Healthy subjects | 231 |
| Asia | Saudi Arabia | | 760 | 5.3 | Healthy subjects | 232 |
| Asia | Taiwan | | 748 | 0.13 | Healthy children | 233 |
| Asia | Taiwan | | 1419 | 0.28 | | 167 |
| Asia | Taiwan | | 294 | 0.34 | Pregnant women | 234 |
| Asia | Taiwan | | 275 | 2.9 | Healthy subjects | 235 |
| Asia | Taiwan | | 108 | 5.6 | Healthy subjects | 236 |
| Asia | Taiwan | Bunun aboriginal community | 712 | 16.9 | gp | 237 |
| Asia | Taiwan | 12 villages | 6059 | 17.0 | gp | 238 |
| Asia | Thailand | Hmong people | 269 | 2.0 | gp | 239 |
| Asia | Uzbekistan | | 1269 | 6.5 | gp | 240 |
| Asia | Vietnam | Hanoi | 511 | 4.0 | gp | 241 |
| Asia | Vietnam | Ho Chi Minh | 491 | 9.0 | gp | 241 |
| Asia | Yemen | | 243 | 2.1 | Pregnant women | 174 |
| Asia | Yemen | | 348 | 2.58 | gp | 242 |
| Europe | England & Wales | | | 1.07 | gp | 28 |
| Europe | France | Limoges | | 0.3 | gp | 243 |
| Europe | France | | 759 591 | 1.1 | gp | 244 |
| Europe | France | | 6283 | 1.15 | gp | 245 |
| Europe | France | Paris suburban | 2367 | 1.73 | gp | 246 |
| Europe | Germany | | | 0.4 | gp | 247 |
| Europe | Greece | Thessaloniki | 2408 | 1.95 | Pregnant women | 248 |
| Europe | Greece | Atenes | 130 | 2.3 | Refugees from Albania and Asia | 249 |
| Europe | Hungary | Budapest | 477 | 2.7 | Hospital workers | 250 |
| Europe | Iceland | | | 0.2 | gp | 251 |
| Europe | Italy | | 5672 | 0.7 | Pregnant women | 178 |
| Europe | Italy | Latinum region | 1142 | 0.9 | Women | 252 |
| Europe | Italy | Bologna | 1646 | 3.46 | gp | 253 |
| Europe | Italy | Particular town | 1352 | 14.4 | gp | 254 |
| Europe | Italy | | 4978 | 0.48 (0.35–0.76) | Air Force overall | 255 |

(*Continued*.)

**Table 25.4** (*Continued*.)

| Continent | Geographical localization | City, region | Sample size | Prevalence (%) | Population cohort | Citation |
|---|---|---|---|---|---|---|
| Europe | Portugal | Coimbra | 657 | 0.46 | gp | 256 |
| Europe | Russia | Moscow | | 1.3 | gp | 184 |
| Europe | Russia | Tuva | | 2.5 | gp | 184 |
| Europe | Russia | Yakutia | | 3.0 | gp | 184 |
| Europe | Russia | Republic Central Asia | | 2.9–5.3 | gp | 184 |
| Europe | Spain | Catalunya | 4551 | 1.0 | Pregnant women | 257 |
| Europe | Spain | Asturias | 2442 | 1.2 | Pregnant women | 258 |
| Europe | Spain | | 2615 | 1.4 | | 259 |
| Europe | Spain | Catalunya | 2142 | 2.5 | gp | 260 |
| Europe | Spain | Catalunya | 2194 | 2.64 (1.7–3.6) | gp | 261 |
| Europe | Sweden | | 563 | 0.3 | Expatriates | 262 |
| Europe | Turkey | | 116 | 0.0 | | 263 |
| Europe | Turkey | | 1005 | 0.1 | Kurd refugees | 264 |
| Oceania | Australia | Victoria | 252 | 0.4 | Women | 265 |
| Oceania | Australia | | 1537 | 1.1 | gp | 190,266 |
| Oceania | Kiribati | | 385 | 4.8 | gp | 196 |
| Oceania | New Zealand | Maori & Pacific Islanders | 3483 | 0.49 | gp | 267 |
| Oceania | Papua New Guinea | | 253 | 1.0 | gp | 268 |
| Oceania | Papua New Guinea | Remote area | 66 | 1.0 | gp | 269 |
| Oceania | Papua New Guinea | Rural near mining town | 48 | 6.0 | gp | 269 |
| Oceania | Papua New Guinea | Provincial capital | 180 | 12.0 | gp | 269 |
| Oceania | Vanuatu | | 138 | <1 | gp | 196 |

gp, general population.

Confirmed anti-HCV-positive blood donors have a history of overt percutaneous exposure to blood (transfusion, intravenous drug abuse, tattooing, or occupational needle-stick exposure) in 30–75% of the cases, this antecedent being higher in areas with a lower prevalence of HCV infection..[93,99,136,271–273] Other factors significantly associated with HCV infection include a prior history of major surgery, the use of non-disposable needles, a history of tuberculosis or prolonged hospitalization before 1970 in older people, and the sharing of straws for cocaine snorting,[4,84,124,125] and certain folk remedies and customs in specific hyperendemic areas.[274–276] The use of paid instead of volunteer blood donations has been implicated as the cause of very high prevalences of HCV in some hyperendemic areas.[43,277,278] The lack of HCV infection, but not of hepatitis B, D or E virus infection, among Amerindian populations[209,279–281] traditionally excluded from any health-care system further supports the proposition that health-care-related covert parenteral exposures may have been an important mechanism of HCV spread in the developed world in the recent past. For instance, the indigenous tribe Parakana (Brazil) had no HCV infection during the first years in which they initiated outside contact in the 1970s and 1980s, but currently Parakana people have a prevalence of 1.4–1.6%.[281]

Because the selection of regular volunteer blood donors (and, to a lesser extent, first-time donors) generally excludes high-risk subjects for blood-borne infections or individuals with surrogate markers for such infections, the extent to which prevalence among blood donors can be extrapolated to the general population remains controversial, because: (1) prevalence among blood donors markedly increases with age and is slightly higher in men than in women;[84,136] (2) changes in blood donor recruitment policies may render data on first-time donors unreliable for extrapolation of prevalence to the general population; (3) prevalence studies in non-donor populations are scarce and usually limited to particular groups such as pregnant women or military recruits, which are biased by age and/or sex preselection or are restricted to small isolated peculiar populations; and (4) different geographical areas or specific populations within the same country may have widely different prevalence rates because of peculiar modes of covert percutaneous exposure. Despite these limitations, however, blood donors seem to have an overall prevalence 25–50% lower than the corresponding general population, which is represented in Plate 25.2 (found between p.786–7) and Table 25.4.

The overall prevalence of HCV among the general population (Table 25.4) reported for each continent gives medium values of 5.17% in Africa, 3.55% in Asia, 1.93% in America, 1.75% in Europe and 1.88% in Oceania. These data give a global prevalence of 2.856%, and this means that around 210 million people are infected by HCV.

## Summary

At least 210 million people are infected by HCV. New human practices together with global expansion of the human population have increased its spread over the world during the last decades. The most important routes of HCV transmission have been parenteral, including administration of therapeutic blood products (transfusion, plasma derivatives for haemophiliacs, immunoglobulin concentrates), intravenous drug use, and nosocomial transmission from patient to patient as in haemodialysis, patient to health-care worker after accidental needle-stick, and health-care worker to patient during surgery or other invasive procedures.

Screening of blood products has almost eradicated transfusion-associated hepatitis C, and transfusion of screened blood should no longer be considered a primary risk factor for HCV infection. In addition, the adoption of efficient inactivation steps in the manufacturing of plasma products has drastically reduced the risk of transmission to haemophiliacs or patients receiving immunoglobulin preparations.

Intravenous drug use continues to carry a high risk of HCV infection, and educational efforts to prevent sharing of drug paraphernalia along with appropriate rehabilitation programmes are the cornerstones of efforts to decrease or eliminate such transmission. Poor infection control practices are suggested as the major cause of transmission within haemodialysis units. With the current safety of the blood supply, nosocomial transmission has become the predominant mode of health-care-associated spread of HCV. Other parenteral routes of HCV transmission that deserve consideration are tattooing and acupuncture when improperly sterilized equipment is used.

The risk of perinatal transmission is very low, although concomitant HIV infection or high viral load in the mother appear to increase the risk. Household transmission may be related to covert percutaneous exposure during health care, folk medicine, exposure to non-disposable sharps and needles, and dental care. Sexual transmission remains controversial, but evidence exists from particular cases and, despite the fact that infection has been considered extremely uncommon, damage of the vaginal mucosa might favour transmission. Evidence of transmission to sexual partners of chronically infected individuals has also been reported, and this has opened the question as to how many spouses have been able to induce an effective lymphocyte immune response that generates durable memory without any other sign of previous infection.

Although the incidence of HCV infection may be decreasing, especially in Western countries, the prevalence of liver disease caused by HCV is on the rise due to the lag (20 years or longer) between onset of infection and clinical manifestation of liver diseases. Improvements in more effective and globally available treatments will help reduce or elongate the period of chronicity and the number of patients requiring liver transplants, which has increased significantly during the last decade. Although the incidence of HCV infection may be decreasing, the prevalence of liver disease caused by HCV is on the rise.[282]

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# Chapter 26
# The immune response to hepatitis C virus in acute and chronic infection

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Hepatitis C virus (HCV) infection has been increasingly recognized as a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. In the United States, it is estimated that the virus infects approximately 1.8% of the population, and worldwide it may infect up to 177 million persons. Following infection, a majority (50–85%) of healthy adults will develop persistent infection. This high rate of persistent infection has hindered the ability to determine the correlates of protective immunity, although recent studies have demonstrated the importance of cellular immune responses in spontaneous clearance of HCV. In immunocompetent individuals, chronic active hepatitis, cirrhosis and HCC typically develop 10, 21 and 29 years after infection, respectively. It is estimated that the number of individuals who have been infected for more than 20 years, thus facing the highest risk of liver cancer, will triple by the year 2010.[1] In fact, a recent study predicted a dramatic rise in the death rate due to HCC in the next two to three decades.[2] However, the likelihood of progression of chronic disease is highly variable, and identification of the host and viral factors that are associated with slow compared to rapid progressors is another area of intense interest.

## Mechanisms of clearance

Determining the correlates of protective immunity in hepatitis C has been limited by several issues. First, the subclinical nature of acute hepatitis C renders prospective studies difficult, especially as studies of cellular immunity require either studies to be done with freshly isolated cells or on large numbers of cells, both of which are difficult to obtain in retrospective studies. Second, there is no small animal model for hepatitis C infection, and use of the chimpanzee model is limited because of the expense and limited availability of these animals (see Chapter 27). Finally, especially in chronic infection, responses are of low magnitude in the peripheral blood, which requires special techniques to identify the cells. Despite these obstacles, a number of recent studies have enhanced our understanding of the immune response that is associated with spontaneous recovery from hepatitis C.

### The role of humoral immunity

Unlike hepatitis B virus (HBV) infection, where antibody against hepatitis B surface antigen (anti-HBsAg) is necessary and sufficient for protection against infection, a role for antibodies in protection against natural HCV infection has been difficult to prove. The first detectable antibody responses against HCV antigens usually target non-structural (NS) protein 3 (NS3; anti-c33) and core (anti-22c) regions of the polyprotein. Later, responses against NS4 and envelope proteins (E1 and E2) develop.[3] Studies in infected humans have failed to identify an antibody reactivity pattern that clearly discriminates individuals with spontaneous recovery from those who go on to chronic infection.[4] An IgM antibody occurs in some individuals with acute hepatitis C but does not appear to predict outcome.[5]

Measurement of the ability of any given antibody to neutralize HCV infectivity is further compromised because of the lack of a robust tissue culture system. One surrogate for a true infectious tissue culture system is the use of pseudotyped viruses, which are chimeric viruses consisting of the replication machinery of one virus, such as human immunodeficiency virus (HIV) or vesicular stomatitis virus (VSV), with the envelope(s) of HCV. Using these chimeric viruses, several laboratories have demonstrated that neutralizing antibodies can be detected that prevent binding of envelope proteins to target cells.[6–9] Direct evidence for a protective role of HCV-specific antibodies derives from limited *in vivo* studies in which chimpanzees were protected against infection with an HCV inoculum that was first incubated with HCV-specific antibodies *in vitro*.[10] However, several investigators have also observed that circulating HCV-specific antibodies do not prevent reinfection of chimpanzees with either homologous or heterologous

isolates.[11,12] Furthermore, antibody responses to the envelope proteins develop slowly, achieve only modest titres and tend to be short-lived, suggesting that neutralizing antibodies may emerge too late or may not be strong enough to prevent chronic infection.[4] However, the chimpanzee model may be suboptimal for evaluation of the effect of antibody neutralization. While the humoral immune response to the non-structural HCV proteins appears to be similar in humans and chimpanzees, antibody responses to HCV structural proteins are observed less frequently in chimpanzees than in humans for reasons not understood.[13] As these proteins would be expected to serve as major targets of a neutralizing antibody response, failure of chimpanzees to develop such responses makes interpretation of preclinical studies of vaccine efficacy difficult in this model. Despite these limitations, a recent pilot study using recombinant anti-E1 antibody demonstrated improvement or stabilization of liver histology.[14]

## The role of cellular immunity

Effective clearance of an acute viral infection typically requires the coordinated function of multiple arms of the immune system, including the innate immune system – interferons (IFNs) and natural killer (NK) and natural killer T (NKT) cells – as well as the adaptive or acquired immune response specific to a given pathogen (CD4+ and CD8+ T cells) (Fig. 26.1). The exact importance of each arm of the immune response is typically dependent on the pathogen. The liver has a larger proportion of cells representative of components of innate immunity, such as NK, γδ T and NKT cells than the peripheral blood.[15,16] There is a relative paucity of literature on these cells in HCV despite their abundance within the hepatic lymphoid system. NK and NKT cells are the major components of innate immunity, and mount the first line of defence within minutes or hours against a number of pathogens by both direct cytolysis of infected cells and production of cytokines such as IFN-γ and interleukin (IL)-12, which have direct and indirect effects on viral replication. The early production of IFN-γ by these cells not only has antiviral effects but also mediates the intrahepatic recruitment of inflammatory cells.[17] These responses are activated within minutes to hours of infection, and so there is very limited information on the role of these responses in acutely infected persons. A single report in the chimpanzee model suggests that clearance of HCV is not dependent on the development of virus-specific CD4+ or CD8+ T cells, but rather upon the development of innate immunity, as measured by phenotypic markers of NK cells.[18] Similarly, although NKT cells have been demonstrated to be important in the clearance of HBV in animal models[19,20] there are no data for animal or human HCV infection.

## CD4+ T cells

CD4+ T cells recognize short antigenic peptides derived from proteolytic cleavage of exogenous antigen in the antigen-binding groove human leukocycle antigen (HLA) class II molecules, which are present on the surface of antigen-presenting cells such as dendritic cells, macrophages and B cells. HLA class II-restricted, antigen-specific CD4+ T cells orchestrate the effector immune response and are broadly divided into two main

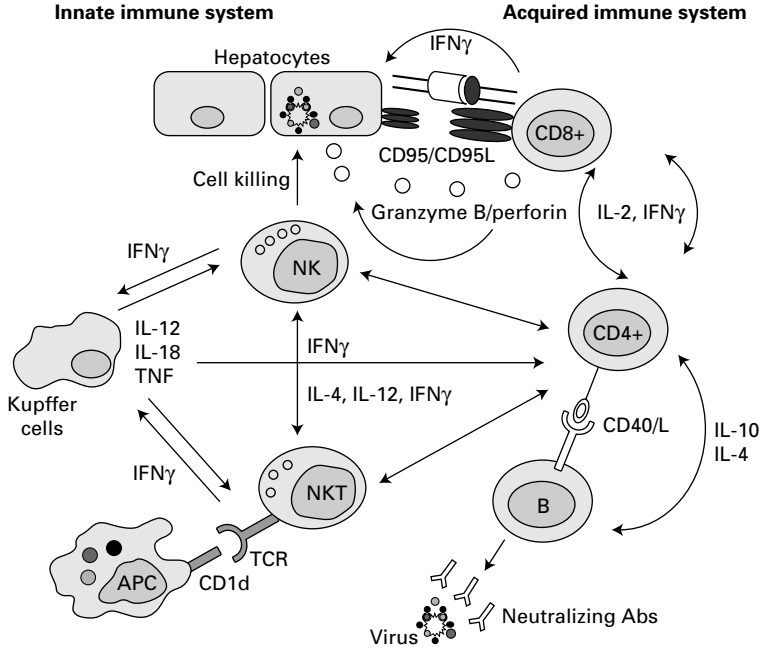


**Figure 26.1** The relationship of the innate and adaptive immune response to viral infection. Infection of hepatocytes by a viral infection leads to activation of natural killer (NK) cells, natural killer T (NKT)) and resident macrophages, which can act as antigen-presenting cells (APCs). Production of cytokines such as interferon-γ (IFN-γ) and interleukin (IL)-12 lead to activation of CD4+ T cells, which in turn direct CD8+ T cells. These CD8+ T cells play a critical role in controlling viral replication through both cytolysis of infected cells and production of cytokines.

categories called T-helper type 1 (Th1) and T-helper type 2 (Th2). Th1 cells produce IFN-γ and IL-2, and are essential for protection against a variety of intracellular infections by promoting cytotoxic T lymphocyte (CTL) activity, whereas Th2 cells produce IL-4, IL-5 and IL-10 and promote antibody responses. CD4+ responses are critical to both the generation and maintenance of antiviral immune responses, as they secrete cytokines that augment antibody production by B cells and prime CD8+ cells specific for virus-infected cells. Without CD4+ cells, induction of new immune responses is impaired and CTL memory cannot be maintained *in vivo*.[21,22]

In both experimental chimpanzee and natural human infection, clearance of hepatitis C is associated with a strong, polyclonal and sustained HCV-specific CD4+ T-cell response.[23–30] The appearance of these CD4+ T-cell responses is temporarily associated with a substantial decrease in viraemia in acute infection of the chimpanzee, and in this model the accumulation of HCV-specific $CD4^+$ T cells in the liver appears to be essential for clearance of HCV.[26,28,30] Individuals who mount a polyclonal HCV-specific CD4+ are more likely to clear HCV, whereas individuals who do not are more likely to become persistently infected.[25,29,31,32] Similarly, in infected persons who go on to resolve hepatitis spontaneously, the clearance of virus is associated with the development of a polyclonal CD4+ response.[24,25,33,34] In humans, CD4+ T-cell responses associated with viral clearance are targeted toward the non-structural proteins, although this may be a reflection of the technical limitations of expressing more highly variable proteins for use in *in vitro* assays.[24,25,33] Strong and multispecific responses of Th1 type directed to structural and non-structural HCV antigens were found to persist in most patients with spontaneous resolution of HCV infections, even when measured 8–17 years after the time of first exposure to the virus.[27] Loss of HCV-specific CD4+ during the initial months of infection is associated with relapse of viraemia,[34] whereas enhancement of CD4+ responses is associated with a higher likelihood of viral clearance after treatment.[35] The kinetics of this response appear to be important, as individuals who clear HCV infection have a more rapid and sustained induction of CD4+ responses than those who develop persistent disease.[36]

## CD8+ T cells

The function of CD8+ CTL requires the interaction of the polymorphic T-cell receptor (TCR) with MHC class I molecules displaying an endogenously synthesized peptide of 8–11 residues on the cell surface of infected cells. The effector function of CTLs consists both of cytolysis of infected cells and production of cytokines, such as tumour necrosis factor (TNF)-α and IFN-γ, that lead to viral clearance.[37,38] The importance of these non-cytolytic effector mechanisms has been shown for HIV and HBV,[39–41] although direct evidence in HCV is lacking. CTLs use two major pathways to eliminate infected cells, perforin Grangyme B and Fas:FasL. *In vitro*, HCV-specific CTLs appear to use both effector mechanisms,[42] although it is not known whether both pathways are operative *in vivo.*

As with the CD4+ response, polyclonal and multi-specific CD8+ CTL responses are also associated with spontaneous clearance in both chimpanzee[24,28,43,44] and human infection.[36,45,46] In the chimpanzee model, resolution of acute HCV was temporally associated with an early polyclonal and vigorous immune response in the liver.[43] Animals who recovered were found to have an early intrahepatic CTL response directed against multiple protein targets, whereas those who went on to chronic infection had a more narrowly focused response during acute infection. HCV-specific IFN-γ-secreting CD8+ T cells are abundant in recovered chimpanzees in comparison with those with chronic infection.[26,47] In animals that recover, protection from reinfection is maintained by the presence of memory CD8+ T cells, as depletion of these memory CD8+ prior to rechallenge resulted in reinfection in previously recovered animals.[44] The importance of the CD4+ memory response in maintaining an effective CD8+ response was demonstrated by Grakoui *et al*., who found that depletion of CD4+ T cells in light of existing memory CD8+ was associated with viral persistence and immune escape.[21]

Prospective studies of the CD8+ response in humans are limited by the low frequency of such responses in the peripheral blood, which requires that large amounts of blood be collected or that sophisticated techniques be applied in freshly collected blood. Therefore, studies to date have either been performed retrospectively, or if prospective, have studied only a few patients. These studies have shown that while CD8+ responses are generated in the majority of acutely infected persons, irrespective of outcome, the distinguishing feature of spontaneous recovery is the ability to maintain such responses over time.[27,46,48,49] The development of newer and more sensitive techniques, such as class I tetramers, which detect CD8+ T cells specific for a peptide epitope, and ELISpot assays, which can detect antigen-specific cells by virtue of their secreted cytokines in response to specific antigen, have provided further tools with which we can more efficiently measure the effectiveness of the early CTL responses.[50] Gruner *et al*. have demonstrated, using ELISpot techniques, a significant CD8+ T-cell response directed against multiple class I-restricted HCV epitopes of structural and non-structural regions of the HCV polyprotein, the appearance of which correlates with elimination of the virus.[45] The frequency reported by Gruner in the peripheral blood of individuals with acute HCV was 0.2% of total CD8+ T cells. Significant

proliferative responses of CD4+ T cells to NS3 and NS4 proteins accompanied the CTL responses and were maintained in those patients with resolving infection.[46] Lechner *et al*., using a combination of class I tetramer staining and ELISpot techniques, have also demonstrated that the successful eradication of HCV is associated with a peripheral blood CTL frequency of >7% at the peak of clinical illness, which is similar to that noted for infectious mononucleosis, and that this CTL response was simultaneously directed against eight different epitopes.[49] This frequency of CTLs contrasts with the low levels (0.07%) found in the peripheral blood of those individuals with chronic infection.[45,51] Taken together, these chimpanzee and human studies suggest that the presence of a broadly directed CTL response is associated with viral clearance.

## Mechanisms of chronicity in hepatitis C infection

Failure to eradicate HCV infection results in the development of chronic HCV infection with resultant chronic hepatitis, after which a significant proportion of individuals will develop cirrhosis and HCC. The mechanism by which chronic HCV infection develops and persists in the majority of infected persons remains unclear, but it does so despite the presence of HCV-specific CD4+ and CD8+ T-cell responses in the peripheral blood and the liver, suggesting that these responses are ineffective for the most part.[52–56] It is likely that HCV has developed a number of means to evade host defences, although most literature has focused on the role of quasi-species variability as a mechanism of immune escape. However, a number of other potential mechanisms have been reported, suggesting that there are multiple means by which this virus interferes with an effective immune response.

### Evasion of the immune response by quasi-species variation

The high degree of genetic variability of HCV probably is a major contributing factor to the development of chronicity. There are six major genotypes, multiple subtypes, and even in a single infected individual multiple strains can be isolated (see Chapter 24, Structure and molecular virology of HCV). The combination of a very high HCV replication rate, estimated at $10^{12}$ virions per day,[57] and the rapidity of viral replication and the lack of error proofing of the viral polymerase[58] probably account for the fact that the HCV RNA genome mutates frequently and appears to favour the selection of variant viruses, which escape recognition by humoral and cellular immune responses. The hypervariable region 1 (HVR1) of E2, in particular, appears to be a region which can mutate in response to immune pressure.[59] Although other B-cell epitopes exist,[60–62] most studies on escape from humoral immune responses have focused on the HVR1 region due to the highly variable nature of this region and its presumed role in binding to cellular receptors.[63] Whereas other regions of the genome develop both synonymous and non-synonymous mutations, the HVR region tends to have preferential accumulation of non-synonymous mutations.[64] In acute HCV infection, resolution of disease is associated with relative homogeneity of the envelope proteins, whereas progressive hepatitis was associated with genetic evolution of the quasi-species, which was temporally associated with the development of antibodies against the HVR1.[65] In patients with depressed humoral immunity, the rate of HVR1 evolution is less than in patients with normal humoral immunity.[66] Similarly, patients with severe recurrence of HCV after orthotopic liver transplantation tend to have a relatively homogeneous population compared with those with less severe recurrence of disease.[67]

### Mutations in CD4+ and CTL epitopes

Given the apparent critical importance of the cellular immune response to spontaneous recovery, several studies have focused on whether or not HCV can escape cellular immune responses. Mutations of MHC class I- or II- restricted epitopes could alter the outcome of infection by preventing, or delaying, clearance of infected hepatocytes by T cells. Mutations leading to abrogation of alteration of CD4+ and CD8+ function have been described. Sequence variants within NS3 have been reported to be associated with failure of clones specific for one viral sequence to proliferate and produce IL-2 upon stimulation with sequence variants.[68] Moreover, variants of the hypervariable region (HVR1) of the putative envelope 2 protein of HCV can act as powerful TCR antagonists for HVR1-specific CD4+ T cells isolated from HCV-infected individuals.[69] Naturally occurring variants can also significantly alter CD4+ function through alteration in cytokines produced upon recognition of variant sequences. In at least one report, sequence variants led to a Th1 to Th2 switch.[70,71] Amino acid substitutions that inhibit CD8+ CTL recognition have been observed in chimpanzees,[72,73] as well as in infected patients.[74–76] In acutely infected chimpanzees, comparison of the viral quasi-species in both epitopes and adjacent regions confirmed that mutations within epitopes recognized by virus-specific CTLs were more frequent than in adjacent regions, consistent with immune escape.[72] However, the frequency of this event has been called into question.[77] One argument against this mechanism as a sole explanation of persistence is the polyclonal nature of the immune response. With such a broadly directed response, it should be difficult for the virus to maintain sufficient

fitness to replicate when faced with the need to mutate at multiple sites.

## Genetic susceptibility to infection

In other diseases it has been possible to identify genetic markers of susceptibility or resistance to infection. Perhaps the best known example of this is the naturally occurring 32-base-pair deletion in the co-receptor for HIV, which results in resistance to HIV infection.[78] Given that the cellular receptor(s) for HCV have not been definitively identified, the search for genetic loci conferring resistance to chronic infection has focused on HLAs and other genes involved in regulation of the immune system. The HLAs are central to the host immune response and thus are ideal candidate genes to investigate for association with HCV outcomes. The HLA molecules are divided into two groups, class I and class II, and present foreign antigens to CD8+ T cells and to CD4+ T cells, respectively. Although a number of studies on the association of HLA antigens and the clinical outcome of HCV infection have been reported, to date there is no clear linkage between any given HLA type and outcome across multiple populations. For example, the study by Thursz and colleagues provides convincing evidence that the HLA class II alleles DRB1*1101 and DQB1*0301 are associated with spontaneous viral clearance,[79] whereas other studies showed that DRB1*01 was associated with clearance[80,81] and DRB1*0301 with persistence.[82] While HLA-B*57 was associated with viral clearance in both Caucasians and African-Americans, the association of HLA-C*w**0102 was present only in Caucasians, while HLA-Cw*04 and HLA-A*2301 were associated with viral persistence.[83,84] In addition to a lack of association across different populations, no study to date has linked functional defects in class I- or II-restricted responses with a given allele. Exploration of other polymorphisms in genes linked to immune responses, such as TNF-α or IL-10, similarly has failed to reveal a clear and consistent pattern, possibly on the basis of inadequate power of most of the studies.[85–88]

## HCV proteins as inhibitors of host immune responses

As the selection of escape mutants to antibodies and HCV-specific CTLs does not fully explain why HCV persists, several studies explored whether HCV proteins directly inhibit host immune responses. The IFN response is a major component of the early response against viral infections, and several studies have demonstrated that HCV proteins interfere with the IFN response (Fig. 26.2). A critical component of the endogenous RNA-dependent protein kinase (PKR) is induced by IFN and activated by double-stranded RNA. Activation of PKR by type I IFN requires its dimerization and autophosphorylation, which in turn phosphorylates the α-subunit of the eukaryotic translation initiation factor 2 α (eIF2 α) and inhibits viral protein synthesis. Although HCV is a strong inducer of type 1 IFN, it is relatively resistant to its antiviral effect. In fact, the glycoprotein E2 of HCV shares a high degree of sequence homology with the autophosphorylation sites of PKR and eIF2α.[89] E2 is able to inhibit PKR activation. This inhibition blocks the phosphorylation of eIF2α, and may contribute to development of resistance during IFN therapy. Other proteins of HCV have been shown to prevent endogenous response to viral infection. Similarly, one of the HCV non-structural proteins, NS5A, also binds to and inhibits PKR through a direct interaction between the C-terminal part of NS5A and the central part of PKR.[90] Recently, NS5A was also found to induce production of IL-8, which in turn partially inhibits the IFN-induced antiviral response *in vitro*.[91] The physiological relevance of this is supported by the finding that hepatitis C patients have shown an elevation of IL-8 in their serum in association with the resistance to IFN therapy,[92] suggesting that NS5A has several potential ways to interfere with the immune response. The HCV NS3/4A serine protease blocks the phosphorylation and effector action of IFN regulatory factor-3 (IRF-3), a key cellular antiviral signalling molecule.[93] Blockade of IRF-3 by NS3/4A results in a decreased transcription of IFN genes. Taken together, these results indicate that HCV has multiple means to inhibit the normal innate immune response to viral infection.

## Impairment of NK and NKT cell functions

Almost all literature to date has focused on the adaptive, antigen-specific response against HCV, despite the abundance of other cell types, such as NK, NKT and γδ T cells in the liver. Two recent reports indicate that HCV E2 protein may inhibit NK cell function and signalling *in vitro* through engagement of CD81, a tetraspanin molecule expressed on the surface of different cell lineage on NK cells.[94,95] Moreover, HCV core protein may impair NK cell activity via p53-dependent upregulation of TAP1 (transporter associated with antigen processing 1) and consequently MHC class I surface expression in liver cells.[96] The inefficient NK cell response could allow virus to evade and counteract a potential NK cell attack. However, studies in persistently infected humans have not consistently demonstrated defects in NK function,[97,98] so further exploration of the role of inhibition of NK function in persistence is needed.

NKT cells are a population that shares features of both classical T cells and NK cells, and are abundant within normal liver.[99] NKT cells actively cause liver destruction in certain models of hepatitis, both directly through their

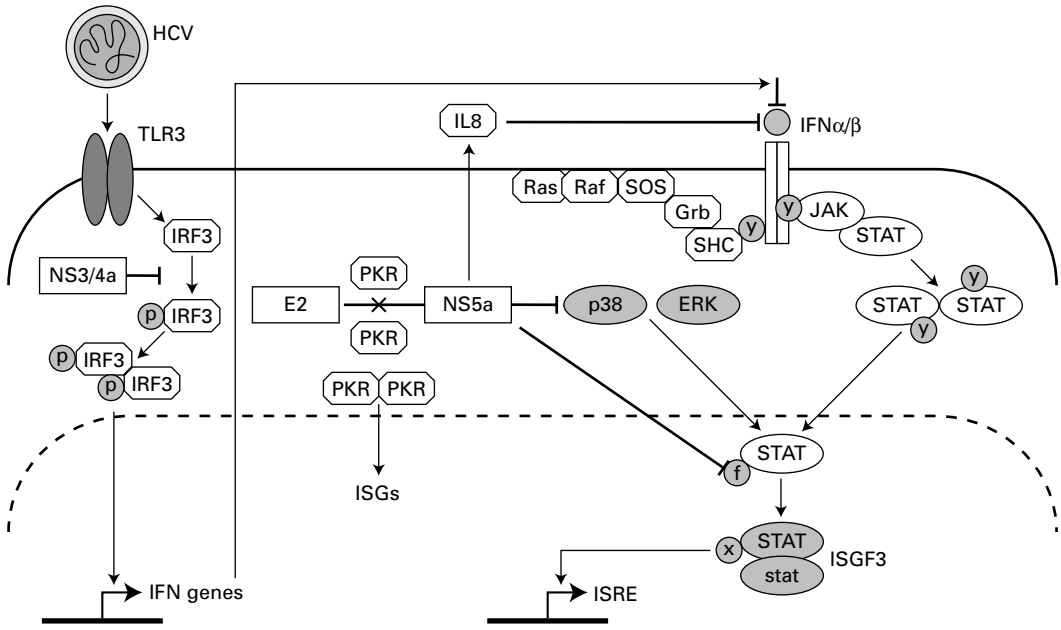


**Figure 26.2** Interference of HCV with the interferon (IFN) response system. Double-stranded RNA stimulates a multi-component complex that phosphorylates IR3, the critical factor required for virus-induced IFN production. Phosphorylated IRF3 dimerizes, moves to the nucleus and activates transcription of IFN genes. HCV NS3/4A results in blockade of IRF3 phosphorylation, and thus prevents IFN transcription. Signalling of IFN-α/β through its receptor is in part mediated by PKR, the active form of which is a dimer. Both HCV E2 and NS5a block dimerization of PKR. NS5a also can interfere with the phosphorylation of STAT proteins, which in turn decreases transcription of IFN responsive elements (ISRE) through interaction with the IFN gene factor 3 (ISGF3). In addition, NS5a stimulates the transcription of IL-8, which is a natural antagonist to IFN-α.

perforin and/or FasL-mediated CD1d-specific cytotoxic activity, and via the ability to activate other immune cells by release of both Th1 cytokines such as IFN-γ as well as Th2 IL-4.[100] As a consequence of their ability to lyse infected hepatocytes and produce cytokines, NKT cells within the liver may play a protective role in clearance of organisms such as *Salmonella* and HBV[20,101,102] as well as contributing to defence against tumours.[103] Conversely, NKT cells are implicated in models of liver injury, as depletion of NK1.1+ NKT ameliorates concanavalin A (ConA)-induced liver disease[104] and CD1d-deficient mice are highly resistant to ConA-induced hepatitis.[105]

There are two distinct populations of CD1d-reactive NKT cells. The first is the classically described 'invariant' TCR expressing Vα24-JαQ in man, paired to a limited set of Vβ chains. Using Vα24, it would appear that CD1d-reactive T cells within the liver are rare, as human liver contains low numbers of cells with this invariant NKT cell-like phenotype (≤1%).[15] In the peripheral circulation, Vα24+ NKT cells are more likely to be apoptotic in patients with chronic HCV than controls.[106] More recent studies using tetramers specific for Vα24+ NKT revealed a very low frequency of these cells in cirrhotic human liver.[107] However, recent results show that measuring only the invariant population may substantially underestimate the true numbers of CD1d-reactive T cells, as a second population expressing 'non-invariant' TCR has been recently been defined.[108] Using functional assays to identify CD1d-reactive T cells, Exley *et al*. have shown that large number of T cells within the human liver are in fact CD1d-reactive T cells.[109] These CD1d-reactive NKT are strongly pro-inflammatory Th1 polarized and are strikingly different from those in the peripheral blood (Th1/2-like) and bone marrow (Th2) of individuals. At present, it is unknown whether this strong Th1 bias is a result of HCV infection or a property of the normal liver, as a recent report found that a greater proportion of NKT intrahepatic-lymphocytes (IHL) in normal human liver produced IFN-γ rather than IL-4 in response to non-specific stimulation.[99]

## Impairment of dendritic cell functions

Dendritic cells (DCs) are important for the initiation of immune system responses to foreign antigens because of their ability to capture and present antigen to T cells. The ability of DCs to induce T-cell responses is also influenced by tissue localization, the antigen dose and the interaction with helper T cells. Recently, it has been shown that HCV may affect the functions of DCs. DCs

expressing HCV core and E1 proteins have an impaired ability to induce CD4+ and CD8+ T- cell responses both *in vitro* and *in vivo*.[110,111] Similarly, DCs from patients with chronic HCV infection showed an impaired ability to stimulate allogeneic T cells and to produce IFN.[112–114] Previous studies on myeloid DCs in HCV-infected patients reported impaired TNF-α-induced maturation that was associated with decreased expression of CD1a, HLA-DR and the co-stimulatory molecules CD83 and CD86.[112] Furthermore, core and NS3 increase production of TNF-α and IL-10 and inhibit DC differentiation in chronic HCV patients.[114] However, not all studies have confirmed that there is functional impairment of DCs.[115,116]

### Failure to generate HCV-specific T cells

In association with a failure of innate immunity or failure of DC function, many have speculated that chronic HCV is due to the failure to generate an HCV-specific T-cell response. For CD4+ responses, as discussed above, the kinetics and breadth of the initial response appears to be directly correlated with the likelihood of clearance. However, for the CTL response this relationship is less clear. While it is true that the magnitude of the response measured in the PBMCs (peripheral blood mononuclear cells) is lower in persons with chronic infection compared with those who have spontaneously resolved HCV, in chronic infection CD4+ and CD8+ T-cell responses appear to compartmentalize to the liver.[51–53,56,117–120] Therefore, persistent infection is not simply a failure to generate HCV-specific T cells, although the kinetics of the response may be different in those who spontaneously resolve compared with those who go on to develop chronic infection. In a recent study, subjects with acute HCV were examined for the kinetics of the immune response in the peripheral blood and liver. Responses were present in the periphery but declined over time whereas they were maintained in the liver tissue in all subjects with chronic infection.[121]

### Impairment of T-cell functions

Why some individuals can mount an effective T-cell response whereas others cannot remains unclear. Certainly, failure of DCs, as discussed above, could alter the kinetics of or the absolute magnitude of the T-cell response. Recent studies have explored whether, in addition to the specificity of the immune response and the kinetics, the function of HCV-specific cellular immune responses is different in those who go on to chronic infection compared with those with spontaneous recovery. For CD4 T cells, most studies have focused on whether there is a Th1 or IFN-γ dominant response, versus a Th2 dominant response. In acute HCV infection, viral clearance is more likely in patients displaying a Th1 profile.[29,31] It has therefore been speculated that an imbalance in Th1/Th2 cytokine production may impair the patient's ability to clear virus. However, examination of T cells *ex vivo* reveals a Th1 bias even in chronic infection.[52,54,56,122]

Other studies have examined whether or not HCV-specific CTLs are capable of effector function. As discussed above, the absolute number of tetramer-positive cells does not permit absolute discrimination of those with chronic evolution versus recovery. In some studies of acute HCV there appeared to be functional 'stunning' in that cells were less capable of producing cytokines thought to be important for the resolution of acute HCV.[46,123] Compared with subjects with acute HBV infection, T cells in acute HCV express significantly lower levels of perforin and decreased proliferation, lytic activity and IFN-γ.[124] However, Takaki *et al*. reported that CD8+ T cells from patients with resolved acute infection exhibit both cytotoxicity and IFN-γ production, but both responses are weak or absent in patients with chronic hepatitis C.[27] These data suggest that the abnormal functional status of virus-specific CD8+ T cells may well account for the failure of these patients to eradicate HCV infection.

What are the mechanisms for this dysfunction? Recent studies have shown that several viral proteins have potential effects on signalling pathways involved in immune response, cell proliferation or apoptosis. HCV core protein, the first protein produced during the early phase of viral infection, can suppress host immune response by inhibiting CTL activity in mice infected with recombinant vaccinia virus expressing core protein. In this model, it appeared that there was interaction between HCV core protein and complement (C1q) receptors on T cells, with resultant inhibition of proliferation and IFN-γ and IL-2 production.[125,126] Core protein increased the sensitivity of the cells to Fas-mediated apoptosis,[127] which may explain the increased Fas-mediated apoptosis observed in PBMCs from patients chronically infected with HCV.[128]

## The role of immune responses in chronic infection

The mechanisms responsible for tissue injury in acute and chronic infection are not well understood. HCV is not considered to be a cytopathic virus because of the absence of classic cytopathic features in liver biopsies, although HCV appears to have important interactions with host cell proteins that might adversely impact on hepatocyte survival or regeneration.[129] The classic understanding of the pathogenesis of liver injury is that it is due to the cellular immune response against the virus. However, there are fundamental problems with this simplistic understanding of the pathogenesis of liver

injury. Clinical experience with immunocompromised hosts indicates that patients with depressed cellular immunity, such as those with AIDS or following orthotopic liver transplantation, are more likely to have progression of liver disease (see Chapter 50). Studies of the role of immune responses in the progression of liver disease are difficult because the chimpanzee model does not develop liver fibrosis, at least in the short term. Moreover, the slow and highly variable rate of disease progression in infected persons limits our understanding of the role of immune responses in chronic infection.

Although it is often stated that immune responses fail to develop in the setting of chronic infection, this is not the case. Instead, responses in chronic infection appear to be preferentially localized to liver tissue. Both CD4+ and CD8+ responses are relatively enriched in liver tissue compared with those observed in peripheral blood.[51–53,56,117–120] Indeed, HCV-specific CTLs can be readily expanded from liver tissue without exogenous antigenic stimulation in both humans and chimpanzees,[52,53,118,130] and are detected at higher frequencies in liver than blood using tetramers.[51,117] Much less is known about the function of these T cells in the liver compared with their counterparts in the peripheral blood. Most studies on the HCV-specific CD8+ T cells in the liver have relied on T-cell lines or clones expanded by *in vitro* stimulation. Studies on these liver-derived CD8+ T-cell clones revealed that their specificity was diverse and comparable to those observed in individuals with acute spontaneous recovery, with some specificities found in the liver that were not identified in PBMCs.[52,53,118,131] This may indicate that the frequency of HCV-specific CD8+ T cells is higher in the liver than in the peripheral blood and/or that the specificity and other properties of the CTLs in the liver may differ from those in PBMCs. Therefore, in order to study the HCV-specific immune response in chronic infection, it is necessary to study responses in both peripheral blood and liver.

There is evidence both for and against a role of cellular immune responses in liver disease and fibrosis. The immune response may play a central role in liver injury, if the magnitude, specificity or effector function is such that viral replication cannot be completely controlled. The role of antigen-specific CTLs in liver cell damage is well established in murine models of viral hepatitis. HBV transgenic mice develop acute hepatitis upon adoptive transfer of syngeneic HBV-specific CD8+ T-cell clones.[132] Several recent studies have confirmed this model in HCV. Animals who only express HCV under the control of an inducible promoter develop inflammation of the liver, whereas those animals who are constitutively tolerant to HCV proteins do not develop inflammation.[133–135] It was recently shown that adoptive transfer of HCV-specific CTLs to transgenic animals bearing HCV structural proteins developed liver injury.[136] In infected persons, the role of cellular immune responses is more controversial, in part because of the difficulties of measuring low frequency responses in the blood and correlating these with progression of disease over time, which requires large numbers of patients to be followed for years. In a small study examining only CTL responses against HCV structural proteins, the presence of liver-derived CTL activity was associated with a higher hepatic necro-inflammatory score, as demonstrated by Nelson *et al*.[137] Reconstitution of the immune system after a period of depressed cellular immune responses, such as occurs after successful engraftment of bone marrow following transplantation, can be associated with dramatic increases in inflammatory activity in the liver, presumably on the basis of enhanced HCV-specific immune responses.[138] Additional supportive evidence for a role for CTLs in liver injury comes from a study where anti-CD8 monoclonal antibodies were administered to a patient with subsequent improvement in serum transaminases.[139] Finally, Nelson *et al*. have shown that recombinant IL-10 improves hepatic inflammation but also leads to increases in viral load via immune modulation.[140] These ineffectual CTLs are thought to exert most of their effect through cytotoxic effects on infected hepatocytes, although indirect effects on nearby, bystander uninfected cells cannot be excluded.[141] Activation of the Fas:FasL system on these activated CTLs may be responsible for a high level of lysis of bystander cells.[142] Production of cytokines by these CTLs may also lead to recruitment of inflammatory cells and activation of stellate cells, although direct evidence for this in HCV is currently lacking.

Alternatively, the cellular immune response may exert a protective effect against disease progression. As discussed above and elsewhere in this volume, patients with depressed cellular immunity have more rapid disease progression, suggesting that some aspects of the cellular immune response, while clearly ineffective at clearing virus, serve to limit liver damage in the presence of viral replication. For example, numerous studies have demonstrated that patients with HIV/AIDS have a higher fibrosis progression rate, particularly in those with CD4+ T-cell counts of <200/mm$^3$.[143–145] Prior to the advent of ART (antiretroviral therapy), several studies described a risk of progressive liver disease in persons with HIV that was in the order of a 3.6-fold increase in the risk of cirrhosis in persons with both infections[145] comparable to the risk of progression in persons with HCV who consume large amounts of alcohol. This rapid progression appeared to be most dramatic in persons with a CD4+ T-cell count <200/mm. Reconstitution of immunity leads to a decrease in the fibrosis progression rate and risk of clinical events due to liver disease.[146] Cross-sectional studies of a small number of subjects with HCV suggest that a more vigorous CD4+ response

is associated with less severe liver histology.[147] Two recent studies by Kamal and colleagues utilized the model of *Schistosoma mansoni* co-infection, in which there is rapid evolution to cirrhosis compared with those with HCV alone and a Th2 bias in co-infected persons. The rate of fibrosis progression was associated with the magnitude of the peripheral and intrahepatic immune response, although the former waned over time, in that subjects who had a vigorous type 1 response in the PBMCs and liver had slower fibrosis progression rates.[121,148] The role of CD8+ T cells is more controversial, with some studies suggesting a relationship between the presence of an HCV-specific CTL response being associated with a lower viral load,[137,149,150] whereas others have failed to find a relationship.[151] As viral load is a poor surrogate for liver damage and does not predict progression of disease in HCV, the clinical importance of the former studies is not clear. None of these cited studies demonstrated a relationship between any CD8+ responses and liver injury, and the number of subjects was relatively small. Therefore, this is another major gap in our understanding of the role of immune responses in liver disease pathogenesis.

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# Chapter 27
# Natural history and experimental models

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The development of animal model systems has provided an invaluable resource for the study of human viral hepatitis.[1] Although the first attempts to transmit this disease to non-human primates date back to the 1950s,[2] the potential value of a laboratory animal model was not clearly recognized until the early 1970s, when the first successful transmission of hepatitis B virus (HBV) infection to chimpanzees was reported.[3] In retrospect, it now appears that many of the failures in transmitting hepatitis to non-human primates occurred because many animals were already immune to the challenge virus. It was not until sensitive serological tests for identifying infections with hepatitis A virus (HAV) and HBV became available that consistent success could be achieved.

Non-human primates have played an important role in furthering our understanding of hepatitis A and B, but their use became widespread only after the discovery of the two causative agents, HAV and HBV.[1] In contrast, much of our understanding of non-A, non-B hepatitis (NANBH), as well as the discovery of its major causative agent, hepatitis C virus (HCV),[4] came from studies in chimpanzees. The first proof that NANBH was indeed an infectious disease caused by a transmissible agent came from almost simultaneous independent reports of successful transmission of human NANBH to chimpanzees,[5–7] and the demonstration of serial transmission to other chimpanzees.[8–10] Chimpanzees were essential to the demonstration that the transmissible agent was filterable[11] and had a lipid-containing envelope.[12] Moreover, chimpanzees also provided an *in vivo* system for the biological amplification of the putative virus to obtain sufficient material for attempts at identification. However, more than 15 years elapsed between the first rigorous description of the disease and the discovery of the aetiological agent.[4] The first specific serological assay for HCV infection was not developed until 1989.[13] This resulted from the partial cloning of the HCV genome after its recovery from plasma of an experimentally infected chimpanzee. In this chapter, we review the role that non-human primate models have played before and after the discovery of the aetiological agent of NANBH.

## The chimpanzee as a model for the study of NANBH in the pre-HCV era

Prospective studies of transfusion-associated hepatitis (TAH) conducted in the United States[14–16] and in Europe[17,18] in the 1960s and 1970s were instrumental in defining the natural history of NANBH. As a result of these studies, it became clear that NANBH was the most common and important complication of transfusion therapy, accounting for the majority of cases of TAH. More important, prospective studies demonstrated that NANBH had a remarkable propensity to progress to chronicity (reviewed by Dienstag[19] and Alter[20,21]). Moreover, besides the transfusion-associated form of NANBH, it was found that a significant proportion of cases, classified as sporadic, community-acquired NANBH, were not associated with identifiable percutaneous exposure.[22,23] Thus, like HBV, both a parenteral and a putative non-parenteral route were identified for the transmission of NANBH.

The discovery of NANBH led to a concerted effort to identify the causative agent. Numerous immunological and serological tests were developed, but none was sufficiently reliable, reproducible or specific to be accepted as a serological marker of NANBH.[24] In this setting, the development of a suitable animal model for NANBH represented an important breakthrough.[25] The animal model was crucial to defining the nature of the causative agent, evaluating suspected epidemiological risk factors and potential sources of infection, and reproducing the natural history of the disease. In addition, the chimpanzee was useful for evaluating the effectiveness of viral inactivation procedures.

Among the potential animal models, chimpanzees were identified as the most promising candidates because they were the only available non-human primates

reproducibly susceptible to both hepatitis A and hepatitis B.[26] The suitability of chimpanzees for hepatitis research stemmed from the close phylogenetic relationship between chimpanzees and humans, as indicated by their high degree of genetic relatedness and similarities in the immune responses and host–pathogen interactions to a variety of human agents.[1,27] The similarity between humans and chimpanzees also had practical applications, such as the ability to use the same reagents to detect proteins of the two species. Indeed, the chimpanzee has proven to be an excellent animal model for NANBH, as the course of primary infection, the acute phase of the disease, the development of the host immune response and the long-term sequelae of HCV infection in chimpanzees mimic well the clinical and immunological observations in humans. The host range of NANBH is remarkably limited.[28] Attempts to transmit the disease to other non-human primate species with inocula of proven infectivity yielded negative or equivocal results.

## Clinical features of NANBH in chimpanzees

Conclusive evidence that the different epidemiological forms of NANBH are associated with a transmissible agent(s) was provided by the successful transmission of NANBH to chimpanzees. Serum or plasma from different sources was used to inoculate the animals. Sources included patients with acute or chronic post-transfusion or community-acquired hepatitis or blood donors implicated in the transmission of hepatitis, as well as concentrates of coagulation factors VIII and IX and fibrinogen (reviewed by Dienstag[24]). After the first experiments in 1978, several laboratories around the world promptly reproduced the transmission of NANBH to chimpanzees. Between 1978 and 1980, using a variety of inocula, 152 animals were inoculated, 110 of which (70%) developed acute hepatitis. The success rate reached 100% in domestically raised chimpanzees that were inoculated with pedigreed infectious inocula.[29]

The clinical features of NANBH in chimpanzees closely reproduce those seen in humans.[24] The incubation period, defined as the time between inoculation and first increase in the alanine aminotransferase (ALT) levels, has ranged from 2 to 20 weeks, with a mean of approximately 7–8 weeks. The acute disease is relatively mild, with peak ALT levels ranging from 100 to 600 IU and, as seen in humans, both monophasic and biphasic ALT patterns can be observed. The incubation period and the severity of acute hepatitis are unrelated to the amount of the inoculum or to the route of administration. Moreover, there is no correlation between the severity of the disease and the number of passages in serial transmission studies. A question raised by the clinical studies of NANBH was the observation of discrete short and long incubation periods. The short incubation period, as brief as 4 days, was predominantly seen in haemophiliacs after therapy with factor VIII concentrates,[30–32] whereas the long incubation period (7–8 weeks) was a regular feature of classical TAH. Based on these observations, some authors hypothesized the existence of two distinct transmissible agents. These speculations, however, were not always supported by transmission studies of viruses from factor VIII concentrates to chimpanzees.[8,33] The incubation period and the clinical and histological characteristics of the disease in chimpanzees inoculated with serum from cases with a short incubation period were indistinguishable from those observed in animals developing NANBH after exposure to viruses associated with long-incubation-period hepatitis.

The development of chronic hepatitis, a hallmark of NANBH, is also a common sequela in chimpanzees. Long-term follow-up of experimentally infected animals demonstrated that NANBH progressed to chronicity in >50% of cases, a rate similar to that reported in humans. Several patterns of ALT elevations were seen in chronically infected chimpanzees. Interestingly, the fluctuating profile of the ALT levels, which is the most characteristic feature of chronic NANBH in humans, has also been observed in chimpanzees. Other patterns include persistent elevations of ALT or recrudescence of the disease several years after the first acute episode.[26,34,35]

## Infectivity studies in chimpanzees

In the absence of any reliable serological tests, the chimpanzee model was instrumental in establishing the duration of viraemia in NANBH virus infection. Successful transmission from sequential serum samples, obtained at various times during the course of acute NANBH, to additional chimpanzees demonstrated that the infectious agent was present in serum as early as 12 days before the first ALT elevation[6] and persisted at least until week 13 after inoculation (1 week after the peak ALT level).[10] Serological re-evaluation of serial subinoculation studies conducted in volunteers in 1969 documented that viraemia appeared within the first week after inoculation.[36] Although the persistence of elevated ALT levels after the acute episode suggested that the disease had progressed to chronicity, it was only through transmission studies in chimpanzees that conclusive proof of the persistence of the NANB agent in blood, in both humans and chimpanzees, could be obtained.[8–10] In addition, successful transmission of NANBH from blood donors or chimpanzees with normal ALT levels provided definitive evidence for the existence of asymptomatic chronic carriers.[37] Evidence was obtained in chimpanzees that a patient was continuously infectious for 6.5 years after the onset of acute hepatitis, even at times when the ALT levels were within normal limits.[37]

The availability of the chimpanzee model permitted the determination of infectivity titres of sera containing NANBH.[26,33] End-point titres of infectivity, defined as the greatest dilution of the inocula at which 50% of the animals became infected, were determined for several clinical samples implicated in the transmission of the disease. The results of these titration studies showed that the end-point infectivity titre of NANBH is generally very low, ranging from $10^0$ to $10^{2.5}$ 50% chimpanzee infectious doses ($CID_{50}$) per mL. Only two inocula among those reported to date had a high infectivity titre ($10^{6.5}$/mL each).[33,34] Thus, the infectivity of NANBH viruses in serum is markedly lower than that commonly observed in HBV ($10^8$)[26] and HDV infection ($10^{11}$).[38] This feature of NANBH may help to explain the difficulties initially encountered in transmission studies, before the introduction of pedigreed inocula, as well as the difficulty encountered in attempting to identify the causative agent.

## Physico-chemical properties of the NANB agent

Most of the physico-chemical properties of the putative agent of NANBH have been determined in experiments utilizing the chimpanzee. Both in humans and in experimentally infected chimpanzees, studies of serum and liver biopsies by electron microscopy (EM) failed to identify virus-like particles specific for NANBH. This failure most probably stemmed from the low infectivity titres of virus in clinical materials. The absence of any serological assays and the inability to grow the virus *in vitro* further hampered the characterization of the causative agent. Infectivity studies in chimpanzees demonstrated that the agent was stable at pH 8.0, but could be inactivated by formalin at a concentration of 1:1000 at 37 °C for 96 hours[39] or 1:2000 at 37 °C for 72 hours.[40] Infectivity could be abolished by heating at 100 °C for 5 minutes[40] or at 60 °C for 10 hours.[39] Complete inactivation of the agent was also achieved by heating at 60 °C for 30 hours after lyophilization,[41] a system that has been widely used for the treatment of pooled coagulation factors for transfusion. The agent is sensitive to a combination of β-propiolactone and ultraviolet light.[42,43] Inactivation by exposure to lipid solvents, such as chloroform, indicated that the agent contained essential lipids, presumably in an envelope.[12,44] Only an isolated report by Bradley *et al*.[44] demonstrated the existence of a chloroform-resistant agent of NANBH, in addition to the common chloroform-sensitive agent, but this could not be confirmed in subsequent tests of the same samples in chimpanzees (Purcell, unpublished data).

To determine the size of the NANBH agent, the virus was subjected to filtration through membranes of defined pore size. Infectivity was retained after filtration through 220-, 80- and 50-nm filters but was removed by a 30-nm filter.[45–47]

The results of the biochemical and physical analyses suggested that the NANBH agent was an enveloped virus with a diameter of 30–60 nm. These properties further suggested that the NANBH virus could belong to the togavirus-flavivirus group, the Hepadnaviridae family, the HDV-like agents, or a previously unrecognized category of virus. That the NANBH agent was not related to hepadnavirus or to HDV was subsequently suggested by nucleic acid hybridization studies.[48,49] Some authors had even proposed that the agent could be a retrovirus, based on the occasional detection of reverse transcriptase activity in the serum of infected patients.[50] However, in subsequent studies with pedigreed sera, these data were not reproduced in other laboratories.[51] Moreover, given the estimated size of the NANBH virus, the hypothesis that it was a retrovirus seemed highly unlikely.

## The role of the animal model in the discovery of hepatitis C virus

The pathway leading to the identification of the causative agent of NANBH was long and tortuous. In retrospect, the length of this process can be explained by the low levels of infectivity, and by the weak and delayed humoral immune response of the host.[52] For these reasons, attempts using conventional virological methods produced only deep frustration for many years. Instead, it was through an unconventional approach, taking advantage of the increasingly refined techniques of molecular biology, that success was eventually achieved. Again, the availability of the chimpanzee model was critical for the discovery of HCV, because it represented the only suitable source for the biological amplification of the putative agent. It was from a chronically infected chimpanzee that large amounts of pooled plasma with an unusually high titre of infectivity ($10^6$) were obtained for the molecular cloning of the viral genome.[4] Litres of plasma were pelleted to concentrate the virus particles, total RNA was extracted from the pellet and retrotranscribed into cDNA. More than 1 million colonies in a lambda gt11 expression system were screened with serum from a chronically infected patient, who served as a source of antiviral antibodies. A single positive clone, designated 5-1-1, which expressed a virus-specific immunogenic peptide was finally identified. As stated by Michael Houghton, the molecular identification of HCV was the culmination of a team effort spanning 7 years, during which 'hundreds of millions of bacterial cDNA clones were screened for a putative NANB hepatitis agent. Only one positive clone was the result of this strenuous effort. If we had missed or lost 5-1-1 from the library, we may still be looking for HCV'.[54] The 5-1-1 clone was then used to identify a larger clone (C100-

3) that was expressed in yeast as a fusion protein with superoxide dismutase. By using overlapping clones, the entire sequence of the HCV genome was subsequently obtained. Thus, HCV represents the first virus in the history of virology that has been characterized primarily by molecular means before it was visualized by EM or isolated in culture.

HCV is a small enveloped, single-stranded positive-sense RNA virus of about 9600 nucleotides in length, which has been classified in a separate genus, Hepacivirus, within the Flaviviridae family.[54] The chimpanzee model has been instrumental for the visualization of intracellular HCV particles, approximately 50 nm in diameter, by EM in liver tissue obtained during the acute phase of hepatitis C.[55] The HCV genome is organized in a manner similar to that of flaviviruses and pestiviruses, the other two genera of the family, and is more closely related to pestiviruses.[56] The viral genome contains a single open reading frame (ORF) with two non-coding regions of approximately 340 and 230 nucleotides at the 5′ end and 3′ end, respectively, and encodes a large polyprotein precursor, approximately 3000 amino acids in length, that is cleaved by cellular and viral proteases into structural proteins – core (c), envelope 1 (E1) and 2 (E2) – and non-structural proteins (designated as NS2–NS5).[57] It is not known whether p7, located between E2 and NS2, is structural or non-structural.[58]

The cloning and sequencing of the HCV genome and the development of serological assays for the detection of specific antibodies to HCV transformed the diagnosis of NANBH from one merely based on exclusion into that of a specific disease, hepatitis C. The application of this assay to clinical practice finally provided the best evidence that HCV is the major aetiological agent of post-transfusion NANBH,[21,59,60] as well as of community-acquired NANBH.[61] The diagnosis of hepatitis C is routinely based on a variety of diagnostic tests, which include indirect markers of HCV infection, such as the humoral response of the infected host, measured by solid-phase enzyme-linked immunoassay (EIA),[13] and the detection of direct markers such as HCV RNA in serum.[62] The development of a third-generation EIA 3, which has replaced the first- and second-generation EIAs, has significantly improved the specificity and sensitivity of detecting anti-HCV antibodies compared with their predecessors.[63] Other EIAs that measure antibodies directed to the E1 and E2 genes have been developed, but they are not yet commercially available, and their application has been limited to experimental studies. Currently, tests to measure HCV antigenaemia are limited to the detection of total HCV core antigen in peripheral blood by means of an ELISA assay.[64] However, the core antigen assay lacks the sensitivity to detect low-level replication (below 10 000–20 000 HCV RNA IU/mL). It has been recently reported that 1 core antigen picogram (pg) per mL is equivalent to approximately 8000 HCV RNA IU/mL and the limit of detection is 1–2 pg/mL.[62] Detection of nucleic acid sequences with the polymerase chain reaction (PCR) or other nucleic acid-based techniques is the most practical method to detect viraemia in the course of HCV infection.[62,65–67] In particular, a sensitive assay such as PCR is critical for evaluating the course of HCV infection, in which low levels of viraemia appear to be the rule, as demonstrated by experimental studies in chimpanzees. The animal model has been fundamental for evaluating the sensitivity of the PCR technique. By analyzing 10-fold serial dilutions of a reference plasma (strain H) whose infectivity was $10^{6.5}$ $CID_{50}$/mL; the PCR assay was capable of detecting HCV with a sensitivity approximately 10-fold greater than the infectivity titre in chimpanzees.[65,68,69]

## Genetic variability

Extensive molecular analysis has demonstrated that HCV is characterized by a high degree of genetic variability, a hallmark of RNA viruses.[70,71] Over the past few years, the study of the genetic variability of RNA viruses has received increasing attention because of the possibility of linking a better understanding of viral evolution to prospects for disease control and prevention.[72,73] The main factor contributing to the genetic variability of HCV is the error-prone nature of genome replication, because the RNA polymerase lacks a proofreading exonuclease activity, which is an important repair mechanism that removes misincorporated bases from newly synthesized RNA strands.[74] This leads to the generation of a high mutation rate during genome replication. Other properties, however, contribute to the extremely high genetic variability of HCV. These include the large population size, the high replication rate and the short generation time.[75]

The genetic variability of HCV is complex and has been classified in four hierarchical strata: genotypes, subgenotypes, isolates and quasi-species. Phylogenetic analysis of full-length or partial sequences of HCV strains collected worldwide led to the identification of six major genotypes and more than 100 subtypes, which differ in their global distribution.[70,76,77] In recent years, however, refined molecular techniques have provided the tools to demonstrate that, not only among different individuals but also within an infected individual, HCV circulates as a population of different, albeit closely related, genomes exhibiting a distribution that follows the model referred to as a 'quasi-species' [78] according to a concept that was first introduced by Eigen in 1971.[79] The quasi-species distribution is a highly dynamic process generating a continuously changing spectrum of mutant viruses, which favours adaptability in the event of environmental change.[80] Variation within an evolving quasi-species

is not only a direct reflection of the mutants generated during replication, but also the result of a competitive selection based on the viability and replicative efficiency of such mutants in a given environment (fitness).[80,81] The quasi-species as a whole, and not its individual components, is the true target of selection. The quasi-species behaves as an evolutionary unit and represents at any given moment the best fitting population that has established a status of equilibrium with the host.[82] The evolution of the viral quasi-species is strictly dependent on the population size of the virus that sustains the infection. Multiple factors act as selective forces, including the specific organ and cellular milieu and the neutralizing effects of both cellular and humoral immunity.[83] The degree of genetic heterogeneity of the HCV quasi-species can be studied at two different levels: the genetic complexity, defined as the total number of viral variants simultaneously present in a single sample, and the genetic diversity, defined as the average genetic distance among the different variants. By cloning and sequencing more than 100 molecular clones of HCV derived at a single time-point during the acute phase of hepatitis C from each of two patients, it was possible to obtain a detailed characterization of the HCV quasi-species.[84] Sequence analysis of the HVR1 demonstrated the simultaneous presence of 53 and 19 different viral variants in patients M and H, respectively. Within the 27 amino acids of the hypervariable region 1 (HVR1) of the E2 envelope glycoprotein of HCV, the genetic diversity among the mutant spectrum ranged from one to ten sites in HCV from patient M and from one to eight sites in HCV from patient H. The plasma source of HCV from patient H and the virus derived from it (H77) have been extensively studied both molecularly and in the chimpanzee model. For example, Ogata *et al*.[85] sequenced HCV isolates obtained from patient H during the acute hepatitis phase and after 13 years of follow-up. Their analysis, based on 50% of the HCV genome, showed a mutation rate of $1.92 \times 10^{-3}$ base substitutions per site per year. A similar study was conducted by Okamoto *et al*.[86] in a chimpanzee experimentally infected with HCV. Sequence analysis of the complete genome of two isolates obtained during the acute phase and 8.2 years later demonstrated a mutation rate of $1.44 \times 10^{-3}$ per site per year. The degree of variability is not homogeneously distributed within the entire viral genome. The 5′ non-coding region is among the most conserved,[87–89] and the E1 and E2 genes are the most variable.[90] The highest variability, both at the nucleotide level and at the amino acid level, has been detected at the amino-terminus of the E2 gene. This domain of 27 amino acids, the HVR1, may result from the continuous immune pressure of the host. [91,92] The HVR1, being the most variable region of the HCV genome, has been used as a fingerprint for identifying individual viral variants and for studying the genetic complexity and diversity of the viral quasi-species. Although the biological function of the HVR1 is only partially understood,[93] this region has attracted considerable interest because it has been shown to contain a neutralization epitope.[94,95] It is a target of antibodies that correlate with viral clearance[96] and it was postulated to serve as a decoy antigen[97] as well as, more recently, a receptor-binding domain.[98] A potential biological role of the HVR1 is suggested by the fact that, despite its extraordinarily high level of variation, there are important structural constraints in this region.[99] Penin and colleagues,[100] by analyzing more than 1300 HVR1 sequences derived from all the different genotypes, provided evidence of a striking similarity in both the hydrophobicity and antigenicity profiles. Moreover, they also documented a conservation of positively charged residues within the HVR1. These findings have been confirmed in other studies,[101] suggesting that the HVR1 contains conserved determinants that may play a critical role in the viral life-cycle, most likely at the level of viral entry.[102]

## The contribution of the animal model to the study of the natural history of HCV infection

Chimpanzees represent a valuable model for the study of the natural history of HCV infection because they reproduce, under carefully controlled experimental conditions, the disease observed in humans. The extensive clinical and experimental studies of NANBH conducted before the discovery of HCV had raised many questions that were difficult to address in the absence of specific diagnostic markers. After the discovery of HCV, antibody assays and sensitive assays for detecting the HCV genome, such as PCR, became available to investigators. These advances provided an opportunity to re-evaluate the early studies of experimental transmission in chimpanzees. Thus, chimpanzees have represented an optimal source of controlled clinical material for studying the kinetics of viral replication and the relationship between viraemia and the host immune response during the course of acute and chronic HCV infection. Moreover, the chimpanzee model has been fundamental for comparing the clinical and virological features of wild-type (polyclonal) HCV infection versus infection with molecularly cloned (monoclonal) HCV.

### Wild-type HCV infection in chimpanzees

Primary HCV infection in chimpanzees is characterized by the early appearance of HCV viraemia, which in most cases becomes detectable within 1 week after inoculation, long before other markers can be detected. [65,103–108] In two chimpanzees in which serum and liver specimens were obtained daily during the first week of

infection, newly synthesized HCV RNA in serum was detected as early as 3–4 days after inoculation.[107] During the acute phase, the peak viral titre ranges from $10^5$ to $10^7$ genome copies per mL. The detection of serum HCV RNA precedes the appearance of ultrastructural changes[107,109] and associated host-derived cytoplasmic antigens, as detected by monoclonal antibody 48-1, in hepatocytes.[107] By direct immunofluorescence, intrahepatic HCV antigens can be found in the cytoplasm of hepatocytes in the early phase of acute hepatitis, before the appearance of the antibody response. [110]

Evidence of hepatitis can be detected on average 7–10 weeks after inoculation, although low-level elevations of liver enzyme values often occur earlier, within the first 1–3 weeks after exposure to HCV.[5,33] This pattern of early ALT elevations resembles that observed in humans and is believed to result directly from HCV infection. The severity of hepatitis, as measured by the ALT levels, does not appear to correlate with the infectivity titre of the inoculum.[111,112] In self-limiting HCV infection of chimpanzees, the viraemia is transient and lasts for a variable period, ranging from 10 to 38 weeks[103,105,106] but occasionally can be longer.[113]

The humoral response to primary HCV infection is usually delayed with respect to the clinical onset of the disease and is highly variable. The biological reasons for the variable immune response observed in primary HCV infection in chimpanzees are unclear at present. Analysis of a large series of chimpanzees has shown that there is no correlation between the first appearance of HCV viraemia and the time of antibody seroconversion,[103,105,106] or between the infectivity titre and the time of seroconversion.[103,111] The time interval between inoculation and antibody seroconversion ranges from a few weeks to several months, and this interval is dependent on the antibody being measured. Antibodies directed to the nucleocapsid protein of HCV (anti-core) are usually the first to appear and can be detected immediately before or coincident with the major ALT peak, although there is an extreme variability among individual chimpanzees.[103,105] Antibodies to the NS3 appear coincidentally with or shortly after antibodies to core,[105] while antibodies to the NS4 are usually the last to appear, on average 10–15 weeks after the major ALT peak.[65,103–106, 114] The shortest interval from the onset of hepatitis to antibody seroconversion has been observed using the second-generation assay,[104–106] and more recently with the third-generation assay.[115,116]

Besides variations observed in the detection of antibodies directed to different HCV antigens, there are also intrinsic differences in the immune response of individual chimpanzees. This variability includes not only the time of seroconversion, but also the pattern of antibody response. In some animals, the antibody response was limited to anti-NS3 and anti-NS4,[104,106] while in others it was associated with the appearance of an isolated anti-core or anti-NS4 response.[103] The importance of individual variability is emphasized by the observation that the same inoculum induces different patterns of antibody response in different chimpanzees.[111] The introduction of the second-generation assay has diminished the variability in the humoral response observed with the single assays and has narrowed the length of the seronegative window (Figs 27.1–27.3) to an average of 5–8 weeks.[104–105] Moreover, it has considerably increased the sensitivity of the assay, as evidenced by the identification of cases that lacked antibodies detectable by first-generation assay (Fig. 27.4).[105,117] This notwithstanding, variability in the time of seroconversion has also been observed with the second-generation assay.

Data obtained both from the animal model and from humans during the early phase of acute hepatitis failed to identify any virological or serological markers that may be of value in predicting the outcome of HCV infec-

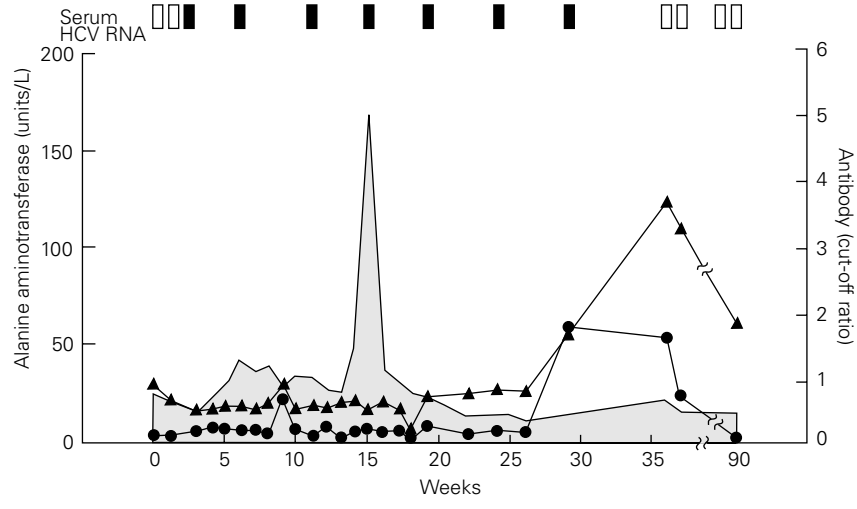


**Figure 27.1** Pattern of antibody response to hepatitis C virus (HCV) in chimpanzee 105 with transient HCV viraemia. Open bars indicate negative assays for serum HCV RNA by PCR, and solid bars positive assays. The grey area indicates the values of serum alanine aminotransferase. First-generation anti-HCV assay is indicated by circles, second-generation anti-HCV assay is indicated by triangles. The cut-off ratio represents the ratio between the absorbance value for the test sample and that for the assay cut-off; values above 1 were considered positive. (Modified from Farci *et al*.[105] with permission.)

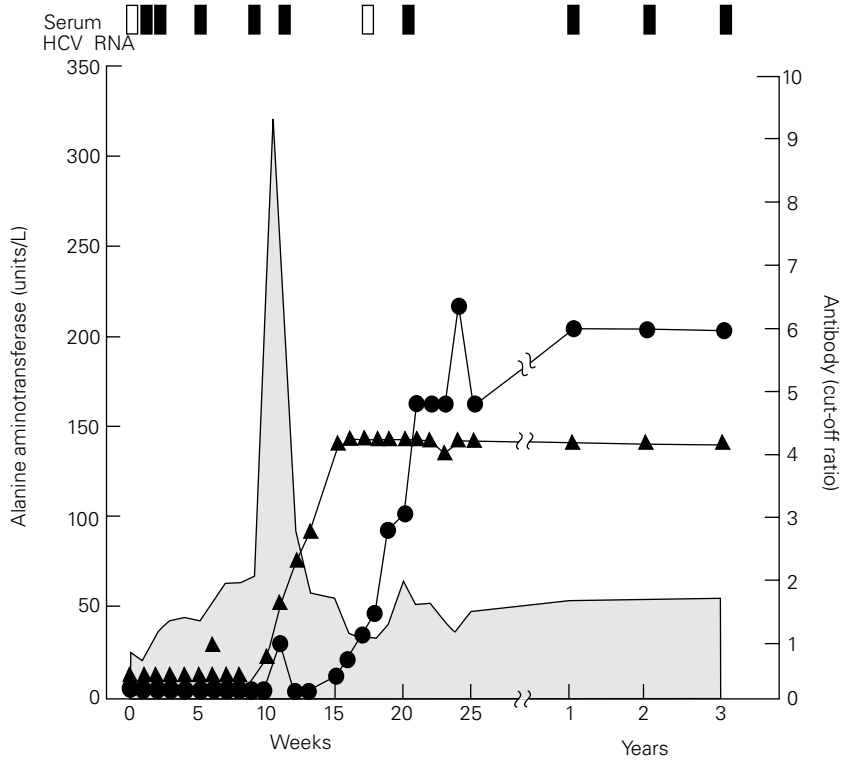


**Figure 27.2** Pattern of antibody response to hepatitis C virus (HCV) in chimpanzee 51 with chronic HCV infection and persistent HCV viraemia. The animal was inoculated with serum from a patient with chronic post-transfusion hepatitis (strain F), fifth passage in chimpanzees. Open bars indicate negative assays for serum HCV RNA by PCR, and solid bars positive assays. The grey area indicates the values of serum alanine aminotransferase. First-generation anti-HCV assay is indicated by circles, second-generation anti-HCV assay is indicated by triangles. The cut-off ratio represents the ratio between the absorbance value for the test sample and that for the assay cut-off; values above 1 were considered positive. (Modified from Farci *et al*.[105] with permission.)

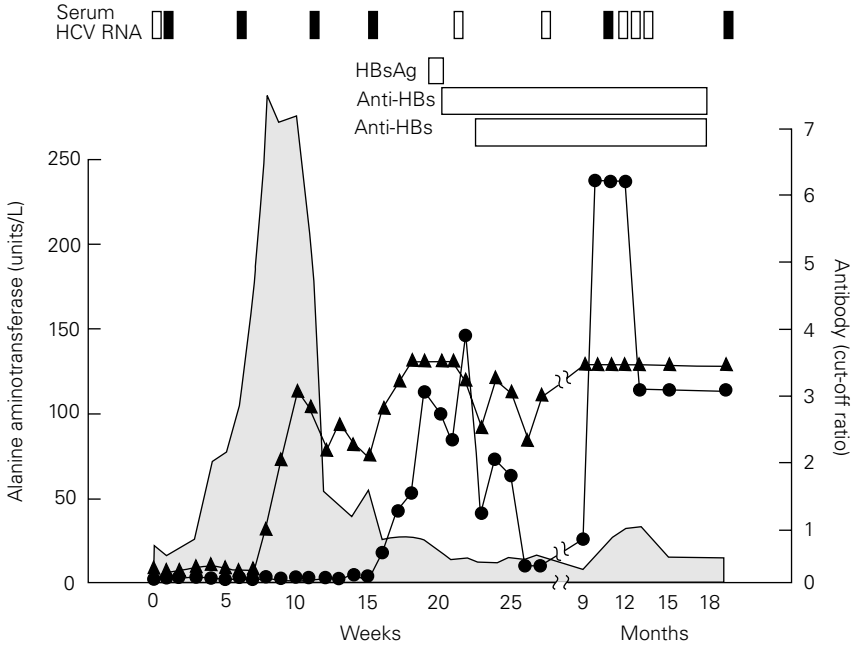


**Figure 27.3** Pattern of antibody response to hepatitis C virus (HCV) in chimpanzee 888 with chronic HCV infection and persistent HCV viraemia. Open bars indicate negative assays for serum HCV RNA by PCR, and solid bars positive assays. The grey area indicates the values of serum alanine aminotransferase. First-generation anti-HCV assay is indicated by circles, second-generation anti-HCV assay is indicated by triangles. The cut-off ratio represents the ratio between the absorbance value for the test sample and that for the assay cut-off; values above 1 were considered positive. Horizontal bars indicate the time during which serum was positive for hepatitis B surface antigen (HBsAg), for antibody to HBsAg (anti-HBs), and for antibody to hepatitis B core antigen (anti-HBc). (Modified from Farci *et al*.[105] with permission.)

tion. The initial antibody pattern was not significantly different between animals that developed chronic HCV infection and those that cleared the viraemia.[106] Thus, the detection of serum HCV RNA is the only currently available test that may provide prognostic information on the outcome of the disease. Sustained clearance of HCV RNA correlates with resolution of the disease, whereas persistence of detectable HCV RNA predicts progression to chronicity.[65] However, the lack of detectable HCV RNA by PCR does not necessarily exclude the presence of tiny amounts of infectious virus.[104] HCV RNA is also the crucial marker for establishing an early diagnosis of primary HCV infection. In fact, although the introduction of the second-generation antibody as-

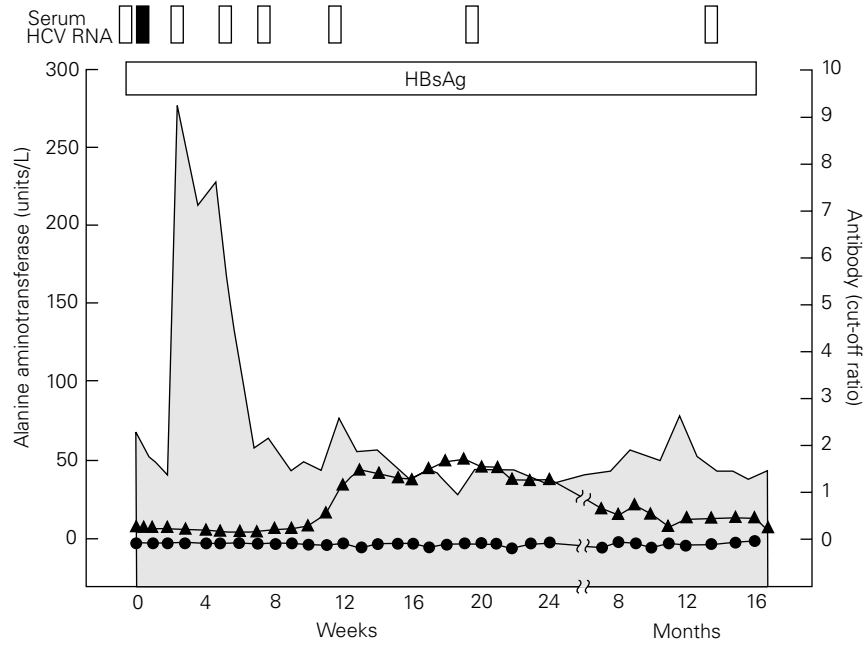


**Figure 27.4** Pattern of antibody response and HCV viraemia in chimpanzee 904, which was inoculated with serum from a patient with acute post-transfusion hepatitis (strain H). The animal was a chronic carrier of hepatitis B surface antigen (HBsAg) at the time of inoculation. Open bars indicate negative assays for serum HCV RNA by PCR, and solid bars positive assays. The grey area indicates the values of serum alanine aminotransferase. First-generation anti-HCV assay is indicated by circles, second-generation anti-HCV assay is indicated by triangles. The cut-off ratio represents the ratio between the absorbance value for the test sample and that for the assay cut-off; values above 1 were considered positive. The horizontal bar indicates the time during which serum was positive for hepatitis B surface antigen (HBsAg).

say has considerably narrowed the seronegative window and increased the sensitivity of the test, there is still a prolonged interval between the first detection of HCV RNA and antibody seroconversion.[105] This seronegative window has not been further narrowed by the introduction of tests for IgM class antibodies against different HCV antigens.[104] With the antibody assays so far available, there is still a serologically silent period, during which HCV RNA, as detected by PCR, is the only marker that permits early diagnosis of primary infection and identification of potentially infectious individuals who would be missed by conventional antibody testing.[65,103–106,109] However, the value of the PCR assay for predicting infectivity of donors of plasma or blood is not absolute, especially in view of the fact that the volumes of plasma tested by PCR are usually lower than those used for experimental inoculation in chimpanzees. In this respect, Beach *et al*.[104] reported successful transmission of HCV infection with 17-mL and 100-mL samples of two inocula that were negative for HCV RNA when 50-µL samples were tested by PCR.

The virological and serological profiles of chronic HCV infection differ from those seen in acute hepatitis. In chronic HCV infection, HCV viraemia usually follows two main patterns: persistent or intermittent (Figs 27.2 and 27.3).[103–106] In contrast to the variable, delayed, and weak antibody response seen in acute hepatitis, the serological pattern in chronic HCV infection is more sustained and consistent. Persistent viraemia is associated with the presence of antibodies against structural and non-structural proteins, both of which increase in parallel with the progression of the hepatitis and persist at high titres as the disease continues (Fig. 27.2).[105] The pattern of continuous viraemia documented for up to 10 years in one chimpanzee[109] and, more recently, for over 20 years in another (unpublished data) resembles the pattern seen in chronically infected humans. In chimpanzees with intermittent viraemia, the antibody profiles may differ from those observed in animals with continuous viraemia.

A correlation was not observed between the pattern of HCV viraemia and the ALT profile during long-term follow-up.[65,104,105] Persistent viraemia may be associated with the characteristic ALT fluctuations, or with mild, nearly normal ALT values. This disparity between HCV RNA and ALT levels indicates that HCV replication is not always associated with serious liver damage, for reasons that are not understood. In both humans and chimpanzees, the absence of ALT elevations for extended periods of time does not exclude the presence of circulating viral RNA and does not imply recovery from disease.[104,118] These clinical features complicate the definition of acute self-limiting hepatitis and suggest that caution should be used in the diagnosis of resolution of HCV infection.

## Monoclonal HCV infection in chimpanzees

The generation of infectious cDNA clones of HCV has represented an important advance in HCV research, providing new insights into the mechanisms of HCV pathogenesis, and permitting more detailed studies of the molecular biology of HCV.[119,120] Infectious cDNA clones of HCV have been generated for strains of genotypes 1a, 1b and 2a. As no reproducible cell culture systems

to grow HCV are currently available, demonstration of the infectivity of such clones would have been impossible without using chimpanzees. Chimpanzees became infected when genomic RNA transcripts synthesized *in vitro* from full-length HCV cDNA clones were inoculated directly into the liver, which can be done in a percutaneous procedure guided by ultrasound.[121] As the RNA inoculated was generated from a single HCV sequence, the animals became infected with a monoclonal virus. The mutation rate per nucleotide site per year ($1.48–1.57 \times 10^{-3}$)[122] was similar to that reported in chimpanzees infected with polyclonal virus ($1.44 \times 10^{-3}$).[86] Monoclonal infection of chimpanzees provides a simplified model for studies of HCV pathogenesis because the viral interaction with the host is not initially complicated by the quasi-species that is invariably present in a wild-type HCV inoculum.[113] Also, in pathogenesis studies, infection with a molecular clone eliminates the possibility that putative co-infecting agents may account for or modify the observed results. It furthermore permits true homologous challenge in studies of protective immunity[123,124] and in testing the efficacy of vaccine candidates.[125] Finally, this *in vivo* transfection system has made possible for the first time molecular analysis of HCV infectivity.[126,127]

The initial pattern of HCV infection after transfection of chimpanzees with monoclonal HCV RNA transcripts does not differ significantly from that observed in animals infected intravenously with the original uncloned virus.[115,119,120,123,128] The animals become viraemic at weeks 1–2 and the viral titres increase to reach a peak of $10^5$–$10^6$ genome equivalents per mL typically during weeks 8–12. The animals develop acute hepatitis, thus formally proving that HCV causes liver disease. A high rate of progression to chronicity was observed in animals with monoclonal HCV infection, indicating that, at least in chimpanzees, the presence of a quasi-species viral population during the early acute phase is not a requirement for viral persistence. Thus, monoclonal infection of chimpanzees provides a suitable model for studying the host factors that determine the outcome of acute HCV infection.[113,115] Major *et al*.[115] studied acute hepatitis C in 10 chimpanzees experimentally infected with the same monoclonal virus, representing the consensus sequence of the prototype HCV strain, H77. Irrespective of the outcome, the animals showed a significant decrease in virus titre after reaching peak levels. All 10 animals had similar levels of interferon (IFN)-γ induction in the liver, which coincided with the initial decrease in virus titre and with the development of hepatitis. As IFN-γ and IFN-induced genes in the liver are expressed as a result of the activation and liver-homing of immune cells, this result suggests the development of significant intrahepatic cellular immune responses in all animals. However, only four animals were able to clear the infection. These animals had 0.5–1 log lower peak titres of viraemia and their initial decrease in virus titres occurred 1–2 weeks earlier. Also, only the animals with resolving infection showed significant induction in the liver of mRNA specific for the epsilon chain of CD3, as well as for macrophage-inflammatory protein 1 (MIP-1) alpha, a chemokine involved in homing and activation of immune cells, which could indicate qualitative and/or quantitative differences in intrahepatic cellular immune responses. Such differences might simply reflect a higher level of immune cell activation, but are most likely related to the nature of the immune cells that are preferentially activated during an effective sterilizing immune response. The timing of these responses might also be crucial. As these animals were all infected with the same virus, the observed differences can with some certainty be associated with the host responses irrespective of the virus sequence.

## HCV and fulminant hepatitis

Before the discovery of HCV, the role of putative NANBH agents in fulminant hepatitis was difficult to establish, as diagnosis was merely based on exclusion criteria (reviewed by Dienstag[19]). In such studies, NANBH agents were thought to account for 30–40% of all cases of fulminant NANBH. However, these values probably overestimated the true incidence, because cases of drug toxicity or fulminant hepatitis B with undetectable HBsAg could have been included in this group. In contrast, prospective studies of post-transfusion NANBH suggest that fulminant NANBH is rare.[129–131]

The discovery of HCV permitted a more accurate investigation of the role of this virus in the aetiology of fulminant NANBH. The evidence thus far accumulated suggests that the association between HCV and fulminant hepatitis differs markedly according to the geographical area studied. In Japan and Taiwan, HCV infection, as determined by the presence of antibodies to HCV or serum HCV RNA, was documented in 40–60% of the patients, [132–138] whereas a number of studies conducted in Western countries failed to demonstrate conclusively an aetiological role of HCV in fulminant NANBH.[139–146] In these studies, the prevalence of HCV infection ranged from 0 to 12%. The only exception was a seroepidemiological survey conducted in California that reported a prevalence of 60%, associated with low socio-economic status and Hispanic ethnicity.[147] An aetiologic link between HCV and fulminant NANBH was established by longitudinal analysis of serum samples collected from a patient enrolled in the National Institutes of Health (NIH) prospective study of transfusion-associated NANBH.[148] Fulminant hepatitis C was associated with continuous HCV replication throughout the course of the disease, while antibody seroconversion

occurred after week 7, on the last few days before the patient's death. Therefore, as previously documented in acute hepatitis C, the detection of serum HCV RNA was the earliest and most valuable marker for the diagnosis of fulminant hepatitis C.

Interestingly, fulminant hepatitis C is characterized by high levels of viraemia associated with a highly homogeneous viral quasi-species population.[149] Although the pathogenic mechanism of virus-induced fulminant hepatic failure is not known, the extent of liver damage correlated with the magnitude of viral replication. In contrast, in patients with fulminant hepatitis B, HBV replication is barely detectable or even undetectable, while antibody titres are high.[150] This suggests that the mechanisms of fulminant liver injury in the two types of hepatitis differ. In fulminant hepatitis C, sequence analysis demonstrated a very low degree of diversity in the viral quasi-species, whereas considerable intra-patient variability has been detected in non-fulminant hepatitis C.[149] Whether this finding reflects the lack of selective immune pressure of the host, particularly the lack of specific antibodies, in this rapidly evolving syndrome remains to be established. Considering the persistence of viraemia observed in fulminant hepatitis C, it is very unlikely that HCV RNA-negative cases of fulminant NANBH could be related to HCV infection. Whether such cases are due to yet unidentified infectious agents or, as recently proposed,[151] represent a syndrome caused by non-infectious hepatotoxic agents is presently unknown. Animal models may be a valuable tool for addressing these questions.

Several attempts have been made to transmit hepatitis from plasma of patients with fulminant NANBH to animal models, but they have been unsuccessful.[33] Subsequently, however, by using a small amount of serum from a patient with fulminant hepatitis C (HC-TN, genotype 1a), HCV infection was successfully transmitted to a chimpanzee (Ch 1422).[152] The acute hepatitis developed by this animal was characterized by the highest serum ALT peak (744 U/L) and by the most severe pathological findings ever observed among more than 20 chimpanzees infected with different HCV strains. The animal developed antibodies to HCV and eventually cleared serum HCV RNA. Sequence analysis indicated that the virus recovered from the chimpanzee was the same as that isolated from the patient who served as the source of the virus. This represented the first experimental animal transmission of HCV from a patient with fulminant hepatitis and provided additional evidence for an aetiological role of HCV in the development of fulminant hepatitis.

Recently, a second animal (Ch 1581) was infected using plasma from chimpanzee 1422 and a third one (Ch 1579) was infected with RNA transcripts from a consensus cDNA clone of HC-TN (unpublished data). In contrast to Ch 1422, these two additional chimpanzees developed a typical acute hepatitis C with an ALT peak of 298 IU/L and 90 IU/L, respectively, and minimal activity grade in liver biopsy, as commonly seen in the chimpanzee model. Although the course of viraemia was similar, Ch 1579 resolved the infection whereas Ch 1581 became persistently infected. Thus, the unusual severity observed in chimpanzee 1422 did not breed true in the other two animals. Virulence depends upon a complex interplay between the virus and the host, and may be influenced by several factors including the dose of infecting virus, route of entry and virus sequence, as well as by the immune response of the host. With regard to the latter, Ch 1581 and Ch 1579 were previously exposed to HCV in attempts to transmit infection using sub-infectious doses of HCV or RNA transcripts from HCV molecular clones, which did not result in detectable infection, but may have primed some type of host immune responses, as previously demonstrated in chimpanzees.[126,153] By contrast, Ch 1422, which developed severe acute hepatitis, did not have such prior exposure. It is also unclear whether serial passage through chimpanzees may affect the biological properties of a particularly virulent strain of HCV. However, when the full-length consensus sequence obtained at the ALT peak from Ch 1422 was compared with those obtained at the ALT peak from the other two animals, only a single point mutation was detected in the sequence recovered from Ch 1581, which was already present at week 1 after infection, while no changes were detected in Ch 1579.

In conclusion, although the pathogenic mechanism of HCV-induced fulminant hepatic failure is not known, the evidence thus far accumulated suggests that HCV is an aetiological agent of fulminant hepatitis. Nevertheless, this is a rare event and in most cases of fulminant hepatitis the causative agents remain elusive. Although to date all attempts to transmit hepatitis from patients with non-A, non-B, non-C fulminant hepatitis to chimpanzees have failed (unpublished data), studies in animal models may be important in the search for yet unidentified factors implicated in the aetiology of this dramatic syndrome.

## Interactions among hepatitis viruses in chimpanzees

The chimpanzee animal model has been instrumental in documenting *in vivo* the phenomenon of viral interference between hepatotropic viruses. This notion has emerged from the observation that simultaneous or sequential infection of chimpanzees with more than one hepatitis virus could alter the course of infection. Several experimental studies have documented that, in chimpanzees with chronic NANBH, superinfection with HAV or HBV is associated with a significantly attenuated acute hepatitis.[154,155] Conversely, chronic HBV

infection does not appear to interfere with the clinical expression of acute hepatitis caused by superinfection with other hepatitis viruses. Instead, superinfection with other hepatitis viruses consistently causes a suppression of HBV replication.[154, 156–158] The most striking example of such an effect is superinfection of chronic HBV carriers with hepatitis delta virus, a unique RNA virus that requires the helper function of HBV for infection.[159] Similarly, superinfection of chronic HBV carriers with HAV results in the transient suppression of serum HBV DNA, usually not associated with variations in the titre of HBsAg. Likewise,[160] superinfection with NANBH virus does interfere with HBV synthesis, as evidenced by suppression of both HBV DNA and HBsAg serum levels.[154,156–158]

The effect of chronic HBV infection on the replication of HCV was unknown until recently. Using PCR, it has been possible to re-evaluate this aspect in sera from previous experimental studies in chimpanzees.[105] One chimpanzee, a chronic carrier of HBsAg, was inoculated with HCV-positive serum from a patient with post-transfusion NANBH. In this animal, serum HCV RNA was detected within 1 week after inoculation, preceding the clinical onset by 2 weeks (ALT peak of 280 U/L), and remained detectable for only 1 week (Fig. 27.4). This unusual pattern of HCV viraemia may be the consequence of an inhibitory effect of HBV infection on HCV replication. The animal developed a transient anti-HCV antibody response detected by the second-generation assay, but failed to develop antibodies to the NS4. It is likely that the short duration of HCV viraemia was responsible for the weak humoral immune response. The animal remained HBsAg-positive throughout the study. Replication of HBV was depressed during superinfection of this chimpanzee with HCV.[156] Thus, there may be a reciprocal inhibition of replication in dual infections by HBV and HCV. Although the short duration of HCV viraemia in this animal did not seem to influence the clinical picture of the acute episode, the acute disease occurred significantly earlier than usual (week 3 after inoculation). Another chimpanzee was inoculated with a commercial source of factor VIII containing both HBV and HCV (Fig. 27.3). This animal developed acute NANBH that progressed to chronicity. Serum HCV RNA appeared 1 week after inoculation, remained positive during the acute phase, and then fluctuated from positive to negative. Nineteen weeks after inoculation and approximately coincident with the disappearance of serum HCV RNA, the chimpanzee became transiently HBsAg-positive (for 1 week). One week later, anti-HBs and anti-HBc became detectable. Liver enzymes remained normal at the time of the appearance of these markers of HBV infection. The delay observed in the incubation period of hepatitis B may result from an inhibitory effect of HCV on HBV replication. This observation is consistent with other studies that documented that co-infection of chimpanzees with inocula containing both HBV and HCV may delay the onset of acute HBV infection.[161]

## HCV and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer.[162,163] It represents a major public health problem because its prevalence has increased worldwide, especially in Europe and the USA over the past decade. [164,165] In the USA, where the development of HCC is mainly related to HCV infection,[166] estimates of the burden of HCC suggest that its incidence will increase, within two decades, to values similar to those reported in Japan.[167] Cirrhosis represents the highest risk factor, as in 80% of the cases HCC develops in patients with cirrhosis.[168]

The aetiological role of HBV in the development of HCC has been well established by epidemiological studies, [169,170] as well as by studies of experimental carcinogenesis in the woodchuck animal model.[171] Virtually 100% of woodchucks chronically infected with woodchuck hepatitis virus (WHV), a member of the Hepadnaviridae family antigenically and structurally related to HBV,[172] develop liver cancer within approximately 4 years of infection.[173,174] The risk of HCC in chronic WHV infection is similar to that calculated by Beasley[175] for chronic HBV infection in humans: >40% of HBsAg carriers are predicted to die of HCC or some other form of liver disease.

The high rate of progression to chronicity of HCV infection, leading to cirrhosis in 20–35% of the cases, [16,118,161,176–180] makes HCV a major cause of HCC worldwide.[162,163] Before the discovery of HCV, several studies supported the epidemiological, clinical and histological evidence of an association between chronic NANBH and liver cancer.[181–183] With the advent of HCV serology and PCR, the evidence that HCV may be aetiologically associated with HCC has grown,[164,165,184–196] although in some HCC patients occult HBV infection has been recently documented in liver tissue. [197] The mechanism of carcinogenesis by HCV is still poorly understood.[198] Unlike other human oncogenic viruses, HCV is not involved in tumorigenesis by integrating into the host genome, as its life-cycle does not involve DNA intermediates. The study of HCV as a causative agent of HCC is hampered by the lack of an efficient *in vitro* system for the propagation of the virus. Moreover, at present, there are no useful animal models for the study of HCV-associated hepatocarcinogenesis.

Chimpanzees can be readily infected with HCV, resulting in either acute or chronic infection, but HCC has been thus far reported only in one chimpanzee experimentally infected with serum obtained from a patient with chronic NANBH.[199] The animal had developed

acute NANBH that progressed to chronicity. HCC was diagnosed 7 years after the initial inoculation. A homogenate of liver obtained from this animal at autopsy transmitted NANBH to another chimpanzee. HCC, however, was independently found in two other chimpanzees in the same facility, and no evidence of HCV infection could be found in these animals, thus weakening the association between HCV infection and HCC in the first animal.[200] Indeed, many chimpanzees chronically infected with HBV or HCV have been followed for more than 18 and 12 years, respectively, without detecting HCC.[201] This is not surprising, however, as the lifespan of chimpanzees is only slightly less than that of humans. Therefore, if HCV is carcinogenic for chimpanzees, the incubation period must be longer, as seen in humans. The long-term observation of chimpanzees following experimental hepatitis C may eventually help to clarify the issue of the putative role of HCV in the aetiology of HCC.

## Immunity to HCV

Shortly after NANBH was identified, clinical and experimental data suggesting the existence of more than one NANBH agent accumulated.[202] The clinical evidence was based on the sequential occurrence of multiple, distinct episodes of acute NANBH in individuals, such as haemophiliacs,[32,203] drug addicts,[122,204,205] or haemodialyzed patients, [206,207] who were repeatedly exposed to blood or its derivatives. Another clinical finding that lent more credence to this hypothesis was the observation, as noted previously, of two distinct forms of NANBH: one characterized by a short (1–3 weeks) and one by a long (7–9 weeks) incubation period. The former occurred more frequently in haemophiliacs.[30–32] Additional evidence for the existence of more than one NANBH virus came from cross-challenge studies in chimpanzees. These studies documented a clinical pattern similar to that seen in humans and characterized by multiple bouts of acute hepatitis in single animals infected with different inocula.[8,208–212]

Despite multiple lines of evidence suggesting the existence of at least two agents of NANBH, other observations in experimentally infected chimpanzees argued against this hypothesis. The availability of an animal model permitting the repetition of experiments under carefully controlled conditions generated data that generally failed to confirm the existence of multiple NANBH agents. The short incubation period seen in haemophiliacs did not always breed true in chimpanzees infected with factor VIII concentrate preparations and previously implicated in the transmission of such forms of NANBH.[8,33] Similarly, most cross-challenge studies in chimpanzees failed to induce a second episode of acute hepatitis.[33,213,214] To further complicate the picture, recurrent episodes of hepatitis were also seen in chimpanzees that were not rechallenged,[213] as well as in animals rechallenged with the same inoculum.[215,216] Thus, explanations other than the existence of multiple NANBH agents could account for these observations.

After the discovery of HCV, extensive seroepidemiological studies have conclusively shown that this virus is the major cause of both post-transfusion and community-acquired NANBH,[13,118,177]and that other hepatitis agents, if they exist, account for only a minority of such cases. Therefore, other explanations had to be found for the episodes of recurrent NANBH seen both in humans and in experimental models, such as the emergence of neutralization-escape mutants or the failure of the host to mount an effective immune response, leading to reactivation or reinfection with the same virus.

### Lack of protective immunity against re-infection and superinfection with HCV

The degree of protection afforded by primary infection against subsequent re-exposure to the virus is a measure of the maximum protection that can be expected from a vaccine against that virus. To examine the question of protective immunity against HCV, experimental cross-challenge studies in chimpanzees, with both homologous and heterologous HCV strains, were re-evaluated.[217] The patterns of viraemia and antibody response were studied in five chimpanzees sequentially inoculated at intervals of 6 months to 2 years with different HCV strains of proven infectivity, obtained from individual patients included in the NIH prospective study of post-transfusion NANBH. Three chimpanzees were challenged twice and two were challenged four times. All were followed for a mean period of 32 months (range 12–51 months). One chimpanzee was rechallenged with the homologous inoculum, and the remaining four were rechallenged with heterologous inocula. After the first virus challenge, all animals developed acute hepatitis C. Viraemia was transient in four animals, but became chronic in one. All chimpanzees seroconverted to HCV, as measured by first- or second-generation antibody assays. In contrast, none of the animals developed detectable antibodies directed to the E2 glycoprotein, and only two had detectable antibodies to the NS5 gene product (anti-NS5). Each rechallenge of a convalescent chimpanzee resulted in the reappearance, within 1–3 weeks, of serum HCV RNA, although the duration of viraemia during rechallenge was shorter (range 1–10 weeks) than that seen during primary infection (range 11–17 weeks). The recurrence of viraemia was always associated with reappearance of antibodies against the non-structural proteins C100-3 or NS5, an indication that viral replication had recurred (Fig. 27.5). Although there was less biochemical evidence of hepatitis after

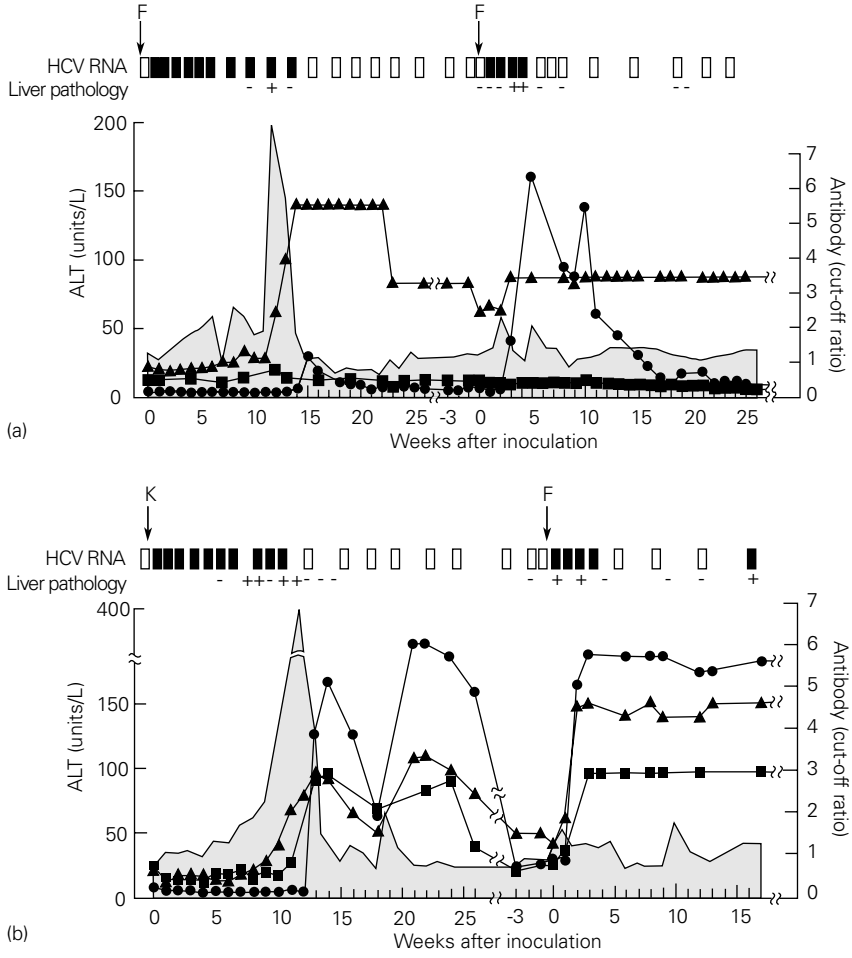


**Figure 27.5** Course of HCV infection in chimpanzee 963 rechallenged with the homologous HCV strain (a) and in chimpanzee 793 with a heterologous HCV strain (b). The grey areas indicate the values of serum ALT. The arrows indicate the time of challenge and the strain used for inoculation. Open bars indicate negative assays for serum HCV RNA by PCR, and solid bars positive assays. Liver pathology indicates necroinflammatory changes rated as negative or positive. First-generation anti-HCV assay is indicated by circles, second-generation anti-HCV assay is indicated by triangles, and squares indicate anti-NS5. The cut-off ratio represents the ratio between the absorbance value for the test sample and that for the assay cut-off; values above 1 were considered positive. (Modified from Farci *et al*.[217] with permission.)

subsequent challenges, the degree of histopathological changes was similar in each bout of hepatitis and the risk of developing a chronic infection was similar to that observed after primary infection. Genetic analysis of the HVR1 demonstrated that the recurrence of viraemia was not due to reactivation of the original challenge strain, but to infection with the HCV strain used for rechallenge (Fig. 27.5). Reinfection occurred several times in a chimpanzee challenged with four different HCV strains and also occurred in a chimpanzee rechallenged with the homologous inoculum. Similar data were reported by Prince *et al*.,[218] who documented the reappearance of HCV viraemia in chimpanzees rechallenged with either homologous or heterologous strains of HCV. Several other studies subsequently confirmed that chimpanzees that had resolved a first episode of acute experimental HCV infection again develop viraemia when rechallenged with either the homologous or heterologous viruses of the same major genotype.[123,124,217–220] As previously observed, however, the duration of viraemia is in most cases significantly shortened following reinfection and biochemical evidence of hepatitis can be observed in only a few cases. Transient infection was also documented following heterologous rechallenge with a different major genotype.[221] The availability of monoclonal virus made possible *bona fide* homologous rechallenge studies, which demonstrated that reinfection is not the result of the emergence of immune escape variants.[123,124] Strikingly, even in this 'best-case scenario' for the host, reinfection can result in persistent infection, as illustrated by studies in two chimpanzees rechallenged with homologous monoclonal virus after resolution of primary infection by monoclonal genotype 1a virus (unpublished data). In one animal, rechallenge resulted in transient viraemia lasting 2 weeks, but in a second animal HCV persisted despite vigorous anamnestic intrahepatic T-cell responses.

Inoculation of both HCV antibody-positive and -negative human immunoglobulin preparations failed to prevent HCV infection in chimpanzees, when administered intravenously 1 hour after virus challenge.[222] Serum HCV RNA was detected in each chimpanzee within a few days after inoculation, regardless of the type of immunoglobulin infused. However, the liver enzyme peak was delayed in the animal that received hepatitis C immune globulin. These data suggest that post-expo-

sure administration of hepatitis C immune globulin can markedly prolong the incubation period of acute hepatitis C, but not prevent or delay HCV infection.

The data obtained in the animal model, that HCV does not induce a protective immune response against reinfection with either homologous or heterologous viral strains, suggested that this mechanism could be the cause of the multiple episodes of NANBH occurring in individual patients, such as polytransfused individuals and intravenous drug users. Evidence that HCV may cause more than one episode of acute hepatitis was obtained in polytransfused thalassaemic children.[223] Three thalassaemic children from Sardinia, Italy, selected among those who experienced more than one episode of acute NANBH, were studied. In each case, the first episode of hepatitis was a classic acute hepatitis C, associated with the appearance of serum HCV RNA and antibody seroconversion. Interestingly, in each case the second hepatitis was as severe as the primary hepatitis and, as previously seen in the chimpanzee model, viraemia persisted in parallel with the development of chronic hepatitis. Sequence analysis of the HVR1 region demonstrated that in two children the reappearance of viraemia was due to reinfection with a different viral strain. Thus, reinfection with HCV may account for the multiple, distinct episodes of acute NANBH documented in thalassaemic children undergoing long-term transfusion therapy. Another important observation related to the ability of HCV to induce a protective immune response in the host is the occurrence of superinfection with heterologous HCV strains, both in humans and in chimpanzees chronically infected with HCV.[224] The first evidence of superinfection was obtained by Kao *et al*.,[225] who documented a second episode of post-transfusion acute hepatitis C in a chronic HCV carrier. By sequence analysis, they demonstrated that the superinfection event was transient and that the new virus was subsequently cleared and replaced by the original strain. Superinfection with HCV has been observed also in the setting of liver transplantation. [152] The superinfection event was again transient, lasting only for 2 weeks. Similar evidence has been obtained in the animal model.[226,227] Okamoto *et al*. demonstrated that chimpanzees chronically infected with HCV genotype 1b can be superinfected with genotype la and subsequently 2a.[226] The superinfecting strain may either replace the original virus or transiently co-exist and eventually replace it. However, the most important observation was that each rechallenge of a chronically infected animal was associated with a new episode of liver damage. These findings have important implications for both the pathogenesis and the differential diagnosis of an acute exacerbation of chronic hepatitis C.

Patients undergoing liver transplantation for HCV-related chronic liver disease have an almost universal recurrence of HCV infection after surgery.[228] The evidence obtained both in humans and chimpanzees strongly suggests that these patients may not be protected against superinfection with different HCV strains. Prospective studies will be important for determining the impact of these events on the natural history of the disease in transplanted patients.

## Neutralizing antibody response against HCV

One of the most important issues related to the immune responses elicited by HCV is the fact that the majority of infected individuals fail to clear the infection despite a competent immune system that produces large amounts of anti-HCV antibodies.[176,229] Only a minority of the patients is able to eradicate the virus. This small subset of individuals might develop an earlier and/or more effective protective immune response. A critical question raised by these observations is whether HCV infection induces in the host the production of neutralizing antibodies (NAbs) and whether such antibodies are involved in the resolution of acute self-limited hepatitis C. It is well established that NAbs play an important role in the prevention of, and recovery from, viral infections and provide the best correlate of protection against several viral infections in vaccinated individuals.

The lack of efficient and reproducible *in vitro* systems for the propagation of HCV in cell culture has severely hampered the study of the host immune response to HCV, in particular the identification and characterization of NAbs. Although some degree of HCV replication has been demonstrated in selected continuous cell lines,[230] none of these *in vitro* systems reproducibly permits the growth of complete, replication-competent HCV. Thus, until recently, the chimpanzee *in vivo* model has remained the only system for the study of antibody-mediated HCV neutralization, although there are obvious limitations related to their routine use, primarily because they are a costly and endangered species. Over the past few years, new systems for testing NAbs to HCV have become available with the development of *in vitro* neutralization assays based on molecular approaches, such as pseudoparticles and virus-like particles. Although the physiological relevance of these systems is currently under investigation, their use has significantly expanded our possibilities to investigate the protective role of humoral immunity to HCV.

## *In vivo* neutralization of HCV in the chimpanzee model

The most conclusive evidence that infection with HCV does elicit NAbs in the host has been obtained in a study performed by *ex vivo* neutralization followed by testing of residual infectivity in the chimpanzee model.[231] How-

ever, the same study clearly demonstrated that the effectiveness of NAbs in resolving HCV infection is limited by a severely restricted spectrum of activity. Plasma from a chronically HCV-infected individual (patient H) was tested for its ability to neutralize HCV obtained from the same patient during the acute phase of post-transfusion NANBH (H77).[232] The infectivity of the inoculum had been previously titrated in chimpanzees.[112] The plasma tested as a putative source of NAbs was collected from patient H at two time-points, 2 and 13 years, respectively, after primary HCV infection.[65] Both plasmas had high titres of antibodies against both structural and non-structural HCV proteins, including anti-envelope 1 (E1) and -E2 antibodies. As a negative control, plasma from a normal blood donor, whose blood repeatedly failed to transmit hepatitis after transfusion into humans, was used. For the neutralization test, the viral inoculum was mixed *in vitro* with each plasma previously heat-inactivated at 56 °C for 30 minutes. The virus–plasma mixtures were incubated overnight at 4 °C and then inoculated into a seronegative chimpanzee. No signs of HCV infection were observed in the animal inoculated with HCV mixed with plasma obtained 2 years after primary infection, providing evidence that infection with HCV does elicit a neutralizing antibody response that can prevent HCV infection in chimpanzees. However, no protection was seen when the virus was treated with plasma collected 11 years later. Thus, the two plasmas derived at different times during the chronic phase of infection differed markedly in their neutralizing capability against H77, although they both contained antibodies against the E1 and E2 envelope proteins.

To investigate the reasons for the differential neutralizing capability of the two plasmas, sequence analysis was conducted on the viral isolates obtained before and after inoculation in chimpanzees. Interestingly, a remarkable degree of genetic divergence was observed both among sequential isolates, documented as early as 2 years after infection, and within the viral quasi-species contained in the H77 inoculum. When the nucleotide sequences of the HVR1 of the viruses recovered from chimpanzees that had received the same virus inoculum were compared, none of the sequences was identical to the consensus sequence of the H77 strain used for inoculation, with differences observed at 2–21 sites. The degree of heterogeneity observed within the HVR1 suggested that the divergent strains were already present within the original viral quasi-species of the inoculum, rather than resulting from an exceedingly rapid mutation rate in the chimpanzees *in vivo*.

Remarkably, similar neutralization data were obtained by Shimizu *et al*.[233] using an *in vitro* system based on a human continuous cell line infected with murine leukaemia virus (MuLV). The H77 virus was neutralized by plasma obtained from patient H for the first 5 years after primary infection, but not by plasma obtained thereafter, including at year 13. Likewise, the virus obtained 13 years after primary infection was not neutralized by plasma obtained early in the disease course, but only by plasma obtained 14 years after infection. Altogether, these *in vitro* and *in vivo* data suggest that HCV does elicit NAbs, but that such antibodies are isolate-restricted and ineffective against some of the strains contained in the complex quasi-species of the inoculum or emerging over time *in vivo*. This strategy may allow HCV to evade the host immune response and may help to explain the development of persistent infection in the majority of infected individuals.

Although these results provided the first formal proof of the existence of NAbs against HCV, historical evidence suggesting their existence was obtained even before the discovery of HCV. Several studies conducted in the 1960s and in the 1970s had indeed documented a reduced incidence and severity of post-transfusion NANBH in subjects treated with serum immunoglobulins.[234–237] Such evidence was subsequently confirmed after the discovery of HCV, especially with the Gammagard accident, when a commercial immunoglobulin product prepared from plasma from which anti-HCV-positive units had been excluded transmitted HCV to recipients.[117,238] Epidemiological and laboratory studies suggested that the exclusion might have removed NAbs and hence compromised the safety of the resulting immunoglobulin product.[117] A direct demonstration of the mechanism by which the safety of Gammagard was compromised was recently provided by Yu *et al*. using chimpanzees and an *in vitro* assay based on neutralization of infectious HCV pseudoparticles.[239] Broadly reactive neutralizing and protective antibodies were detected in Gammagard lots made from unscreened plasma that did not transmit hepatitis C, as well as in experimental immunoglobulin preparations made from anti-HCV-positive donations, but not in Gammagard lots prepared from anti-HCV-screened plasma, which did transmit hepatitis C.

## Identification of the hypervariable region 1 as a major neutralization domain of HCV

As stated above, the limited spectrum of activity documented for anti-HCV NAbs may be directly related to the quasi-species nature of this virus, with the continuous emergence of viral variants that can escape antibody-mediated neutralization. Consistent with this concept, several lines of evidence indicate that the most variable region of the entire HCV genome, the HVR1,[91,92] is a critical target of NAbs. This assumption is based on the fact that the HVR1 contains linear epitopes recognized by patient antibodies,[240–245] and undergoes sequential mutations over time *in vivo*, suggesting that it is subjected to the selective immune pressure of the host.[85,86,246–250] That

an effective humoral immune pressure is required for driving HVR1 variability was elegantly demonstrated by the lack of genetic variation documented in patients with agammaglobulinaemia.[251,252] Moreover, using an *in vitro* test of virion-cell binding, antibodies specific for the HVR1 present in human sera were shown to block the binding of HCV to cells.[253] Another study has recently identified virus binding-neutralizing antibodies directed against putative conserved E2 epitopes, presumably located outside the HVR1.[254] Such antibodies were present at low titres in patients infected with different genotypes of the virus, but at high titres in chimpanzees vaccinated with recombinant E1 and E2 envelope glycoproteins expressed in mammalian cells in a vaccinia system,[255] which were protected from challenge with 10 chimpanzee infectious doses ($CID_{50}$) of the homologous virus.[256] Finally, the HVR1 has a marked similarity to the V3 domain of the gp120 envelope glycoprotein of HIV-1, a disulfide-bonded loop of 35–37 amino acids, which plays a critical role in the HIV life-cycle by directly interacting with the cellular co-receptor and by determining the viral co-receptor preference.[257] Like the HCV HVR1, the V3 loop mutates rapidly *in vivo* under the selective pressure of the host humoral immune response and is a major target of type-specific NAbs.

Conclusive evidence that the HVR1 is a critical target of NAbs was obtained in a study conducted in chimpanzees, which also provided the first *in vivo* model for the emergence of neutralization escape mutants.[94] A hyperimmune rabbit serum was raised against a synthetic HVR1 peptide of 21 amino acids (aa) representing aa 390–410 of the sequence of the predominant strain contained in the H77 inoculum. This antiserum was then used for *in vitro* neutralization of the homologous viral stock, and the residual infectivity was tested by intravenous inoculation of two HCV-seronegative chimpanzees. One animal did not show any signs of HCV infection, whereas the second developed acute hepatitis that progressed to chronicity. Strikingly, the analysis of the viral quasi-species recovered from the latter chimpanzee demonstrated that none of the molecular clones had the sequence identical to the predominant strain that was used to raise the hyperimmune rabbit anti-HVR1 serum. Instead, the viruses emerging *in vivo* were identical to the sequence of two minor variants that were already present within the original H77 inoculum (where they represented 6% and 2%, respectively, of the quasi-species). These findings demonstrated that the anti-HVR1 serum had neutralized the predominant variant bearing the sequence used for immunization but was ineffective against minor variants that were already present at the time of inoculation. These data support the concept that the genetic heterogeneity of the viral quasi-species is involved in immune escape.

The *in vivo* neutralization data are consistent with those reported by Shimizu *et al*.[95] using an *in vitro* system based on HPBMa10.2 human lymphoid cells. The hyperimmune serum raised against the homologous HVR1 peptide neutralized the H77 virus, but not a genetically divergent isolate (H90) obtained from the same patient 13 years after primary infection. This study also identified a smaller segment within the HVR1, spanning amino acid positions 398–410, as the epitope recognized by the hyperimmune anti-HVR1 antiserum.

## *In vitro* neutralization of HCV: pseudotype particle assays

Testing for anti-HCV NAbs has recently been expanded with the introduction of various *in vitro* assays based on pseudoparticles (pp)[258,259] and virus-like particles (VLP) bearing HCV envelope glycoproteins.[260] These recent developments have provided important new tools to determine the titre, breadth and duration of antibody-mediated neutralization against different HCV isolates, to identify the major neutralization epitopes, as well as to investigate the role of humoral immunity in the outcome of HCV infection (viral clearance or persistence). In addition, these assays will be a valuable tool for evaluating potential passive and active immunization approaches against HCV. One of the major concerns regards the cell types used for testing the pp or VLP infectivity, such as the hepatocarcinoma cell line Huh-7 or cultured primary hepatocytes, which are not the same as hepatocytes *in vivo*.

Bartosch *et al*. generated infectious HCV pp that were assembled in cell culture by displaying unmodified and functional HCV glycoproteins onto MuLV or HIV core particles.[258] These pseudotyped retroviruses were infectious for certain cell lines of hepatocyte origin, primarily Huh-7 cells, as well as for primary human hepatocytes maintained in culture. High infectivity of the pp required the presence of both the E1 and E2 glycoproteins of HCV. A similar system, utilizing pseudotyped HIV, was used by Hsu *et al*.[259] who confirmed the infectivity of the pp for hepatoma cells (Huh-7 and PLC/PR5). In both assay systems, specific rodent monoclonal antibodies against the E2 protein were able to specifically neutralize the infectivity of the pp.[259,261,262]

An encouraging result for the clinical validation of the *in vitro* pp assay is the fact that Bartosch *et al*. were able to reproduce most of the previously reported neutralization findings.[261] Using some of the same reagents employed in the chimpanzee studies,[231] as well as in the T-cell assay by Shimizu *et al*.,[233] they were able to confirm the existence of NAbs to HCV in patient H during the chronic phase of infection. The only discrepancy was the result obtained with serum H90, which strongly neu-

tralized the infectivity of H77 in the pp assay, but failed to neutralize in the chimpanzee and T-cell assays, even though all the tests were based on the neutralization of the same H77-derived viruses.[261] This discrepancy is most likely due to the fact that the pp threshold of positivity is only 50% neutralization, whereas the chimpanzee model requires a complete, 100% neutralization, as a single surviving particle escaping neutralization can initiate infection. Interestingly, no anti-HCV NAbs were detected by the pp assay in the serum of patients with resolving infections but relatively high titres were measured in chronically infected patients. In some patients, NAbs were broadly reactive as they cross-neutralized pp bearing different subgenotypes (1a and 1b).[261] The pp neutralization assay was also instrumental in confirming that the HVR1 is a neutralization domain.[259,261] However, NAbs were shown to be directed not only to epitope(s) in the HVR1 of the E2 envelope protein but also to one or more epitopes elsewhere in the viral envelope, suggesting the existence of other, more conserved, neutralization epitopes within the E2 protein outside the HVR1.[259] Thus, besides confirming the existence of NAbs in plasma or serum from chronically HCV-infected patients, these studies have also documented the broad spectrum of activity of such NAbs, suggesting the existence of conserved neutralization epitopes in the HCV envelope.

Using the HIV/HCV pp assay, the role of NAbs has been recently investigated in relation to the outcome of HCV infection in chimpanzees. Logvinoff *et al*.[263] examined the development of NAbs over time in 10 chimpanzees infected with the monoclonal H77 virus. Strikingly, no NAbs were detected in three animals that cleared the infection, whereas strain-specific NAbs were found in six of seven chronically infected animals after about 50 weeks of infection.[263] Likewise, NAbs were detected in only two of seven acutely infected health-care workers, without any association with subsequent viral clearance, while they were present in the majority of chronically HCV-infected patients. In the prototype patient H, longitudinal analysis showed an early but weak NAb response restricted to the autologous virus (H77), whereas cross-reactive NAbs appeared only after 33 weeks of infection.[263] Similar results were recently obtained by Meunier *et al*. who tested both chimpanzee and human sera against heterologous pp (unpublished data). Low-titre NAbs were detected in one of five acutely infected chimpanzees that later developed chronic infection, as well as in patient H during the acute phase of infection, but in only one of four chimpanzees and in none of three patients with acute resolving hepatitis C. By contrast, relatively high titres of NAbs were found in two of five chimpanzees and in patient H during the chronic phase. Interestingly, NAbs raised during infection with genotype 1a cross-neutralized HCV pp constructed with genotype 4a or 5a envelopes, but showed only a limited activity against genotypes 2a and 3a. Finally, both chimpanzee and human sera were shown to contain a still unknown factor(s) that enhances the infectivity of HCV pp.

## Cell-mediated immune responses to HCV in chimpanzees

The role of cell-mediated immune responses in the control of HCV infection has been the focus of extensive research over the past decade both in natural infection in humans and in the experimental chimpanzee model.[264–267] Even though there is a general consensus that the development of early, broad and persistent CD4+ and CD8+ T-cell responses is essential for the *in vivo* containment of HCV replication and, in the best scenario, for viral clearance, the data reported in the literature are not univocal, at least in part due to differences in the study design, in the selection criteria for patients and animals, and in the experimental techniques utilized. The two major settings in which cell-mediated immune responses have been investigated are the resolution of acute hepatitis C and the control of virus replication in patients with chronic HCV infection. In both instances, the primary aim of the studies was to ascertain whether specific CD4+ or CD8+ T-cell responses provide a reliable correlate of protection associated with viral clearance or with a sustained control of viraemia.

## Acute hepatitis C and its long-term outcome

Studies in chimpanzees have been essential for our understanding of the cellular immune responses during acute hepatitis C. In experimentally infected chimpanzees, unlike naturally infected humans, liver tissue can be collected frequently during the acute phase of hepatitis. Cells can be directly isolated from the organ where the virus primarily replicates, and used to quantify and functionally characterize HCV-specific T-cell responses. Furthermore, RNA can be extracted from liver tissue and the mRNA profiles can be determined either by microarray technology, which provides an exhaustive analysis of thousands of genes, or by RNase protection or real-time PCR assays, which permit analysis of a limited number of selected genes.

In acute HIV infection, which shares many similarities with the HCV model, the dramatic reduction of viraemia that commonly follows the peak viral replication has been temporally correlated with a remarkable expansion of virus-specific CD8+ T lymphocytes in blood (up to 10% of total CD8+ T cells, often with prominent clonal Vβ populations), which are therefore believed to play an essential role in virus control. This notwithstanding, definitive HIV clearance is never achieved,

possibly due to the lack of a sufficient helper function of CD4+ T cells, which represent the primary target for HIV replication.[266] In HCV infection, the study of the protective mechanisms and correlates is facilitated by the possibility of comparing individuals (humans or chimpanzees) who spontaneously clear the virus with those in whom the infection progresses to chronicity. The results obtained from the study of cells derived from chimpanzee peripheral blood have not been univocal. Whereas Cooper *et al*. showed a correlation between the appearance in blood of multi-epitope-directed CD8+ T-cell responses and subsequent resolution of primary HCV infection,[267] Thomson *et al*. failed to confirm such a correlation.[268] Strikingly, comparative analysis of liver-infiltrating and peripheral blood-derived HCV-specific CD8+ T lymphocytes demonstrated that only the former, but not the latter showed a correlation with disease outcome.[269] However, some animals in which the virus persisted also had vigorous intrahepatic CD4+ and CD8+ T-cell responses. In these animals a significant decrease in virus titre during the acute phase was followed by persistence at relatively low titres. A subset of animals in which the virus persisted at the same titres found during the acute infection did not have detectable intrahepatic T-cell responses. In contrast, all animals had detectable peripheral T-cell responses. In the animals with detectable intrahepatic responses, these were typically detected after several weeks of infection, prior to the initial decrease in virus titres. Overall, vigorous intrahepatic T-cell responses correlated with a significant decrease in virus titre during the acute phase, but they did not always result in viral clearance.

The relative role played by CD4 ('helper') and CD8 ('cytotoxic') T-cell responses in the control and clearance of acute HCV infection is still a matter of controversy. Experiments of multiple HCV challenges in chimpanzees have been essential in addressing this fundamental issue. The importance of CD8+ T lymphocytes in the resolution of HCV infection has been challenged by the observation that their *in vivo* depletion using a specific monoclonal antibody did not impede the eventual clearance of HCV reinfection in multiply challenged chimpanzees, even though the duration of the viraemic period was prolonged with respect to previous infections.[270] By contrast, antibody-mediated depletion of CD4+ T cells was shown to result in an incomplete control of HCV replication, with invariable progression to chronicity, in spite of the presence of functional intrahepatic CD8+ T lymphocytes, providing compelling *in vivo* evidence of the critical role played by this cellular subset in protective immunity.[271] Consistent with this observation, the development of HCV-specific CD4+ T-cell responses was shown to correlate with a positive outcome of HCV infection following rechallenge.[124,219,272] Strikingly, it has been documented that specific memory CD4+ T-cell responses persist for several years after definitive resolution of primary infection, and vigorous anamnestic responses are observed following rechallenge, whereas $CD8^+$ T-cell responses tend to disappear.[270,273]

The introduction of microarray techniques for the analysis of gene expression profiles has provided new tools for exploration of the early molecular events in the acutely HCV-infected liver, which is beginning to reveal the complexity of the pattern of virus–host interaction.[115,269,274,275] A strong induction of markers of the innate immune response, such as IFN-α-stimulated genes, was documented during the early phase of acute infection in chimpanzees, regardless of the infection outcome.[275] The role of these early responses both in the control of HCV replication and in the eventual outcome of HCV infection is still unclear. Conversely, during the phase of declining viraemia, an upregulation of markers of the acquired immune responses was observed, with high-level expression of IFN-γ-induced genes and other genes involved in antigen processing and presentation.[274,275] Such genes include MHC class II, TAP, selected immunoproteasome subunits (e.g. LMP2), CD8, granzyme A and various chemokines (e.g. Mig, IP10, RANTES).[275] Yet, it is noteworthy that such responses developed both in animals with subsequent viral clearance and in animals that became persistently infected. The difference in outcome could be associated with subtle qualitative and/or quantitative differences in the intrahepatic cellular immune responses, which may be difficult to appreciate using current methodologies. In particular, the nature of the immune cells that are specifically activated during this critical phase and their level of activation may be critical. Also, the timing of these adaptive responses, in relation to the virological events as well as to the early innate immune responses, might be crucial. Finally, it should be emphasized that a number of other genes not related to the innate or adaptive immune responses have been found to be upregulated during the early acute phase of HCV and could potentially play an equally important role for the early control of HCV viraemia or for the outcome. In particular, host genes involved in lipid metabolism were also upregulated in chimpanzees displaying the most effective immune responses, although the significance of these findings is still uncertain.

The emergence of escape mutants from CD8+ T cells may represent one of the most effective viral mechanisms for establishing persistent infection in the host. The relevance of genetic variability, which is one of the hallmarks of HCV, in the outcome of acute hepatitis C has been investigated using the chimpanzee model. An association between failure to resolve HCV infection in chimpanzees and the emergence of CTL-escape variants has been demonstrated. In acutely infected animals in which HCV persists, T-cell escape mutants are rapidly

selected following the initial intrahepatic HCV-specific T-cell responses. Weiner *et al*. described the early emergence, at week 4 after inoculation, of a virus variant bearing a single point mutation within a CTL epitope in the NS3 protein.[276] Such mutation was sufficient to determine loss of recognition by specific CTLs extracted from the liver and persisted for several years. Erickson *et al*. provided evidence that multiple CTL escape mutants appeared simultaneously in MHC class I-restricted epitopes during the acute phase of hepatitis C, remaining fixed in the surviving viral quasi-species for several years without further diversification.[277] Two epitopes were located in NS3, one in NS2 and one in NS4. By contrast, animals with acute resolving infection showed no amino acid changes in most of the CTL epitopes examined. These observations suggest that the CTL response, similar to neutralizing antibodies, may have a viral isolate-restricted spectrum of activity, which would be ineffective against mutant strains that continuously emerge *in vivo*. Thus, it is possible that failure to clear acute HCV infection may result from the selection of virus mutants that escape both humoral and cellular immune responses.

The host immune response was recently studied in two chimpanzees infected with an HCV strain derived from a patient with fulminant hepatitis C (HC-TN), in whom inoculation induced a classical acute hepatitis C that resolved in one animal (Ch 1579) while it progressed to chronicity in the other animal (Ch 1581). Despite their different clinical outcomes, both chimpanzees developed vigorous peripheral and intrahepatic cellular immune responses. Interestingly, intrahepatic CD4+ and CD8+ T-cell responses were stronger and appeared earlier in the animal that developed persistent infection. Sequence analysis of the virus recovered from the two chimpanzees at various time-points during the first year of follow-up showed high mutation rates with high proportions of non-synonymous substitutions, indicating that immune pressure and positive selection occurred in both animals. Changes were not selected until after the initial decrease in virus titres and until after the development of immune responses and hepatitis. Subsequently, however, mutations emerged repeatedly in both animals. Several of these were located within known T-cell epitopes. Thus, this study confirmed that the emergence of HCV mutants might represent an important mechanism for viral persistence, although it does not necessarily predict the final outcome. The emergence of mutant strains within the continuously evolving viral quasi-species would thereby lead to an endless 'cat-and-mouse' game between the host and the virus, with an invariably frustrating outcome for the former.

In conclusion, there is little doubt that the development of an early and broad-spectrum adaptive T-cell immunity constitutes a critical defence mechanism against HCV, which can lead to a sustained containment of viral replication. Convincing evidence has been provided that CD8+ T lymphocytes cannot properly develop and function in the absence of a strong CD4+ T-cell response. Nevertheless, there is also a growing conviction that such T-cell responses may not be sufficient to achieve ultimate clearance of viral infections, nor to maintain long-term control over viral replication in the absence of an equally broad antibody response, as clearly illustrated in the lymphocytic choriomeningitis virus mouse model.[278] Likewise, it is difficult to conceive that effective adaptive immune responses can be generated and sustained without the crucial help of innate immune mechanisms. These considerations emphasize the need to maintain a wide-angle perspective in the study of protective immunity.

## Functional analysis of the HCV genome

The ability to test infectivity of molecular HCV clones in chimpanzees has permitted functional analysis, in which the role of genomic regions believed to harbour important determinants of HCV pathogenesis, or relevant targets for drug development, can be determined. In the first such study, Yanagi *et al*.[127] analyzed the importance of various elements of the 3′ UTR of HCV. The 3′ UTR begins immediately after the ORF stop codon and consists of a short variable region, a polypyrimidine tract and a highly conserved sequence, which constitutes the 3′ terminus of the HCV genome. The conserved sequence of the 3′ UTR potentially forms three stem-loop structures, including a stable stem-loop of 46 nucleotides at the 3′ terminus. A chimpanzee was sequentially inoculated with RNA transcripts of mutants containing large deletions in the 3′ UTR. Mutants in which the entire 3′ terminal conserved region was deleted were non-viable. Furthermore, mutants with only the 3′ terminal stem-loop structure or with deletion of both proximal stem-loop structures of the conserved region failed to replicate in the chimpanzee, indicating that the conserved region was critical for infectivity *in vivo*. A mutant with deletion of the entire poly U-UC region was also non-viable. By contrast, a construct in which the proximal 24 nucleotides of the variable region of the 3′ UTR were deleted, was infectious. Thus, the conserved region and the poly U-UC region of the 3′ UTR, but not the variable region, appear to be critical for infectivity. Kolykhalov *et al*.[126] also found that deletion of the conserved region was deleterious for infectivity *in vivo*. The data obtained in these chimpanzee studies are in agreement with recent data generated in Huh-7 cells with the HCV replicon system,[279,280] in which the various structures of the conserved region of the 3′ UTR were shown to be critical for HCV RNA replication.[281] Furthermore, it was found that the polypyrimidine tract had to consist of at least 26 resi-

dues to permit replication, whereas the variable region of the 3′ UTR was not essential for replication.

The E2 HVR1 region is believed to play an essential role in HCV infection.[84,282] This region evolves rapidly in infected individuals, and there is evidence that the virus is able to escape the host immune response by accumulating mutations in HVR1, which does contain critical neutralization epitopes. Recent evidence in humans has demonstrated a significant increase in HVR1 diversity in acute progressing hepatitis C, whereas resolution was associated with a marked decrease in viral diversity.[149,283] In contrast with the pattern seen in humans are the observations made in chimpanzees infected with HCV molecular clones,[119,120] which demonstrated that chronic HCV infection may develop even in the absence of a viral quasi-species in the inoculum[128] and without the emergence of mutations within the HVR1. These data may appear difficult to reconcile with the evidence accumulated in humans on the role of HVR1 variation as a mechanism of immune escape. However, there are some differences between humans and chimpanzees, which may account for such a discrepancy. Despite similar HCV RNA levels, the HVR1 region in infected chimpanzees undergoes very little sequence variation compared with humans,[284,285] suggesting that chimpanzees exert a weaker positive selective pressure on this region. Also, although the chimpanzee model mirrors well the infection in humans, the disease is commonly milder: whereas the rate of chronicity is similar in both species,[286] in chimpanzees the ALT peak is significantly lower, necroinflammatory lesions are consistently mild and fibrosis is most often absent. Understanding the similarities and differences of hepatitis C in the two species will be important for the study of pathogenesis and the development of preventive and therapeutic measures to control HCV infection.

The biological role of the HVR1 was directly investigated by Forns and colleagues,[287] who inoculated a chimpanzee with RNA transcripts from an infectious clone lacking the HVR1. Serum HCV RNA was detected at week 1 and the animal became persistently infected. The animal, however, had an attenuated infection with unusually low titres of viraemia during the first several weeks after inoculation. The ALT remained completely normal and antibody seroconversion was delayed, occurring at week 37. Mild necroinflammatory lesions did not appear until week 34. However, coinciding with the appearance of five amino acid changes, including two changes in E2, the viral titres increased progressively to reach ~$10^4$ genome copies per mL of plasma, and remained at this level throughout follow-up. These amino acid changes were interpreted as compensatory mutations, as the replication of the deletion mutant without the mutations was impaired. The compensatory role of these mutations has been recently demonstrated by Scarselli *et al*.,[98] who investigated the binding of wild-type E2 and of E2 lacking the HVR1 to a novel putative HCV receptor, the human scavenger receptor class B type 1 (SR-B1). The mutant lacking the HVR1 had a reduced ability to bind to SR-BI+ human hepatoma cells, but this ability was restored by the introduction of one of the compensatory mutations observed in the chimpanzee study, presumably by inducing an HVR1-independent functionally competent conformation of the E2 glycoprotein. Thus, these findings suggested a functional role of HVR1 and confirmed that the genetic variability of HCV provides a rapid and effective mechanism to compensate for a decrease in biologic fitness. The virus recovered before the appearance of consensus mutations was transmissible to a naïve chimpanzee. The chimpanzee became viraemic at week 2 even though the virus titre was low. However, coinciding with the appearance of four amino acid changes in the viral genome, the titre increased rapidly to peak levels of $10^4$–$10^5$ genome copies per mL. One mutation in E2 was the same found in the original transfected chimpanzee. The infection nonetheless resolved at week 18. This *in vivo* study of HVR1 provided definitive evidence that the HVR1 is not essential for the life-cycle of HCV. However, the ΔHVR1 virus was markedly attenuated, indicating an important functional role of this region for HCV infectivity and/or pathogenesis. After compensatory mutations were selected, infection with the ΔHVR1 virus resembled wild-type HCV infection. This study also proved that a host immune response to HVR1 is not essential for viral clearance, as one chimpanzee infected with the ΔHVR1 virus was able to clear the virus. In contrast, HCV persisted in the other chimpanzee infected with ΔHVR1 virus, demonstrating that this region is not required either for clearance or for persistence of HCV in chimpanzees.

The *in vivo* function of the HCV p7 protein is unknown. However, recent *in vitro* data indicate that this 63 amino acid polypeptide has two transmembrane domains connected by a cytoplasmic loop. The amino- and carboxy-terminal tails are oriented toward the ER lumen.[288] In addition, it was reported that this protein has a hexameric form within cellular membranes and might function as a viral ion channel.[289] In a study by Sakai *et al*.,[290] p7 of an infectious genotype 1a clone was mutagenized and RNA transcripts of mutants were tested for infectivity in chimpanzees. Mutants with deletions of all or part of p7 and a mutant with substitutions of two conserved positively charged residues in the cytoplasmic loop were non-viable, demonstrating that p7 is essential for infectivity *in vivo*. Furthermore, the observation that a genotype 1a clone in which p7 was replaced with that of an infectious 2a clone was non-viable indicated a genotype-specific interaction between p7 and other genomic regions. To define which portions of p7 played the most significant role in this interaction, additional chimeras

with the 1a backbone in which only specific domains of p7 had the 2a sequence were tested. Only a p7 chimera with 2a transmembrane domains and cytoplasmic loop and 1a terminal tails was viable. The initially recovered viruses maintained the chimeric sequence. These data indicate that the amino- and/or carboxy- terminal intraluminal tails of p7 contain sequences that play an essential, genotype-specific function.

Kolykhalov *et al*.[126] confirmed the critical importance of the active sites of various enzymatic functions within the HCV genome, such as the NS3 protease, NS3 helicase and the NS5B polymerase. Also, infectious chimeric viruses have been generated from different strains or genotypes, which will permit further analysis of the function and interaction of different genetic components.[121,290,291] Finally, the role of adaptive mutations in the HCV replicon system,[279,280] the development of which was an important breakthrough for basic research on HCV RNA replication, has been examined *in vivo*. A study in chimpanzees, using an infectious clone of the HCV strain from which the replicon was developed, demonstrated that such adaptive mutations markedly attenuated the HCV infectivity *in vivo*.[292] This finding has implications for understanding the biological relevance of data obtained in this *in vitro* replication system and emphasizes the importance of confirming in a biologically relevant system data generated in the test tube or in cell culture systems. Despite important progress in developing novel investigation models, the chimpanzee continues to represent the only biologically relevant system for the study of HCV.

## The future

It is likely that chimpanzees will continue to play an important role in research on hepatitis C. In the absence of suitable cell culture systems in which HCV can be propagated, the chimpanzee model will continue to provide the only means of biologically amplifying rare or interesting strains of HCV. Although the recent development of *in vitro* assays based on pseudoparticles bearing HCV glycoproteins has provided new and valuable tools for studying the humoral immune response evoked by HCV in the host, the chimpanzee model will continue to be essential in validating the results obtained *in vitro* by comparison with *in vivo* studies using the same reagents. Moreover, it likely will continue to be essential for defining the role of immunoglobulins in preventing HCV infection and for testing the efficacy of passive immunoprophylaxis. The chimpanzee model has also been of unique importance for testing the infectivity of molecular HCV clones, thereby permitting for the first time studies of the importance of the genetic elements for virus infectivity, as well as pathogenesis. The high endemicity of HCV infection throughout the world and its high rate of progression to chronicity dictate that prevention should be a high priority in the control of hepatitis C. The chimpanzee model will undoubtedly be critical in attempts at vaccine development. Finally, it is likely that the future characterization of new candidate hepatitis agents will be facilitated by taking advantage of the unique biological amplification system provided by the chimpanzee model.

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# Chapter 28
# Autoimmune disorders

Elmar Jaeckel, Michael P Manns

Acute and chronic viral infections have been implicated in the pathogenesis of numerous autoimmune diseases. Autoimmune diseases are defined by loss of tolerance of the adaptive immune response to self-antigens. Usually, autoreactive T cells recognize peptides of self-antigens in the context of self-MHC (major histocompatibility complex).

Our current concept as to the initiation of autoimmune diseases assumes that mostly unknown environmental factors can trigger autoimmune responses in genetically susceptible individuals. The genetic trait is polygenic and the disease-associated genes mostly represent polymorphic variants and not disease-defining genes (Fig. 28.1).

Most peripheral antigens are expressed in specialized epithelial cells in the thymus and the peripheral T-cell repertoire is negatively selected, e.g. self-reactive T cells with high affinity for self-antigen are deleted during thymic development. Therefore, autoimmune T cells usually have a very low precursor frequency and their T-cell receptor has a low avidity for the MHC peptide complex. This might explain why autoimmune diseases develop over extended periods of time (months to years), e.g. autoantibodies to β-cell antigens can be detected years before the onset of type I diabetes. It is therefore difficult to judge if a viral infection is initiating or just precipitating an already ongoing autoimmune disease (Fig. 28.2).

Thus, many viruses have been implicated in the pathogenesis of autoimmune diseases, but there is usually no single agent causing a certain autoimmune disease and a given pathogen can be implicated in various autoimmune disorders, indicating that the 'one organism–one disease' paradigm that is central to Koch's postulates might not invariably apply to microbially induced autoimmune diseases.

Compared with other hepatotropic viruses, hepatitis C virus (HCV) has been implicated in many extrahepatic/hepatic and autoimmune manifestations. The best documented associations are mixed cryoglobulinaemia and the presence of autoantibodies, while the rest remain largely controversial (Table 28.1). Lympho-

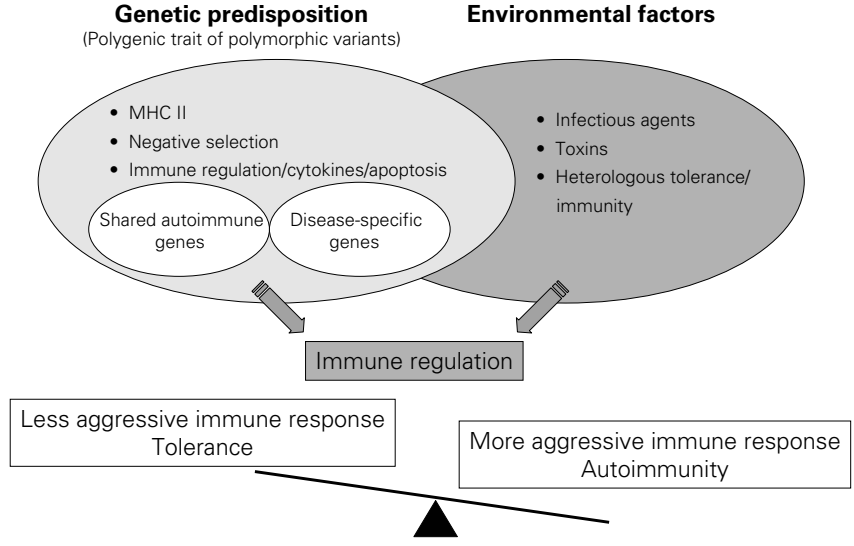


**Figure 28.1** Pathogenesis of autoimmune diseases.

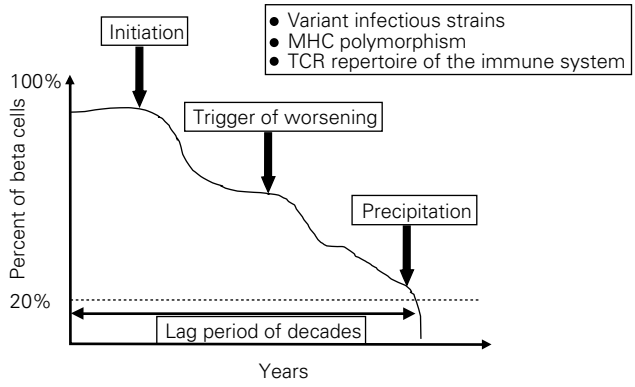


**Figure 28.2** The role of viral infection in autoimmune disease. There is usually a long interval between acute viral infection and start of the autoimmune disease. Therefore, the viral infection might initiate, worsen or precipitate an autoimmune disease. TCR, T-cell receptor.

**Table 28.1** Autoimmunity associated with HCV infection

| |
|---|
| Antigen-specific thyroiditis |
| Type I diabetes |
| Antiphospholipid syndrome |
| Vitiligo |
| Autoimmune thrombocytopenic purpura |
| Antigen-non-specific/B-cell stimulation |
| Mixed cryoglobulinaemia |
| Membranoproliferative glomerulonephritis |
| Leukocytoclastic vasculitis |
| Sialoadenitis (Sjögren's-like) |
| Arthralgia, neuropathy, pulmonary vasculitis |
| B-cell lymphoma/MALT lymphoma |
| Antigen non-specific/unknown mechanism |
| Lichen planus |
| Polyarteritis nodosa |
| Sicca syndrome |
| Mooren's corneal ulcer |
| Others |
| Porphyria cutanea tarda |

ma, renal disease, neuropathy and Sjögren's syndrome manifest incomplete overlaps with the cryoglobulinaemic syndrome. Unfortunately, most reports on autoimmunity caused by HCV used small numbers of patients, inappropriate controls or patient selection bias. In this chapter, we will discuss the mechanism of viral-induced autoimmunity followed by the autoimmune phenomena seen in chronic HCV infection and their therapeutic options.

## Mechanisms of viral-induced autoimmunity (Table 28.2)

It is very difficult to link an exact mechanism for induction of autoimmunity with HCV infection. This is because of the long time lag between HCV infection and onset of autoimmunity (Fig. 28.1), due to differing viral genotypes and quasi-species, and due to genetic heterogeneity and MHC polymorphisms of the host. Furthermore, the T-cell repertoire of patients at the time of HCV infection is not 'naïve', meaning that the pool and specificities of memory T cells has already been shaped by numerous infections during the lifetime of an individual.

The virus may share a sequence homology with self-proteins. Virus reactive lymphocytes might therefore trigger autoimmune responses to a mimicking self-peptide. The epitopes probably involved in molecular mimicry usually share a high sequence homology (especially of the MHC anchoring and the T-cell receptor [TCR] contact residues). Such epitopes are usually defined by searching protein databases followed by testing of cross-reactivity. Likewise a cross-reactive sequence between the hepatic autoantigen cytochrome P450 IID6 and NS5 of HCV has been detected (Fig. 28.3).[1] While such studies may define cross-reactivities of linear humoral epitopes, the disease relevance of this humoral cross-reactivity remains questionable. Molecular mimicry on the T-cell level is much more complex. The mere demonstration of peptide cross-reactivity is not sufficient to explain autoimmunity.[2]

It first has to be shown that the mimicking peptide is indeed generated from the virus and the self-protein and that it is presented by MHC molecules on the surface of antigen-presenting cells (APCs). Next, it has to be shown that T cells recognizing the mimicking peptide are indeed generated during the antiviral immune response. The last two mandatory experiments to prove molecular mimicry can just be performed in animal models. It is necessary to show that the cross-reactive T cell is capable of destroying the target tissue, and that altering the

**Table 28.2** Possible mechanisms of autoimmunity induced by infectious agents

| |
|---|
| **Molecular mimicry** |
| **Heterologous immunity**: altered repertoire of naïve and memory T cells |
| **Bystander activation**: T cells, antigen-presenting cells, innate immunity (cytokines, co-stimulation, epitope presentation, release/modification of sequestered antigens) |
| **Superantigens**: unspecific activation and expansion of autoreactive T cells |
| **Block of regulatory** immune response |
| **Virus-specific factors** interacting with host immune system |

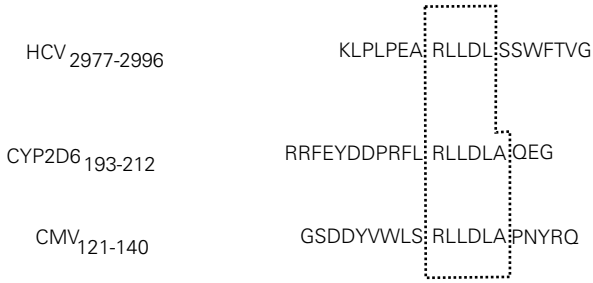


**Figure 28.3** Molecular mimicry between HCV, cytomegalovirus (CMV) and the cytochrome P450 2D6. Comparison of amino acid motifs.

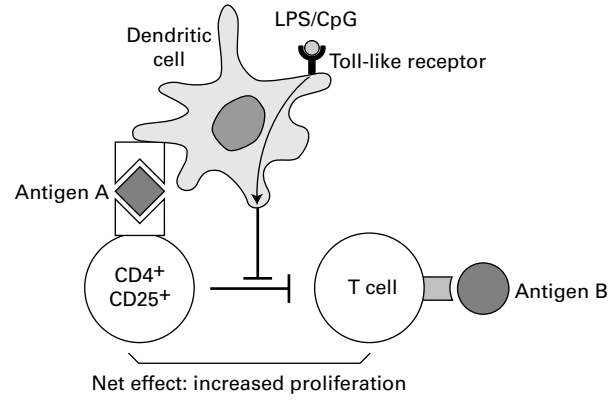


**Figure 28.4** Blockade of CD4+CD25+ T-cell mediated suppression of naïve and activated T cells by toll-like receptor ligation of dendritic cells.

viral epitope without altering viral replication abolishes the autoimmune disease.[2] Proof for an involvement of a viral infection by such criteria stems from infection with herpes simplex virus in autoimmune keratitis.[3] As for HCV, it has been shown that T cells expanded on HCV core protein can recognize and lyse target cells expressing the hepatic antigens cytochrome P450 2A7 and 2A6[4] – the latter is also the target of the humoral immune response in a few patients with chronic HCV infection.[5] The frequency and relevance of these cross-reactive T cells in HCV infection remain enigmatic. So far, there is no conclusive proof for molecular mimicry as the cause of autoimmunity in HCV infection apart from some antibody cross-reactivities.

While molecular mimicry describes cross-reactivities of lymphocytes to similar epitopes, heterologous immunity[6,7] means cross-reactivity of a given T cell to largely unrelated peptides which can even be presented by different MHC molecules.[7,8] Such a largely unpredictable cross-reactivity of virus-specific T cells is due to the degeneracy of the T-cell receptor (TCR)–MHC interaction, where a single TCR can react with up to $10^5$–$10^6$ various peptides. However, such interactions can be studied by use of positional scanning libraries and biometrical analysis.[9] T cells with high avidity, as usually seen in antiviral immune responses, are probably less degenerate in their antigen recognition than low avidity T cells.[10] Although such T-cell cross-reactivities are difficult to study in chronic HCV due to host and virus variants, cross-reactivities between influenza virus and HCV epitopes have been described,[11] suggesting that similar cross-reactivities with self-proteins might exist.

A further mechanism of induction of autoimmunity might be bystander activation of autoreactive T cells by cytokines or proinflammatory stimuli associated with the chronic hepatic inflammation. These non-cross-reactive T cells might be activated by altered and more mature APCs, by cytokine release through components of the innate immune response, e.g. natural killer T (NKT) cells and natural killer (NK) cells. The inflammation caused by HCV infection might further lead to release or modification of sequestered antigens. Although autoimmunity through bystander activation during virus infection can be experimentally demonstrated in animal models,[12] its role in viral infections in humans remains unknown.[13,14]

Microbial superantigens can activate a subset of T cells by cross-linking the MHC molecule with a certain Vβ-subset independent of the antigen specificity of the T cell. This might also lead to activation of autoreactive T cells. Although such superantigens of endogenous retroviruses were implicated in human autoimmune diseases, these results were not reproducible by several other groups. So far, no superantigen seems to be encoded in the HCV genome.

Autoreactive T cells are controlled by active, dominant tolerance involving regulatory cells besides central and peripheral deletion. It was recently shown that microbial infection engaging toll-like receptors on dendritic cells can block such a regulation (Fig. 28.4).[15] This loss of regulation could possibly lead to activation of autoreactive T cells, although this has not been investigated for HCV infection.

## HCV-specific factors predisposing to autoimmunity (Table 28.3)

It has been suggested that the chronic HCV infection with its evolving generation of quasi-species and immune escape mutants might lead to profound activation of the immune system, producing large amounts of immunoglobulins. On the other hand, it should be noted that T-cell responses in chronic HCV are usually rather low, arguing against a broad antigen-specific activation.

**Table 28.3** HCV-specific factors predisposing for autoimmunity

| |
|---|
| Chronic inflammation with elevated cytokine levels |
| Variability of HCV causing constant immune evasion |
| HCV E1/E2–CD81 interaction |
| B cell, T cell and NK cells |
| (HCV core/apoptosis) |
| (Lymphotropism of HCV) |

Of great interest is the interaction between the tetraspannin molecule CD81 and HCV envelope protein. CD81 is expressed on most human tissues except red blood cells and platelets. A common characteristic of tetraspannins is their propensity to physically associate with a variety of other membrane molecules such as integrins, lineage-specific molecules and other tetraspannins.[16] In the immune system, they are involved in the organization of membrane microdomains of T, B and APCs,[17] thereby modulating the immunological synapse.[18] It has been shown that residues 613–618 of HCV E2[19] bind to the large extracellular loop of CD81,[20] although binding to the E1/E2 heterodimer seems to be stronger.[21]

On B cells, CD81 is part of the B-cell co-receptor besides CD19, CD21 and Leu-13 (Fig. 28.5). CD81 has a short cytoplasmic tail lacking signalling motifs. It is therefore thought that CD81 promotes cellular adhesion.[22] Studies with monoclonal antibodies have indicated that engagement of CD81 can lower the activation threshold of B cells in the presence of suboptimal concentrations of anti-IgM.[23] In T cells, cross-linking of CD81 provides a co-stimulatory signal to CD3, thereby lowering the activation threshold at low antigen concentration.[24] Likewise, it was shown that HCV-E2 can co-stimulate human T cells at suboptimal anti-CD3 stimulation.[25]

For NK cells, it was shown that cross-linking of CD81 by the major envelope protein of HCV (HCV-E2) or anti-CD81 antibodies blocks NK cell activation, cytokine production, cytotoxic granule release and proliferation.[26,27] Conversely, on NK-like T-cell clones, including those expressing NK cell inhibitory receptors, CD81 ligation delivered a co-stimulatory signal.[27]

All the above mechanisms (Table 28.4) might explain a lower activation threshold of B and T cells, possibly leading to autoimmunity. However, the CD81–E1/E2 interaction might even shape the repertoire of activated T cells in favour of self-reactive T cells by reorganizing microdomains on APCs.[17] Recent evidence suggests that the APC is equipped to preorganize MHC-peptide complexes in the absence of T cells (Fig. 28.6). To this end, MHC molecules become incorporated into two types of membrane microdomains: (1) cholesterol- and glycosphingolipid-enriched domains, denoted lipid rafts, that preconcentrate MHC class II molecules; and (2) mi-

**Table 28.4** CD81/HCV–E1E2 interaction

| | |
|---|---|
| B cells | Adhesion; activation threshold lowered |
| T cells | Co-stimulation lowering activation threshold |
| NKT cells | Co-stimulation lowering activation threshold |
| NK cells | Inhibiting NK function |
| Antigen-presenting cells | MHC molecules enriched for self-peptides |

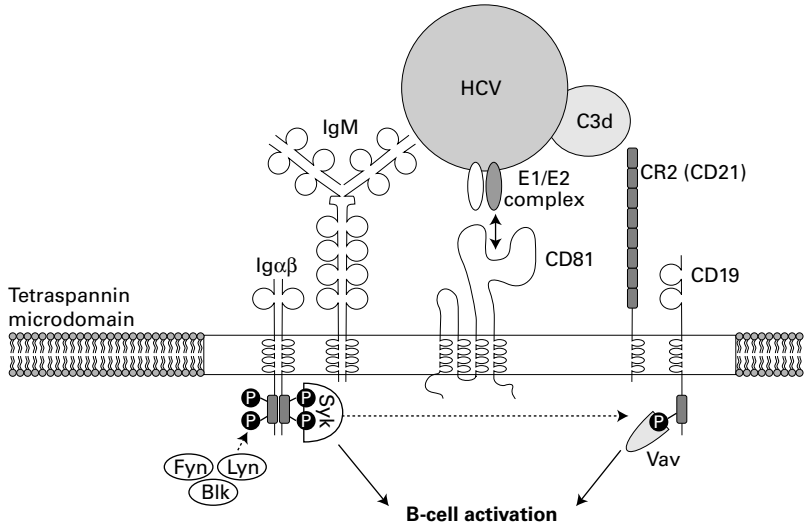


**Figure 28.5** Signal transduction of B cells through the B-cell receptor complex. Cross-linking of the membrane-bound IgM will lead to intracellular activation transduced by Igα and Igβ containing immune receptor tyrosine-based activation motifs (ITAM). A second signal is provided by binding of breakdown product of complement (C3d) to the type 3 complement receptor (CR2). This recruits CD19 into the complex, which contains additional ITAM motifs. The complex of CD19, CR2 and CD81 is called the B-cell co-receptor. CD81 organizes B-cell receptor microdomains and mediates adhesion. It cannot transduce signals by itself.

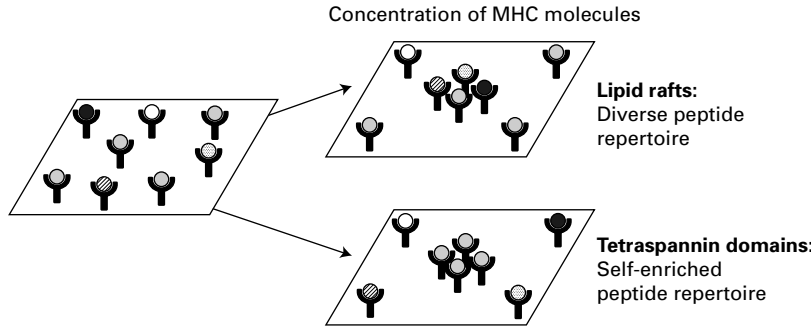


**Figure 28.6** Microdomains on antigen-presenting cells (APCs) are concentrating MHC-peptide complexes for more efficient presentation to T cells. While lipid rafts are concentrating MHC molecules with an unselected set of peptides, tetraspannin microdomains are enriched for MHC molecules containing self-peptides.

crodomains made up of tetraspannin proteins, such as CD9, CD63, CD81 or CD82 that mediate enrichment of MHC class II molecules loaded with a select set of peptides. This narrow repertoire of peptides in tetraspannin microdomains is enriched for self-peptides, thereby presenting self-antigens highly concentrated to T cells (Fig. 28.6).[28] As CD81 is overexpressed in CD5+ B cells of individuals with chronic HCV infection, this might further contribute to the above-mentioned effects of CD81–HCV/E2 interaction on B cells.[29]

It is still controversial as to whether CD81 acts as co-receptor for uptake of HCV. Likewise, it is questionable if there is a relevant lymphotropism of HCV. Finally, even if HCV replicates in lymphocytes the significance of this for virus-induced autoimmunity is uncertain. In various epithelial and mesenchymal cells lines, HCV core protein was able to promote or inhibit apoptosis,[30,31] although these functions have not been evaluated in cells of the immune system.

## Antigen-specific autoimmunity

### Autoantibodies in chronic HCV

The occurrence of autoantibodies is relatively common in patients with chronic hepatitis C infection[32] (Table 28.5). Although initial studies might contain some sampling bias and not represent the autoantibody prevalence in the general population of HCV-infected patients, the prevalence of patients with autoantibodies is higher in chronic HCV infection than it is in chronic HBV infection. However, there are significant variations in prevalence of patients with positive autoantibodies, which might represent ethnic or geographical differences. Also, the determination of autoantibodies is not standardized between laboratories. Most autoantibody titres in chronic HCV infection are lower than those reported in organ-specific autoimmune disease and their relevance for the course of HCV infection, the response to antiviral therapy and the development of organ-specific autoimmunity is generally low. There is, however, the chance that patients with the propensity to develop an autoimmune disease and patients with undiagnosed autoimmune diseases become infected with HCV. In these cases, therapy with interferon (IFN)-α might worsen the underlying autoimmune disorder. This is especially important if the autoantibodies are indicative of autoimmune liver disease, as deterioration of liver inflammation may occur despite reduction of viral load.

### Autoantibodies found in autoimmune liver disease

Anti-nuclear and anti-smooth muscle autoantibodies (aab) have the highest prevalence in chronic HCV infection (Table 28.5). Although these aabs are the hallmark of autoimmune hepatitis (AIH) type I,[33,34] they are frequently found in other autoimmune diseases and chronic inflammation. Their titre in chronic HCV is usually lower than in AIH and most of them have non-homogenous immunofluorescence staining pattern of the antinuclear antibodies (ANAs) and most anti-SMAs

**Table 28.5** Prevalence of autoantibodies (aab) in chronic HCV

| Strong association | Weak or no association |
|---|---|
| Antinuclear aab (ANA): 9–38% | IgG and IgM anti-cardiolipin aab (ACA) |
| Smooth muscle aab (SMA): 5–91% | Anti-neutrophil cytoplasmic aab (ANCA) |
| Liver-kidney microsomes I (LKM1): 0–10% | Anti-gastric parietal cells aab (GPC) |
| Liver cytosol aab type I (LC1): 0–5% | |
| Rheumatoid factor: 8–76% | |
| Anti-thyroid: 9–20% | |

(anti-smooth muscle autoantibodies) are not reactive with actin-containing microfilaments,[35] both diagnostic features of AIH. The exact antigen-specificity of ANAs in chronic HCV remains unknown. It has been suggested that autoantibody-positive patients have a more severe course of their HCV infections.[35,36] However, these findings have not been confirmed by others.

Autoantibodies to liver and kidney microsomes reactive with cytochrome P450 IID6 are one major diagnostic determinant in patients with AIH type II,[37] an autoimmune disease preferentially affecting children. However, it became clear that a substantial proportion of anti-LKM1-positive patients were infected with HCV[38] and did not suffer from AIH type II. The overall prevalence of anti-LKM1 in chronic HCV is low in adult patients (0–6%)[35,39–41] and tends to be higher in children (8–11%).[42,43] Anti-LKM1 aabs are less often seen in American[32,44] and Japanese[41] populations with HCV. The prevalence data might be due to a selection bias and variant expertise in autoantibody testing, as the highest prevalence has been reported from centres involved in studies of autoimmune liver diseases. Prevalence in unselected populations may be around 1%. Development of anti-LKM is not linked to viral genotypes.[45] Anti-LKM1 aab in chronic HCV usually present with similar titres to those seen in AIH type II (Fig. 28.7). However, they less frequently recognize the linear epitope amino acid 257–265 of CYP450 2D6, which is recognized by >60% of patients who are HCV-negative AIH type II (Fig. 28.8).[46,47] It thus seems that AIH type II and anti-LKM1-positive HCV infections present diverse disease entities with different therapeutic regimens.[48]

However, there might be an overlap between both entities in rare cases, which probably have an undiagnosed autoimmune liver disease in addition to HCV infection. One such patient is shown in Fig. 28.9.[47] She presented with chronic liver disease and was HCV RNA-positive. Anti-LKM1 titres, serum transaminases and serum IgG deteriorated under therapy with IFN-α-2b. After discontinuation of IFN therapy, the patient responded proper-

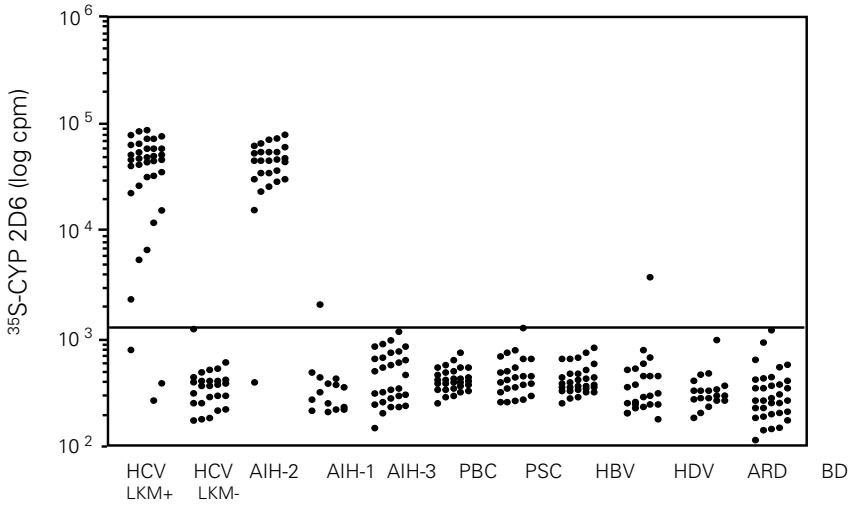


**Figure 28.7** Anti-LKM titres measured by immune precipitation with radiolabelled CYP 2D6 protein. There are similar LKM titres in AIH-2 and hepatitis C.

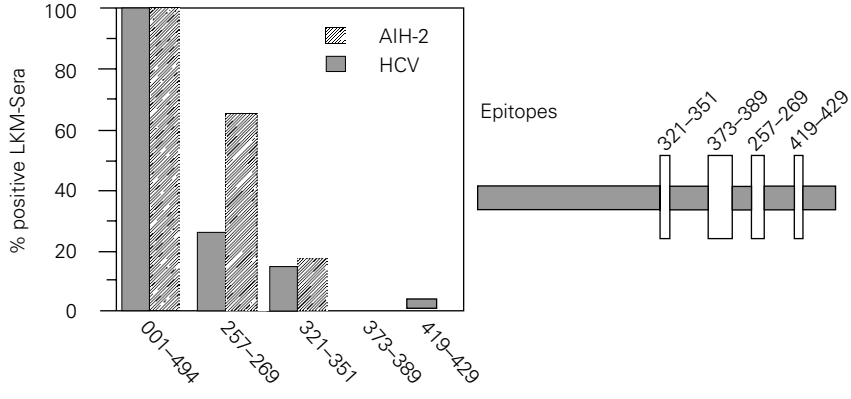


**Figure 28.8** The linear epitope of aa 257–269 is preferentially recognized in sera of patients with AIH type 2.

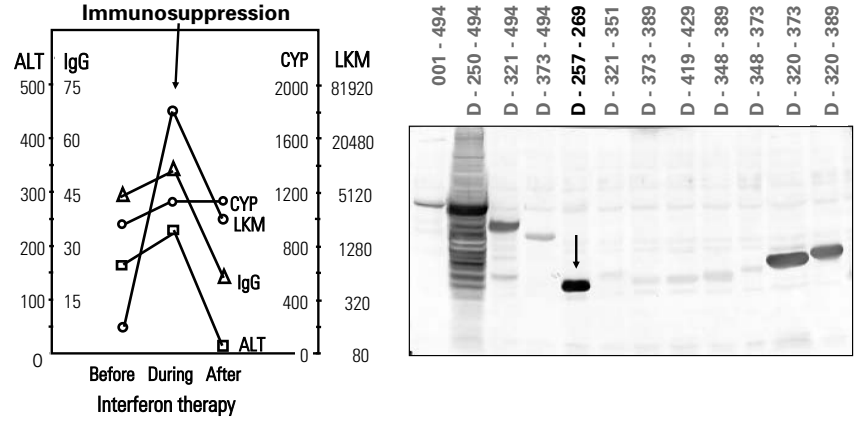


**Figure 28.9** Exacerbation of hepatitis under IFN therapy. The patient presented with chronic liver disease and was HCV RNA-positive. Anti-LKM1 titres, serum transaminases and serum IgG deteriorated under therapy with IFN-α-2b. After discontinuation of IFN therapy, the patient responded properly to immunosuppressive therapy with steroids. Epitope mapping confirmed reactivity with the linear 257–269 epitope, as usually seen in AIH.

ly to immunosuppressive therapy with steroids. Epitope mapping confirmed reactivity with the linear 257–269 epitope, as usually seen in AIH.[47]

In addition, anti-liver cytosol antibodies (LC1)[49] and anti-LKM3 aabs directed against UGT-1.1[50] have been reported in rare cases of patients with HCV infection.

### Thyroid-specific antibodies

Thyroid-specific antibodies (usually anti-thyroid peroxidase or anti-thyroglobulin) have been reported in up to 20% of patients chronically infected with HCV. However, many of these studies lacked proper age- and gender-matched controls. More recent studies using healthy controls rather showed that the prevalence of thyroid antibodies is comparable to age-matched controls.[51] Only 2–5% of these patients will develop thyroid dysfunction which is not different in age-matched controls. However, the prevalence of anti-thyroid autoantibodies increases during IFN therapy (up to 30%),[52,53] and 3–9% of patients develop clinical thyroid dysfunction during or after IFN therapy.[52,53] Positive autoantibodies before therapy, female gender and HLA-A2 are risk factors for the development of thyroid dysfunction under IFN therapy.[54,55] Screening for thyroid disease is therefore recommended in all HCV patients before IFN-α therapy.

### Other autoantibodies

Anti-cardiolipin antibodies occur with low titres in patients infected with HCV, they are not associated with thrombocytopenia or thrombotic events, and the anti-β2-glycoprotein antibodies usually associated with clinical manifestations are not present.[56,57]

Taken together, most autoantibodies found in HCV are probably due to chronic inflammation and B-cell stimulation as described above. However, a few organ-specific antibodies, if occurring at high titres, should raise suspicion of underlying autoimmune diseases. This is of special importance if therapy with IFN-α is planned, as this might lead to exacerbation of the autoimmune disorder.

Likewise type I diabetes, vitiligo and autoimmune thrombocytopenic purpura have been seen in patients with HCV. However, these cases are extremely rare in HCV-infected patients and a pathogenetic role of HCV has not been established in those cases.

Taken together, positive autoantibody titres for aab frequently seen in other diseases and usually presenting with low titres rather present an epiphenomenon. Nevertheless, detection of rare aab of higher titre which are usually specific for a small clinical entity warrants close observation under antiviral therapy, as these might be indicative of underlying autoimmune diseases.

## Antigen non-specific autoimmunity

### Cryoglobulinaemia

Cryoglobulinaemia is defined by the presence of circulating immunoglobulins (Ig) that reversibly precipitate at temperatures below 37 °C. Mixed cryoglobulinaemia (MC) describes cryoglobulinaemia types II and III, which contain a mixture of two different kinds of antibodies as compared with cryoglobulinaemia type I which is found in lymphoproliferative and myeloproliferative diseases where a monoclonal IgG is found in precipitates (Table 28.6). Cryoglobulins can precipitate *in vivo* in small blood vessels (venules, capillaries, arterioles), causing a vasculitis. The link between HCV and cryoglobulinaemia is strong and is supported by epidemiological, molecular and virological studies.

### Prevalence

HCV accounts for almost 70% of all cases of cryoglobu-

**Table 28.6** Classification of cryoglobulinaemias

| Cryoglobulinaemia type | Immunoglobulin | Disease |
|---|---|---|
| I (10%) | Monoclonal Ig, no RF activity | Primary cryoglobulinaemia<br>Lymphoproliferative and myeloproliferative diseases |
| II (60%) | Polyclonal IgG + monoclonal IgM with RF activity IgA, Bence Jones | Secondary mixed cryoglobulinemia; often associated with HCV |
| III (30%) | Polyclonal IgG + polyclonal IgM | Secondary mixed cryoglobulinaemia; associated with infectious, autoimmune and lymphoproliferative diseases |

RF, rheumatoid factor.

**Table 28.7** Prevalence of cryoglobulinaemia in HCV-infected individuals

| Country | HCV-positive (n) | Cryoglobulinaemia-positive (%) | RF-positive (%) |
|---|---|---|---|
| Sweden | 21 | 0 | ND |
| Israel | 90 | 11 | 44 |
| Germany | 132 | 28 | 42 |
| France | 58 | 36 | 70 |
| France | 321 | 56 | 38 |
| Korea | 49 | 59 | 14 |

RF, rheumatoid factor. ND, not done.

linaemia; 0–60% of patients chronically infected with HCV have cryoglobulins (Table 28.7). Besides regional differences in prevalence with high prevalence in southern Europe, some differences might be related to the various sensitivities in testing for cryoglobulins. MCs in HCV are often present at low concentrations (<1%) and are therefore not detected with standard methods. Long-term incubation (8 days) at low temperatures with extensive washing might enhance sensitivity of detection.[58] Meanwhile in situations in which the sensitivity of the cryoglobulin test is known to be low, determinations of rheumatoid factor (RF) may be used as a surrogate marker, as the IgM component of the cryoprecipitate possesses a high level of RF activity. However, if the sensitivity of the cryoglobulin test is expected to be high, the additional determination of RF will not detect many more cases of MC.

There is a slight female predominance in HCV-related MC (60–70%), and patients with MC tend to be older with a longer disease duration.[59] While the presence of HLA-DRB1*11 (DR11) was positively correlated with development of MC, HLA-DR7 seemed to be protective.[60]

## Symptoms

The clinical signs of purpura, arthralgia and weakness were originally described as Meltzer's triad.[61] However, the disease manifestations caused by this systemic vasculitis are more diverse (Table 28.8). Lymphoma, renal disease, neuropathy and Sjögren's syndrome manifest an incomplete overlap with the cryoglobulinaemic syndrome (Table 28.1). However, many HCV patients with MC are asymptomatic. The classical sign of leucocytoclastic vasculitis is palpable purpura of the lower extremities, although other locations might be found as well. A severe systemic vasculitis as seen in polyarteritis nodosa (PAN), often associated with HBV infection, is rarely seen (Table 28.9). MC-related arthritis is usually an intermittent, non-destructive mono- or oligo-arthritis affecting the interphalangeal and metacarpophalangeal joints and the knees. Occasionally, joint pain might be precipitated by exposure to cold. It is important to note that arthralgias are common in HCV, while MC-related arthritis is not. Likewise, weakness might be due to HCV infection rather than to MC-related symptoms.

Peripheral neuropathies caused by MC are frequently under-recognized. They usually present as a peripheral moderate axonal sensory polyneuropathy involving bilateral nerves symmetrically or multiple isolated nerves. They are often painful long before motor deficits develop. As compared with PAN, motoneuropathies are less

**Table 28.8** Symptoms of cryoglobulinaemia

| Symptom | % |
|---|---|
| Skin disease (purpura, urticaria, ulcers, oedema) | 50–90 |
| Arthralgia | 16–60 |
| Weakness | 65 |
| Raynaud phenomenon | 3–40 |
| Neuropathy | 8–40 |
| Renal disease | 25–30 |
| Sicca syndrome | 20 |
| Lymphadenopathy | 3 |
| Fever | 3 |

**Table 28.9** Clinical features of polyarteritis nodosa and mixed cryoglobulinaemia in patients infected with HCV

| Polyarteritis nodosa | Mixed cryoglobulinaemia |
|---|---|
| Life-threatening systemic disease | ... |
| Severe multifocal, asymmetric neuropathy | Moderate, distal, symmetrical neuropathy |
| Malignant hypertension | ... |
| Cerebral angitis | ... |
| Kidney and liver microaneurysms | ... |
| Renal insufficiency | Renal insufficiency |
| Medium size artery | Small and medium size artery |
| Necrotizing vasculitis (occlusion) | Perivascular infiltrates |

common, lesions are distal symmetrical without necrotizing vasculitis (Table 28.9). As involvement is discontinuous lesions may be missed by biopsy.

Renal involvement is common and usually caused by membranoproliferative glomerulonephritis (MPGN) and to a lesser degree by membranous glomerulonephritis or focal segmental sclerosis: 15% (USA)[62] to 60% (Japan)[63] of all MPGN might be related to HCV infection and most but not all HCV-related renal disease is related to MC. Liver disease in patients with MC-related MPGN may be occult,[64,65] and just 40% have other systemic manifestations of MC. Therefore, the diagnosis of MPGN should always be followed by investigation of markers of HCV infection. Patients usually present with nephrotic syndrome. Often, the serum albumin is <3 g/dL with mild renal insufficiency. Few patients progress to dialysis.[62,65] Renal biopsy reveals distinct morphological features consistent with immune complex disease.

The numbers of patients with fever and lymphadenopathy is small. These patients might present the progression of the MC syndrome to non-Hodgkin's lymphoma.

Renal disease, arthralgia and neuropathy may also occasionally be found in HCV-positive patients without MC or RF activity.

## Pathogenesis

Pathology of MC is caused by vascular deposits of cryoprecipitate containing HCV RNA, low-density lipoprotein, IgG and a highly restricted IgM with RF activity (Table 28.10). Virus and anti-HCV concentrations are 10- and 100-fold higher in cryoprecipitates than in serum, respectively.[66] In all, 80% of the monoclonal IgMs found in HCV patients share a major complementarity region named WA (initials of the patients in which these were first described). This WA cross-idiotype is associated with a high degree of rheumatoid activity (formation of immune complexes by avid binding to IgG). These antibodies often express a Vκ light chain derived from a single germinal gene (K325 VL gene). Thus, the repertoire is highly limited with the same cross-reactive idiotype and is encoded by few genes, probably due to close antigenic stimulation. Most of the IgM RF is generated in the liver and in the bone marrow.[67] It is of interest that formation of intrahepatic lymphoid follicles is a characteristic feature of chronic HCV infection[68] and that most intrahepatic mononuclear cells in chronic HCV are B cells expressing IgM.[69] Besides a controversial lymphotropism of HCV,[70] the interaction of CD81 with E1/E2 proteins described above may contribute to the generation of B-cell activation and production of IgM by lowering the activation threshold of B cells. To this end, it is interesting that the CD81 expression of B cells is increased in chronic HCV[71] and highest in patients with MC.[72] Besides this, certain amino acid sequences within the E2 region possessing a high binding affinity to CD81 *in vitro*[19] are associated with the development of MC.[72]

By itself, this process could lead to a type III (polyclonal) MC. Emergence of a dominant clone would subsequently result in a type II (monoclonal) MC. Therefore, type III MC might be the precursor of MC II in some patients.

Emergence of dominant B-cell clone might be due to alterations enhancing B-cell survival. To this end, translocation of the bcl-2 gene from chromosome 18 to 14 results in overexpression of the anti-apoptotic bcl-2 (Fig. 28.10). This translocation has been found in 88% of patients with HCV-related MC compared with 8% in HCV-positive patients without MC and 2–3% in control populations of chronic liver or autoimmune disease.[73] A further genetic alteration by a stochastic hit like a c-myc

**Table 28.10** Content of cryoprecipitate

| |
|---|
| Polyclonal IgG |
| Anti-HCV IgG (1000× conc.) |
| HCV RNA (10× conc.) |
| Lipoproteins (VLDL, LDL) |
| Monoclonal IgM highly restricted |

VLDL, very low density lipoprotein; LDL, low density lipoprotein.

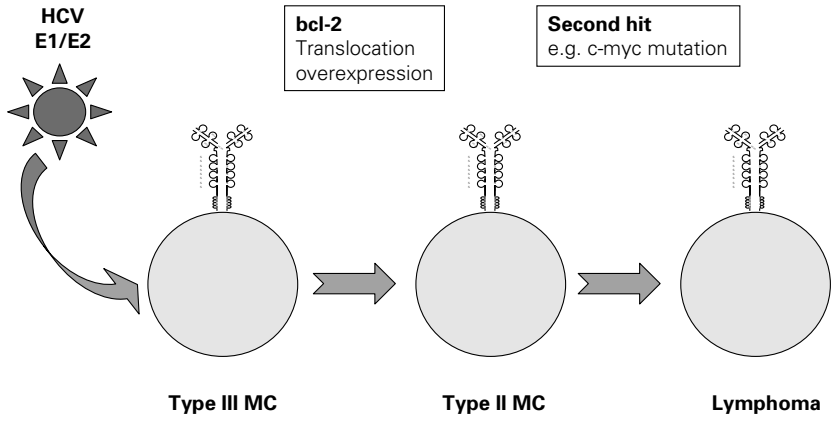


**Figure 28.10** Possible mechanism of HCV-related lymphoma.

mutation might then be sufficient to transform the B cell into a malignant lymphoma blast (Fig. 28.10). Although the aetiological role of HCV in the development of B-cell non-Hodgkin's lymphomas (B-NHL) is controversial, it was shown that up to 56% of chronic HCV patients with MC present with abnormal bone marrow morphology.[74,75] A recent meta-analysis estimated the HCV prevalence in patients with B-NHL to be approximately 15%, higher than that reported not only in the general population (1.5%) but also in patients with other haematological malignancies (2.9%), suggesting a role of HCV in the aetiology of B-NHL. The striking geographic variation in this association suggests that genetic and/or environmental factors are also involved in the pathogenesis of this disorder.[76] Extranodal involvement is common with significant over-representation of liver and salivary glands.[77] Another extranodal site being reported is the stomach, and HCV has been suggested to be a possible cause of mucosa-associated lymphoid tissue lymphoma (MALT).

## Therapy

Before chronic HCV was discovered as the major cause of MC, symptomatic disease was treated with plasmapheresis and/or steroids and/or cyclophosphamide to decrease production of cryoglobulins and inhibit vascular inflammation. More than 50% of patients with HCV-related MC will respond to antiviral therapy with IFN-α, with decreased cryocrit, RF level and improvement in symptoms. However, almost all patients not achieving a sustained response (lasting HCV clearance) will relapse with their MC symptoms after therapy is stopped. The treatment response to IFN in terms of viral clearance is independent of the presence of MC. Most studies were performed with IFN monotherapy using 3 million units tiw. So far, there have been no large studies with pegylated IFNs and ribavirin. However, in most small trials improvement of symptoms was usually linked to suppression of viral replication. It can therefore be assumed that response rates to modern therapeutic regimes might be substantially better than IFN monotherapy. Although a small trial reported an effect of ribavirin monotherapy on MC symptoms,[78] these results have never been confirmed by others. Long-term IFN therapy is effective in controlling symptoms of MC in partial virological responders, particularly for symptoms of cutaneous vasculitis. In the latter patients, a combination of IFN with steroids does not improve results compared with IFN monotherapy. However, combined antiviral and immunosuppressive therapy may be indicated in patients with severe renal disease.

Various disease manifestations respond differently to antiviral therapy. Cutaneous vasculitis responds best to antiviral therapy. Skin lesions disappear in sustained viral responders and improve significantly in the rest under long-term therapy. Improvement is usually seen early.

The influence of HCV treatment on the course of HCV cryoglobulinaemic MPGN is controversial.[79] A recent published study demonstrated that after first-line treatment with prednisone, furosemide or plasmapheresis, antiviral therapy with standard or pegylated IFN-α and ribavirin could improve proteinuria and stabilize creatinine clearance in sustained virological responders.[80] However, in the presence of acute cryoglobulinaemic glomerulonephritis, IFN does not prevent progression of renal damage. Instead, combination therapy with cytotoxic and anti-inflammatory drugs, and sometimes plasma exchange, is recommended.[79] Combined antiviral and immunosuppressive therapy may be indicated in patients without sustained virological response.

The response of MC-related peripheral neuropathy to therapy has been discouraging. Most patients showed either no response or continued with the progression of symptoms under antiviral therapy, although occa-

sionally an improvement may be observed in sustained virological responders.[81] Some gradual improvement might also be expected with long-term IFN therapy (>24 months).[82,83] It might be possible that the poor response of neuropathies compared with skin lesions might be due to the slow speed of regeneration of the target organ. Patients with MC-related neuropathies should be carefully monitored under antiviral therapy, as several cases of dramatic worsening under IFN therapy have been reported. Plasmapheresis might be effective in halting the disease in severe cases.[84]

Low grade B-NHL can regress with IFN therapy and viral clearance, very much like MALT lymphomas may respond to *Helicobacter pylori* eradication.[85] This further supports a possible role for HCV in the pathogenesis of NHL. However, high grade malignancies require systemic chemotherapy. The anti-CD20 antibody rituximab has been shown to be an option in selected patients.[86]

Rare cases of renal disease, skin disease and neuropathies may be seen in chronic HCV infection without the presence of MC. The treatment response in these cases is variable.

### Sialadenitis

A lymphocellular infiltration of the salivary glands has been described in chronic HCV.[87] It resembles Sjögren's syndrome, although it presents with lymphocytic pericapillaritis of milder intensity. The characteristic anti-Ro/SSA and anti-La/SSB autoantibodies are normally lacking, thereby calling into question the autoimmune origin. Up to 10% of patients with chronic HCV infection (mostly of them female) might have xerophthalmia or xerostomia,[88,89] and there might be an incomplete overlap with sicca symptoms seen in MC patients (Table 28.1.). On the other hand, only 5% of patients with classical Sjögren's syndrome are infected with HCV,[90] suggesting that HCV-related sialadenitis and Sjögren's represent distinct entities.

A direct role of the virus has been suggested by studies expressing the HCV envelope proteins E1, E2 in liver and glands; 84% of these mice develop sialadenitis, compared with 2% in control littermates and 0% in HCV core transgenic animals.[91]

### Porphyria cutanea tarda (PCT)

PCT is the most common form of porphyria. The reported prevalence of HCV in patients with PCT varies considerably, but averages around 45%. However, the pathogenesis of the disease is not autoimmune. HCV may be the trigger for clinical expression but is by itself insufficient to cause porphyrin metabolic derangements. Alcohol consumption is an important co-factor to HCV for the development of PCT. In addition, hepatic iron and fat accumulation and increased oxidative stress during chronic HCV infections might be involved in the pathogenesis.

### Diabetes

It is now clear that HCV conveys a risk of developing diabetes mellitus (DM), particularly type 2 DM.[92–94] Hepatic steatosis, insulin resistance and oxidative stress caused by HCV might be involved in pathogenesis, which is not autoimmune. In terms of type I DM, antibodies against β-cells (anti-GAD65, anti IA-2) and against adrenals (anti-21OH) have been found in patients with HCV infection and their titres increased during therapy. However, none of these patients developed clinical disease.[53] Although there are single case reports of type I DM under IFN therapy, these cases seem to be rare[95] and rather represent HCV infection in patients with underlying β-cell autoimmunity.

### Other disorders

Lichen planus, Mooren ulcer's of the eye, idiopathic pulmonary fibrosis, rheumatoid arthritis, autoimmune hepatitis, fibromyalgia and many more have been reported in HCV-positive patients. The prevalence of many of these diseases has either not been studied in larger populations, or has not been investigated in control populations or not confirmed by others.

## Conclusion

In summary, HCV affects many other organ systems besides the liver. Long-term infection is usually necessary for the development of extrahepatic manifestations, some of which are of autoimmune origin. Development of autoantibodies and MC is strongly associated with HCV infection. Renal disease and skin disease, as well as sialadenitis, neuropathy and lymphoma, represent incomplete overlaps with the MC syndrome and some of these entities respond well to antiviral therapy. Organ-specific autoantibodies and other organ-specific autoimmune diseases might represent underlying previously unrecognized autoimmunity rather than induction of autoimmunity by the HCV infection itself. These cases have to be followed closely, as autoimmunity might deteriorate under IFN therapy. The interaction of the HCV envelope proteins E1/E2 with CD81 might be of major importance in the pathophysiology of HCV-related autoimmunity.

Future reports of disease associations with HCV should contain control groups well matched for age, sex, weight, alcohol consumption, disease duration and severity of the liver disease, because these are confounding variables.

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# Chapter 29
# Central nervous system complications

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Chronic hepatitis C virus (HCV) infection is a major cause of liver-related morbidity and mortality, with hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC) as the dominant clinical sequelae.[1] Hepatic encephalopathy as a consequence of cirrhosis and portal-systemic shunting is the clearest example of central nervous system (CNS) involvement in chronic HCV infection.[2] Neurological involvement due to HCV-associated mixed cryoglobulinaemia most commonly presents as a peripheral sensory or motor neuropathy,[3] but there are also rare case reports of cryoglobulin-associated CNS vasculitis.[4,5] Recently, there has been interest in the possibility of a link between chronic HCV infection and cerebral dysfunction, occurring at an early stage of chronic infection, prior to the development of cirrhosis and unrelated to cryoglobulinaemia. In this chapter, we review the current evidence for such a link and examine the possibility of CNS infection by HCV.

## CNS involvement in HCV-related vasculitis

Chronic HCV infection accounts for between 80% and 98% of cases of mixed cryoglobulinaemia,[6] a condition characterized by the presence of immune complexes between monoclonal or polyclonal immunoglobulin (Ig) M (rheumatoid factor) and polyclonal IgG. These complexes precipitate at low temperatures *in vitro* and contain anti-HCV IgG, HCV RNA and rheumatoid factor.[6] The prevalence of cryoglobulinaemia in chronic HCV infection varies widely and has been reported to be between 0.8% and 66%.[7] This variation is probably explained by varying sensitivity of the methods used to measure cryoglobulins, geographical variation of cryoglobulinaemia and, most importantly, case mix. Cryoglobulinaemia is associated with longer durations of infection and more advanced liver disease. In one study, cryoglobulins were present in 33% of HCV-infected patients with cirrhosis but only in 1.8% of patients with chronic persistent hepatitis.[8] Although cryoglobulins may be frequently detected, the majority of patients do not show clinical or physical signs of the cryoglobulinaemia syndrome, which include vasculitic skin rash, arthralgias, glomerulonephritis and peripheral neuropathies. CNS involvement is unusual but there have been sporadic reports of vasculitic involvement of the brain in HCV-positive patients, usually in the context of detectable cryoglobulins. Reported presentations have included hemiparesis or tetraparesis,[9,10] dysarthria,[11] encephalopathy,[5,12,13] dizziness[14] and communicating hydrocephalus.[15] The main pathophysiological mechanisms are ischaemia or haemorrhage due to diffuse or segmental vasculitis of the small cerebral vessels.[4] Ischaemic lesions may be detectable with cerebral magnetic resonance imaging as hyperintensities, most commonly in the white matter.

Non-specific symptoms of fatigue, arthralgias and weakness are common in patients with detectable cryoglobulins, but in the absence of clinical signs or symptoms of vasculitis, these patients are not labelled as having cryoglobulinaemia syndrome. No relationship has been found between the presence of cryoglobulins and severity of fatigue when vasculitis is absent.[16,17] The clinical significance of low levels of cryoglobulins in chronic HCV infection is not known.

## Evidence for a cerebral effect in HCV infection

The suggestion that chronic HCV infection might cause cerebral dysfunction resulted from initial, anecdotal observations that HCV-infected patients complain of a variety of non-specific symptoms, often in the absence of histologically advanced liver disease.[18] Complaints of fatigue, depression, mental clouding ('brain fog') and a perceived inability to function effectively led to a number of published reports documenting the prevalence of such symptoms and their impact on health-related quality of life (HRQL) scales in cohorts of patients with HCV infection.[17,19–25] However, the presence of these symptoms in the context of HCV infection does not nec-

essarily imply causality, as there are many associated factors which may independently affect patients' perceptions of well-being, e.g. anxiety regarding diagnosis, prognosis and treatment, previous or ongoing substance abuse and associated emotional problems or personality traits.[26] The evidence for a causal relationship between HCV infection and these predominantly neuropsychological symptoms is reviewed below.

## Health-related quality of life

Health related quality of life (HRQL) questionnaires have been used extensively to study both the effect of HCV infection on patients' well-being and the effect of antiviral therapy. The SF-36 questionnaire, a generic health instrument, has been used most widely in this context and generates a health profile, divided into eight separate categories, reflecting physical and emotional performance.[27] The results from several large studies challenge the perception that HCV infection is an 'asymptomatic' disease, with general agreement that HRQL is significantly reduced in HCV-infected patients, independent of the severity of the liver disease.[19,22,23,28] Furthermore, in one study, SF-36 scores were lower in patients with HCV infection compared with those with chronic hepatitis B virus (HBV) infection and were unrelated to the mode of acquisition, i.e. the presence of previous intravenous drug usage.[21] These findings, together with large studies which have shown significant improvements in quality of life (QOL) in combined cohorts of many thousands of patients after successful antiviral therapy, suggest that the viral infection itself is an important determinant of reduced QOL.[22,23,29] There are, however, other relevant determinants of HRQL, which have been described in the literature.[30–32] For clear practical reasons most of the above studies did not blind their subjects to HCV PCR status. The impact of diagnosis is likely to impair HRQL, as has been shown in a small study where patients who were unaware of their diagnosis reported less impaired QOL than patients who knew their HCV status.[30] Despite this, the patients who did not know their diagnosis were still impaired on three scales of the SF-36 questionnaire. It is also possible that some of the improvement in HRQL, seen after successful antiviral therapy, is due to the patients' knowledge of their response. Other studies have demonstrated an association between depression and other co-morbidities and reduced HRQL.[31,32] HRQL studies have been useful in quantifying the impact of chronic HCV infection on patient well-being and in monitoring a response to treatment. However, these studies tell us little about the nature of the symptoms or of the underlying cause of the perceived disability, and are influenced by a large number of factors, many of which are difficult to control for in clinical studies

## Fatigue

Fatigue is the commonest symptom in patients with chronic HCV infection. Numerous surveys have reported the prevalence of fatigue to be between 20% and 80% in HCV-infected patients[16,17,20,24,25,33–35] and have found no association between the severity of fatigue and the degree of hepatitis[17,21,25,31] Furthermore, a number of studies have reported improvements in fatigue after treatment,[16,34,35] although it appears to persist in some individuals despite a virological response. These studies are subject to the same shortcomings as described earlier, as fatigue is a multidimensional symptom and is influenced by multiple inter-relating social, behavioural, psychological and personality factors[31,36,37] and the relative contribution of a biological mechanism remains unclear. Thus, in the context of investigating the presence of a cerebral effect of HCV infection, measured fatigue is likely to be a poor marker.

## Depression

Depression is a common finding in HCV-infected patients.[20,31,32,38–40] It is of considerable clinical importance, as depression may limit the tolerability of treatment with α-interferon (IFN-α)[41] and reduce compliance.[42] The relationship between HCV and depression is complex. Patients with depression may have a higher incidence of HCV infection. The greatest reservoir of HCV infection is in intravenous drug users, many of whom have clinical depression.[43] Conversely, depression may exist as a secondary phenomenon to HCV infection. This may take the form of a reactive depression related to the diagnosis and concerns over long-term health or may be secondary to symptoms such as fatigue and cognitive impairment.[36,40] Finally, a biological effect of HCV infection itself may underlie depression. There is currently little evidence for this theory, although in one blinded study of intravenous drug users, HCV-positive individuals had significantly lower positive affect scores than HCV-negative drug users.[43] In another study, there was no difference in psychological morbidity between HCV-positive and HCV-negative drug users,[44] although in both these studies the effect of HCV may have been masked by the high background prevalence of depression in active drug users. Although depression is an important variable in the context of HCV infection, its occurrence alone is unlikely to be useful in determining whether HCV has a biological effect on cerebral function.

## Cognitive function

A number of studies have evaluated whether HCV infection has an impact on cognitive function.[40,45–48] It is

well established that cognitive impairment is frequently detectable in patients with cirrhosis, even in the absence of clinical encephalopathy and is termed minimal encephalopathy.[2] It is therefore imperative to either exclude patients with advanced liver disease or to study sufficiently large numbers to allow subgroup analyses in any study that tests for a direct effect of HCV infection on cognitive function.

Using a computer-based cognitive battery, selective impairments of concentration and working memory were reported in patients with histologically proven minimal HCV hepatitis.[40] These impairments were not seen in patients who had recovered from HCV infection, either spontaneously or after successful therapy. Furthermore, the presence of fatigue, depression or a history of substance abuse did not explain these findings. In a study by Hilsabeck and colleagues, impairment of various neuropsychological tasks was seen in up to 49% of HCV-infected patients without cirrhosis.[46] The authors found no excess of neuropsychological impairment in HCV-infected patients compared with patients with liver disease from other causes. However, the comparison group included patients with alcoholic liver disease and was small, with only six patients without cirrhosis. Perhaps unsurprisingly, patients with HCV infection and a second chronic medical condition, such as alcoholic hepatitis or human immunodeficiency virus 1 (HIV1) infection, showed greater levels of cognitive dysfunction. Furthermore, patients with more hepatic fibrosis were more likely to show greater cognitive impairment. Whether this was a direct consequence of increased fibrosis or whether it was somehow related to duration of infection was not addressed in the study. The findings of this study were replicated by the same investigators in an independent sample of HCV-infected individuals.[49] Impairments were detected in areas of complex attention, concentration and working memory, with a frequency of between 9.5% and 38%. There were no associations between subjective complaints of cognitive dysfunction or fatigue and performance on the neuropsychological tests, although a relationship was shown between depression, fatigue and cognitive complaints. Krause and colleagues studied a cohort of patients with chronic HCV infection and normal liver function tests (who were therefore unlikely to have cirrhosis) and found distinct attention deficits which were related to measurements of fatigue severity.[45] More recently, the prevalence of mild cognitive impairment was found to be 39% in a cohort of patients recruited for a pharmaceutical trial of antiviral therapy.[48] This study found no relationship between cognitive impairment and hepatic fibrosis or duration of infection. Furthermore, there was no relationship between cognitive impairment and substance abuse, depression and anxiety, in agreement with an earlier study.[40] The pattern of cognitive dysfunction in the above studies is similar, with mild impairments in the domains of attention and working memory. Such findings have also been reported in the medically asymptomatic stages of HIV infection[50] and are consistent with the involvement of subcortical brain systems.[51]

In contrast to these studies, Cordoba and colleagues found no evidence of cognitive impairment in HCV-infected patients without cirrhosis and in those with compensated cirrhosis.[47] Impairments were only detected in patients with previous hepatic decompensation, which were almost certainly due to hepatic encephalopathy. A number of factors may explain this finding. The majority of patients in the non-cirrhotic and compensated cirrhotic groups were enrolled after HCV infection was diagnosed at blood donation. Hence, these groups were positively selected for good health, as symptomatic individuals are unlikely to volunteer to give blood. Furthermore, this study used more stringent criteria for cognitive impairment than the other studies, by performing statistical analyses on raw scores compared with a healthy control group, rather than defining impairment by comparison to population norms. Therefore, a number of questions remain, regarding the prevalence, pattern and significance of cognitive impairment in patients with chronic HCV infection and further studies are required.

Two recent studies have investigated the neuropsychiatric impairment of HCV co-infection in HIV-infected patients. Ryan and colleagues tested the hypothesis that in patients with AIDS, co-infection with HCV results in greater cognitive impairment than matched HCV-negative patients.[52] There was a trend for co-infected patients to perform worse neurocognitively, with impairments of executive functioning. The co-infected patients were also more likely to be diagnosed with HIV-associated dementia. However, this group also had higher rates of previous opiate or cocaine dependence than the HIV-positive, HCV-negative patients. A second study that examined HCV/HIV co-infected patients reported significant impairments in psychomotor speed compared with uninfected controls.[53] However, similar impairments were also seen in HIV and HCV mono-infected patients.

In summary, the above studies show evidence of mild neurocognitive impairment in a proportion of patients infected with HCV, with complex attention, concentration and working memory being most affected. However, with respect to determining whether HCV infection *itself* can cause cognitive dysfunction, the studies described above were all complicated by one or more of a large number of potential confounding factors, e.g. previous substance abuse, co-morbid conditions, HIV co-infection. Although strict entry criteria were observed in some studies and appropriate controls have been employed, it could still be argued that the observed

cognitive impairment in HCV infection resulted from confounding factors, rather than the virus itself, and that an objective physical or physiological measure of cerebral function is required.

## Cerebral magnetic resonance spectroscopy

Magnetic resonance (MR) techniques provide one of the most important tools for quantitative analysis of composition and structure. Importantly, the technique is non-invasive and over the last two decades MR techniques have been applied *in vivo* to image the human brain (magnetic resonance imaging – MRI) and to study biochemical processes (magnetic resonance spectroscopy – MRS). Interpretation of a clinical MR spectrum from the brain can provide information about cellular energetics, neuronal function, osmoregulation, selected neurotransmitter activity, inflammation, membrane turnover and intracellular pH.[54] The proton ($^1$H) nucleus is the commonest nucleus in biological systems and has the highest absolute sensitivity for MR studies. Cerebral metabolites that can be measured with $^1$H MRS include N-acetylaspartate (NAA), an amino acid derivative thought to be located in neurons, choline-containing compounds (Cho) such as phosphoryl- and glycerophosphoryl-choline which participate in membrane synthesis and breakdown, creatine and phosphocreatine (Cr), glutamine and glutamate, which usually form a composite resonance (Glx) and *myo*-inositol (mI), a sugar involved in osmotic regulation within the brain.[55] Semi-quantitative measurements of MR metabolites are often expressed as ratios to Cr.

Several studies have reported characteristic spectral appearances from the brains of patients with cirrhosis, typified by a reduction in the mI and Cho resonances and an increase in the Glx composite resonance relative to the Cr resonance.[56–64] This pattern has only been reported in cirrhosis, usually complicated by hepatic encephalopathy and in patients with hyperammonaemia due to urea cycle diseases.[58] A resonance cannot be specifically assigned to ammonia using cerebral proton MRS but the increase in Glx is likely to reflect ammonia incorporation into glutamine. Elevated cerebral glutamine levels have been interpreted as a consequence of chronic hyperammonaemia. Reduced mI levels may reflect the function of inositol as an osmolyte regulating cell volume in astrocytes. Reduced Cho levels may also be related to a disturbance in cerebral osmoregulation.[65]

To date there have been three cerebral $^1$H MRS studies in patients infected with HCV but without cirrhosis.[40,66,67] In the first study of 30 HCV-infected patients with histologically mild liver disease, there were significant elevations of Cho/Cr in the basal ganglia and frontal white matter, compared with both normal controls and patients with chronic hepatitis B infection (Fig. 29.1).[66] There were no differences in NAA/Cr. Similarly, in the cerebral white matter, Cho/Cr was significantly increased and NAA/Cho was significantly reduced, compared with both other groups. These results suggest that Cho-containing compounds were increased in the basal ganglia and white matter in the HCV patients. There were no statistically significant differences between the HBV group and the healthy volunteers. The elevation in Cho/Cr was unrelated to a history of drug abuse or hepatic encephalopathy. In a second study, patients who performed worse on neurocognitive testing

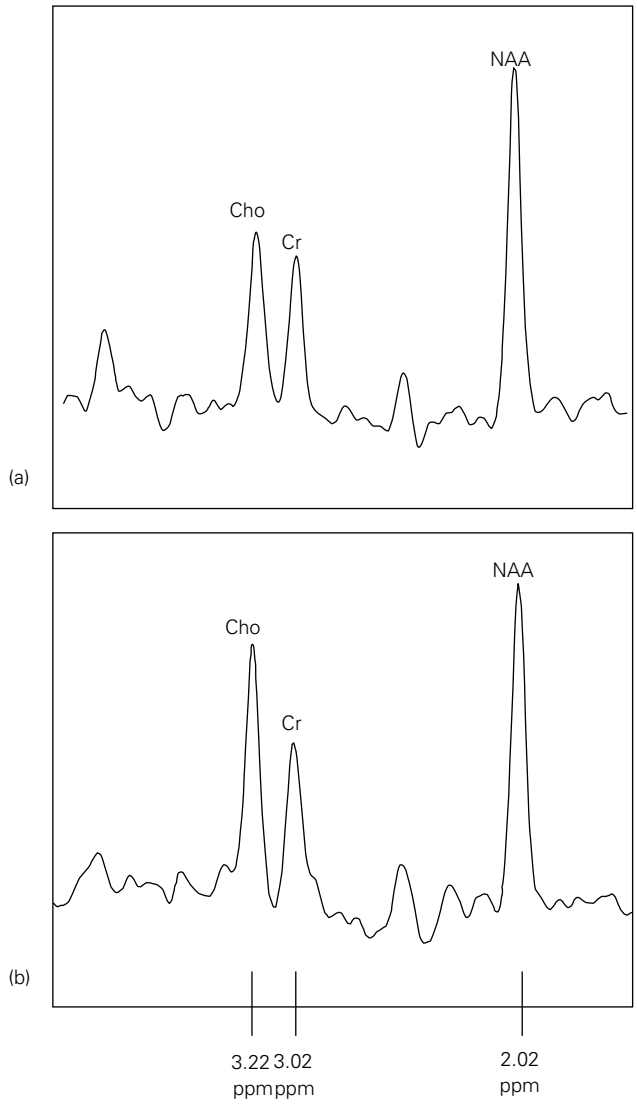


**Figure 29.1** Representative proton magnetic resonance spectra acquired from the frontal white matter. (a) A healthy control. (b) A patient with histologically defined mild hepatitis due to hepatitis C infection. The choline/creatine ratio is elevated in the patient with hepatitis C infection. Cho, choline; Cr, creatine; NAA, N-acetylaspartate; ppm, parts per million.

had significantly higher basal ganglia Cho/Cr ratios, although there were no significant associations between MRS metabolites and specific cognitive functions.[40] The MRS sequences used in these studies precluded the measurement of mI. However, in a subsequent study of HCV-infected patients with mild liver disease, abnormal elevations in frontal white matter mI have been detected, which are significantly correlated with impairments in working memory (unpublished data).

These results suggest, for the first time, that a cerebral metabolic abnormality does exist in a proportion of patients with histologically proven mild HCV infection.[40] It is therefore possible that chronic HCV infection may cause both cognitive impairment and altered brain metabolism by an, as yet, unknown mechanism.

Taylor and colleagues used MRS to determine whether HCV infection may exacerbate methamphetamine-associated neuronal injury.[67] In a small study, reductions in frontal white matter NAA were detected in abstinent HCV-positive methamphetamine users compared to HCV-negative users. No abnormalities were seen in Cho or mI. The authors conclude that HCV may exacerbate drug-induced brain injury, but in the absence of an HCV-positive, metamphetamine-negative group, they were unable to examine the effect of HCV infection in isolation.

Similar $^{1}$H MRS metabolite abnormalities to those reported in HCV infection have been extensively documented in cerebral HIV infection, both in neurosymptomatic and neuroasymptomatic individuals.[68] MRS has been applied to cerebral HIV infection more extensively than any other infection, although data exist on subacute sclerosing panencephalitis (measles),[69,70] cerebral nocardiosis,[71,72] pyogenic and tuberculous brain abscesses,[73] Nipah virus encephalitis[74] and herpes simplex encephalitis.[75] In general, the small number of studies that have examined non-HIV viral encephalitides report reduced NAA/Cr and increased Cho/Cr, which may or may not be reversible.[69,70,74,75]

In cerebral HIV infection, subcortical grey matter and frontal white matter are the principal sites of the metabolic abnormalities, detected using $^{1}$H MRS.[76,77] These findings are consistent with the neuropsychological deficits in minor cognitive-motor disorder (MCMD) and HIV-associated dementia (HIAD) and the predilection of HIV for the basal ganglia.[50,78] In early disease, the most common finding is increased mI/Cr in the frontal white matter.[79,80] Elevated Cho/Cr and decreased NAA/Cr in both the white matter and basal ganglia have also been reported, even in asymptomatic patients.[68,76,77,81] In advanced disease, these metabolic abnormalities are all commonly reported.[82–84] The interpretation of the MRS data has been consistent with the neuropathological findings, i.e. neuronal loss (reduced NAA) and CNS inflammation (increased mI and Cho).[77,82,83]

A finding in the study by Forton and colleagues was the absence of associations between fatigue, depression or QOL measures and cerebral metabolite ratios, despite the presence of impairments in these domains.[66] Although, at first sight, this finding appears to run counter to the possibility of a causative relationship between abnormal cerebral biochemistry and neuropsychological symptoms in these patients, it is important to note that MRS metabolites and neuropsychological test scores do not represent the same phenomenon. MRS metabolites are a direct, biological measure reflecting 'brain pathology' when abnormal. Although neuropsychological tests and symptom scores may be sensitive for detecting brain dysfunction, they do not directly reflect brain injury and may be affected by education, socio-economic and cultural influences, all of which may be relevant confounding variables in this patient group.

Further evidence of subclinical impairment of brain function in chronic hepatitis C infection comes from Kramer and colleagues, who used P300 event-related potentials, a neurophysiological test of cognitive processing, in a large cohort of patients with chronic HCV infection.[85] Although cirrhotic patients were not excluded, subgroup analyses were made. HCV-infected patients had delayed P300 peak latencies and reduced amplitudes compared with age-matched healthy subjects; 17% of HCV-infected patients had P300 latencies outside the age-adjusted normal range, suggesting slight but significant neurocognitive impairment. Subgroup analyses indicated that the findings were not accounted for by a history of substance abuse, cirrhosis or alcohol. Prolonged P300 latencies have also been reported in HIV infection.[86]

The parallels in the MRS and P300 data between HCV and HIV infection, where infection of cerebral microglia,[78] possibly via infected monocytes entering the brain, and subsequent microglial activation occur, raises the prospect that the HCV might also infect the CNS.[18]

## Virological studies

### Evidence for extrahepatic replication

The detection of markers of HCV infection and replication in human tissue is complicated by a number of factors. First, the level of viral replication in the liver, the major site of productive HCV infection, is relatively low. Viral kinetic studies have shown that, although approximately $1 \times 10^{12}$ virions are produced daily by the infected liver, this only corresponds to a virion production rate of 50 particles per hepatocyte per day, if it is assumed that a liver contains $2 \times 10^{11}$ hepatocytes and 10% are infected.[87] Second, cytoplasmic viral RNA degrades rapidly, necessitating rapid freezing of tissue specimens after biopsy. Madejon and colleagues have shown that

a delay of between 4 and 30 minutes before freezing of fresh liver biopsies resulted in a 20% and 64% loss in detection of positive- and negative-strand HCV RNA, respectively, by RT-PCR.[88] The rapid degradation of viral RNA is especially relevant when analyzing post-mortem tissue, where there is invariably a significant period between death and sample collection. The detection of HCV RNA in tissue is further complicated by relatively high titres of circulating virus. Demonstration of HCV RNA by RT-PCR in a tissue or cell lysate does not prove viral infection and replication in that tissue as contamination from serum-derived virus is likely.

Two main strategies have been developed to address these issues, each with its own methodological difficulties.

### *HCV quasi-species*

The analysis of HCV quasi-species has been used to support the hypothesis that HCV replicates in various extrahepatic sites, including B and T lymphocytes, monocytes/macrophages and dendritic cells.[89–98] These studies presuppose that different HCV variants are tropic to different cellular compartments, as is the case for HIV.[99,100] Hence, the demonstration of different viral sequences in extrahepatic cell or tissue extracts compared to liver and serum is considered indicative of viral replication in those sites. An alternative explanation is that certain HCV variants are adsorbed to or are phagocytosed by PBMCs, and that the mere presence of different viral sequences does not prove viral replication. This issue has been addressed by some of the studies outlined below.

Molecular cloning of RT-PCR products, followed by sequencing of individual clones, is the most accurate method currently available for analysing quasi-species. Although labour-intensive, this method allows precise analysis of the genetic diversity of the quasi-species, provided that sufficient clones are sequenced. It has been calculated that 20 clones are necessary to prevent artifactual simplification of the quasi-species.[101] The genomic region most commonly cloned has been the hypervariable region 1 (HVR1) within the envelope protein E2. This technique has generated conflicting results regarding the relative complexity of the quasi-species distribution in liver and serum. Cabot and co-workers reported that the viral complexity of the HCV quasi-species in liver was greater than in serum.[102] In a later study, the same group argued against quantitatively important extrahepatic replication on the basis of their sequence analysis.[103] In contrast, Navas *et al*. reported greater genetic complexity and diversity of the HCV quasi-species in serum compared with both liver and peripheral blood mononuclear cells (PBMCs), implying that extrahepatic sites may release HCV into the circulation.[92] Using the same methodology, Okuda *et al*. reported unique HCV amino acid sequences in PBMCs in eight of thirteen patients with chronic hepatitis C.[94] To exclude an effect of viral adsorption to PBMCs, this study pretreated the PBMCs with trypsin and used appropriate negative controls. The distribution of HCV within subsets of PBMCs was addressed by a study which found a non-random distribution within B lymphocytes and monocytes/macrophages, consistent with viral replication.[95] However, the negative strand was not detected by RT-PCR in these subsets. More recently, phenetically distinct variants have been reported in B cells and monocytes. HCV variants complexed to immunoglobulin were removed from these preparations and differed from the cell-derived sequences, suggesting that variants complexed to immunoglobulin and bound to Fc receptors on the cell surface were not being detected.[97]

In common with a number of other positive-stranded RNA viruses within and outside the Flaviviridae, the 5′ untranslated region (UTR) of the HCV genome contains a highly conserved element, the internal ribosomal entry site (IRES).[104,105] This element consists of three stem-loop structures (domains II, III, IV) and a pseudo-knot, and is recognized by ribosomes, thus allowing translation of the viral polyprotein in a cap-independent manner.[106,107] Although this region is the most invariant of the HCV genome, it does exhibit a quasi-species distribution in human serum.[108] Naturally occurring mutations within this region are likely to be of biological significance and have been shown to lead to important differences in translation efficiency, both in cell-free systems and in cell culture.[108–111] Furthermore, the effect of these mutations may be to either enhance or abrogate translational efficiency in different cell lines, suggesting that interactions between specific cellular factors and the IRES may be important in determining cellular tropism.[108,109,111] A parallel may be drawn with poliovirus (PV) where mutations within the PV IRES result in reduced translational efficiency in neural but not HeLa cells, in association with a reduction in neurovirulence.[112]

Several groups have reported nucleotide substitutions in the HCV IRES, which are associated with lymphoid replication.[90,109,113,114] Long-term cultures of HCV in lymphoblastoid lines (HPBMa and Daudi cells) resulted in the selection of three nucleotide substitutions (G to A at position 107, C to A at position 204, G to A at position 243), which were not detected in the wild-type inoculum.[113] In a subsequent experiment, these mutations increased IRES translational activity in lymphoid cell lines.[109] These polymorphisms have also been detected in HCV sequences from extrahepatic compartments in patients with wild-type sequences in the serum. The A204 polymorphism has been detected in PBMCs[114] and monocyte/macrophages,[115] and the A107, A204, A243

polymorphisms have been detected in monocyte-derived dendritic cells.[111]

Single-stranded conformation polymorphism (SSCP) is an alternative, less laborious method to analyze HCV quasi-species. It relies on the differential gel mobility of PCR products containing different nucleotide mutations, and thus provides a general representation of the quasi-species complexity but not diversity. Laskus *et al*. used this technique in addition to sequencing in patients co-infected with HCV and HIV, and demonstrated unique 5′ UTR sequences in PBMCs, adrenal, pancreas and lymph nodes.[116] Negative-strand HCV RNA was detected in the same tissues by strand-specific RT-PCR. This widespread distribution of HCV was attributed to immunosupression due to HIV infection. However, the same group has more recently reported SSCP data in immunocompetent individuals, supporting the presence of HCV in PBMCs, thyroid and pancreas.[114] Experiments were also performed to exclude the possibility that PBMCs can concentrate a subset of circulating virus, differing in the 5′ UTR region. The authors concluded that adsorption of specific variants to PBMCs and other cells did not explain their results. Interestingly, they were unable to conclude that this is also the case for the HVR1. Several other studies have used SSCP to demonstrate quasi-species differences between serum and PBMCs.[89,91,93] Finally, SSCP has been used to analyze HCV quasi-species detected in cerebrospinal fluid (CSF) in both HIV-positive and HIV-negative patients.[117] Although there was no microscopic evidence of blood contamination of the CSF, the quasi-species analysis suggested that the virus was serum-derived.

### *Detection of negative-strand HCV RNA*

The very low levels of HCV found in infected individuals have meant that RT-PCR is often the only suitable method to detect viral RNA. There are few reports of HCV RNA detection from liver extracts by Northern blot hybridization analysis.[118,119] In order to demonstrate viral replication in the liver, many workers have demonstrated the presence of HCV negative-strand RNA, the postulated replicative intermediate, by RT-PCR.[120,121] Indeed, soon after the discovery of HCV, the negative strand was widely reported to be present in serum and PBMCs.[120,122–124] However, the strand specificity of these early studies was questioned in subsequent work.[125,126] Three main mechanisms were proposed to explain the lack of strand specificity, based on the demonstration that excess positive-strand HCV RNA may act as a template for the synthesis of a 'false' negative strand: false priming of the positive strand, self-priming of the positive strand and random priming by extraneous nucleic acids.[126] These factors were particularly important as the reverse transcriptase reactions were generally performed at 42 °C, a temperature at which the secondary structure of the template RNA is preserved. This has special relevance for the 5′ UTR, which has a hairpin secondary structure that predisposes to self-priming.[127]

Several solutions to these problems have been proposed including the use of rTth, a thermostable enzyme with both reverse transcriptase and DNA polymerase activity under different conditions. False priming is prevented because cDNA synthesis can occur at high temperatures,[128] allowing a 10 000-fold discrimination between the detection of the correct and incorrect strands.[129]

Although the initial reports that HCV readily replicates in substantial numbers of PBMCs and serum have been questioned,[128] data from other strand-specific RT-PCR studies and other experimental evidence have led to an emerging consensus that HCV either replicates at a low level in PBMCs or that the proportion of infected PBMCs is low, in both immunocompetent[127,130,131] and immunocompromised individuals.[115,132] Other studies have demonstrated HCV-negative strand in bone marrow and haematopoietic progenitor cells.[133,134] It has recently been suggested that even after apparently successful clearance of HCV RNA leading to undetectable serum HCV RNA using standard clinical assays and normal liver function tests, PBMCs may still harbour low levels of replicating strains.[98] In this study, positive- and negative-strand HCV RNA was detected in PBMC fractions using a highly sensitive RT-PCR nucleic acid hybridization technique and strand-specific RT–PCR of PBMCs after culture with a T-cell proliferation-stimulating mitogen. These results imply that HCV RNA can persist at very low levels in PBMCs, and that an intermediate replicative form of the HCV genome can persist for many years after apparently complete spontaneous or antiviral therapy-induced resolution of chronic hepatitis C.

## Detection of HCV RNA in the CNS

The above techniques have recently been applied to examination of autopsy brain samples to seek evidence of HCV replication in the CNS. Radkowski and colleagues used a strand-specific method and detected HCV negative-strand RNA in a number of brain structures including subcortical white matter, medulla oblongata and cerebellum in three of six patients who died from complications of chronic HCV.[135] In one of these patients, the A204, A243 polymorphisms were present in the positive and negative strands isolated from the cerebellum but were absent from serum. In this patient, a negative-strand sequence obtained from a mesenteric lymph node was identical to the cerebellum-derived sequence, suggesting a lymphoid origin for the brain variants. In a second patient, who was HIV-positive, analysis of the NS5 region indicated that the genotype

of the brain strain was 3a, whereas the circulating serum genotype was 1b. In a second autopsy study, the same group detected negative-strand RNA in subcortical white matter and cerebral cortex from two patients who died after liver transplantation for chronic HCV infection.[136] In these two HIV-negative patients, the A204, A243 polymorphisms were present in both tissue and serum sequences.

The same group have also analyzed cell pellets from cerebrospinal fluid (CSF) obtained from HIV-positive patients with meningitis or cerebral toxoplasmosis.[137] In three patients the A107, A204, A243 polymorphisms were detected in CSF cell pellets and circulating PBMCs. Genotyping by sequencing of the NS5b region revealed different genotypes in the CSF pellets and PBMCs compared with serum in two of three cases (serum 3a, PBMC/CSF cells 1a; serum 1a, PBMC/CSF cells 4c; serum 1b, PBMC/CSF cells 1b). Although the authors suggest that their results show that HCV neuroinvasion could be related to trafficking of infected leucocytes across the blood–brain barrier by a mechanism analagous to HIV infection, it seems likely that in these patients, the sequences were derived from PBMCs that had crossed a blood–brain barrier, compromised by the unrelated meningitis/toxoplasmosis.

In another autopsy study which used cloning and sequencing to define quasi-species for the IRES and HVR1 in brain, liver, lymph node and serum samples, there was evidence of tissue compartmentalization of sequences in the brain in two of three patients[138] Between 24% and 55% of brain-derived IRES sequences were not seen in the serum, and there was significant phylogenetic and phenetic clustering of the brain and lymph node HVR1 sequences, consistent with tissue derivation as opposed to serum contamination. The A204, A243 polymorphisms were again detected exclusively in the brain in one patient. A dicistronic reporter vector was used to test whether brain-derived variants showed altered IRES-mediated translational efficiency, which might favour CNS infection. The translational efficiencies of the brain-derived IRES sequences were generally reduced compared with the master serum and liver sequences in rabbit reticulocyte cell lysates and human liver and brain cell lines: HuH7 (liver) and CHME3 (microglial). The A204, A243 mutations showed preserved translational efficiency in HuH7 but reduced efficiency in CHME3 cells. These data suggest that IRES polymorphisms, including A204, A243, may be important as a viral strategy of reduced translation to favour latency in the CNS as well as other extrahepatic sites, such as dendritic cells.[111]

## Conclusions

The possibility of a cerebral effect of HCV has been investigated at three different levels: neuropsychological, metabolic and virological. The findings support the concept of a biological effect of HCV infection on cerebral function, which may be mediated by low-level infection of the CNS by HCV (Fig. 29.2). However, there are many unresolved questions, which can only be addressed by further research.

The significance of mild impairments in cognitive function, which do not progress to dementia, remains unclear and may cause some to question their clinical significance. These impairments may, however, begin to explain the high prevalence of neuropsychological symptoms in this patient group, many, if not the majority of whom, do not have liver dysfunction. As a result, chronic infection with HCV may come to be regarded as an infectious disease, with liver complications, rather than a primary liver disorder and the indications for antiviral therapy may be broadened. The long-term effect of antiviral therapy on cognitive function is unknown. Studies to examine this could shed further light on the relationship between HCV viraemia and cognitive function. Such studies would be complicated by the neurological effects of IFN-α treatment itself[139] and would require a long wash-out phase after the end of treatment. Indeed, there is emerging evidence that gains in QOL and symptoms only occur some time after cessation of treatment.[16] However, evidence that successful viral eradication reverses the mild cognitive impairments in HCV infection would provide convincing evidence that the virus is key in their aetiology.

The demonstration of elevated cerebral Cho/Cr ratios in patients with chronic HCV infection, using $^{1}H$ MRS, again supports the central biological hypothesis. However, these findings are not highly specific for any pathological process. Elevations in choline-containing compounds have been reported in cerebral HIV infection,[140] but also in bipolar depression[141] and chronic fatigue syndrome.[142] Although it is postulated that increased cerebral Cho/Cr ratios may be due to altered membrane fluidity in activated microglia, as in HIV infection,[143] the data do not prove this. At present, it can only be concluded that a cerebral metabolic abnormality occurs in some patients with histologically mild hepatitis C and that the cellular mechanism is unknown. Longitudinal studies to investigate the effect of antiviral treatment on cerebral metabolism could provide evidence regarding reversibility of the observed effects but, again, could be complicated by the neurotoxicity of IFN-α.

The presence of unique HCV RNA sequences in autopsy brain tissue, which are phylogenetically distinct from liver and serum variants, raises the possibility that brain-specific HCV variants may infect the CNS. However, there is currently no experimental evidence to suggest how HCV may enter the CNS. The evidence for HCV

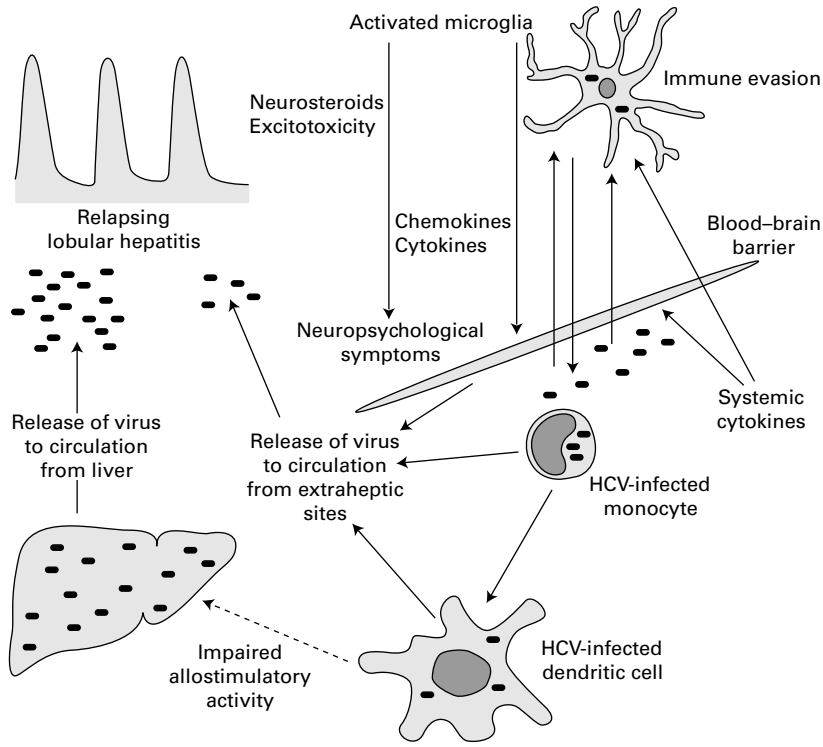


**Figure 29.2** Proposed model for hepatitis C infection of cells of monocytic lineage. Infection of circulating monocytes and/or bone marrow precursors, microglial cells and dendritic cells may explain a number of clinical and virological features of chronic hepatitis C infection. Low-level immune activation within the brain may explain to some degree the neuropsychological symptoms and cognitive impairments observed in some patients.[40,46,49,66,85] CNS immune activation may result from direct infection of the brain,[135,136,138] possibly introduced from infected monocytes[95,97] to microglial cells and/or as a result of elevated circulating and cerebral cytokine levels.[26] Together with other potential sanctuary sites, such as dendritic cells,[96,111] the brain may allow low-level viral replication to occur without eliciting an immune response sufficient to terminate it. The pattern of relapsing lobular hepatitis seen in HCV infection may represent a repeated cycle of immune lysis of infected hepatocytes, followed by repopulation by virus from low replication sites.[138] Similarly, it is possible that the relapse seen after initially successful antiviral treatment may be a result of persistent low-level replication in sites such as the brain and dendritic cells, which may be less responsive to therapy.[8]

infection of monocytes and bone marrow precursors has been reviewed. There is good evidence that microglial cells and resident perivascular macrophages within the brain turn over continuously and originate from bone marrow-derived precursors and monocytes.[144,145] It is therefore conceivable that HCV-infected monocytes or progenitor cells may introduce the virus into the CNS by a 'Trojan horse' mechanism, triggering a number of pathways that may result in neuronal dysfunction.

The immune response to HCV viral proteins within the CNS may underlie the cerebral dysfunction, as is the case in early HIV infection. Activated microglia are thought to liberate neurosteroids such as pregnenalone,[146] which may have an upregulatory role on neuroinhibitory pathways in the brain. Activated microglia also release excitatory amino acids, which can induce neuronal apoptosis through a process known as excitotoxicity,[147] and are potent producers of neurotoxins such as nitric oxide. These processes may be amplified by the release of cytokines and chemokines,[148,149] as well as recruiting further virally infected PBMCs across the blood–brain barrier.

Peripherally derived cytokines may also be implicated, either in the facilitation of viral transfer across the blood–brain barrier[150] or independently, in the absence of HCV infection of the CNS. Although cytokines are large peptides, animal studies have demonstrated passage of cytokines across the blood–brain and blood–spinal cord barriers.[151] Peripherally derived cytokines may also be passively transported into the brain at circumventricular sites which lack a blood–brain barrier. Intracerebral cytokines have been associated with immunological, neurochemical, neuroendocrine and behavioural activities,[152] probably through the pathways described above. It is clear that treatment with IFN-α is associated with depression and complaints of memory impairment and cognitive slowing,[153] but whether elevated endogenous cytokines in chronic inflammatory and infective conditions exert a significant cognitive effect is unclear. A study found no correlation between

levels of circulating interleukin (IL)-1, IL-6, tumour necrosis factor (TNF) and patients' fatigue in chronic HCV infection,[154] although no study exists to date on circulating cytokine levels and cognitive function in chronic HCV infection.

The above mechanisms remain hypothetical. Clinical, imaging and further virological studies may ultimately answer these questions.

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# Chapter 30
# *In vitro* replication models

Ralf Bartenschlager, Sandra Sparacio

## Introduction

In 1989 Choo and co-workers discovered the hepatitis C virus (HCV) as the causative agent of a liver disease which at that time was called non-A, non-B hepatitis.[1] Since then, numerous HCV isolates have been cloned molecularly and grouped together in the genus Hepacivirus that belongs to the *Flaviviridae* family. Based on the genomic heterogeneity, six major genotypes and a large number of subtypes have been defined. While genotype 1 viruses have almost worldwide prevalence, HCV of genotypes 4–6 can only be found in distinct geographical regions.

HCV is mainly transmitted via blood, blood products and parenteral risk factors. Blood screening assays developed soon after the discovery of the virus reduced the rate of transfusion-related transmissions significantly.[2,3] Nevertheless, HCV is still a major public health problem, as 170 million people worldwide are infected with this virus. Up to 80% of all infections become persistent, with approximately 20% of these patients developing liver cirrhosis within 20 years post-infection. The current antiviral treatment in most cases consists of the combination of a polyethylene glycol (PEG) conjugated form of interferon (IFN)-α and ribavirin.[4] However, the success rate depends very much on the genotype of the infecting virus. While up to 90% of HCV genotype 2- and 3-infected patients develop a sustained virological response, only about 45% of patients infected with genotype 1 viruses do so. Furthermore, neither a protective nor a therapeutic vaccine is available, emphasizing the requirement for alternative antiviral treatments.

The development of new HCV-selective drugs or vaccines requires a detailed understanding of the viral lifecycle as well as a profound knowledge of the interplay between the virus and the host. An essential prerequisite for studies in this direction is the availability of a robust and convenient cell culture system. This chapter gives a brief introduction to the biology of HCV and then describes the various cell-based replication models.

## Genome organization and replication cycle of HCV

HCV has a single-stranded RNA genome of positive polarity with a length of about 9.6 kb. The 5′ non-translated region (NTR) contains an internal ribosome entry site (IRES), mediating translation of an open reading frame (ORF), which encodes a polyprotein of about 3000 amino acids. The order of the individual proteins within the polyprotein is C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Fig. 30.1a). The structural proteins core, envelope protein 1 (E1) and E2 are located within the amino-terminal part of the polyprotein, whereas the non-structural (NS) proteins NS2 to NS5B are encoded in the remainder. With the exception of the F protein, the 10 viral products arise from co- and post-translational processing of the polyprotein by cellular and viral enzymes. Translation of the F protein initiates also at the AUG start codon of the ORF, but due to ribosomal frame shifting, an alternative reading frame is utilized.[5,6] The 3′ NTR is composed of a variable region, a polypyrimidine tract and the 98 nucleotides long highly conserved 3′ terminal X-tail. Both the 5′ NTR and the 3′ NTR contain RNA elements that are required for replication (reviewed by Bartenschlager *et al*.[7]).

The HCV particle consists of at least the genomic RNA, the core protein and the two envelope proteins E1 and E2 that are embedded into the viral envelope. The core protein forms the internal viral capsid that shelters the RNA genome. The two envelope proteins mediate attachment and entry of the virions into the host cells. *In vitro* studies suggest that the p7 protein can function as a virus-encoded ion channel and therefore it was grouped into the viroporin-protein family.[8,9] Moreover, a recent chimpanzee study demonstrated that this protein is essential for infectivity of HCV.[10] The NS2 protein together with the amino-terminal protease domain of NS3 is responsible for the autocatalytic cleavage of the NS2–NS3 junction. The same NS3 protein domain carries a serine-type protease that after association with its co-factor NS4A

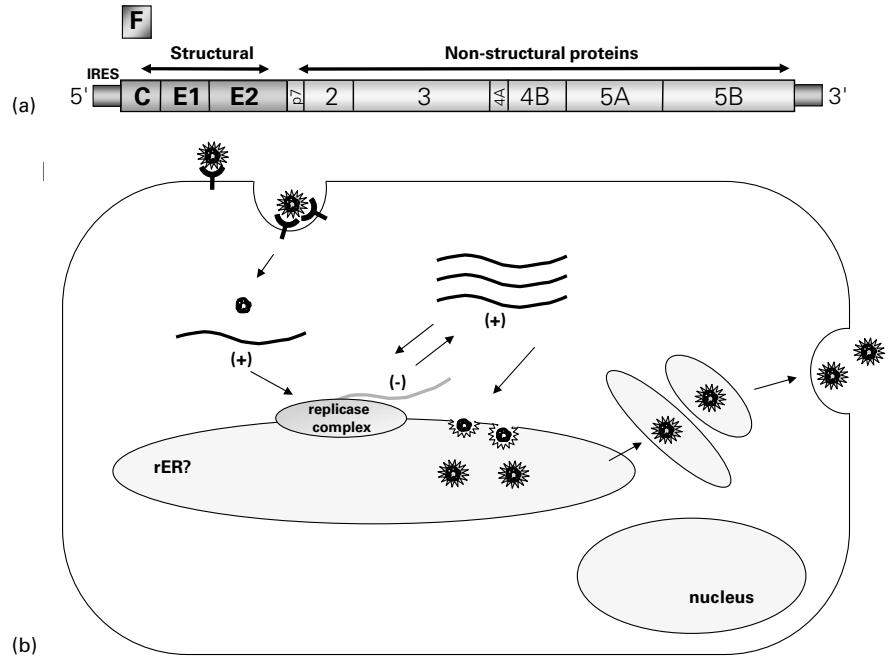


**Figure 30.1** HCV genomic organization and hypothetical HCV replication cycle. (a) Schematic representation of the HCV genome indicating the positions of the structural and non-structural proteins within the polyprotein as well as the 5′ NTR that contains the internal ribosome entry site (IRES) and the 3′ NTR. The F protein generated by ribosomal frame shifting is drawn above. (b) Hypothetical model of the HCV replication cycle. Upon binding of the virus particle to the appropriate cellular receptor and entry by receptor-mediated endocytosis, the genomic plus-strand RNA is liberated into the cytoplasm. After translation and processing of the viral polyprotein a membrane-associated replicase complex is formed. Minus-strand RNA is synthesized by this complex and serves as a template for production of excess amounts of plus-strand RNA. The newly synthesized RNAs either are translated or are packaged into the nucleocapsid. After budding into the lumen of the rough endoplasmic reticulum (rER) progeny viruses are presumably transported via the Golgi complex to the cell surface, where after fusion of the transport vesicle with the cellular membrane the virus particles are released.

cleaves the remaining junctions between the NS proteins. Two additional enzymatic activities, RNA helicase and nucleoside triphosphatase, reside in the carboxy-terminal two-thirds of NS3. Alterations of intracellular membranes, in particular membranous vesicles, are induced by NS4B. These alterations, which are the site of viral RNA synthesis, are called the membranous web.[11,12] So far, the role of the highly phosphorylated NS5A protein in the viral life-cycle is unclear. In addition to a potential function in RNA replication, NS5A may contribute to IFN-α resistance that is often observed with genotype 1 and 4 viruses (reviewed by Tan and Katze[13]). The membrane-associated NS5B protein is the RNA-dependent RNA polymerase (RdRp) and like other viral RdRps contains a 'GDD' amino acid sequence motif.

A brief summary of the HCV life-cycle may read as follows (Fig. 30.1b). Infection of the host cell starts by binding of the envelope glycoprotein E1/E2 complex to its cognate receptor(s), presumably leading to receptor-mediated endocytosis.[14] Upon release of the RNA genome into the cytoplasm the polyprotein is translated via the IRES in the 5′ NTR at the rough endoplasmic reticulum (rER). After or during cleavage of the polyprotein, the membrane-associated replication complex forms, which catalyzes the RNA amplification via negative-strand RNA intermediates. Newly synthesized plus-strand RNAs either are used for translation or serve as templates for further RNA syntheses or interact with the core protein to form the viral nucleocapsid. The E-proteins are retained at rER membranes, indicating that viral envelopes are generated by budding into the lumen of this organelle. Progeny particles are thought to be exported by the secretory pathway and after fusion of the transport vesicle with the plasma membrane, virions are released. It should be pointed out that most of these steps are poorly understood or hypothetical, and most often based on studies with heterologous expression systems and analogies to closely related viruses.

## HCV cell culture systems

In principle, three different approaches have been used to propagate HCV in cell culture and to study its replication cycle: first, the inoculation of primary cells or continuous

cell lines of hepatic or lymphoid origin with serum from HCV-infected individuals; second, the transfection of cell lines with genomic HCV constructs or *in vitro* transcribed genomic RNA; third, the transfection of cell lines with subgenomic or genomic replicons. These experimental approaches will be described in detail below.

## Infection of hepatocytes

As hepatocytes are considered to be the natural target cells for HCV *in vivo*, several attempts have been undertaken to establish an efficient HCV replication system by infecting primary hepatocytes from humans and chimpanzees with HCV-positive patient sera.[15–19] Although successful HCV replication has been shown in primary cell cultures, the common drawback is the extremely low efficiency. Moreover, when using primary cells from different donors experimental results are subject to variations that are difficult to control for. Finally, problems in handling and limited availability of primary cells impede their usefulness for experimental analyses. Thus, it is not surprising that much effort has been invested in developing cell culture systems based on immortalized hepatocytes. Several liver cell lines have been tested for their ability to support HCV replication. Among these, PH5CH cells immortalized with the simian virus large T antigen were found to be most susceptible.[20] Single cell clones were generated that are capable of supporting HCV replication more persistently than the parental PH5CH cells. In fact, when these cells were cultured at 32 °C, HCV RNA was detected up to 100 days post-infection.[21]

The human liver cell line FLC4 cultured in a three-dimensional radial-flow bioreactor (RFB) is the basis for an HCV infection system that was described by Aizaki and co-workers.[22] Upon inoculation with serum from an HCV-positive patient, viral RNA was detected by RT-PCR with peak RNA titres between day 19 and 23 in the range of $10^4$–$10^5$ HCV RNA copies/mL. However, this system is extremely inefficient, because at best only about 0.0001 HCV RNA copies/mL were released. Moreover, the equipment required to support cell growth in these 3D cultures is expensive, technically difficult and not readily available.

## Infection of lymphocytes

Although hepatocytes are considered the primary target cells of HCV, peripheral blood mononuclear cells (PBMCs) as well as B- and T-cell lines have also been used for infection studies. For instance, Cribier *et al*.[23] incubated HCV-positive sera with PBMCs isolated from healthy donors. Using RT-PCR and *in situ* hybridization, viral RNA was detected in infected cells as well as in cell culture supernatant until day 25 post-infection. HCV RNA contained in these supernatants could be transferred to naïve PBMCs, indicating the presence of infectious particles. However, the overall replication level of HCV in PBMCs is very low and not always reproducible.

Several human lymphocytic cell lines including the B-cell lines CE, TOFE and Daudi as well as the T-cell lines MOLT-4, HPB-Ma and MT-2 have also been used for the establishment of *in vitro* HCV culture systems. Among these, HPB-Ma and the MT-2 cells are the best characterized. The first is a murine retrovirus-infected human T-cell line that was shown to support HCV replication to some extent.[24] The isolation of single cell clones of HPB-Ma cells by limiting dilution revealed clonal differences in permissiveness. HCV replication, which was sensitive to IFN-α and IFN-β,[25] could be maintained in an isolated single cell clone (HPB-Ma clone 10–2) for more than 1 year.[26]

MT-2 cells, a human T-cell line immortalized with human T-lymphotropic virus 1 (HTLV-1), are also susceptible to HCV infection.[27] By means of limiting dilution, five MT-2 cell clones were obtained that support HCV replication more persistently than the parental MT-2 cells. One of these five clones, designated MT2-C, was shown to support HCV RNA replication until day 11 post-infection. Furthermore, negative-strand RNA was detected and an HCV-specific antisense oligonucleotide reversibly blocked replication. Treatment of cells with IFN-α for 2 days led to a reduction of HCV RNA down to 90%. In accordance with what was described for PH5CH, when cells were cultured at 32 °C instead of 37 °C, HCV RNA persisted much longer and could be detected up to 198 days post-infection.[28–30]

Very recently, a B cell line derived from the spleen of an HCV-infected patient was established.[30a] These cells support continuous replication and production of viruses that appear to be infectious in PBMCs and the B cell line Raji. However, the efficacy of this system is also limited with HCV RNA copy numbers being in the range of 0.01 to 0.1 per cell.

## Transfection of cell lines with genomic HCV constructs

In comparison with the infection of cells with authentic HCV particles contained in serum samples, the transfection of cell lines with viral cDNA constructs or *in vitro* transcripts corresponding to full-length HCV genomes has the major advantage of using a well-defined homogenous starting material that can be generated in large quantities. Moreover, mutational analyses can be performed to gain insights into the viral life-cycle. One prerequisite of this approach is the availability of cloned infectious genomes. Although several such HCV genomes have been described, productive replication in cells transfected with these RNAs turned out to be very difficult.[31–33] Initial reports describing HCV replication in the cell lines HepG2 and Huh-7 upon transfection with near full-length HCV RNAs must be viewed with caution because the used genomes lacked the 3′ terminal

X-tail.[34,35] This RNA element, however, is essential for replication *in vivo* and in cell culture (see below).

More recently, Aizaki and colleagues[22] performed the transfection of FLC4 cells with full-length HCV RNA of the genotype 1a isolate H77. Upon passage of these cells in a radial-flow bioreactor, intracellular viral RNA was detected. Culture supernatant transferred to naïve FLC4 cells initiated a second round of infection, indicating the production of infectious HCV particles in transfected cells. However, this culture system is complicated not commonly available and, the overall yield of HCV is very low.

In 2001, Chung and co-workers reported a DNA-based HCV replication system.[36] A plasmid containing the T7 promoter upstream of a full-length HCV genome of the genotype 1a and the ribozyme of the hepatitis delta virus at the 3′ end that generates an authentic HCV 3′ end was transfected into CV-1 and HepG2 cells. Transfected cells were subsequently infected with a recombinant vaccinia virus expressing T7 RNA polymerase. In these cells HCV negative-strand RNA was detected by strand-specific RT-PCR and ribonuclease protection assay. Furthermore, replication of HCV was sensitive to IFN-α and the emergence of distinct quasi-species in transfected cells was consistent with an error-prone replication mediated by the NS5B RNA polymerase. However, no evidence for the production of infectious HCV particles from these cells was obtained, arguing that this system does not support a full replication cycle. Further studies are required to elucidate the usefulness of this system, as the cytopathic vaccinia virus that delivers the T7 RNA polymerase may interfere with HCV.

## Transfection of cell lines with replicons

Up to now, the most efficient and reliable *in vitro* replication model for HCV is the replicon system that is based on the autonomous replication of selectable subgenomes in the human hepatoma cell line Huh-7.[37] These replicons were originally derived from a genotype 1b consensus genome, designated Con-1, that was isolated and cloned from a chronically infected patient who had undergone liver transplantation. The overall structure of the replicons is depicted in Fig. 30.2a. The sequence encoding for core to p7 or core to NS2 was replaced by a gene cassette consisting of the selectable marker neomycin-phosphotransferase (*neo*) which confers resistance to the cytotoxic drug G418 and by the IRES of the encephalomyocarditis virus (EMCV) that directs the translation of the NS3 to NS5B coding region of HCV. Translation of the *neo* gene is controlled by the IRES in the HCV 5′ NTR. Authentic 5′ and 3′ ends are generated by the proper positioning of the T7 RNA polymerase promoter at the 5′ end and by introducing a unique restriction site at the 3′ end of the replicon sequence. After electroporation into

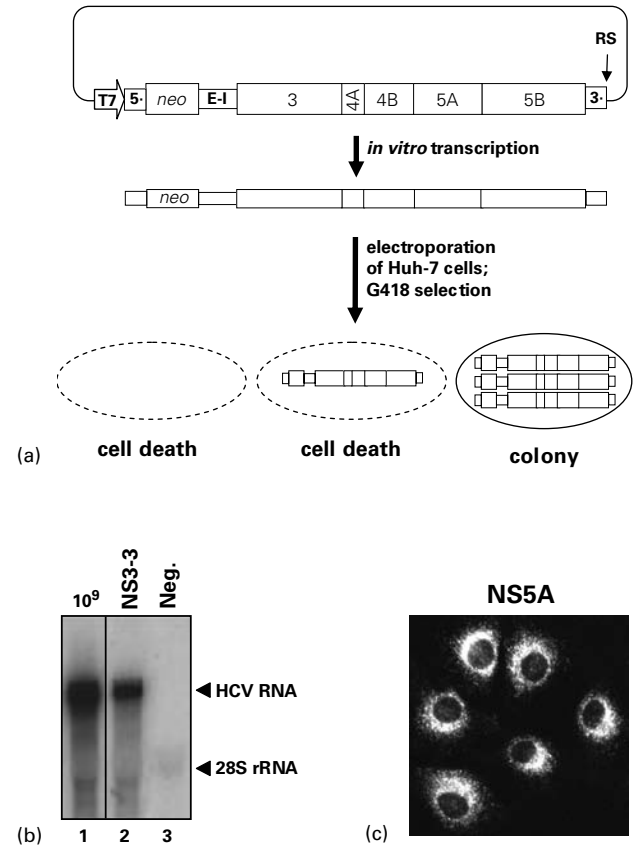


**Figure 30.2** Experimental strategy to generate stable replicon cell clones and demonstration of HCV RNA and protein in Huh-7 replicon cells. (a) Schematic illustration of the method used to establish subgenomic replicon harbouring Huh-7 cells. The DNA construct consists of a T7 RNA polymerase promoter (T7), the HCV 5′ NTR (5′), the selection marker neomycin phosphotransferase (*neo*), the internal ribosome entry site of the encephalomycarditis virus (E-I), the NS3 to NS5B coding sequence and the 3′ NTR (3′). After linearization of the DNA construct at an engineered restriction site (RS), *in vitro* transcription is used to synthesize replicon RNA molecules, which are transfected via electroporation into Huh-7 cells. Upon selection with G418, only those cells that support efficient RNA replication will survive. Cells which were either not transfected or which do not support efficient RNA replication are eliminated. (b) Detection of plus-strand RNA in subgenomic replicon harbouring Huh-7 cells. Total RNA isolated from a replicon cell clone (lane 2) and from naïve Huh-7 cells (lane 3) was analyzed by Northern blot using a plus-strand specific RNA probe. As a size marker, $10^9$ molecules of the corresponding *in vitro* transcripts were analysed in parallel (lane 1). The positions of the HCV-specific RNA and the 28S rRNA are indicated to the right. (c) Demonstration of NS5A in a cell clone carrying a subgenomic replicon by indirect immunofluorescence.

Huh-7 cells, replicon RNAs are translated and the HCV proteins build up active replication complexes that catalyze the amplification of the replicon RNAs. Positive-strand RNA progeny are used as mRNA for translation

of further HCV proteins as well as neomycin-phosphotransferases. Thus, when transfected cells are cultured in the presence of G418, untransfected cells and cells in which the replicons do not amplify to sufficient levels will die. Only those cells supporting RNA replication to high levels will express sufficient neomycin-phosphotransferase to confer resistance against G418. These cells will grow under such selective pressure and form colonies (Fig. 30.2a).

Thorough analyses of these colonies confirmed the presence of self-replicating HCV RNAs. About $10^8$ positive-strand RNA copies per µg RNA were detected, corresponding to approximately 1000–5000 molecules per cell (Fig. 30.2b). A 5–10-fold lower amount of negative-strand RNA was determined, supporting the assumption that this RNA species serves as a template for the generation of excess amounts of positive-strand RNA genomes. Consistent with the fact that replication is dependent on the NS5B RdRp, replicon RNA synthesis was not affected by actinomycin D, whereas the synthesis of cellular RNAs was blocked. Viral proteins could easily be detected, e.g. after metabolic radiolabelling and immunoprecipitation or by immunofluorescence analyses (Fig. 30.2c). These results demonstrated the high efficiency of the replicon system as compared with the previously described *in vitro* infection systems, where these kinds of assays did not allow the detection of viral proteins and RNAs due to the low replication levels of HCV RNAs in these cells.

### *Cell culture adaptation*

As described by Lohmann and co-workers,[37] only a low number of colonies were obtained after transfection with replicon RNA transcripts and G418 selection, even though a high copy number of replicon RNA was detected within a selected cell clone. There are two potential explanations for this discrepancy. First, only a small subpopulation of Huh-7 cells supports RNA replication to a level that is sufficient to confer G418 resistance. This would be consistent with the observation made with *in vitro* infection systems (see above) that subcloning of cells leads often to more permissive cell clones. Second, the replicon RNA has to gain cell culture adaptive mutations that enhance RNA replication. Indeed, detailed analyses of RNAs isolated from several replicon cell clones revealed amino acid exchanges in the HCV coding region that were conserved among the majority of replicon RNAs isolated from a given cell clone (Fig. 30.3).[38,39–41] Depending on the particular mutation, its introduction into the parental replicon increased the number of G418 resistant cell colonies to various extents in comparison with the wild-type replicon, showing that these mutations enhance RNA replication and confer cell culture adaptation. Taking advantage of this discovery, highly efficient replicons were developed that could be analysed in transient replication assays. For instance, replicons were constructed in which the selectable marker *neo* was replaced by easily detectable reporter genes like the one encoding the luciferase of the firefly (Figure 30.4a**)**. In this setting, RNA replication is measured by using luciferase assays and it was shown that luciferase activity closely correlates with replicon RNA levels.[39,42]

Although cell culture adaptive mutations have been found at various positions in the HCV coding sequence, with the exception of a single amino acid substitution in NS5B (R2884G), they cluster in three distinct regions (Fig. 30.3). The major cluster resides in the centre of NS5A protein,[38,41] often affecting serine residues that are involved in NS5A hyperphosphorylation (reviewed in Pawlotsky and Germanidis[43]). This result implies that a reduction of hyperphosphorylation enhances RNA replication, at least in Huh-7 cells. In fact, it was shown recently that reduction of heperphosphorylation by using an inhibitor of the NS5A kinase enhances replication of wild type RNAs.[38b] However, a minimum of hperphosphorlated NS5A is required as treatment of adapted replicons with the same kinase inhibitor blocked RNA replication.[38b]

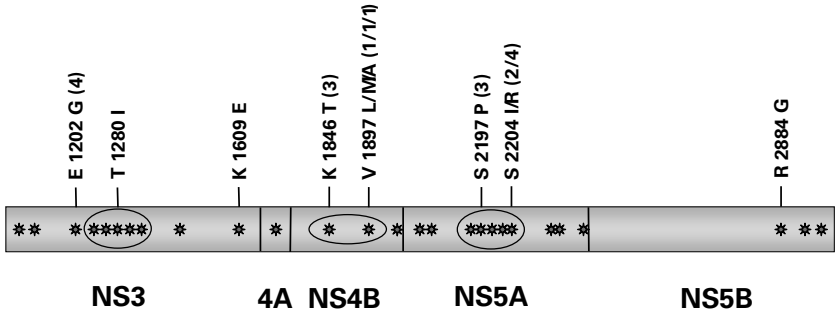


**Figure 30.3** Identification of clusters of cell culture adaptive mutations. Amino acid substitutions that were found to be conserved in 26 independently selected replicon cell clones are indicated by (✶). Distinct mutations described in the text are specified above with numbers referring to the polyprotein of the HCV Con-1 isolate. Numbers in parenthesis indicate the frequency with which a given mutation was found in independent replicon cell clones. The circles illustrate clusters of adaptive mutations.

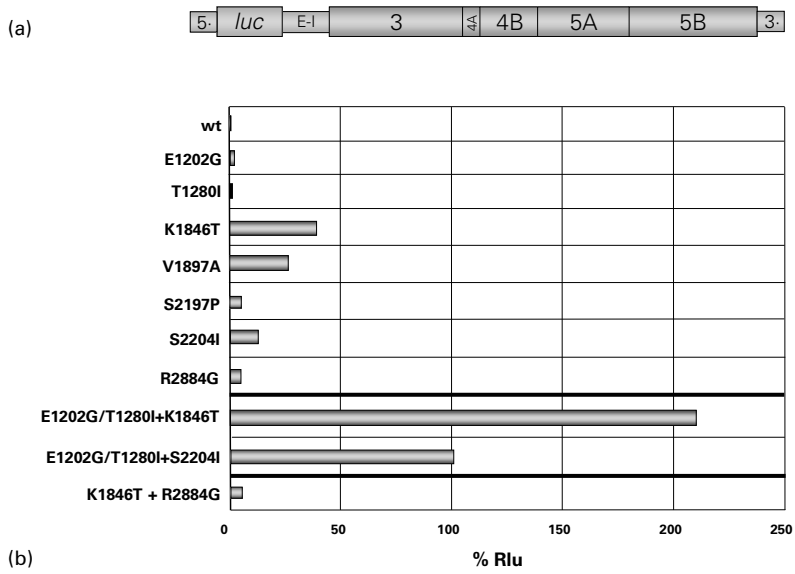


**Figure 30.4** Effect of amino acid substitutions in the HCV coding sequence on transient RNA replication. (a) Schematic representation of the luciferase reporter replicon. Its structure is analogous to the selectable replicon shown in Fig. 30.2a, but the selection marker *neo* is replaced by the gene encoding the luciferase from the firefly (*luc*). (b) Huh-7 cells were transfected with replicons containing the mutations specified on the left and harvested 4 hours (h) and 48 h later. Luciferase activity is expressed as percentage relative light units (% Rlu) determined in lysates of cells at 48 h compared with those at 4 h after electroporation. Note that the 4-h value reflects translation of the input RNA and, therefore, is a marker for transfection efficiency.

Two distinct positions in NS4B represent the second hot spot for cell culture adaptive mutations (Fig. 30.3). It was found that single substitutions affecting these positions (amino acid 1846 or 1897 of the Con-1 polyprotein) enhance RNA replication dramatically (Fig. 30.4b).[41] The third cluster resides in the helicase domain of the NS3 protein (Fig. 30.3).[39,42] Mutations in this region have only a low impact on RNA replication (Fig. 30.4b).[39–41] However, when highly adaptive mutations in NS4B (e.g. K1846T) or NS5A (e.g. S2204I) were combined with mutations in NS3, the combination was beneficial and replication of the replicon was enhanced cooperatively (Fig. 30.4b), at least with Con-1 derived replicons.[39,41,42,44] In fact, the combination of the K1846T substitution with two NS3 mutations (E1202G and T1280I) yielded the most efficient Con-1-derived replicon (Fig. 30.4b). This is consistent with the observation that mutations in NS3 were only found in combination with other adaptive substitutions in the NS4B to NS5B region. However, when highly adaptive mutations in the NS4B to NS5B region were combined with each other, RNA replication was much lower compared with replicons that carried these mutations individually (Fig. 30.4b).[41] These results are consistent with the fact that none of the highly adaptive mutations in NS4B, NS5A or NS5B were found in combination with each other in the same HCV replicon RNA. This led to the suggestion that mutations in this region mediate cell culture adaptation via the same or a very similar mechanism.

One particular amino acid exchange within NS3, K1609E, was found independently by three groups that used two different genotype 1b[40,45] and a chimeric 1a/b replicon.[46] In the latter, the 5′ NTR and 75 amino-terminal residues of the NS3 protein were taken from the Con-1 (1b) isolate and the remainder from the H77 (1a) isolate. Another remarkable adaptive mutation within NS3 is E1202G that resides at the very carboxy-terminus of the NS3 protease domain. This mutation is present in about 5% of published HCV sequences and it represents the only adaptive mutation that is also found *in vivo*. All other substitutions affect highly conserved residues and have not been observed within HCV genomes isolated from patients.

At present, we can only speculate about the mechanisms underlying cell culture adaptation. Based on the available X-ray crystal structures of NS3 and NS5B, the locations of some of the substitutions have been mapped. Interestingly, most of them reside on the solvent-exposed surface of the molecules.[39,40,42] These sites might therefore be involved in interactions with other viral or cellular proteins. The identification of such interaction partners will be an important task.

To analyze the impact of adaptive mutations on infectivity *in vivo*, the two mutations E1202G and T1280I in NS3 and S2197P in NS5A were introduced into the infectious HCV genome Con-1 and RNA transcripts were inoculated into the liver of a chimpanzee. In three independent attempts, no productive infection could be established.[47] When a chimpanzee was inoculated with a Con-1 genome containing the single adaptive mutation in NS5A, the animal became viraemic after a delay of about 1 week. However, all HCV genomes isolated from this chimpanzee had reverted to wild-type. This led to the conclusion that cell culture adaptation is closely associated with attenuation *in vivo*. In line with this observation is the fact that the HCV-N isolate (also genotype 1b) has a low infectivity *in vivo*,[47,48] but replicons derived from this isolate are highly efficient.[49,50] A four amino acid insertion within the NS5A protein is responsible for the inherent ability of the HCV-N isolate to replicate

efficiently without further adaptive mutations.[50] These findings imply an inverse correlation between cell culture adaptation and attenuation *in vivo*, but this view has recently been challenged by the development of a novel genotype 2a replicon (see below) that replicates with high efficiency without the need for adaptive mutations. Although the infectivity of the HCV genome from which this replicon was derived has not yet been tested, the genome was isolated from a patient with a fulminant hepatitis C, arguing that it might be replication-competent *in vivo*.

### *Host cell permissiveness*

Recently, it was shown that in addition to cell culture adaptive mutations the host cell itself contributes very much to the efficiency of RNA replication. Blight and co-workers as well as Murray and colleagues demonstrated that within a given population of naïve Huh-7 cells only a subpopulation is capable of supporting high-level RNA replication.[51,52] When replicon-harbouring cells were treated with IFN-α or a selective inhibitor to remove the replicon, the resulting cured cells supported HCV replication more efficiently than naïve control cells. This result suggests that the selective pressure used to generate a replicon cell clone leads to the selection of both cell culture adaptive replicons and to Huh-7 cells that are more permissive.

Different levels of permissiveness were also observed between various passages of naïve Huh-7 cells originating from the same cell stock.[41] These differences (up to 100-fold) were not due to variations in IRES-dependent translation or RNA stability. One explanation might therefore be that the expression levels of cellular factors required for RNA replication differ between these passages. In line with this assumption is the fact that an increase in the amount of transfected replicon RNA leads to a decrease of replication efficiency per transfected replicon RNA molecule,[41] indicating that cellular factor(s) required for the formation or activity of the HCV replicase complexes limit viral RNA replication.

### *Genomic replicons and cell culture adapted full-length genomes*

In an attempt to produce infectious replication-competent HCV particles in cell culture, selectable genomic replicons were constructed and used to generate stable Huh-7 cell clones. Ikeda and colleagues[50] demonstrated the replication competence of a selectable genomic replicon derived from the HCV-N isolate by using Northern blot and immunofluorescence analyses. However, the efficiency of genomic RNA replication was reduced in comparison with replication of subgenomes and the transfer of filtered supernatant from genomic replicon cells to naïve Huh-7 cells did not lead to a detectable infection (Table 30.1).[42,50,52,53]

Pietschmann and colleagues conducted a more detailed analysis. They took advantage of three cell culture adaptive mutations that cooperatively increase RNA replication (E1202G and T1280I in NS3 and S2197P in NS5A) and constructed a selectable genomic replicon (Fig. 30.5A).[53] However, despite efficient RNA replication and protein expression in several independent Huh-7 cell clones, no conclusive evidence for the production of virus particles has been obtained (Fig. 30.5b, c). Transfer of cleared supernatant from these cells to naïve Huh-7 cells did not lead to a detectable infection. Although nuclease-resistant HCV RNA with the same low-density profile reported for infectious HCV[54] was found in the supernatant of genomic replicon cell clones, HCV RNA with the same characteristics was also detected in the supernatants of cell clones with subgenomic replicon RNAs. Several rea-

**Table 30.1** Summary of genomic replicon systems for HCV

| Genotype | Isolate | Assay* | Replication detection† | Particle detection‡ | Reference no. |
|---|---|---|---|---|---|
| 1b | HCV-N | Stable | (+) RNA; IF | Infection assay (Huh-7) | 50 |
| 1b | Con-1 | Stable; transient | (+) RNA; IF; IP; WB | Infection assay (Huh-7); density gradient | 53 |
| 1b | Con-1 | Transient | (+) RNA; IP; FACS | NA | 52 |
| 1a | H77 | Transient | (+) RNA; IP; FACS | NA | 42 |

NA, not available.

*Stable, establishment of replicon harbouring cell clones by a selection process; transient, transient replication assay.

†Method used to verify HCV replication: (+) RNA, detection of plus-strand HCV RNA; IF or IP or WB or FACS, detection of HCV antigens by immunofluorescence or immunoprecipitation after metabolic radiolabelling or Western blot or FACS, respectively.

‡Method used for detection of virus particles: infection assay, transfer of cleared supernatant from cell clones carrying selectable full-length replicons to naïve Huh-7 cells and subsequent selection for G418 resistance; density gradient, supernatant from cell clones carrying selectable full-length replicons were subjected to density gradient centrifugation and analyzed by quantitative RT-PCR.

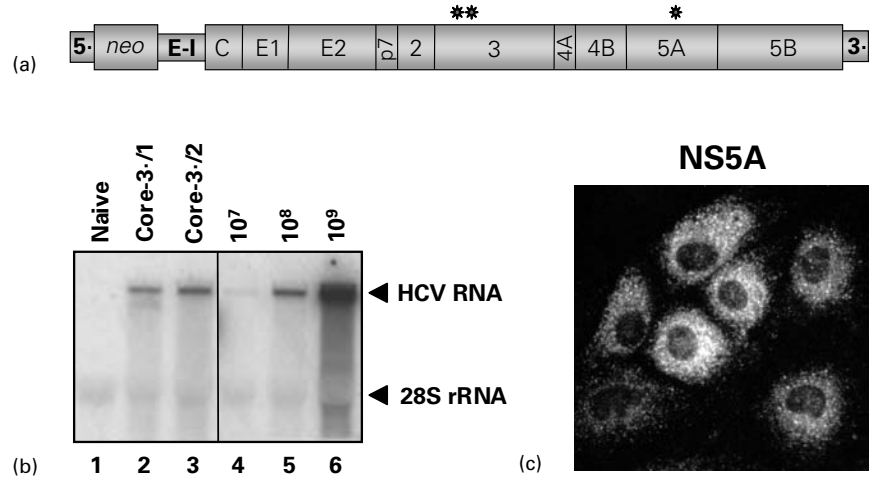


**Figure 30.5** Structure of genomic replicons and demonstration of HCV RNA and protein in Huh-7 cells. (a) Schematic representation of a selectable full-length replicon composed of the 5′ NTR (5′), the selection marker neomycin phosphotransferase (*neo*), the internal ribosome entry site of the encephalomycarditis virus (E-I), the core to NS5B coding sequence and the 3′ NTR (3′). The asterisks (✶) represent the positions of adaptive mutations that were introduced into the HCV coding sequence. (b) Detection of self-replicating genomic replicon RNA. Total RNA isolated from two full-length replicon cell clones (lanes 2 and 3) and from naïve Huh-7 cells (lane 1) were analyzed by Northern blot using a plus-strand specific RNA probe. As a size marker, $10^7$ up to $10^9$ molecules of the corresponding *in vitro* transcripts were analyzed in parallel (lanes 4–6). The positions of the HCV RNA and 28S rRNA are indicated to the right. (c) Demonstration of NS5A in a cell clone carrying a genomic replicon by indirect immunofluorescence.

sons may account for the lack of virus production in stable cell clones carrying genomic replicons. For instance, the presence of heterologous sequences in the 5′ region of these replicons may have destroyed an RNA packaging signal or led to an increase of the genome length beyond the packaging limit. Alternatively, it is possible that the high genetic drift rendered the structural proteins non-functional due to the accumulation of inactivating mutations. To circumvent these problems a transient replication assay of cell culture adapted full-length genomes was established. However, despite efficient transient replication, no infectious particles could be detected.[54]

By using an analogous approach, Blight and co-workers assessed the ability of cured Huh-7 cells to support replication of a Con-1 genome containing an adaptive mutation in NS5A (S2204I).[52] Furthermore, a genotype 1a full-length genome derived from the HCV isolate H77 and carrying two cell culture adaptive mutations (P1496L in NS3 and S2004I in NS5A) was established in the same laboratory.[42] Replication of both genomes was demonstrated by RNA quantification and metabolic radiolabelling of HCV-specific proteins. However, release of replication-competent HCV was not observed in any of the described approaches (Table 30.1). So far, it is not clear where the block for virus particle production or secretion resides. For instance, the lack of host cell factors in Huh-7 cells might lead to an inefficient assembly process. Alternatively, cell culture adaptive mutations may interfere with asembly and/or virus release. As described below, this appears to be the the primary reason for the lack of virus production.

### *Replicons derived from non-genotype 1 HCV isolates*

An inherent complication of the HCV replicon system is the unpredictability as to whether or not an HCV isolate will replicate. As deduced from the appearance of cell culture adaptive mutations, slight variations in the HCV sequence can have dramatic effects on replication fitness. Until recently, only replicons derived from genotype 1 isolates have been established and attempts to develop replicons of other genotypes have failed (reviewed in Lanford and Bigger;[55] Windisch and Bartenschlager, unpublished results). An important contribution was therefore the establishment of a genotype 2a replicon derived from an HCV isolate (JFH-1) that was obtained from a patient with fulminant hepatitis C.[56] This replicon has a remarkably high efficiency of RNA replication, in fact up to 20-fold higher than Con-1-derived adapted replicons, and does not require cell culture adaptive mutations. The reasons for this highly efficient replication are unclear, but it seems possible that the JFH-1-derived replicon carries some inherent adaptive mutations that were already present in the isolate cloned from the patient. If that is true, one might expect that these adaptive mutations are compatible with infectivity *in vivo*. In fact, the genotype 1b HCV-N isolate carries a natural

four amino acid insertion in NS5A that confers cell culture adaptation and that seemingly does not completely block a productive infection *in vivo*.[50]

### *Host range of HCV replicons*

In addition to Huh-7 cells, replicon cell clones derived from HuH6 were recently established using cell culture adapted subgenomic HCV replicons of the Con-1 isolate (Windisch *et al*., 10th International Meeting on the Hepatitis C Virus and Related Viruses, Kyoto, Japan, 2003). Furthermore, Zhu *et al*.[57] demonstrated HCV replication in a non-liver cell line as well as in a liver cell line of murine origin (Table 30.2). The approach that they used was based on the assumption that cell culture adaptive mutations observed in Huh-7 cells represent an adaptation to a specific cellular environment, and that further mutations might be required to adapt HCV replicons to other cell lines. Consequently, they screened a panel of different cell lines using total RNA isolated from replicon-containing Huh-7 cell clones. Owing to the high error rate of the HCV replicase, replicon RNA that is present in a given cell clone has a high genetic complexity. Therefore, the chances of selecting an adapted variant are higher as compared to the use of rather homogenous *in vitro* transcripts that are derived from a cloned template. By using this approach, Zhu *et al*. were able to establish clones from HeLa cells, a human cervix carcinoma cell line, and from Hepa1-6 cells, a murine liver cell line.[57] Sequence analysis of the RNA isolated from these HeLa and Hepa1-6 clones revealed a number of mutations that might be involved in fine-tuning of replicon adaptation.[57] These results show for the first time that HCV RNA replication does not depend on liver cell-specific factors and they may open a way to develop a mouse model for HCV replication by using adapted HCV sequences.

Overall, the properties of HeLa replicon cells (only these have thus far been analyzed in some detail) are very similar to those described for Huh-7 cells. However, distinct differences exist. First, in Huh-7 cells the replicon RNA copy number very much depends on host cell confluency. RNA levels are highest in well proliferating cells, but drop significantly when cells are resting.[61] In contrast, no such dependence was found with replicons in HeLa cells.[57] Whether this is due to some different requirements for host cell factors that vary in abundance in Huh-7 cells but not in HeLa cells remains to be determined. Alternatively, HeLa cells may have a much less pronounced cell contact inhibition. Another difference between Huh-7 and HeLa replicon cell clones relates to their IFN-α sensitivity. No morphological changes but efficient inhibition of replicons has been described for Huh-7 cells, whereas about 30% of HeLa cells undergo apoptosis.[57] The fact that inhibition of replicons by an NS5B selective drug prior to treatment with IFN-α pre-

**Table 30.2** Summary of subgenomic replicon systems for HCV

| Cell type | Species | Genotype | Isolate | Detection method* | Reference no. |
|---|---|---|---|---|---|
| Huh-7 | Human | 1b | Con-1 | (+), (–) RNA; IF; IP | 37 |
| Huh-7 | Human | 1b | Con-1 | IP; IFN-α | 38 |
| Huh-7 | Human | 1b | HCV-N | (+) RNA; IFN-α | 49 |
| Huh-7 | Human | 1b | HCV-N | (+) RNA; IF | 50 |
| Huh-7 | Human | 1b | 1B-1 | (+) RNA; WB | 45 |
| Huh-7 | Human | 1b | Con-1<br>HCV-N | (+) RNA; IF, tat-dependent SEAP expression | 44 |
| Huh-7 | Human | 1a | H77 | IP; (+) RNA; FACS | 42 |
| Huh-7 | Human | 1b | Con-1 | *bla* | 51 |
| Huh-7 | Human | 1b | HCV-BK | *Bla* | 58 |
| Huh-7 | Human | 1a/b | H77/Con-1 | (+) RNA; WB; IF; IFN-α | 46 |
| Huh-7 | Human | 1b | 1B-2 | (+) RNA, WB,<br>IFN-α,-β,-γ | 59 |
| Hepa1–6 | Mouse | 1b | Con-1 | (+) RNA; IF | 57 |
| HeLa | Human | 1b | Con-1; JFH-1 | (+) RNA; IF | 57, 60b |
| HuH6 | Human | 1b | Con-1 | (+) RNA; IF; WB; IFN-α | Windisch *et al*., 2003† |
| 293 | Human | 1b | Con-1; JFH-1 | (+) RNA; IF; WB; IFN-α; NS3 inhibitor | 60a, 60b |
| Huh-7 | Human | 2a | JFH-1 | (+), (–) RNA; WB; IF | 56 |
| HepG2 | Human | 2a | JFH-1 | (+) RNA, IF, WB | 60b |
| IMY-N9 | Human | 2a | JFH-1 | (+) RNA, IF, WB | 60b |

*Method used to verify replication of HCV replicons; (+) RNA, detection of plus-strand HCV RNA; (–) RNA, detection of negative-strand HCV RNA; IF or IP or WB or FACS, detection of HCV antigens by immunofluorescence or immunoprecipitation after metabolic radiollabeling or Western blot or FACS, respectively; IFN-α,-β,-γ, inhibition of RNA replication by treatment with interferon-α, -β and -γ; NS3 inhibitor, inhibition of RNA replication by a specific NS3 protease inhibitor; *bla*, β-lactamase reporter used for transient replication assay; tat-dependent SEAP expression, secretion of placental alkaline phosphatase (SEAP) under control of the HIV-LTR.
†The 10th International Meeting on Hepatitis C Virus and Related Viruses, 2003, Kyoto, Japan.

vents apoptosis suggests that HCV RNA replication or proteins stimulate an innate cellular response that sensitizes HeLa cells towards apoptosis.

Beside HeLa cells, the human embryonic kidney cell line 293 was also shown to support HCV RNA replication.[60,60a] In one approach by co-cultivation of Huh-7 cells with 293 cells, rare replicon-containing colonies were obtained, which morphologically resembled 293 cells. Total RNA isolated from these cells and transfected into naïve 293 cells gave rise to replicon harbouring cell clones, as shown by antigen and RNA detection assays as well as inhibition of HCV replication with IFN-α (Table 30.2). These findings corroborate that HCV replication is not dependent on hepatocyte- or primate-specific factor(s) and point out the unpredictable nature of the search for new cell lines that support HCV RNA replication. Nevertheless, these novel replicon cell lines will be instrumental in understanding the mechanism of cell culture adaptation and, eventually, help to produce authentic HCV particles in cell culture.

### *Applications of the HCV replicon system*

Taking advantage of cell culture adaptive mutations and highly permissive cell lines, transient replication systems were developed that avoided the cumbersome and time-consuming process of selection for G418 resistant colonies when using selectable replicons. To facilitate transient replication assays, an easily detectable reporter gene like the one encoding the firefly luciferase was inserted instead of the selectable marker *neo* (Fig. 30.4a). In this setting, RNA replication can be measured by luciferase assays and it was shown that luciferase activity correlates well with the level of RNA replication.[39] An alternative assay for transient replication is based on replicons that carry the *tat* gene of the human immunodeficiency virus (HIV), which activates the transcription of a tat-dependent reporter gene that is stably integrated into the Huh-7 cell.[44] Alternatively, replicons that express the beta-lactamase gene have been developed. In this case, the reporter activity can be measured with a cell-permeable substrate, which greatly facilitates the analyses.[51] Finally, transient replication of cell culture adapted replicons can be measured by using nuclease protection or Northern blot assays as well as quantitative RT-PCR.

The availability of these assays enabled for the first time detailed molecular studies of HCV RNA replication. For instance, *cis*-acting elements in the 5′ and 3′ NTRs required for RNA replication have been mapped and characterized.[62–66] Another interesting application is to study the mode of action of antiviral cytokines such as IFN-α, -β, or -γ and tumour-necrosis-factor (TNF)-α, and to identify cellular factors involved in viral replication.[38,49,67–70]

Finally, the replicon system encodes all viral enzymes that are required for HCV replication and thus are potential targets for therapy. In this respect, the replicon system is an important tool for the screening and evaluation of antiviral drugs. Furthermore, it also provides a means to develop novel therapeutic strategies (reviewed in Bartenschlager[71]). One example that nicely illustrates the latter is the inhibition of HCV replication by RNA interference (RNAi). Small duplex RNA molecules, so-called small interfering RNAs (siRNAs) target homologous sequences for degradation (reviewed in Hannon[72]). In plants, this is an important antiviral defence mechanism, which so far has not been discovered in mammalian cells. However, the artificial introduction of siRNA molecules into mammalian cells can inhibit replication of several viruses in cell culture (reviewed in Joost Haasnoot *et al*.[73]). In case of HCV, the replicon system provided a suitable model to test this antiviral approach. It was found that HCV replication was sensitive to siRNA molecules generated either by chemical synthesis, or by intracellular transcription of short hairpin RNAs (shRNAs) from transfected plasmids or stably integrated retroviral vectors.[74–77] Alternatively, HCV-specific esiRNAs can be prepared by enzymatic digestion of *in vitro* transcribed, double-stranded RNAs with RNaseIII from *Escherichia coli*. These highly heterogeneous esiRNAs that can target multiple sites within an mRNA were shown to be efficient in inhibition of HCV replication in Huh-7 replicon cells.[77]

## Surrogate models of HCV

As so far genomic replicons and cell culture adapted full-length HCV genomes do not lead to virus production, until very recently aspects of early stages (virion attachment, entry and uncoating) and late stages (assembly, egress) of the HCV life-cycle could not directly be addressed. Therefore, several laboratories used surrogate model systems. The GB viruses (especially GBV-B) and the pestiviruses are the closest relatives of HCV. In addition to a productive infection and replication in tamarins (*Saguinus* species), GBV-B replicates efficiently in primary tamarin hepatocytes.[78] The genome of this virus has a genetic organization that is very similar to that of HCV including an IRES in the 5′ NTR, an almost superimposable genomic organization of the coding region and a 3′ NTR that is indispensable for replication and *in vivo* infectivity.[79–81] The functional similarities between these viruses were demonstrated by the correct processing of the HCV polyprotein by the GBV-B NS3 protease and the creation of functional chimeric NS3 proteins between HCV and GBV-B.[82,83] However, there is a striking difference in the course of infection between HCV and GBV-B. The latter usually causes an acute self-limiting

hepatitis in tamarins, whereas HCV infection of humans frequently becomes persistent. Whether this is due to differences in the viruses or the host is not known.

Pestiviruses, especially the bovine viral diarrhoea virus (BVDV), have also been used as surrogate model systems. BVDV shares a similar genomic organization with HCV and both viruses generally cause persistent infections in their respective hosts. Furthermore, it is easy to culture BVDV *in vitro*, where the virus performs a complete replication cycle both after infection of cells and after transfection of RNA derived from cloned infectious genomes. However, also in this case, there are some differences in the biology of both viruses. For instance, BVDV has a much broader host range in cell culture than HCV and, in contrast to HCV, cytopathic and non-cytopathic BVDV strains exist.

HCV chimeric viruses have been used for genetic and biochemical analyses of the functions of HCV gene products and RNA structures. For instance, chimeric HCV/polioviruses have been constructed in which the poliovirus replicates under the translational control of the HCV IRES.[84] Another example is a BVDV/HCV chimera in which BVDV replication is conditionally dependent on the activity of the NS3/4A protease that was fused with the amino terminus of BVDV core protein.[85] However, HCV protein functions and RNA structures involved in replication can now be studied in the more authentic context of replicons and cell culture-adapted full-length genomes (see above).

A major step towards the study of early steps in the HCV life-cycle is the HCV pseudoparticle system that was recently described by Bartosch and colleagues.[86] These pseudoparticles are composed of the HCV envelope glycoproteins E1 and E2 and either retroviral or lentiviral nucleocapsids. They were generated by co-transfection of 293T cells with the following three different plasmids (Fig. 30.6). First, the packaging construct coding for the structural and enzymatic proteins of the corresponding retrovirus; second, a viral transfer vector encoding a reporter gene like the green fluorescence protein (GFP); and third, an expression vector carrying the two HCV envelope glycoproteins E1 and E2. Upon transfer of the pseudoparticles to a panel of different target cells, a preferential tropism for liver-derived cell lines or primary hepatocytes and a requirement for both E1 and E2 was observed.[86] Furthermore, the infection process was blocked by antisera from HCV-positive patients and by monoclonal antibodies directed against E2. Taken together, these data clearly show that the pseudoparticles carry functional E1 and E2 complexes, thus allowing the analysis of the early steps in the HCV life-cycle.

By using the HCV pseudoparticle system, it was demonstrated that CD81, an E2-binding molecule,[87] is an important component of the receptor complex, but is insufficient to confer entry of the pseudoparticles into the cell.[14,86] Hsu and colleagues also studied the role of two further HCV receptor candidates, namely scavenger receptor class B type 1 (SR-B1) and the low density lipoprotein receptor (LDLR).[14] They found that infection of target cells by HCV-E1/E2 pseudoparticles was not blocked by antibodies directed against SR-B1 or LDLR. However, the inability of LDLR-specific antibodies to inhibit pseudoparticle infection may be attributed to the fact that LDL is not associated with the particles that were generated in 293T cells. Irrespective of that, these pseudoparticles were useful to confirm the existence of HCV neutralizing antibodies. Surprisingly, these antibodies turned out to be broadly cross-neutralizing and to block the infection of pseudoparticles carrying genotype 1a or 1b envelope glycoproteins. Such neutralizing antibodies were only found in chronic but not in resolving infections.[88]

### A novel system for the production of infectious virus in cell culture

As mentioned above, the reason for the lack of virus production might be an interference of adaptive mutations with particle assembly and/or release. Based on this assumption it was interesting to see whether the newly established genotype 2a isolate JFH-1 (see above), which replicates efficiently in the absence of adaptive mutations, will allow virus production. Indeed, very recently, the production of infectious HCV in cell culture was achieved by transfection of Huh-7 cells with the full length 2a JFH-1 genome as well as chimeric genomes that carry the JFH-1 replicase and the core to NS2 region from other genotypes.[89,90] Sucrose density gradient centrifugation of the supernatant from the transfected cells revealed cosedimentation of viral RNA and core protein at a density of 1.17 g/ml. Furthermore, supernatants from such transfected cells contain infectious HCV that enters naive Huh-7 cells in a CD-81 dependent manner. Electron microscopy showed virus particles of 50-65nm in diameter reacting with an anti-E2 antibody. Moreover, inspection of a luciferase reporter gene into the JFH-1 genome allowed the production of virus particles that upon infection of naive target cells express high levels of the reporter gene.[91] This method should allow detailed analyses of the early steps in the HCV life cycle and facilitate the development of antiviral compounds interfering with the infection process.

## Conclusion

Significant progress has been made in the development of cell culture models for HCV. Among the cell-based replication models, the replicon system is most widely used. It is robust and efficient and it allows the study of

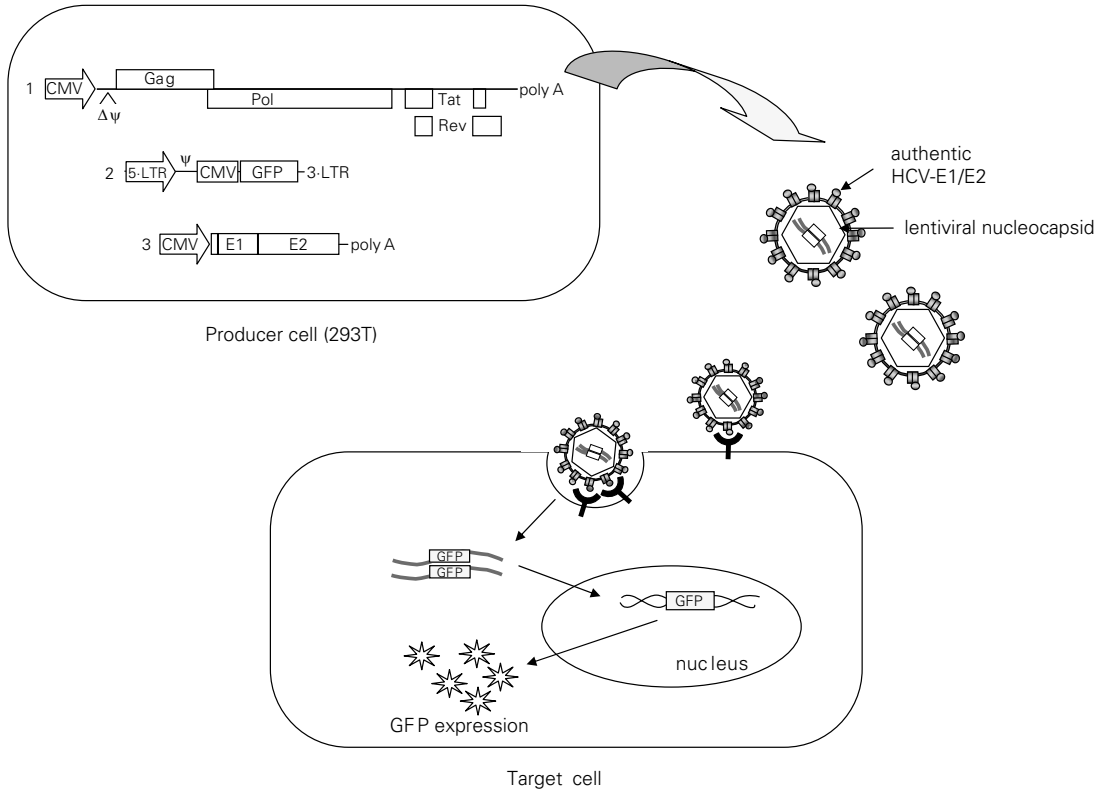


**Figure 30.6** Experimental approach for the production of HCV pseudoparticles. Producer cells (293T) are co-transfected with three different plasmids: (1) the HIV-1-based packaging plasmid composed of the human cytomegalovirus (CMV) promoter, the structural proteins (Gag), the enzymatic proteins (Pol) and the two regulatory proteins Tat and Rev upstream of the polyadenylation signal (poly A). RNA generated from this construct lacks the packaging signal ψ (Δψ) and therefore is not incorporated into the HIV-1 nucleocapsid; (2) the transfer vector composed of the two long-terminal repeats (LTR), the RNA packaging signal ψ and an expression cassette containing the CMV promoter upstream of the GFP marker gene; (3) the plasmid for the expression of the HCV glycoproteins E1 and E2. Upon transfection of 293T cells, the GFP-encoding RNA is packaged into the HIV-1 nucleocapsid, which acquires by an unknown mechanism its viral envelope into which native E1/E2 complexes are incorporated. Supernatant of these cells contains the HCV pseudoparticles that are used for infection of appropriate target cells, e.g. Huh-7 cells. Pseudoparticles enter the cell, the transfer vector is reverse transcribed and integrated into the genome of the target cell. Successful transduction of the reporter gene GFP, which is a marker for successful infection, can then be measured by FACS analysis of the target cells.

different aspects of the HCV life-cycle. In addition, production of infectious HCV particles in cell culture from a cloned genome has been recently achieved allowing studies of the complete viral life-cyle. These achievements open new avenues for the development of novel antiviral therapies and they will hopefully stimulate further research which will help to gain a better understanding of the interplay between HCV and its host.

## Acknowledgements

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# Chapter 31
# Progression of fibrosis

Thierry Poynard, Vlad Ratziu, Yves Benhamou, Dominique Thabut, Joseph Moussalli

## Natural history of hepatitis C fibrosis progression

The major hepatological consequence of hepatitis C virus (HCV) infection is the progression to cirrhosis and its potential complications: haemorrhage, hepatic insufficiency and primary liver cancer.[1]

Current understanding of HCV infection has been advanced by the concept of liver fibrosis progression (Figs 31.1 and 31.2).[2,3] Fibrosis is the deleterious but variable consequence of chronic inflammation. It is characterized by the deposition of extracellular matrix component leading to the distortion of the hepatic architecture with impairment of liver microcirculation and liver cell functions. It is increasingly recognized that HCV can directly exert profibrogenic effects on the liver. Recent and exciting experimental data provide evidence that the core protein of HCV acts on hepatic stellate cells resulting in increased proliferation, release of profibrogenic cytokines and enhancement of type I collagen secretion.[4] Moreover non-structural proteins of HCV contribute to the local inflammatory reaction by triggering the synthesis of stellate cell-derived chemokines and increasing the expression of adhesion molecules involved in the recruitment of inflammatory cells.[4]

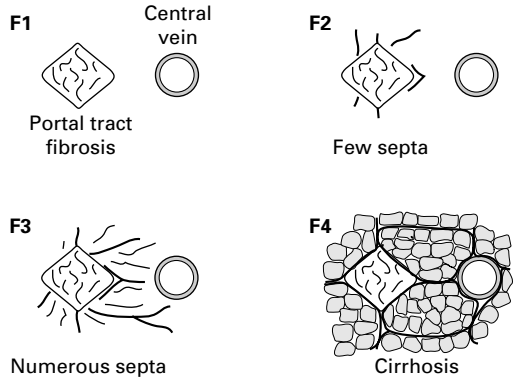


**Figure 31.1** The METAVIR fibrosis staging system. F0 is normal liver (no fibrosis); F1 = portal fibrosis; F2 = few septa; F3 = many septa; F4 = cirrhosis

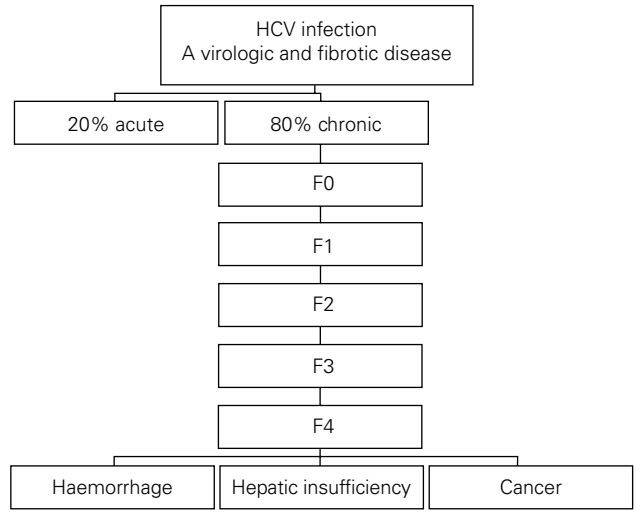


**Figure 31.2** The model of fibrosis progression from infection to complications. Estimated key numbers of HCV natural history from literature and our database are as follows. The median time from infection (F0) to cirrhosis (F4) is 30 years. The mortality at 10 years for cirrhosis is 50%. The transition probability per year from non-complicated cirrhosis to each of the complications is around 3%.

Infection by HCV is usually only lethal when it leads to cirrhosis, the last stage of liver fibrosis. Therefore, an estimate of fibrosis progression represents an important surrogate end-point for evaluation of the vulnerability of an individual patient and for assessment of the impact of treatment on natural history.[2,3]

## Fibrosis stages and necroinflammatory activity grades

Activity and fibrosis are two major histological features of chronic hepatitis C, which are included in different proposed classifications.[5–8] One of the few validated scoring systems is called the METAVIR scoring system.[5,8] This system assesses histologic lesions in chronic hepatitis C using two separate scores, one for necroinflammatory grade (A for Activity) and another for the stage of

fibrosis (F). These scores were defined as follows. Stages of fibrosis (F) (Fig. 31.3): F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with rare septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis. Grade for activity (A): A0 = no histologic activity, A1 = minimal activity, A2 = moderate activity, A3 = severe activity. The degree of activity was assessed by integration of the severity of the intensity of both piecemeal (periportal) necrosis and lobular necrosis as described in a simple algorithm.[5] The intra- and inter-observer variations of this METAVIR scoring system are lower than those of the widely used Knodell scoring system.[7] For METAVIR fibrosis stages there is an almost perfect concordance (kappa = 0.80) among pathologists. The Knodell scoring system has a non-linear scale. There is no stage 2 for fibrosis (range 0–4) and the activity grade ranges from 0 to 18 with the sum of periportal necrosis, intralobular and portal inflammation grades. The modified Histological Activity Index is more detailed, with four different features and continuous grades, and the modified fibrosis staging includes six stages.[6]

Activity grade, which represents the necrosis feature, is not a good predictor of fibrosis progression.[2,9] In fact, fibrosis alone is the best marker of ongoing fibrogenesis.[2,3,9,10] Fibrosis stage and inflammatory grade are correlated, but for one-third of patients, there is discordance. Clinicians should not take a 'significant activity' as a surrogate marker of 'a severe disease'. The clinical hallmarks of major necrosis and inflammation, i.e. severe acute hepatitis and fulminant hepatitis, are finally very rare in comparison with hepatitis B. Even in immunologically compromised patients there are very few acute flare-ups in patients with chronic hepatitis C.

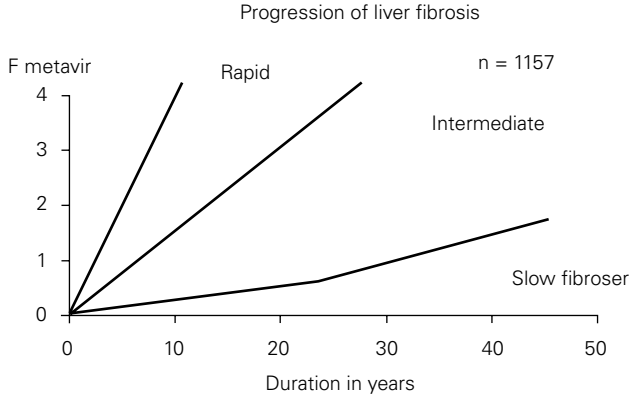


**Figure 31.3** Progression of liver fibrosis in patients with chronic hepatitis C. Using the median fibrosis progression rate, in untreated patients, the median expected time to cirrhosis is 30 years (intermediate fibroser); 33% of patients have an expected median time to cirrhosis of <20 years (rapid fibroser); 31% will progress to cirrhosis in >50 years, if ever (slow fibroser). Adapted with permission from Poynard *et al*.[2]

## The dynamic view of fibrosis progression

Fibrosis stage summarizes the vulnerability of a patient and is predictive of the progression to cirrhosis (Fig. 31.3).[2,3,10,11] There is a strong correlation for fibrosis stages, almost linear, with age at biopsy and duration of infection. This correlation was not observed between activity grades.[2,3,10]

Because of the informative value of fibrosis stage it is of interest for the clinician to assess the speed of the fibrosis progression. The distribution of fibrosis progression rates suggests the presence of at least three populations: a population of 'rapid fibrosers', a population of 'intermediate fibrosers' and a population of 'slow fibrosers' (Fig. 31.2). Therefore, the expression of a mean (or median) fibrosis progression rate per year (stage at the first biopsy/duration of infection) and of a mean expected time to cirrhosis does not signify that the progression to cirrhosis is universal and inevitable. Using the median fibrosis progression rate, in untreated patients, the median expected time to cirrhosis is 30 years; 33% of patients have an expected median time to cirrhosis of <20 years and 31% will progress to cirrhosis in >50 years, if ever (Fig. 31.1).[2,3,10,11]

Limitations of any estimate of fibrosis include (1) the difficulty in obtaining paired liver biopsies, (2) the necessity for large numbers of patients to achieve statistical power and (3) the sample variability in fibrosis distribution. Because the time elapsed between biopsies is relatively short (usually between 12 and 24 months), the number of events (transition from one stage to another) is rare. Therefore, the comparisons between fibrosis progression rates require a large sample size to observe significant differences. The slope of progression is difficult to assess because there is no large database with several biopsies. Therefore, the real slope is currently unknown and even if there is a linear relationship between stages and age at biopsy or duration of infection, other models are possible.[12–14] On a larger database we confirmed that the fibrosis progression was mainly dependent of the age and the duration of infection with four different periods with very slow, slow, intermediate and rapid slopes[10,11] (Fig. 31.2).

Furthermore, liver biopsy has its own limits in assessing liver fibrosis. Although it was the gold standard for scoring fibrosis, its value is limited by very high sample variability.[15–17] Future studies using validated non-invasive biochemical markers, such as FibroTest, should improve modelling of fibrosis progression.[18–21]

## Factors associated with fibrosis progression

Factors associated and not associated with fibrosis are summarized in Table 31.1. Several factors have been shown to be associated with fibrosis progression rate:

**Table 31.1** Factors that are or are not associated with fibrosis progression

| Associated in univariate and multivariate analysis | Not sure | Not associated |
|---|---|---|
| Fibrosis stage | Inflammation | Last serum viral load |
| Age at infection | Haemochromatosis heterozygote | Genotype other than genotype 3 |
| Duration of infection | Cigarette consumption | Mode of infection |
| Age at biopsy | Moderate alcohol consumption | Liver viral load |
| Consumption of alcohol >50 g per day | Genotype 3 | |
| HIV co-infection | Schistosomiasis | |
| CD4 count <200/mL | | |
| Male gender | | |
| Necrosis | | |
| Body mass index and/or diabetes and/or steatosis | | |

duration of infection, age, male gender, heavy consumption of alcohol, human immunodeficiency virus (HIV) co-infection, low CD4 count and necrosis grade.[2,2,10,11,22–24] The progression from infection to cirrhosis depends strongly on age as expressed by duration of infection, age at infection or age at last biopsy.[10,11] Metabolic conditions such as overweight, steatosis and diabetes are emerging as independent co-factors of fibrogenesis.[25,26]

### *Age*

The role of ageing in fibrosis progression could be related to higher vulnerability to environmental factors, especially oxidative stress, to reduction in blood flow, in mitochondria capacity, or in immune capacities.[27]

The effect of age on fibrosis progression is so important that modelling the hepatitis C epidemic without taking into account the age is not possible (Table 31.2). The estimated probability of progression per year for men aged between 61 and 70 years was 300 times greater than that for men aged between 21 and 40 years (Fig. 31.4).[10,11] Age of the transplanted liver has also been associated with higher fibrosis progression rates.[28]

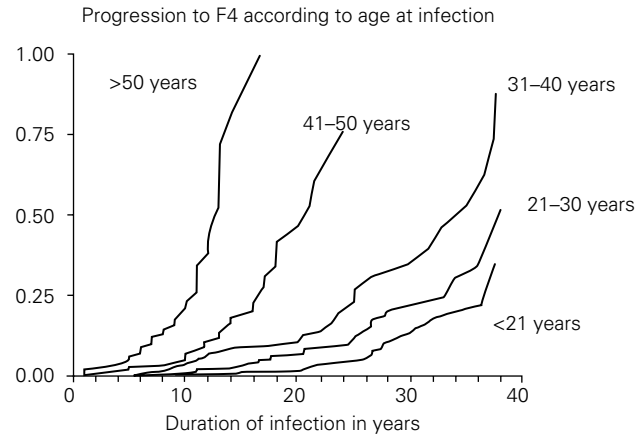


**Figure 31.4** Probability of fibrosis progression to cirrhosis (F4) according to age at infection. Modelling in 2313 patients with known duration of infection. Adapted with permission from Poynard *et al*.[10]

### *Male gender*

Male gender is associated with 10 times more rapid progression to cirrhosis than female, whatever the age.[11] Oestrogen modulates fibrogenesis in experimental in-

**Table 31.2** Multivariate analysis of risk factors by proportional hazards regression model for each fibrosis stage 20 years after HCV infection in 2313 patients

| | Stage F1 | | Stage F2 | | Stage F3 | | Stage F4 | |
|---|---|---|---|---|---|---|---|---|
| Risk factor | Relative hazard | *p* value | Relative hazard | *p* value | Relative hazard | *p* value | Relative hazard | *p* value |
| Infection after 30 years | 4.4 | <0.001 | 4.8 | <0.001 | 11.5 | <0.001 | 27.1 | <0.001 |
| Infection for 21–30 years | 2.3 | <0.001 | 1.8 | <0.001 | 2.5 | <0.001 | 5.3 | <0.001 |
| Alcohol >50 g | 1.3 | 0.20 | 3.0 | <0.001 | 2.3 | 0.008 | 4.5 | 0.001 |
| Male gender | 1.0 | 0.76 | 1.3 | 0.03 | 1.9 | <0.001 | 2.0 | 0.003 |
| Intravenous drug use | 1.6 | <0.001 | 1.2 | 0.22 | 1.4 | 0.11 | 1.2 | 0.55 |
| Activity A2,A3 | 0.8 | 0.009 | 1.2 | 0.21 | 2.0 | <0.001 | 1.4 | 0.16 |

Adapted with permission from Deuffic-Burban *et al.* [11]

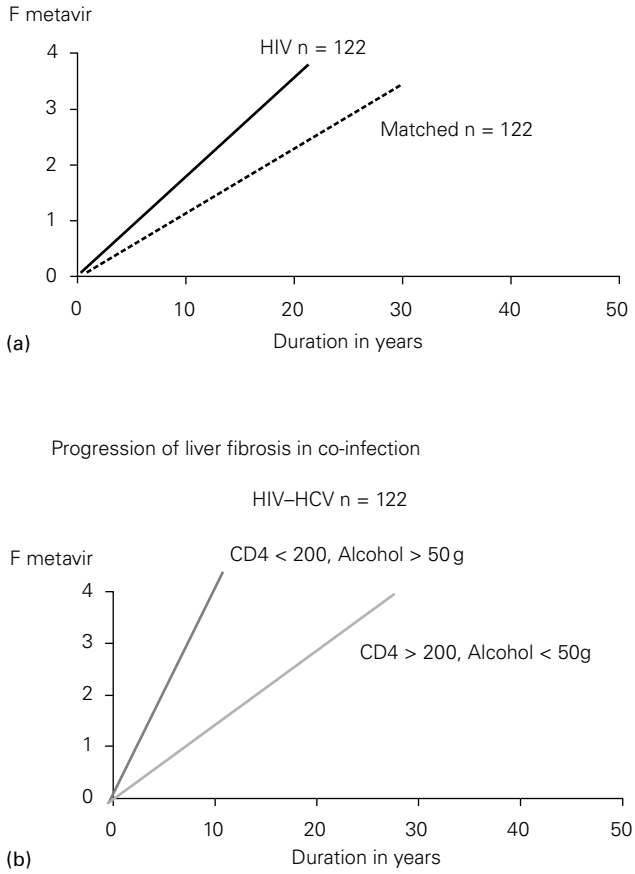


**Figure 31.5** (a) Progression of liver fibrosis among patients co-infected by HCV and HIV. There is a significant increase of fibrosis progression rate among HIV in comparison with matched controls infected by HCV alone. (b) Progression of liver fibrosis among patients co-infected by HCV and HIV. There is a very significant increase of fibrosis progression rate among patients with CD4 <200 per $mm^3$ and drinking >50 grams of alcohol per day. Adapted with permission from Benhamou *et al*.[33]

jury. Oestrogen blocks proliferation and fibrogenesis by stellate cells in primary culture. Oestrogen could be modifying the expression of transforming growth factor and other soluble mediators.[23] Recently, we observed that when the metabolic factors were taken into account the association between male gender and fibrosis was reduced.[29]

### *Alcohol*

The role of alcohol consumption has been established for daily doses >40 or 50 grams per day.[2,10,22,24] For lower doses there are discordant results, with even preliminary studies suggesting a protective effect of very small doses. Alcohol consumption is difficult to quantify and conclusions must be prudent. However, it seems from these studies that the influence of alcohol is independent of other factors, weaker than that of age, and is exerted only at toxic levels of intake.

### *HIV co-infection*

Several studies have demonstrated that patients co-infected with HCV and HIV have one of the most rapid fibrosis progressions in comparison with mono-infected patients or other liver diseases even after taking into account age, sex and alcohol consumption (Fig. 31.5a).[10,30–33] An HIV-infected patient with <200 CD4 cells/μL and drinking >50 grams of alcohol daily has a median expected time to cirrhosis of 16 years versus 36 years for an HIV-infected patient with >200 CD4 cells/μL, drinking 50 grams or less of alcohol daily (Fig. 31.5b).[22]

### *HCV viral genotype*

Viral factors such as genotype, viral load at the time of the biopsy and quasi-species are not associated with fibrosis.[2,10,31,34,35] Only genotype 3 is suspected, because of the steatosis associated with this genotype.[10,36,37]

### *Risk of fibrosis in patients with normal transaminases*

Patients with repeated normal serum transaminase activity have lower fibrosis progression rate than matched control patients with elevated transaminases (Fig. 31.6).[10,38,39] However, 15–19% of these patients do have moderate or high fibrosis progression rates. Therefore, we recommend assessment of the fibrosis stage by performing liver biopsy or biochemical markers in these polymerase chain reaction (PCR)-positive patients. If the patient has septal fibrosis or portal fibrosis with a high fibrosis rate, a treatment should be considered. FibroTest

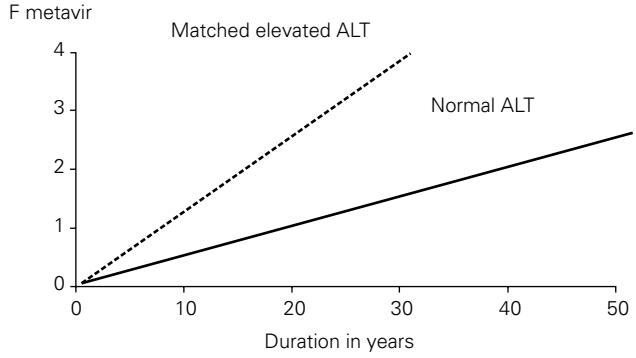


**Figure 31.6** Progression of liver fibrosis in patients who are HCV PCR-positive with repeated normal alanine aminotransferase (ALT). There was a significant reduction of fibrosis progression rate in comparison with matched controls with abnormal ALT. Adapted with permission from Mathurin *et al*.[39]

has the same predictive values in patients with normal or elevated transaminases.[19] In patients aged 65 years or older, there are even more patients with extensive fibrosis and normal transaminases and this population is particularly at risk of high fibrosis progression rates.[40]

## Metabolic factors

### *Impact of steatosis on the pathogenesis of chronic hepatitis C*

With a few exceptions,[41,42] steatosis is associated with a more severe necroinflammatory activity[43,44] or fibrosis.[1,43–48] Steatosis is associated with more advanced fibrosis, even after adjustment for age.[42] In a small number of patients with a known duration of infection, the fibrosis progression rate appeared to be higher when marked steatosis was present than when steatosis was mild or absent.[42] In addition to these transversal studies, some studies with follow-up biopsies in untreated patients are available. A faster progression of fibrosis in patients with steatosis on the first biopsy has been observed, but the small sample size precludes an analysis according to the genotype.[47] It is possible that these relationships can be specific for unknown HCV characteristics, as differences were observed for genotype 3.[47] Other studies suggested that the increase in steatosis rather than its amount could be indicative of fibrosis progression,[49] although a convincing demonstration of this challenging hypothesis is lacking.[50]

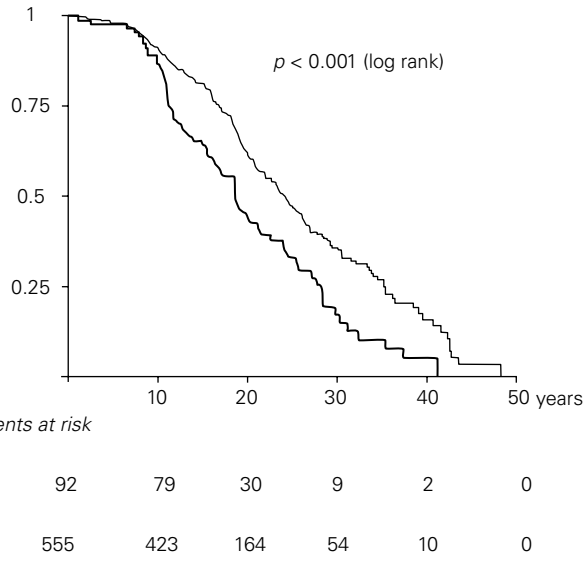


**Figure 31.7** Fibrosis progression according to serum glucose levels. The time-dependent variable was the duration of infection in years. The thick and thin lines represent patients with high or normal serum glucose, respectively. The percentage of patients free of significant fibrosis (F2, F3, F4) is plotted against the duration of infection. Adapted with permission from Ratziu *et al*.[29]

No study documented an association between steatosis and fibrosis independent of other confounding factors such as body mass index (BMI), blood serum glucose levels or serum triglycerides. In one study, the strong association between steatosis and fibrosis disappeared after adjustment for serum glucose and BMI, thus casting doubt on the true relevance of steatosis *per se* to fibrogenesis.[29]

In a single study, steatosis was associated with a higher cumulative incidence of hepatocellular carcinoma, independent of age, cirrhosis and treatment by interferon (IFN).[51]

### *Impact of diabetes on the pathogenesis of chronic hepatitis C*

Although many studies have documented the epidemiological association between hepatitis C and type II diabetes, only a few have focused on its consequences for liver injury.[52] In a small series, the necroinflammatory activity was higher in diabetics than in non-diabetics.[52] Fibrosis stages are usually higher in diabetics, although results are discordant when taking into account other well identified risk factors of liver fibrosis.[46,52–55] In the largest study to date, performed on 710 patients with a known duration of infection, a high serum glucose (as well as overt, drug-treated diabetes) was associated with more advanced liver fibrosis as well as a higher fibrosis progression rate, independent of other risk factors for fibrosis such as age at infection, duration of infection, male sex and alcohol consumption (Fig. 31.7).[29] The fibrogenic impact of a high serum glucose was higher than that associated with overweight, suggesting that measurement of blood glucose could provide more accurate information on the fibrogenic potential of underlying insulin resistance than the mere measurement of the BMI.[29]

A common caveat of these transversal observational studies is that cirrhosis-induced alterations of glucose homeostasis may confound the relation between a high serum glucose/diabetes and liver fibrosis. While this cannot be ruled out, some studies still documented a significant association after exclusion of cirrhotic patients.[29] High serum glucose is associated with intermediate and advanced stages of fibrosis but not with early stages, thus suggesting a more important role in the perpetuation and progression of fibrogenesis rather than its initiation.[29] This should be confirmed in further studies.

### *Impact of obesity on the pathogenesis of chronic hepatitis C*

Overall, obesity seems to worsen liver histology in

chronic hepatitis C. One study has documented a highly significant association between obesity and steatosis as well as between steatosis and fibrosis, although no direct association was found between obesity and fibrosis.[56] Obese patients have more advanced fibrosis stages than lean patients, but this does not appear to be independent of other confounding factors such as a high serum glucose/diabetes.[29,53,54] These discrepancies could result from the fact that none of these studies make the distinction between visceral and peripheral obesity: only the former entity is correlated to insulin resistance and its complications, hepatic steatosis in particular.[57,58]

As a consequence of the complex interactions between insulin resistance and liver injury, it is difficult to analyze the specific contribution of obesity. Therefore, several authors have attempted to identify, on histological grounds alone, the presence of lesions compatible with non-alcoholic steatohepatitis in obese patients with chronic hepatitis C.[46,59,60] Their assumption is that these two fibrogenic conditions would increase liver fibrosis when occurring together, which in turn would demonstrate the contribution of obesity in fibrosis in HCV-infected patients. Unfortunately, the two conditions share many common histological lesions, which make it difficult to assert the presence of non-alcoholic steatohepatitis (NASH) in chronic hepatitis C. The attributable risk of NASH in liver fibrosis of obese patients with hepatitis C may not be determined until more specific markers of NASH than histology or exposure to risk factors such as obesity or diabetes are identified.

Some preliminary data on the possible contribution of obesity to liver injury in chronic hepatitis C come from the demonstration that after a 3-month period of controlled weight loss through diet and exercise, nine of ten patients with hepatitis C had reduced steatosis and five of ten had less fibrosis.[61] Weight loss was associated with improved insulin sensitivity. Although sampling variability of liver biopsy is a significant concern with such a small sample size, the demonstration that cellular markers of stellate cell activation were also turned off in patients with reduced weight and less fibrosis further strengthens the hypothesis of a deleterious impact of obesity in chronic hepatitis C. Similarly, it has been observed that surgical treatment of obesity decreased fibrosis.[62]

### *Interaction between genotype and metabolic factors*

It was observed that fibrosis was associated with steatosis only in genotype 3-infected individuals, and with past alcohol abuse and (marginally) diabetes only in patients infected with genotypes other than genotype 3.[37] Another study confirmed that HCV may induce insulin resistance and accelerate fibrosis progression, and this effect seems to be genotype 3-specific.[63]

### *Other factors*

There are very few studies for the following factors and more studies with a high sample size are needed: fluctuations of HCV RNA, intrahepatic cytokine profiles, HLA class genotype, C282Y heterozygote haemochromatosis gene mutation and cigarette consumption.

## Impact of treatment: regression of liver fibrosis

Many studies have now demonstrated that treatment of hepatitis C with IFN alone or in combination with ribavirin can stop the progression of liver fibrosis or even induce a significant regression of fibrosis.[3,11,21,33,64–73]

We pooled individual data from 3010 naïve patients with pretreatment and post-treatment biopsies from four randomized trials.[69] Ten different regimens combining standard IFN, pegylated IFN (PEG-IFN) and ribavirin were compared. The impact of each regimen was estimated by the percentage of patients with at least one grade improvement in the necrosis and inflammation (METAVIR score), the percentage of patients with at least one stage worsening in fibrosis METAVIR score and by the fibrosis progression rate per year. Necrosis and inflammation improvement ranged from 39% (IFN 24 weeks) to 73% (PEG-IFN 1.5 μg/kg + ribavirin >10.6 mg/kg/day). Fibrosis worsening ranged from 23% (IFN 24 weeks) to 8% (PEG-IFN 1.5 μg/kg + ribavirin >10.6 mg/kg/day). All regimens significantly reduced the fibrosis progression rates in comparison with rates before treatment. This effect can be observed even in patients without sustained virological response.

The reversal of cirrhosis was observed in 75 (49%) of 153 patients with baseline cirrhosis.[69] Six factors were independently and significantly associated with the absence of significant fibrosis after treatment: baseline fibrosis stage (odds ratio, OR = 0.12), sustained viral response (OR = 0.36), age <40 years (OR = 0.51), BMI <27 kg/m$^2$ (OR = 0.65), no or minimal baseline activity (OR = 0.70) and viral load <3.5 million copies per mL (OR = 0.79). Other studies have also documented reversal of cirrhosis in patients treated with PEG-IFN.[74]

## Conclusion

Tremendous progress has been achieved in the comprehension of fibrosis progression in patients with chronic hepatitis C. The roles of ageing and metabolic factors are particularly important for therapeutic decisions. The treatment of hepatitis C with IFN and ribavirin in combination is very effective for blocking fibrosis progression and can even induce reversal of cirrhosis, a concept that would have been unbelievable 10 years ago.

Unfortunately, even in developed countries, death due to hepatitis C is increasing because of inadequate detection and treatment.

## Conflict of interest

T.P. is a consultant and has a capital interest in Biopredictive, the company marketing FibroTest-Actitest.

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# Chapter 32
# The natural history of hepatitis C and hepatocellular carcinoma

Edward Tabor

The disease caused by hepatitis C virus (HCV) infection is a continuum that extends over a period of decades. It ranges from acute infection early in the course to chronic infection in the majority of patients, and in some patients leads to hepatocellular carcinoma (HCC) near the end. It is estimated that 3.9 million Americans have antibody to HCV (anti-HCV), indicating current or prior infection with HCV, and 2.7 million of them have detectable HCV RNA in their serum, indicating a current infection (most of which are chronic infections). Most of these individuals are unaware that they have chronic HCV infections. Some will come to medical attention during the next two decades when HCV infections in the peak prevalence age group begin to cause life-altering symptoms, and a small but definable percentage will eventually develop HCV-associated HCC.

HCV infection is an important factor in the aetiology of HCC throughout the world. In Japan, the incidence of HCC, mostly associated with HCV infection, ranges from 11 to 32/100 000 among males (and from four to 8/100 000 in females).[1] In contrast, the incidence of HCC among males in the general population of the United States and Canada is 2/100 000 and 3/100 000, respectively (1/100 000 for females in both countries),[1] and only about 14–32% are associated with HCV infection.[2,3]

In all of these areas, cases of HCC associated with hepatitis B virus (HBV) infection also continue to occur. In Japan, HBV-associated HCC remains at a background level that is relatively constant from year to year, 24–27% of HCC patients.[4–6] In Africa and in some parts of Asia, such as China, HBV-associated HCC remains the predominant form of HCC.[1] In southern China, 70% of HCCs are associated with chronic HBV infections and only 5% with HCV infections.[7]

## The natural history of HCV infection

### Acute HCV infection

Acute HCV infection is characterized by the appearance of HCV RNA in the blood beginning 1–3 weeks after exposure. Symptoms can include jaundice, malaise, weakness and anorexia. However, symptoms are not observed in many individuals, even if liver enzymes in serum (e.g. alanine aminotransferase, ALT) are elevated. In most patients, symptoms resolve, but only about 23% truly recover, as defined by HCV RNA becoming undetectable in their serum.[8]

### Chronic HCV infection

HCV infections evolve into chronic hepatitis in 60–85% of acutely infected patients. The definition of 'chronic hepatitis C' is generally accepted as the detection of HCV RNA in serum for a period of at least 6 months. Most chronic HCV infections are life-long in duration without effective therapy. Spontaneous clearance of HCV occurs in patients with chronic HCV infections at a rate of 0.5% per year.[9]

The existence of chronic HCV infection may first be noted when elevated ALT levels are found on routine screening. As a result, many chronic HCV infections are not recognized until very late in the course. Many patients with chronic HCV infection will develop fibrosis of the liver and some will progress to cirrhosis. The task of predicting who among them will develop cirrhosis is daunting and new approaches are still being developed.

About 70% of individuals with chronic HCV infection are asymptomatic. ALT levels can also be normal

in some asymptomatic patients. Among all individuals with chronic HCV infection, 30% have persistently normal ALT levels, and another 40% have minimal or intermittent minimal elevations of ALT levels. Most of these individuals with normal or minimally elevated ALT levels are believed not to be at risk for progression to cirrhosis or HCC. However, careful observation is advisable because of the theoretical possibility that some could progress to cirrhosis or HCC.[10] Most patients with HCV RNA in their serum for a period of years and normal ALT levels have been shown to have persistently normal liver histology in liver biopsies obtained 2–5 years later.[11]

Life-long immunity to HCV appears to develop in almost all patients in whom spontaneous resolution occurs (i.e. loss of detectable HCV RNA). This combination of resolution and lasting immunity apparently requires the presence of an effective CD4 T-helper cell response. Conversely, the absence of an effective CD4 response has been shown to be associated with persistent viraemia and the emergence of escape mutants (based on studies in chimpanzees with chronic HCV infections in which the CD4 response was experimentally blocked).[12] If the CD4 response is adequate but is not accompanied by adequate interferon (IFN)-γ production, chronicity of HCV infection and development of HCC may be more likely to occur.[13]

## Development of cirrhosis

From 4% to 24% of persons with chronic HCV infection develop cirrhosis. The rate depends on the population, with lower rates (4–7%) being found in community-based and blood donor cohorts, and higher rates (>20%) being found in populations of HCV-infected patients followed in liver clinics.[14,15] Risk factors for development of cirrhosis include acquisition of HCV infection after age 30 years[15] and heavy ingestion of alcohol.[14]

As many as 70–80% of HCC patients have cirrhosis in the adjacent non-tumorous liver. The high rate of co-existing cirrhosis in HCC patients and the emergence of HCC in prospectively followed cirrhosis patients have led to the assumption that pre-existing cirrhosis is an important prerequisite for hepatocarcinogenesis, although some HCCs do arise in the absence of cirrhosis. It is generally believed that the majority of HCCs develop in a progression from acute hepatitis through various stages of chronic hepatitis, to cirrhosis, to HCC. In HCV-associated cirrhosis, the cumulative development of HCC increases steadily with time, reaching as high as 75% at 15 years in one study.[16] In contrast, in HBV-associated cirrhosis,[2] the cumulative prevalence increases with time but levels off (in one study, at 27% of patients after 8 years of follow-up[16]). Higher rates of HCC have been reported in cirrhosis inpatient studies.[17] There is an even greater risk of a patient with cirrhosis developing HCV-associated[18] but not HBV-associated[16] HCC if alcoholism is present in addition.

## Development of HCC

The incidence of HCC developing in patients with HCV-associated cirrhosis has been reported to be 2% per year (5-year cumulative incidence of 10%).[19] Others have reported an incidence of 5% per year, although this is generally considered to be unusual (cumulative incidence of 75% after 15 years).[16] In a study of haemophiliac patients with HCV cirrhosis, the incidence of HCC was 2% per year (12% during a 6-year study; most did not have co-infection with human immunodeficiency virus).[20] HCC is most likely to develop in those haemophiliacs who became infected with HCV as adults, particularly after age 40 years.[21,22]

It has been estimated that the mean interval from infection to diagnosis of cirrhosis is 20–24 years, and from infection to diagnosis of HCC is 27–29 years, based on a variety of studies.[8] Heavy ingestion of alcohol accompanying chronic HCV infection with cirrhosis results in a greater incidence of HCC, compared with cirrhosis patients with HCV and no alcohol use, or compared with those with heavy alcohol use alone (with no serological evidence of HCV).[18] (The same has not been found with regard to HBV-associated cirrhosis and alcohol.[16]) This could be due to additive damage to the liver from the two toxic agents (HCV and alcohol), or it could be due to a potentiation of HCV expression by alcohol by means of activating the transcription factor NF-κB, which has been demonstrated to occur in *in vitro* studies.[23]

Notwithstanding that HCV infection is believed to have a substantial negative effect on the quality of life and life expectancy of the patient, it is important to mention two reports that did not find increased mortality due to HCV, even though they may not be generalizable to other populations. In one study, a 67% all-cause mortality was observed in patients with HCV-associated ALT elevations acquired by transfusions after approximately 25 years. However, this mortality rate was no greater than that (65%) in a population of transfused controls who had not developed post-transfusion elevation of ALT (but who also were anti-HCV positive).[8] In this study, there was also a relatively low (3.3%) liver-related mortality in cases, compared with 0.3% in controls.[8] In another study, a 25-year follow-up of community-acquired HCV infection in Australia, excess mortality was observed in HCV-infected persons but was not due to liver disease.[24] It is not clear why these observations are so much at variance with the findings in other studies.

The possibility that mutations in HCV could play a role in the course of chronic HCV infection leading to HCC has not been fully investigated. No unique muta-

tions have been identified in HCV obtained from HCC patients.[25] An association between a greater number of HCV quasi-species in patients and the development of HCC has been suggested.[26] This could be consistent with a role for HCV mutations in carcinogenesis, but there are no data to support such a role at present.

## The rising incidence of HCC

During the past half-century, the incidence of HCC has doubled approximately every 20 years in Japan;[27–30] an increase that has been shown to be due primarily to cases associated with HCV infection.[27,30] Today, the prevalence of of HCV infection (anti-HCV without concomitant HBsAg) among HCCs in Japan is 54–70%.[4–6]

Essentially, the rise in HCC incidence is a direct reflection of the rise in HCV incidence in Japan. The high incidence of HCV in Japan is felt to be a post-World War II phenomenon, due to the reuse of medical syringes with inadequate sterilization, in an era before the availability of disposable plastic syringes. However, this widely held explanation is not based on systematic scientific or historical analyses, and the possibility that other societal changes in that era may be responsible for the high incidence of HCV should not be ruled out. Nevertheless, studies conducted among persons of Japanese ancestry living in Hawaii support the concept that the HCV incidence in Japan is at least a 20th Century phenomenon.[31]

It is perhaps consistent with this theory that areas in Japan with high endemicity of HCV appear to be very focal. Although the prevalence of HCV infection in the general population in Japan is high compared with the United States (about 1–2% of potential blood donors in large cities in Japan),[29,32,33] the prevalence of HCV is significantly higher in some towns and villages in Japan. Three such pockets have been reported: 'Village A' in Miyazaki prefecture, in which 23% of the population have anti-HCV,[34,35] 'Town H' in Fukuoka prefecture, in which 23% of the population have anti-HCV,[36] and 'K Town' in Saga prefecture, in which 22% of the population have anti-HCV.[37]

Preliminary evidence suggests that the incidence of HCC also may be increasing in the United States and other Western countries. Although none of those studies has so far included virological test results that could be used to determine whether HCV is an aetiological factor (most were retrospective analyses of large databases), many authorities have assumed that the increase in HCC incidence in Western countries also might be associated with HCV infection because of the epidemic increases in HCV incidence in recent years in the countries involved. An analysis of the SEER database revealed that the incidence of HCC in the United States increased from 1.4/100 000 in the period 1976–1980 to 2.4/100 000 in the period 1991–1995, a 41% increase.[38] Others reported a 140% increase in HCC incidence in the 47 years between 1950–1996 in the USA, also based on the SEER database.[39] A doubling of the incidence of HCC in the US state of Colorado between 1988 and 1997 has been reported.[40] Similarly, a nearly fourfold increase in HCC incidence (1.9 to 7.6/100 000) was reported in Göteborg, Sweden between 1958 and 1979.[41] A nearly fourfold increase in HCC incidence in men was reported in France between 1979 and 1994 (an increase from 3.2 to 11.1/100 000), along with a doubling in women (an increase from 1.2 to 2.5/100 000).[42]

Infection with both HBV and HCV (presence of both HBsAg and anti-HCV) has been reported in 3–13% of HCC patients.[2–7] In addition to these patients, a few additional patients with HCV infection may also have so-called 'silent HBV' infections, in which HBV DNA can be detected in serum and/or liver despite the absence of detectable serological markers of HBV infection.[43–46] These should also be considered to be co-infections with HBV and HCV, in considering the aetiology of their HCCs.

Nevertheless, it is not clear whether simultaneous infection with HBV and HCV truly increases the risk of developing HCC. The findings of both viruses in HCC patients could reflect the existence of common risk factors for exposure to both viruses, for instance because both viruses are blood-borne. Recent work has not answered the question. Some studies have supported an additive role and others have not. For instance, in one retrospective cohort study of 459 HCV-infected patients for an average of 6.6 ± 3.3 years, 23% of patients with anti-HBc developed HCC compared with only 9% of those without anti-HBc,[47] although no testing of HBsAg was reported. Another study, however, reported that the prevalence of anti-HBc in HCV-infected HCC patients was similar to that among HCV-infected candidate blood donors (i.e. without HCC) in Japan (54–56%).[48] In addition, simultaneous infections with HBV and HCV appear not to affect the severity or aggressiveness of HCC.[49]

## The effect of therapy for HCV: 'chemoprevention' for HCC

Treatment of HCV infection, particularly early in the course of infection, theoretically could prevent the development of HCC as an outcome of HCV infection. At present, the only data on prevention of HCC by treating the HCV infection involve IFN-α. Even though the combination of IFN-α and ribavirin has been shown to be far more effective in treating chronic HCV infection than IFN-α alone, the combination has not been evaluated for its impact on preventing HCC.

A reduction in HCC incidence in HCV-infected persons treated with IFN-α has been reported by many investigators[50–53] (reviewed by Tabor[54]). Nevertheless, most of the published studies have had defects in study design such as pooling of patients who received a variety of IFN-α treatment regimens, short periods of follow-up (relative to the incubation time for HCC), large numbers of drop-outs, and retrospective study design with historical controls. As a result, one must be cautious in evaluating chemoprevention of HCC with IFN-α until further studies are available. Preferably, future studies should evaluate the combination of IFN-α and ribavirin.

In many of these studies, it has appeared that more HCCs developed among non-responders to HCV treatment with IFN-α (i.e. patients in whom ALT or HCV RNA did not decrease or whose ALT or HCV RNA levels fluctuated after IFN treatment) than among responders. For example, in one study, HCC developed in 10/30 (33%) non-responders to IFN-α compared with 2/15 (13%) with a complete or partial response.[55–57] (In addition, the reduction in risk of HCC was greatest in patients without ALT elevations.[57]) In another similar study, HCC developed in 16% of 38 non-responders to IFN-α compared with none of 25 sustained responders, among patients with chronic active hepatitis due to HCV, all initially without cirrhosis (five of the six who developed HCC also eventually developed cirrhosis).[58] In another study, IFN-α treatment of HCV-infected patients with cirrhosis was reported to result in a sixfold reduction in developing HCC.[59]

## Projections for the future

We can expect a substantial increase in the incidence of HCV-associated HCC throughout the world, and probably also in the United States. The high prevalence of chronic HCV infections in many countries, combined with aging of those cohorts, is expected to lead to a greater prevalence of HCC. At present, the prevalence of HCC is directly proportional to the incidence of HCC, as the vast majority of HCC cases are fatal within 6 months of diagnosis.

It has been projected that the prevalence of HCV infections in the USA will peak (and begin to decline) by 2040.[60] By 2020, the prevalence of cirrhosis among HCV-infected persons will increase to a level of 32%. By 2020, the prevalence of HCC will nearly double (nearly 81% over current levels).[60] This consists of an increase in HCV-associated HCC from 7271 cases in 2000 to 13 183 in 2020. This model takes into account the aging of the cohort currently infected with HCV and the increased efficacy of antiviral therapy for HCV infection.

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# Chapter 33
# Treatment of chronic hepatitis C

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## Introduction

Acute infection with hepatitis C virus (HCV) is followed by chronic infection in 50–80% of those infected.[1,2] Individuals with chronic hepatitis C without treatment rarely spontaneously clear virus unless there is a change in their immune status.[3] Chronic infection is associated with multiple morbidities, not all due to liver disease. Data from post-transfusion studies indicate that the mortality from hepatitis C appears to be no more than 10–15%.[4]

It has been estimated that 2.2% of the world's population, i.e. 130 million individuals, are infected with hepatitis C. The majority have been infected via parenteral exposure with contaminated injections, either related to injection drug use (illegal) or contaminated injections or transfusion with blood products received as part of an individual's health care (iatrogenic). The majority of infected individuals are hard to access, thus, were primary prevention possible, it would be difficult to achieve in these particularly hard to reach populations. Secondary prevention, i.e. preventing an acute infection from becoming chronic, is effective but again is hard to achieve unless the individual is being closely monitored and the infection resulted from occupational exposure.

The current standard of care for hepatitis C is pegylated interferon-alpha (PEG-IFN-α) (given once weekly) in combination with oral ribavirin (given daily).[5] When interpreting the results of phase III studies, it must be realized that the participants included were a select patient population without significant co-morbidities, who were agreeable to regular follow-up in tertiary referral centres, and for the most part had only mild chronic hepatitis, i.e. probably not representative of the population at large chronically infected with hepatitis C.

## Treatment of acute hepatitis C infection

Because the majority of individuals infected acutely with hepatitis C are either asymptomatic or, if symptomatic, have other more serious life issues to contend with, it is rare outside the context of occupational exposure or close monitoring of injection drug users (IDUs) for an acute infection with hepatitis C to be identified. Recent data indicate that these two particular populations who are repeatedly exposed to small doses of hepatitis C may have evidence of cell-mediated immunity in the absence of viraemia and/or antibody (about a third), indicating that far more people than have been realized in the past have in fact been infected with hepatitis C and have spontaneously cleared the virus.[6–8] However, on re-exposure they may become transiently reinfected or chronically infected.[9]

Those who have been identified as being acutely infected need to be monitored closely for the first 12 weeks to establish whether or not they will undergo spontaneous viral clearance. Serum HCV RNA (qualitative, i.e. very sensitive) needs to be measured as close to baseline as possible, and this test should be repeated at least before 12 weeks have passed.[10] Rapid viral decline in titre after the initial identification of viraemia may predict those who will spontaneously clear virus, although this is not 100% reliable.[11] In those who remain viraemic at 12 weeks after initial seroconversion, antiviral therapy is recommended if there are no absolute contraindications to its use. The largest uncontrolled study which employed induction dosing with standard IFN-α-2b indicated that 95% of treated patients achieved a sustained virological response with only 6 months of therapy.[12] It is unlikely that the newer forms of IFN, namely PEG-IFN-α in combination with ribavirin, are going to be much more successful!

## Antiviral therapy for chronic hepatitis C

The standard of care as of 2002 is weekly subcutaneous PEG-IFN-α given in combination with daily oral ribavirin, but the optimal duration of treatment remains unclear.[5] Two large phase III studies, one employing PEG-IFN-α-2b plus ribavirin,[13] and the other PEG-IFN-α-2a plus ribavirin[14] – both given for 48 weeks – have demonstrated enhanced efficacy when compared with the earlier standard of care, i.e. standard IFN-α-2b given three times per week subcutaneously combined with

daily oral ribavirin. Sustained virologic response (SVR) rates, defined as undetectable HCV RNA (using a qualitative technique) in serum 6 months after cessation of therapy were 54% and 56%, respectively (Figs 33.1 and 33.2),[13,14] but there were some genotype-specific differences between the two forms of PEG-IFN-α. These differences may have resulted from what has now been clearly shown to be an inadequate dose of ribavarin (800 mg daily) when given to individuals infected with genotype 1, in the PEG-IFN-α-2b plus ribavirin study. So in the 48-week trial which involved PEG-IFN-α-2b plus ribavirin, antiviral efficacy in genotype 1 in the low dose and high dose IFN regimens was 34% and 42%, respectively, whereas for genotypes 2 or 3 it was 82% and 80% (SVR for genotypes 4/5/6 were 33% and 50%).

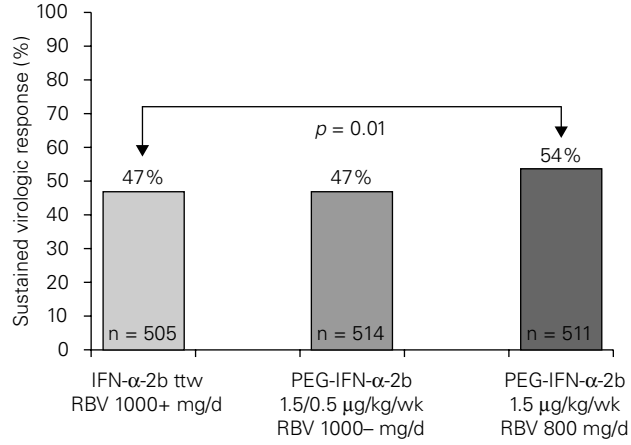



**Figure 33.1** PEG-IFN-α-2b (12 kDa) + ribavarin: 48 weeks sustained virologic response (SVR). RBV, ribavarin. (From Manns *et al*., *Lancet* 2001;**358**:958.[13])

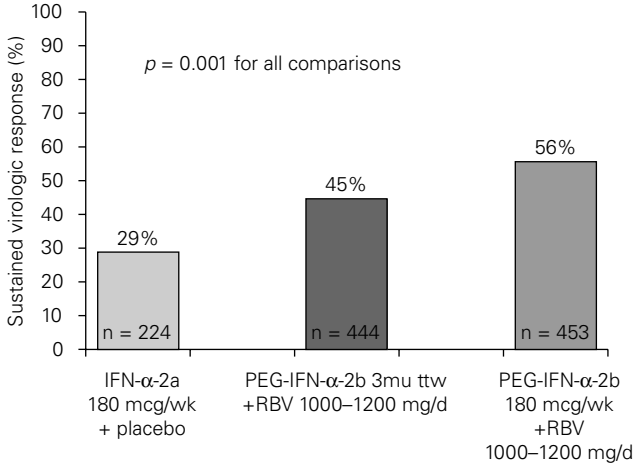



**Figure 33.2** PEG-IFN-α-2a (40 kDa) + ribavarin: 48 weeks sustained virologic response (SVR). RBV, ribavarin. (From Fried *et al*., *N Engl J Med* 2002;**347**:975.[14])

In the 48-week phase III trial of PEG-IFN-α-2a plus ribavirin, only one dose, 180 μg/wk of IFN and 1000–1200 mg ribavirin, was evaluated. In those with genotype 1, high viral load ($>2 \times 10^6$ c/mL) overall SVR was 41% (compared with 33% for standard IFN-α-2b plus ribavirin), whereas the SVR in those infected with either genotype 2 or 3 was 76% compared with 61% in those who received standard IFN-α-2b plus ribavirin.

It is likely that 24 weeks of treatment is sufficient for those infected with genotypes 2 and 3. Because of small numbers, the most appropriate duration of therapy for genotypes 4, 5 and 6 is unknown, whereas it is felt that for those infected with genotype 1 therapy for a full 48 weeks is required. Durability of SVR is excellent[15] and unless the patient is known to have cirrhosis pretreatment, long-term follow-up after achieving an SVR is probably unnecessary.

## Pharmacokinetics of PEG-IFNs

There are intrinsic differences between the two PEG-IFN-α preparations, causing the molecular weight and hence their pharmacokinetics to differ quite considerably[16] (Table 33.1). As the dose of PEG-IFN-α-2b, the 12-kDa molecule, needs to be weight-based (as does ribavirin), efficacy in part depends on the correct dose being given for a particular body weight. The larger size of the 40-kDa PEG-IFN-α-2a molecule means that it has a smaller volume of distribution, and thus it is unlikely that weight-based dosing is required.

## Who should be treated for chronic hepatitis C?

As treatment with PEG-IFN-α and ribavirin is curative in only about 50% of patients and this treatment regimen is associated with a wide spectrum of quite severe side-effects, treatment is not recommended for all infected individuals. In treatment-naïve individuals, those with a persistent hepatitis, as judged by elevated serum alanine aminotransferase (ALT) levels and a liver biopsy which shows some evidence of progressive disease in terms of hepatic fibrosis and who have no absolute contraindications to the standard of care, are thought to be the most appropriate to undergo antiviral therapy.[5]

There are many individuals who do not fulfil these criteria but who wish to be treated, e.g. those with persistently normal ALT levels, despite chronic infection. The response to treatment in those with normal ALT is no different from those who have persistently elevated ALT levels.[17] Others may have co-morbid complications of their hepatitis C, e.g. those with active vasculitus, secondary to an associated cryoglobulinaemia who require treatment for their vasculitus more than for their liver disease. In other situations, an infected

**Table 33.1** Pharmacokinetics standard and PEG-IFN-α

| | Standard PEG-IFN-α | IFN-α PEG-IFN-α–2b (12 kDa) | IFN-α PEG-IFN-α–2a (40 kDa) |
|---|---|---|---|
| Absorption half-life (h) | 2.3 | 4.6 | 50 |
| Elimination of half-life (h) | 2–5 | ≈ 40 | 77 |
| Volume of distribution (L) | 31–98 | 80 | 6–14 |
| Renal clearance (mL/h) | 11 800–16 170 | 1540 | 80 |

h, hours.

individual may be better off being treated sooner rather than later as it can be anticipated that they will be unsuitable candidates for treatment in the future. For example, individuals with minimally impaired renal function in whom subsequent development of end-stage renal failure can be anticipated, when effective antiviral therapy for hepatitis C will be much harder to institute.

The association of human immunodeficiency virus (HIV) infection and HCV is common, as both are acquired via similar routes. Since the advent of highly active antiretroviral therapy (HAART) for HIV, there has been a marked improvement in disease progression of HIV and with this, the relevance of the co-infection with hepatitis C has become more apparent. Disease progression in those with co-infection is much more rapid,[18,19] and death from liver failure in individuals with both controlled and uncontrolled HIV infection occurs.[20] Individuals co-infected with HIV and hepatitis C present a particularly urgent need for treatment, but the co-infection and the treatment for the same present very specific issues with regards to therapy and thus often the expertise from several disciplines, e.g. infectious diseases, hepatology and addiction medicine, is required[21] (see Chapter 50).

## Assessment of Individuals with chronic hepatitis C prior to antiviral therapy

There are some very definite contraindications to treatment with IFN and/or ribavirin that preclude certain individuals from receiving this therapy. In addition, there are other relative contraindications which, if they can be ameliorated, may allow antiviral therapy to be initiated. There are also a number of pretreatment conditions which affect treatment outcome in terms of antiviral responsiveness. Some of these – such as viral genotype – cannot be altered, but others may be modified, e.g. body weight, alcohol intake. Hence, it is important that the assessment of infected individuals includes not only screening for absolute contraindications, but also education of the patient so that their therapeutic outcome can be optimized.

## Absolute contraindications to PEG-IFN-α and/or ribavirin (Table 33.2)

### Hepatic decompensation

Any evidence of hepatic decompensation precludes treatment with IFN-α combined with ribavirin (except in rare circumstances just prior to a liver transplant).[22] It is important that the treating physician appreciates the sometimes subtle findings of hepatic failure, e.g. any elevation in serum bilirubin (in the absence of haemolysis or Gilberts' syndrome), an elevation of the international-alized ratio (INR) or a lowering below the lower limit of normal for serum albumin. The more obvious signs of liver failure such as jaundice, variceal haemorrhage, hepatic encephalopathy and ascites are not difficult to miss! If they are suitable candidates, it is better to refer such individuals to a liver transplant centre.

### Autoimmune disease

Both IFN and ribavirin modify the immune response, and it is likely that it is the combination of this stimulation of the immune response together with the antiviral activity of IFN (less so with ribavirin) that promotes viral clearance. Thus, in individuals who have an autoimmune disease, treatment with IFN and ribavirin could worsen their autoimmune condition,

**Table 33.2** Contraindications to antiviral therapy for hepatitis C

| |
|---|
| Interferon |
| Hepatic decompensation |
| Cardiac arrhythmia |
| Uncontrolled seizures / depression |
| Psychosis |
| Autoimmune disease (except thyroid) |
| Platelets < 70, ANC <1500 |
| Ribavirin |
| Renal failure |
| Ischaemic vascular / heart disease |
| Unwillingness to practise birth control |

so it is not recommended that individuals with active autoimmune disease undergo antiviral therapy, except in situations where simple drug therapy, e.g. thyroid replacement, is all that is required to control the effects of the autoimmune disease. Additionally, the need for immunosuppressive therapy to control autoimmune disease causes HCV RNA titres to be very high, which may reduce the chance of an SVR. Hence, the management of hepatitis C in individuals with autoimmune liver disease requires careful assessment of the risk: benefit ratio.

## Cardiac arrhythmias

Although rare, IFN therapy may induce a reversible cardiac arrhythmia[23] or cardiomyopathy,[24] and it is inadvisable to use IFN in individuals with ventricular arrhythmias or any other form of significant cardiac disease. It is rare for such individuals to be contemplated for antiviral therapy, as their cardiac disease usually presents a much greater risk of death than their hepatitis C.

## Anaemia/ischaemic vascular disease

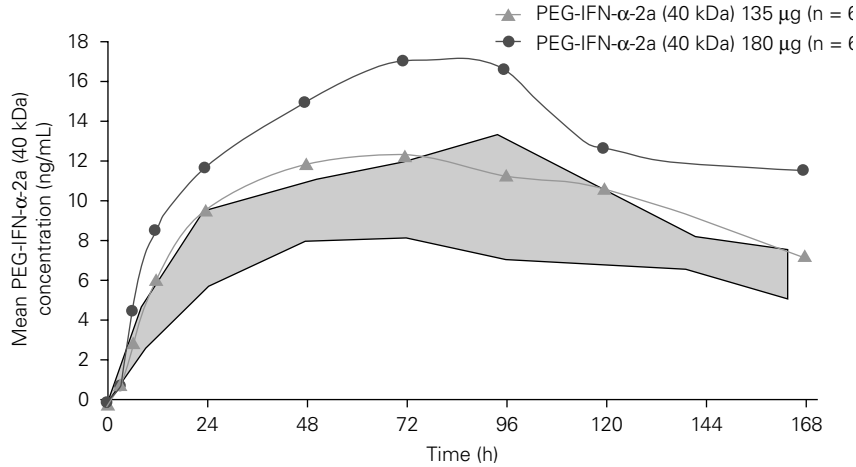
Ribavirin therapy is complicated by varying degrees of haemolysis in at least one-third of individuals undergoing combination therapy,[25] which may induce a sudden fall in haemoglobin. As it is a bone marrow suppressant, IFN monotherapy is also associated with a more gradual fall in haemoglobin. Thus, individuals who are anaemic at baseline need correction of their anaemia prior to the initiation of therapy. The risk incurred by a sudden fall in haemoglobin upon introduction of ribavirin means that individuals with ischaemic vascular disease (cardiac in particular) are unwise to undergo therapy for their chronic hepatitis C, if it involves the use of ribavirin.

## Renal failure

Ribavirin is excreted via the kidney, and so any renal impairment will promote high blood levels (particularly within erythrocytes) and as the usual half-life of ribavirin is 120 days, the presence of renal failure promotes severe ongoing haemolysis, and thus its use is contraindicated in renal failure. Very low-dose ribavirin has been utilized in end-stage renal disease in a few studies.[26] PEG-IFN, because its molecular size is much larger than that of standard IFN, has less renal excretion and may be better tolerated in patients with end-stage renal failure. Pharmacokinetic studies conducted in patients with varying degrees of impairment indicate that when the creatinine clearance is <30 mL/min blood levels of IFN when given as PEG-IFN-α-2a become markedly elevated,[27] not so with PEG-IFN-α-2a (the larger molecular weight form of PEG-IFN-α) (Fig. 33.3).

## Pregnancy

Ribavirin is teratogenic, whether it is taken by the male or the female in a partnership, and thus it is essential that all treated patients and their sexual partners practise safe sex, not only during the entire course of therapy, but for at least 6 months after the cessation of therapy because of the prolonged half-life of ribavirin.




*Shaded area denotes concentration of PEG-IFN-α-2a (40 kDa) for both doses in patients with normal creatinine clearance

**Figure 33.3** Pharmacokinetics of PEG-IFN-α-2a (40 kDa) in end-stage renal disease. The shaded area denotes concentration of PEG-IFN-α-2a (40 kDa) for both doses in patients with normal creatinine clearance. (From Lamb *et al.*, *Hepatology* 2001;**34**(Pt 2):266A.)

## Relative contraindications to antiviral therapy with PEG-IFN-α and ribavirin

### Pancytopenia

IFN is a bone marrow depressant, and it causes a dose-related neutropenia, thrombocytopenia and anaemia.[25] Adjunctive therapy to support blood components and high dose IFN therapy is efficacious in individuals with either a low absolute neutrophil count and/or haemoglobin at baseline.[28] However, there are no data yet that indicate that this very expensive therapy enhances antiviral responsiveness! The need for adjunctive therapy to support blood components is common in patients with HIV/HCV co-infection and/or in those with cirrhosis complicated by hypersplenism. Although the administration of granulocyte colony stimulating factor (G-CSF) will induce a marked rise in the peripheral neutrophil count, it remains to be seen whether this therapy reduces the chance of a bacterial infection. In the published phase III trials, in the few patients who had their PEG-IFN-α and ribavirin therapy complicated by an episode of sepsis, none developed sepsis at a time when their absolute neutrophil count was below $1.5 \times 10^9$ cells/mm$^3$.[13,14] Erythropoietin will increase the haemoglobin in anaemic patients suffering from chronic renal failure or those on HAART for their HIV infection. Another patient population frequently infected with HCV comprises those with congenital haemoglobinopathy, e.g. thalassaemia. Such individuals may be supported by blood transfusions during the course of antiviral therapy very successfully.[29]

### Psychiatric disorders

IFN, a cytokine, causes flu-like symptoms, malaise and mood changes and frank depression,[30] and thus individuals who already suffer from mood instability before treatment can be made much worse by antiviral therapy for their hepatitis C. In the past, depression has been considered a contraindication to treatment with IFN. In a large study of patients with melanoma undergoing IFN therapy, a randomized control trial of paroxetine (a selective serotonin-reuptake inhibitor, SSRI) clearly indicated that this drug was able to prevent IFN-associated depression.[31] The use of SSRIs in any individual who has a past history of depression has allowed many such individuals to undergo successful antiviral therapy without dire consequences. There are early reports suggesting that individuals with other psychiatric conditions may also undergo antiviral therapy for their hepatitis C safely[32] – individuals with schizophrenia or bipolar disorder were excluded from this study.

**Table 33.3** Factors influencing response to antiviral therapy – hepatitis C

| Viral | Host |
|---|---|
| Viral genotype | Race |
| HIV co-infection | Age |
| Viral load | Body weight |
| Early evolution of quasi-species | Alcohol |
| | Fibrosis |

### Seizure disorders

Whereas epilepsy may be exacerbated by the use of IFN therapy, those individuals whose seizure disorder is well controlled with drug therapy can safely be treated for their hepatitis C if this is felt to be necessary.

## Factors influencing response to antiviral therapy for hepatitis C (Table 33.3)

### Non-adjustable factors

#### *Viral genotype*

Viral genotype is the major viral factor which determines the likelihood of achieving an SVR following a complete course of antiviral therapy for hepatitis C. All studies, whether employing standard or pegylated IFN, given with or without ribavirin, indicate that the patients most likely to respond to therapy are those infected with genotype 2 and slightly less so, those with genotype 3. Unfortunately, the majority of individuals worldwide are infected with less responsive genotypes, namely genotypes 1 and 4.

#### *Viral load*

When antiviral therapies were less effective than they are now, viral load played an important role in determining whether the outcome of therapy was successful. However, the maintenance of high IFN levels week-long with PEG-IFNs has reduced the influence of viral load on antiviral responsiveness. But in immunosuppressed individuals, the viral load can be very high (as is found in HIV co-infected or post-liver transplant patients) and in these circumstances, the high viral titre combined with poor tolerance to antiviral therapy for hepatitis C reduces the likelihood of an SVR. Studies indicate that the pattern of quasi-species development following introduction of therapy also correlates with virological outcome.[33]

### *Ethnicity*

For reasons that still remain unclear, African-Americans respond poorly to antiviral therapy, regardless of infecting genotype.[34] This may or may not relate to specific genetic polymorphisms of IFN-associated proteins.

### *Degree of hepatic fibrosis*

Consistently across all published trials, the greater the degree of hepatic fibrosis the lower the response to antiviral therapy. When standard IFN monotherapy was used, there was a negligible response seen in patients with cirrhosis,[35] but upon the introduction of the long-acting PEG-IFN, a marked improvement was observed, such that 30% of patients with bridging fibrosis or cirrhosis achieved SVR with PEG-IFN-α-2a monotherapy.[36] Combination of ribavirin with PEG-IFN has improved the chance of SVR to just over 40%.[13,14] However, this figure still remains less than may be achieved in individuals without background cirrhosis.

## Adjustable factors

### *Hepatic steatosis and/or body mass index*

Recent data indicate that both the degree of fat within hepatocytes[37] and overall body weight, better still, body mass index (BMI), appear to influence treatment response.[38] An aggressive weight loss programme induced by a combination of dietary modification and exercise has been associated with a reduction in hepatic fat and a reduction in hepatic fibrosis in a small sample of individuals with hepatitis C.[39] It is logical to assume that this may lead to enhanced responsiveness to antiviral therapy, but this will be hard to prove.

It remains unclear whether all PEG-IFN doses should be weight-based. Weight-based dosing is recommended for the 12-kDa PEG-IFN-α-2b, but because of the much smaller volume of distribution of the larger molecule (limited to the vascular space) of the 40-kDa PEG-IFN-α-2a, theoretically this agent should not have to be weight-based.[40]

### *Alcohol consumption*

Retrospective analyses have shown that alcohol consumption both prior to and during treatment may influence the outcome of therapy, above and beyond the effects of alcohol in causing more rapid disease progression and influencing adherence.[41] Thus, it is recommended that patients abstain from alcohol prior to and during antiviral therapy for hepatitis C.

### *Age*

The likelihood of achieving SVR diminishes by about 5% per decade.[42] Thus, it has been suggested, particularly in those who are difficult to treat because they are infected with genotype 1, that treatment should be considered sooner rather than later, so as to not reduce the chance of achieving an SVR any more than is possible. There is no pharmacologic explanation for this age effect.[43]

# Adherence

## The effect of adherence on response to antiviral therapy for hepatitis C

### *Achieving an early virologic response (EVR)*

The factor that has the greatest effect on the outcome of antiviral therapy remains the ability of the patient to maintain full dose or close to full dose therapy for the prescribed duration. Trials of standard IFN and ribavirin indicated that a figure of ≥80% maintenance of therapy was critical. In the more recent studies of PEG-IFN and ribavirin, it has been calculated that individuals who are able to maintain at least 80% of the PEG-IFN dose required and 80% of the ribavirin dose required have an 80% chance of achieving an EVR,[44] defined as a ≥2 log drop in HCV RNA between baseline and 12 weeks of treatment. EVR determines the subsequent likelihood of an SVR. In patients who can maintain 80% of their prescribed PEG-IFN dose, but who take <80% of the ribavirin dose, the EVR rate falls to 60%. Alternatively, if 80% of the PEG-IFN dose cannot be achieved, but 80% of the ribavirin is achieved, the EVR rate is 70%. The worst outcome is when <80% of both PEG-IFN and the prescribed dose of ribavirin are taken; then the EVR rate falls to 30%!

## Enhancing adherence to therapy for hepatitis C

There are many factors which play a role in facilitating adherence to treatment with PEG-IFN and ribavirin.

### *Time of initiation of therapy*

This needs to be optimal for the patient. Thus, for students it is important to avoid examination time. For others, it is important to avoid the time of a major life event such as marriage, new job, moving home, etc.

### *Pre-emptive treatment of side-effects*

Predictable side-effects may be minimized. Of greatest benefit to the patient is for their ongoing treatment

to be closely monitored by an individual who has the time to interact and support them throughout treatment. Physicians are rarely able to achieve this, and this role is best performed by a specially trained nurse. Under their tutelage, the patient can learn to control the fevers associated with IFN by taking prophylactic acetaminophen. The irritating cough and dry skin associated with ribavirin therapy can be considerably reduced by encouraging the patient to maintain a high fluid intake. The troublesome depression that particularly affects individuals whose pretreatment history indicates past depressive illness, can be greatly helped by prophylactic use of antidepressant medications.

#### *Appropriate dose reduction of IFN and/or ribavirin*

Dose reduction is most often required because of adverse laboratory events, particularly a fall in haemoglobin and/or absolute neutrophil count. Sometimes symptoms, particularly overwhelming fatigue and shortness of breath, may occur when the haemoglobin is not very low but the haemolysis caused by ribavirin has been particularly rapid. Under these circumstances, it may be necessary to reduce ribavirin dosage for a short period of time.

#### *Addiction medicine counselling*

A recent study of antiviral therapy given to patients with hepatitis C who fell into various psychiatric risk groups indicated that adherence rates could be easily maintained in individuals with well controlled psychiatric illnesses or in those with prior addiction habits that were controlled on methadone. But in those who were former injecting drug users (IDUs), but not on methadone, the drop-out rate was higher and hence SVR rates were much lower.[32] Unfortunately, methadone is only useful in injecting drug users who are addicted to opioids, and at present there is no medication which helps curb addiction to other compounds such as cocaine. In some centres, antiviral treatment given to current IDUs is said to be successful,[45] although few current IDUs are motivated to seek and maintain medical help.

## Predicting response to antiviral therapy for hepatitis C

Retrospective analysis of outcomes following treatment with standard IFN and ribavirin suggested that it was possible early on in treatment to predict who may not attain an SVR by examining the fall in viral load from baseline to 4 weeks.[46] It was shown that 50–60% of subsequent non-responders could have been predicted early into treatment.[47] Prospective analysis of PEG-IFNα-2a plus ribavirin has shown that the likelihood of achieving an SVR can also be reliably predicted at 12 weeks into therapy.[14]

### Clinical implications of improved predictability of antiviral treatment in hepatitis C

Our ability to reliably predict outcome at 12 weeks into treatment means that the burden of therapy to patients can be limited to those who are most likely to respond. In those who do attain an EVR, this information may help to sustain them through the ongoing unpleasant side-effects of treatment required for a further 36 weeks, i.e. it acts as an incentive for patient counselling and education. An improvement in cost-benefit ratio will also be achieved if therapy is stopped at 12 weeks in those who do not achieve an EVR. However, as all those infected with genotype 2 or 3 achieve an EVR, testing at 12 weeks into treatment is not recommended, rather 6 months of treatment is given to all.

## Barriers to antiviral therapy for hepatitis C

### Accessibility

The major pool of infected individuals worldwide live in situations which are either hard to access, e.g. homeless individuals, impoverished individuals in the developing world, those living in isolated areas such as on reservations, and individuals not isolated, but in a social situation where the need for therapy is often ignored, i.e. individuals incarcerated in prisons. An equally disturbing situation is the inability of an individual to afford the cost of antiviral therapy in any part of the world because their insurance system fails to cover their costs.

The complex requirements of both repeated injections and serial blood monitoring, with the need to access patients immediately if the drug dose needs to be reduced or stopped, really precludes employing the current therapies in many individuals. But unless this large source of potential transmission for virus is controlled, there is no hope that we will significantly impact the burden of hepatitis C. Thus, clean needle exchange programmes have been initiated in many countries. Many governments have recognized the inadvisability of withholding antiviral therapy from the prison population. In such a controlled environment, there is no reason why antiviral therapy should not be successful.[48,49]

### Contraindications to current therapies

There remain many infected individuals who either have co-morbidities that preclude current antiviral therapies, e.g. severe seizure disorder which cannot be

adequately controlled, or who are unable to tolerate the treatment-associated side-effects. It is hoped that such individuals will be able to be successfully treated with the second generation of antiviral therapy for hepatitis C, namely enzyme inhibitors. There are others who cannot tolerate current therapies because their liver disease is too advanced, in which case if there are no other contraindications, liver transplantation may be the optimal route.

### Need for adjunctive therapy

With appropriate adjunctive and supportive therapy, the number of patients in whom treatment is recommended, but in whom co-morbid conditions have in the past precluded therapy, may be significantly reduced with the use of agents to control depression and support blood components.

## Special populations of patients with hepatitis C requiring antiviral therapy

### Patients with cirrhosis

Potentially, individuals with cirrhosis due to hepatitis C have the most to gain in the short and possibly the long term, were they to be successfully treated for their hepatitis C. Whereas therapy with standard IFN monotherapy was very disappointing, particularly in cirrhotic patients, the chance of achieving an SVR with PEG-IFN-α and ribavirin is markedly better, i.e. 40–50%. Those most likely to die from their hepatitis C are those with cirrhosis. Both early[50] and long-term[51] follow-up liver biopsy data indicate that regression of hepatic fibrosis may be observed following antiviral therapy most often when an SVR is achieved. If liver transplantation is still required, recurrence of hepatitis C post-transplant is much less in those who have undetectable HCV RNA.[22]

Analysis of very large numbers of patients with hepatitis C treated with all forms of antiviral therapy do suggest that the rate of subsequent hepatocellular carcinoma (HCC) is significantly less in those who at the time of therapy had a hepatic fibrosis score of 3 out of 4.[52] However, the benefit seems to be mainly in Japanese patients, in whom the risk of HCC in chronic hepatitis C is so much higher. A significant benefit in terms of reduction in HCC in Caucasians is disputed.[53,54] There are reports that following successful antiviral therapy the rate of hepatic decompensation is reduced in both Japanese and Caucasians – but all these data are from observational studies, where the less severe patients tend to be those treated.[55,56] It is logical to assume that if the inciting agent is removed, there will be a long-term benefit – just as there is when appropriate reduction in copper or iron overload is achieved in individuals with Wilson's disease or haemochromatosis.

### HIV/HCV co-infection

Liver-related mortality in individuals with HIV/HCV co-infection has become more overt since the introduction of effective therapy for HIV infection. Although the mortality from liver disease remains highest in co-infected individuals *not* treated for their HIV infection, liver disease remains a significant problem in those whose HIV infection is well controlled.[20] There is an urgent need to introduce effective antiviral therapy for hepatitis C in the co-infected individual, because the rate of liver disease progression in co-infected individuals is almost double that seen with HCV infection alone.

However, there are many problems encountered during treatment for hepatitis C in those who also are infected with HIV. Because of the background immune suppression, HCV RNA titres tend to be high. There are reports of untoward drug interactions between ribavirin and various components of HAART therapy, particularly with the 'd' drugs, which are probably related to competition for intracellular metabolism.[57,58] Such drug interactions may cause severe mitochondrial injury, hepatic steatosis and even liver failure, but less overt drug interactions may just destabilize the HAART. As many co-infected individuals either have been or currently remain IDUs, the issue of adherence to both HIV and HCV antiviral therapy is problematic.

Co-infected individuals generally have as their primary physician an individual who is not trained in the management of liver disease or skilled in the practice of liver biopsy. Without a liver biopsy, patients with cirrhosis will be missed, as compensated cirrhosis cannot be reliably assessed with non-invasive techniques. Antiviral therapy in HCV/HIV co-infection in an individual with cirrhosis who has even only mild evidence of hepatic impairment in terms of minimal elevation of serum bilirubin and/or low serum albumin, may lead to rapid hepatic decompensation and death.[59] Thus, it is advised that co-infected patients be managed in a multidisciplinary clinic which includes experts in HIV and liver disease as well as addiction medicine.[60]

#### *Timing of treatment for hepatitis C in individuals co-infected with HIV*

Management guidelines suggest that in individuals co-infected with HCV and HIV, it is generally best to consider the need for treatment of the HIV first and if anti-retroviral therapy is not required, then this is the ideal time for introducing treatment for hepatitis C. If anti-retroviral treatment is required, then this should be started before any antiviral therapy for hepatitis C is

given, and the HIV infection should be stabilized before introducing treatment with IFN and ribavirin (this is discussed in more detail in Chapter 50).

## Those with normal ALT with or without other non-hepatic, hepatitis C-related co-morbidities

At least 25% of individuals infected with hepatitis C have consistently or intermittently normal ALT levels, over a period of several years. Some may have significant hepatic fibrosis on liver biopsy, indicating that they are at risk for further disease progression and thus may warrant therapy.[61] Efficacy of PEG IFN-α-2a 180 μg/week plus ribavirin (800 mg/day) for 48 weeks indicates that SVR rates are not dissimilar to those observed in individuals with elevated ALT.[17]

There are other individuals who have significant co-morbidity caused by hepatitis C which may improve were the virus to be eradicated. Serum ALT values may well be normal, and liver biopsy may show only mild liver disease, but nevertheless treatment is warranted, particularly if the patient suffers from symptomatic vasculitus caused by cryoglobulinaemia with or without a complicating non-Hodgkin's lymphoma (NHL). A recent meta-analysis suggests that the association between hepatitis C and NHL appears real.[62]

Symptoms of cryoglobulinaemia caused by a vasculitus affecting most often the skin, kidneys or peripheral nerves are well controlled in 70% of those undergoing antiviral therapy, even when viraemia persists.[63] Relapse after cessation of therapy occurs if viral clearance has not been achieved, but symptoms may remain controlled even in the presence of persistent viraemia in many[64] without the need for immunosuppressive therapy and/or plasmapheresis. This particular co-morbidity may be one of the few indications for prolonged antiviral therapy in the face of persistent viraemia.

Complete resolution of splenic lymphoma associated with a chronic hepatitis C infection may be observed following a sustained viral response, not so if SVR is not achieved.[65]

The seroprevalence of hepatitis C in patients with end-stage renal disease is high.[66] As IFN is generally not recommended in patients with a kidney transplant because this drug may promote rejection of this small organ,[67] antiviral therapy needs to be given as early into the course of an individual's chronic renal disease as possible, as antiviral therapy is poorly tolerated in end-stage renal disease. However, the pharmacokinetics of the PEG-IFN-α-2a is such that these drugs may be better tolerated than standard IFN-α (Fig. 33.3)

## Liver transplant patients

End-stage liver disease caused by chronic hepatitis C is now the major indication for liver transplantation in Europe and North America. With no active intervention, graft reinfection is inevitable and, in the setting of the anti-rejection therapy, rapidly progressive disease can occur – threatening graft and patient survival.

### *Pre-transplant*

Ideally, the physician should aim for a sustained viral clearance before transplant, but this is rarely feasible as the patients frequently have decompensated disease and, in patients with HCC, the urgent need for transplant limits the use of effective antiviral therapy prior to the procedure.

With careful supervision and dose modification some patients can benefit from pre-transplant treatment.[22]

### *Post-transplant*

Despite the immunostimulatory effects of IFN-α and the theoretical risks of graft rejection, IFN is successfully used in many recipients of liver transplants. Most of the published studies have featured trials of standard IFN and ribavirin, and there is increasing use of PEG-IFN with ribavirin. Overall, the success rates are lower in the post-transplant patients and discontinuation rates are much higher than those seen in the non-transplant setting.

In one French study,[68] 52 liver transplant recipients were randomized to receive standard IFN in combination with ribavarin or placebo. The SVR was 21% in those who received the combination and 0% in those who received placebo. Significant side-effects, particularly severe anaemia, led to treatment discontinuation in 43%.

Another study compared treatment with IFN and ribavirin for 6 months versus 12 months.[69] Fifty-seven patients (68% genotype 1b) were treated, and the SVR was 22% in those who received therapy for 6 months and 17% in those who received therapy for 1 year, suggesting that a subgroup of patients may benefit from a relatively short course of treatment. This study also demonstrated that in the virological non-responders with a biochemical response there was also histological improvement, and there may be a role for 'maintenance therapy' in this group of patients.

In view of the poor tolerance of antiviral regimens and the low antiviral response in this group, other approaches have been tried such as ribavirin monotherapy. Short-term use of ribavirin monotherapy[70] has been shown to lead to a biochemical response and a decrease in the inflammatory score on liver histology, but longer-term follow-up will be required to ascertain whether this is also associated with a reduction in fibrosis development.

Most studies now feature PEG-IFN and ribavirin. One pilot study[71] described high levels of discontinuation

because of side-effects (43.6%) but an SVR of 66.7% in those who completed therapy.

For those patients unable to tolerate IFN and ribavirin, there are prospects of future therapy with protease and helicase inhibitors or alternative approaches with anti-fibrotic rather than antiviral agents.

## Ongoing drug use

The patient who continues to inject drugs can vary from the stable patient on a supervised (oral or injected) methadone or heroin programme to the erratic patient who injects from time to time and who continues to share needles and syringes with others. Delivery of care to the more chaotic patient is a considerable challenge and requires substantial resources. In the more erratic patient, there are risks of missing doses and monitoring visits and also the risk of reinfection at any time during the treatment course. A multidisciplinary approach is vital. Most would advise that the priority with such a patient is to work closely with the drug treatment centres and to encourage the patient to follow their prescribed drug stabilization programme before embarking on a treatment course for hepatitis C. Alcohol consumption, polydrug use, nutritional problems, anxiety and depression are common in this group and psychiatric input is therefore valuable. Many of the studies reporting beneficial effects of IFN and ribavirin in drug users exclude those with active drug or alcohol use.[72] In a Norwegian study[73] 27 ex-intravenous drug users who had been successfully treated for hepatitis C were followed up. Although 33% of the group had returned to injecting drugs, only one had evidence of reinfection, suggesting that at least some behaviour modification had occurred. It is also argued that treating drug users may have some benefits beyond the individual patient, and that reducing the viral load in a drug user reduces the chance of infection spreading to others.

Early treatment guidelines counselled against treating active drug users, and the wisdom and ethics of this have been questioned by Edlin and others.[74] They emphasize that adherence to treatment regimens is reduced in many chronic illnesses and that the risk versus benefit of side-effects, risk of reinfection and timing of treatment must be assessed for each individual.

Supervised drug treatment programmes may involve daily visits and can be combined with directly observed hepatitis C treatment programmes. This approach could also be adapted to the prison environment. The longer half-lives of PEG-IFNs lend themselves to the concept of once-weekly supervised therapy.

## African-American patients

African-American patients are more likely to have genotype 1 infection, lower ALT values and less rapid disease progression.[75] African-Americans also have lower neutrophil counts, which may lead to exclusion from therapy or to inappropriate dose reductions.[76] They are also less likely to have an elevated alpha-fetoprotein with HCC development.[77] The chance of responding to antiviral therapy is reduced and this is not fully understood, although the prevalence of 'hard-to-treat genotypes' tends to be high. Two clinical trials[78] were pooled to analyze this further. There were 53 black patients among the total of 1744 patients, and 96% of them had genotype 1 infection compared with 65% of the white patients. The overall response rate was 27% in the white patients and only 11% in the black patients. The SVR rates in the black patients were 20% with 24 weeks of standard IFN and ribavirin, 23% with 48 weeks of this therapy, and no patients responded to IFN monotherapy. For genotype 1 infection, the sustained response rates of 23% in the black patients and 22% in the white patients suggest that in this study the genotype was a major factor in the overall responses. Increased iron stores have been reported in African-Americans,[79] which may influence immune response.

One study compared the virological kinetics in 19 African-American and 16 white patients with genotype 1 infection and reported a decreased viral load reduction in the African-American group.[80] Similarly, intensification of the IFN dosing with daily administration also demonstrated a decreased response in this group.[81]

## Children

Earlier experiences with higher doses of IFN in children with chronic hepatitis B have shown that children tolerate such therapy surprisingly well. There are limited clinical trial data for children, and many of the children treated have underlying conditions such as haemophilia, thalassaemia or malignancy, which may impair their response and reduce their chance of tolerating antiviral therapy. In one study of 41 children, an SVR of 61% was reported following 1 year of standard IFN and ribavirin.[82] As yet, there are limited data on the use of PEG-IFN regimens in this group.

## Thalassaemia and iron overload

Thalassaemia patients with hepatitis C are challenging to treat as they may be unable to tolerate the haemolysis associated with ribavirin and may have advanced liver disease and a substantial iron overload at baseline – factors which generally reduce the chance of responding to treatment.

In one IFN monotherapy study,[83] six of thirteen children responded and a favourable response was more likely in those with less severe liver disease and lower

baseline ferritin levels. An Australian study,[84] however, found that the baseline hepatic iron concentration did not affect the chance of a sustained response.

In a pilot study,[85] 11 patients with thalassaemia were treated with a combination of standard IFN and ribavirin. Five patients had a sustained response although, probably as a result of the ribavirin, transfusion and iron chelation requirements were increased during the treatment period. In another study,[86] 18 patients (14 with genotype 1b) received 12 months of combination of standard IFN and ribavirin and the SVR was 72.2% with a 30% increase in supportive blood transfusion.

Studies are underway with PEG-IFN and ribavirin, and PEG-IFN monotherapy may be an option for those who are unable to tolerate ribavirin.

### Non-responders and relapsers to standard IFN monotherapy or combination regimens with ribavirin

Several studies have shown that for those who have previously failed to respond to IFN monotherapy there is a reasonable chance of responding to combination therapy.

The benefit is particularly seen in those who initially responded and then relapsed. In one study[87] of 345 patients who had relapsed following IFN monotherapy, the sustained response rate was 49% in those who received IFN and ribavirin combination therapy and only 5% in those who were retreated with IFN monotherapy.

Response rates in previous non-responders are much lower at 15–20%.[88–92] Other studies have tried other approaches such as an induction period with high dose IFN.

Another approach has been the addition of amantadine to the regimen. In a large study of 225 non-responders,[93] IFN and ribavirin was compared with IFN, ribavirin and amantadine. There was a trend towards a higher response rate in the group who received triple therapy (25% versus 18%).

The use of other types of IFNs has also been tried in this group of patients. A pilot study of treatment with consensus IFN and ribavirin[94] in patients who had failed to respond to standard IFN and ribavirin reported SVRs of 33% in the previous non-responders and 42% in the previous relapsers.

There are limited data on PEG-IFN ribavirin regimens as retreatment, but preliminary results suggest significant benefits for those who have received IFN monotherapy and more modest gains for those who have already received IFN and ribavirin. The early virological response will hopefully also prove useful in determining the chance of an SVR in these patients, but more data are required to ascertain whether the predictors in previously treated patients undergoing retreatment remain valid.

Overall, the factors associated with a higher chance of responding to retreatment are previous relapse (rather than non-response), genotypes 2 and 3, lower levels of HCV RNA and an early virological response to treatment. Negative factors include being African-American and/or cirrhotic.

Those with only mild liver damage can afford to wait for the development of new antiviral agents, but for those with more severe disease a more realistic priority may be to inhibit fibrosis and maintain liver function. Long-term maintenance regimens may need to be considered.

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# Chapter 34
# New drugs for the management of hepatitis C

John G McHutchison, Jennifer M King, Amany Zekry

## Introduction

Approximately 170 million people worldwide are infected with hepatitis C virus (HCV).[1] Current therapies for chronic HCV infection (Table 34.1) are only effective in approximately 50% of patients. In addition, therapies are costly, prolonged, associated with significant side-effects, and not suitable for all patients.

Fortunately, many new treatments for HCV infection are currently being designed and evaluated in preclinical, animal and human studies. For patients with chronic hepatitis C infection, the ideal therapy would be highly effective, orally bioavailable, without major side-effects and affordable. While such a drug is unlikely to be developed in the near future, a number of novel and promising compounds may significantly improve current options for therapy. Many were designed on the basis of insights into the mechanics of HCV replication, entry into cells and host cellular immune responses. Others may slow the progression of liver disease. In this chapter, we highlight and summarize many of these ongoing developments and research.

**Table 34.1** Hepatitis C drugs in various phases of development

| Drug type | Compound | Phase of development | Comments |
|---|---|---|---|
| Small molecules and viral enzyme inhibitors | | | |
| NS3 helicase inhibitors | | Preclinical and phase 1 | Numerous agents in preclinical stages. Development of one compound halted in early dose-ranging studies |
| NS5B polymerase inhibitors | JTK-003 | Phase 1 and 2 | Inhibits HCV RNA replication in cell culture |
| | JTK-109 | Preclinical | Inhibits HCV RNA replication in cell culture |
| | NM-283 | Phase 1 and 2 | Inhibits HCV RNA replication in cell culture. Up to 1 log reduction preliminarily reported in early short-term clinical trials |
| p7 inhibitors | Long-alkyl-chain iminosugar derivatives | Phase 2 | Have antiviral activity against bovine viral diarrhoea virus. Clinical data pending in HCV-infected patients |
| Ribozymes | Directed at IRES | Phase 1 and 2 | Further development halted because of animal toxicology findings |
| Antisense oligonucleotides | ISIS-14803 | Phase 2 | >1 log reductions observed in some HCV non-responder patients, associated with transient asymptomatic ALT elevations |
| siRNA and eiRNA | | Preclinical | Uses double-stranded RNA to downregulate post-translational gene expression |
| NS3 serine protease inhibitors | | Preclinical and phase 1 | A multitude of agents from different classes are in preclinical development. One agent, BILN-2061, has entered a phase 1 trial and is associated with profound viral inhibition, but further development has been halted |

(*Continued*.)

**Table 34.1** (*Continued*)

| Drug type | Compound | Phase of development | Comments |
|---|---|---|---|
| Non-specific immune activators | | | |
| Interferons | IFN-α-2a | Approved | Roferon-A (Roche), 3 MU three times/week, SC |
| | IFN-α-2b | Approved | Intron A (Schering-Plough), 3 MU three times/week, SC |
| | IFN-alfacon-I | Approved | Infergen (InterMune), 9 μg three times/week, SC |
| | PEG-IFN-α-2a | Approved | Pegasys (Roche), 180 μg once/week, SC |
| | PEG-IFN-α-2b | Approved | Peg-Intron (Schering Plough), 1.0–1.5 μg/kg once/week, SC |
| | Albuferon | Phase 2 | Fusion of interferon with albumin to increase half-life. Preliminary data suggest prolonged half-life and similar antiviral efficacy |
| | Omega-interferon (IFN-ω) | Phase 2 | Alternate type 1 IFN. Early studies suggest similar antiviral effects to type 1 IFNs and similar side-effect profile |
| | Gamma-interferon (IFN-γ) | Phase 2 | Combination studies with type 1 IFNs for non-responder patients in progress |
| | Oral IFNs | Phase 1 | Absorption of IFN via oral route |
| Oral interferon inducers | Imiquimod | Preclinical | Approved for use as a topical agent in dermatology |
| | Resiquimod | Phase 2 | Studies show no antiviral effects or cytokine induction, but doses may have been inadequate |
| | ANA 245 | Phase 1 | Low molecular weight nucleoside analogue |
| | ANA 971 | Phase 1 | Delivers ANA 245 to plasma of various animals |
| Nucleoside analogues | Ribavirin* | Approved | Copegus (Roche), 0.8–1.4 g/day, orally† Rebetol (Schering-Plough), 0.8–1.4 g/day, orally† |
| | Levovirin | | Development is halted because of absorption and delivery issues |
| | Viramidine | Phase 3 | Ribavirin 'prodrug' with preferential liver uptake. Large-scale international multicentre trials in combination with PEG-IFNs in progress |
| | ANA 246 | Preclinical | Oral compound that enhances the type 1 cytokine response |
| IMPDH inhibitors | VX-497 | Phase 2 | Specific IMPDH inhibitor, no haemolysis, further trials with IFNs and ribavirin in progress |
| | Mycophenylate mofetil | Phase 2 | Evaluation of efficacy combined with IFN-α in non-responders in progress |
| Broad-spectrum antivirals | Amantadine | Phase 2 | Numerous clinical trials evaluating efficacy with IFN or IFN and ribavirin in naïve and non-responder populations |
| | Rimantadine | Phase 2 | Similar trials to those for amantadine |
| Other immunomodulators | Histamine dihydrochloride | Phase 2 | NK cell activator. Combined with PEG-IFN and ribavirin in clinical trials in non-responder patients |
| | Thymosin α-1 | Phase 2 and 3 | Two large-scale trials comparing PEG-IFN alone or with thymosin α-1 in non-responders |
| | IL-10 | Phase 2 and 3 | Anti-inflammatory cytokine. Randomized controlled trial failed to show benefit in terms of fibrosis; IL-10 was associated with decreased ALT values and hepatic inflammation, but increased viral concentrations |
| | IL-12 | Phase 2 and 3 | Proinflammatory cytokine. Phase 2 trial indicated lack of efficacy and significant toxicity |
| Passive immunization | | | |
| | HCIg | Phase 1 and 2 | Inactivated pooled high-titre HCV RNA-negative immunoglobulin. Initial trial to evaluate recurrence of HCV post-liver transplantation |
| Therapeutic vaccination | | | |
| | E1 therapeutic vaccine | Phase 2 | Administration of E1 vaccine in HCV patients after 28 weeks produced detectable levels of E1 antibody and specific T-cell responses |

(*Continued*.)

**Table 34.1** (*Continued*)

| Drug type | Compound | Phase of development | Comments |
|---|---|---|---|
| | E1/E2 therapeutic vaccine | Preclinical and phase 1 | Recombinant E1/E2 vaccine has been effective in preventing infection or chronic illness in chimpanzees |
| | NS3–NS4–NS5–core fusion protein | Phase 1 | Combined with an adjuvant, primed broad CD4+ and CD8+ T-cell responses in chimpanzees |
| Antifibrotics | | | |
| | IFN-γ-1b | Phase 2 | Large-scale antifibrotic trial did not reach its end-points in terms of antifibrotic effects |

ALT, alanine aminotransferase; HCIg, hyperimmune anti-HCV immunoglobulins; HCV, hepatitis C virus; IMPDH, inosine monophosphate dehydrogenase; IRES, internal ribosome entry site; MU, million units; NK, natural killer; PEG-IFN, pegylated interferon; SC, subcutaneously.
*Ribavirin is not approved as a monotherapy, but as part of a combination therapy with IFN-α.
†According to HCV genotype and body weight.

## The HCV life-cycle and potential inhibitors

HCV is an enveloped positive-sense RNA virus (Fig. 34.1). The genome (Fig. 34.2) has a single open reading frame (ORF) of approximately 9.6 kb flanked by 5′ and 3′ untranslated regions (UTRs) that are required for replication and protein synthesis. The 5′ UTR of the HCV genome contains an internal ribosome entry site (IRES) for the initiation of translation.[2,3] Translation of the HCV ORF leads to synthesis of a polyprotein that varies in length between 3010 and 3033 amino acids depending on the strain. The polyprotein is processed into 10 mature structural (core, E1, E2 and p7) and non-structural (NS2, NS3, NS4, NS5A and NS5B) proteins.

A number of macromolecules involved in viral replication, protein translation and post-translational modification are potential inhibitory targets for drug development. Exogenous macromolecules may in theory also be effective inhibitors of such functions.

### RNA replication

#### *NS3 helicase*

The primary function of the HCV NS3 helicase is to unwind viral genomic RNA during replication. Recent insights into the mechanics of polynucleotide unwinding have led to new strategies for drug discovery.[4] In addition, three-dimensional helicase structure determinations have helped to pinpoint the functionally important regions of NS3 helicase and domain motions that accompany the progressive unwinding of nucleic acid duplexes.[5,6] A few small molecules inhibit NS3 helicase *in vitro*. Also in this setting, recombinant antibody fragments at nanomolar concentrations can inhibit NS3 helicase enymatic activity.[7] Their specificity and potential efficacy in the clinical setting remain unclear.[8]

#### *NS5B*

NS5B, or the HCV RNA-dependent RNA polymerase (RdRp), is a 68-kDa protein with a hydrophobic tail that anchors to internal cellular membranes and catalyzes RNA synthesis during replication. The three-dimensional structure of the active RdRp[9–11] has revealed several inhibitory targets. The RdRp has the classical 'finger/palm/thumb' motif of many other single-chain nucleic acid polymerases. However, it has a unique, fully encircled, preformed active site where nucleotides can bind in the absence of the template. The active site is a target for nucleoside/nucleotide analogue inhibitors.[6] In addition, on the surface of the enzyme there is a specific guanosine triphosphate (GTP) binding site that is more than 30 Å away from the active site.[12] Non-nucleoside inhibitors of HCV RNA-dependent RNA polymerase (RdRp) can bind very close to the surface GTP site, suggesting that these drugs may inhibit a conformational change needed for RNA elongation.[13]

Inhibitors of viral polymerases can be classified into three categories: nucleoside (substrate) analogues, non-nucleoside inhibitors and pyrophosphate (product) analogues. In the cytoplasm of infected cells, nucleoside analogues (cyclic or acyclic) become triphosphorylated nucleotides. Nucleotides are then incorporated by the viral polymerase during processive nucleic acid synthesis, causing premature termination of replication. Nucleoside inhibitors of viral polymerases are used therapeutically for HIV (human immunodeficiency virus), hepatitis B (HBV) and herpes viruses. The only non-nucleoside inhibitors of clinical interest act against HIV-1 reverse transcriptase. These compounds bind to an allosteric site on the enzyme surface away from the active site, possibly distorting the precise geometry of the active site. Phosphonoformic acid (Foscarnet) is the only pyrophosphate analogue approved for treatment of herpes viruses. Pyrophosphate analogues are believed

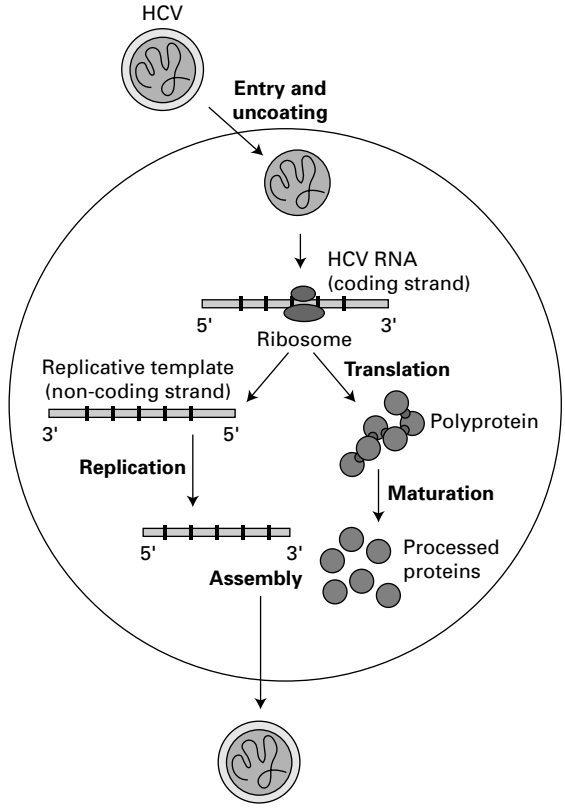


**Figure 34.1** Life-cycle of the hepatitis C virus (HCV). The virus selectively fuses with liver cell plasma membranes. This interaction probably involves the HCV envelope glycoproteins E1 and E2, making them potential targets of inhibitory drugs. Once fusion of the viral lipid coat and host plasma membrane is complete, the viral core enters the host cell. The HCV genome is a sense strand of RNA, which can be directly read by the host's ribosomes. During translation, the ribosomes produce a polyprotein that is processed into 10 proteins (four structural and six non-structural – NS). Potential specific inhibitors of HCV translation include antisense oligonucleotides and RNA interference. The enzymes NS3 serine protease and NS2/NS3 protease cleave the polyprotein and are thus targets for inhibitors. When adequate RNA transcriptase is produced, an antisense version of HCV RNA is made to serve as a template for RNA replication. The enzyme NS3 helicase unwinds the RNA during replication, and NS5B, or the HCV RNA-dependent RNA polymerase, catalyzes RNA synthesis. Both are also targets for inhibitors. The newly produced RNA and processed proteins assemble to form viruses that travel to the inside portion of the plasma membrane and then exit the host cell.

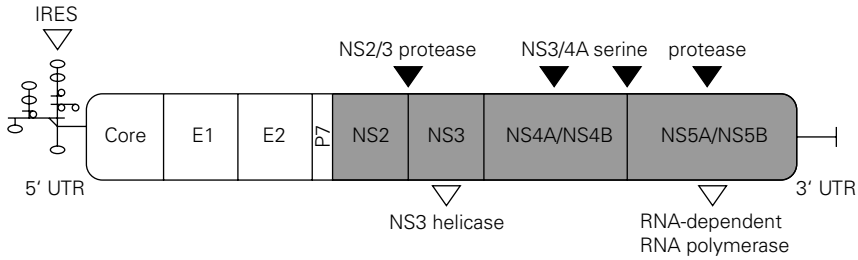


**Figure 34.2** Schematic representation of the hepatitis C genome. Unshaded region indicates the major structural proteins: core, envelope (E1 and E2) and p7. The shaded region indicates the non-structural proteins NS2, NS3, NS4A/NS4B, NS5A/NS5B. At the 5′ untranslated region (UTR) resides the internal ribosome entry site (IRES), which is a potential target of translation inhibitors such as antisense oligonucleotides and ribozymes. NS3 encodes a specific helicase, and the NS5A region encodes RNA-dependent RNA polymerase, both important in viral replication. Other potential enzyme targets include the HCV-specific proteases (NS2/3 and NS3/4), which are involved in processing the viral polyprotein at specific sites (closed arrows).

to interact directly with the pyrophosphate-binding site of the viral polymerases.

Use of high throughput screening and rational drug design has led to the identification of HCV RdRp inhibitors that belong to each of the above classes.[14] Several compounds inhibit HCV RNA replication in cell culture. Moreover, a few orally bioavailable inhibitors of HCV RdRp, such as JTK-003, JTK-109 and NM-283, are being studied in early clinical trials. However, their mechanism(s) of action remain unclear, and preliminary *in vitro* results suggest that resistance to RdRp inhibitors might occur in the clinical setting.

### *p7*

Although its function is partly unknown, p7 has ion channel activity,[15,16] and there is preliminary evidence that p7 localizes to plasma and endoplasmic reticulum membranes[17] and is necessary for HCV replication.[18] Long-alkyl-chain iminosugar derivatives, which have antiviral activity against bovine viral diarrhoea virus,[19] can inhibit HCV p7 ion channels.[16] Therefore, such iminosugar derivatives that have low toxicity profiles in animals might in theory be used in treating chronic hepatitis C. One such compound is currently being tested in a phase 2 trial.

### *Ribozymes*

Ribozymes, catalytic RNA molecules that cleave specific RNA sequences, represent another potential mechanism to interrupt HCV genomic replication. Ribozymes contain a catalytic core region flanked by binding arms with nucleotide sequences complementary to the target RNA. An HCV-specific ribozyme was developed to treat chronic hepatitis C: Heptazyme™, a synthetic, stabilized 33-mer that is chemically modified for resistance to enzymatic and chemical degradation. In cell culture, Heptazyme selectively cleaves hepatitis C RNA within the IRES, significantly inhibiting viral replication.[20] In a phase 2 clinical trial, Heptazyme given alone led to reduced serum HCV RNA concentrations in 10% of patients.[21] Unfortunately, further development has been halted because of an observation in toxicology studies in animals. Instead, a product with an improved therapeutic index for stability and targeting to specific tissues is being developed.

## Protein translation

### *Antisense oligonucleotides*

Viral genomes contain numerous unique nucleic acid sequences that are drug targets because they are not present in the human genome. Because of the high affinity and selectivity of nucleic acid hybridization, antisense oligonucleotides can be used to develop highly specific drugs. Several antisense oligonucleotides inhibit the translation of HCV RNA in cell-free systems and cell culture models.[22,23] Among them is ISIS 14803, a 20-nucleotide antisense oligodeoxynucleotide that is complementary to the IRES surrounding the translation initiation codon. ISIS 14803, which is in clinical development, decreases HCV RNA and protein levels in various *in vitro* and *in vivo* models through RNase H cleavage of HCV in heteroduplexed regions of oligonucleotide and genomic RNA. In two clinical studies of ISIS 14803 monotherapy, plasma HCV RNA concentrations were reduced in three of ten patients treated at 2 mg/kg (–1.3 to –2.2 $\log_{10}$) and in six of twenty patients dosed twice weekly at 6 mg/kg (–1.0 to –3.8 $\log_{10}$).[24,25] However, patients with and without plasma HCV RNA reductions had transient asymptomatic alanine aminotransferase (ALT) flares 1–30 times the upper limit of normal range. The mechanism(s) by which HCV RNA reductions and the ALT elevations occurred is unclear, and further studies are required.

### *RNA interference*

Another potential therapy involves using RNA interference (RNAi). RNAi is a process in which cells downregulate gene expression through destruction of a specifically targeted mRNA.[26] The RNAi process is mediated inside the cell by a naturally occurring protein complex that uses double-stranded RNA as a molecular guide to downregulate expression post-translationally. In human cells, small interfering RNA (siRNA) are biologically active short fragments of 20–23 residues. Stabilized siRNA and eiRNA (expressed interfering RNA) compounds are currently being evaluated in the preclinical setting for their potential inhibitory activity of HCV genes. In a human hepatoma (HepG2) cell line, iRNAs targeted against specific sites in the HCV genome dramatically reduced virus-specific protein expression and RNA synthesis.[27,28] Similarly, recent data indicate that viral vectors can be used successfully for transducing HCV-specific siRNA. The latter approach effectively inhibits HCV replication in Huh-7 cells.[29,30]

### *Small molecules*

The increased understanding of the atomic structures of the 5′ and 3′ non-coding RNA segments[31–33] should aid rational design of small molecules that bind specifically to structures within these regions and that could serve as inhibitors of translation or of translation initiation and/or HCV RdRp recognition. In both cases, the target would be a relatively large RNA structure that most probably has multiple contacts with macromolecular protein assemblies, such as the 40S ribosome

subunit or the replication complex.[31,33] For example, small RNA molecules corresponding to the different stem-loop domains of the HCV IRES efficiently inhibit HCV IRES-mediated translation.[34] Whether small molecules could effectively disrupt the resulting high affinity RNA–protein interaction remains under investigation.

## Post-translational modification

### *NS3 serine protease*

The non-structural (NS) viral protein 3 encodes a multifunctional protein that contains a serine protease (NS3 protease) in the N-terminus. The NS3 protease requires the relatively small NS4A sequence as a co-factor for proteolytic activity and mediates essential polyprotein processing through cleavage of junctions NS3–NS4A, NS4A–NS4B, NS4B–NS5A and NS5A–NS5B. NS3 cleaves an initially synthesized viral polyprotein into functional proteins. The NS3 protease structure has been recently determined by X-ray crystallography.[6]

A number of peptide-based or peptidomimetic inhibitors of NS3 serine protease have been developed and tested *in vitro*. Most fall into one of three classes: (1) substrate analogues, (2) serine-trap inhibitors (or transition-state analogues), or (3) product analogues.

Efforts to discover non-peptide inhibitors have also been made.[8] BILN 2061 is a small, selective and potent inhibitor of the NS3 serine protease.[35] Inhibitor constant values of 0.30 nM and 0.66 nM with a non-covalent, competitive mode of inhibition were obtained for genotypes 1a and 1b, respectively. BILN 2061 retains its inhibitory efficacy in human cells and showed low nanomolar inhibition of HCV RNA replication through blockade of the NS3 protease-dependent polyprotein processing. In early clinical development studies, BILN 2061 was administered for 48 hours to HCV-infected patients. Administration resulted in a rapid, dose-dependent HCV RNA decrease up to 4 $\log_{10}$ that happened within 2 days for the highest doses. Within a week after treatment withdrawal, HCV RNA concentrations progressively returned to baseline.[36,37]

### *NS2/NS3 protease*

The NS2/NS3 protease is a zinc-dependent metalloprotease that cleaves the non-structural proteins between the NS2 and NS3 polypeptides. This region overlaps with the NS3 serine protease. The search for inhibitors of the NS2/NS3 cleavage reaction has been hampered by the hydrophobic nature of the protein and by the autocatalytic nature of the cleavage.

## Virus attachment and entry into cells

The mechanism by which HCV enters target cells is currently unknown. The envelope glycoproteins E1 and E2 probably determine selective interaction of HCV with specific cell-surface receptors. E1 and E2 are released from the viral polyprotein by signal peptidase cleavages and expressed as non-covalent heterodimers at the surface of viral particles. E2 is thought to initiate virus attachment, and E1 has been hypothesized to be responsible for viral and cellular membrane fusion.[38] An early interaction of envelope glycoproteins with glycosaminoglycans has been suggested to play a role in cell recognition and tropism.

Truncated soluble versions of E2 can bind specifically to human cells. The truncated versions have been used to identify interactions with tetraspanin CD81,[39] with scavenger receptor class B type I,[40] and with dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule 3-grabbing integrin (L-SIGN).[41] The low-density lipoprotein (LDL) receptor is also an entry point for HCV *in vitro*.[42] Whether these ligands are receptors for HCV infection *in vivo* and could be targets for future therapies is currently unknown. A recently described *in vitro* interaction between cholesterol metabolism and HCV replication that was inhibited by 3-hydroxy-3-methyglutaryl (HMG) CoA reductase inhibitors highlights the potential importance of these interactions and the potential of future metabolic pathways to help in designing drugs effective against HCV.[43]

Testing inhibitors of cell entry has been limited by a lack of conventional cell culture systems for HCV. A recent effective approach is to generate infectious HCV pseudoparticles expressing HCV glycoproteins on the surface of another virus.[44,45] This system should help dissect the early events of HCV infection, identify novel HCV receptors or co-receptors, and test alternative therapeutic approaches based on inhibiting viral entry. The system could also be used to assess neutralizing responses *in vitro* and to test therapies based on viral neutralization, such as specific anti-HCV immunoglobulins.

# Host immune responses and immune therapies

The cellular immune response plays a major role in HCV infection. Vigorous and multi-specific CD4+ and CD8+ T-cell responses during acute hepatitis C are associated with viral clearance and recovery.[46] Memory T-cell responses specific to HCV are detectable in the peripheral blood for decades[47] and can mediate rapid viral clearance upon re-exposure.[48,49] In contrast, insufficient T-cell

response, particularly in combination with viral mutations, is associated with persistent infection and chronic hepatitis.[50] Once infection is persistent, typically the number of HCV-specific T cells is low and their proliferative, cytokine and cytotoxic effector functions appear to be impaired.[51] Whether antiviral therapy can lead to reconstitution of cellular immune responses is unclear.[52] Nonetheless, the cellular immune response remains an attractive target for therapeutic intervention.

## Non-specific immune responses

### *Interferons*

Human interferons (IFNs) are classified based on the cell surface receptor that they bind. Type 1 IFNs bind to the IFN-α heterodimeric receptor IFNAR1/IFNAR2 and include the 21 non-allelic subtypes of IFN-α, IFN-β, IFN-ω and IFN-τ.[53] IFN-γ, a type 2 IFN, binds a unique cell surface receptor and has antiviral, antifibrotic and immunomodulating activity that stimulates the T-helper (Th) 1 response.[54] Three novel cytokines share sequence identity with type 1 IFNs and bind to a novel cell surface receptor. These putative type 3 IFN molecules, called interleukin (IL)-28A, IL-28B and IL-29, are induced by viral infections and have marked antiviral activity *in vitro*.[55]

α-IFNs have been used for more than a decade to treat patients with hepatitis C. Yet IFNs can have poor pharmacokinetic profiles, limited biological activity and suboptimal therapeutic indices. And knowledge is limited as to the efficacy of non-α, non-β IFNs. Several groups have modified naturally occurring IFNs to improve their performance. Modifications include altering the primary amino acid sequences, adding polyethylene glycol (PEG; i.e. pegylated IFNs), altering glycosylation patterns and making fusion proteins. Consensus IFN-α (IFN-alfacon1) is an engineered second-generation cytokine that contains the most frequently occurring amino acids of the non-allelic IFN-α subtypes. IFN-alfacon1 is more effective than naturally occurring type 1 IFNs in cell culture models and equally effective in clinical trials.[56,57] Other novel α-IFNs have been produced by shuffling the family of 20 human IFN-α DNA encoding sequences.[58] Gene shuffling has led to the production of a novel non-naturally occurring type 1 IFN with a 285 000-fold increase in antiviral activity compared with IFN-α-2b. Whether this highly active IFN can be used to treat chronic hepatitis C is unknown.

Adding PEG to therapeutic IFN-α proteins can dramatically increase plasma exposure following dosing and lead to increased response rates.[59,60] An IFN-β molecule conjugated to a linear 20-kDa PEG molecule[61] is entering clinical trials for treating chronic hepatitis C. A pegylated IFN-alfacon1 will also be clinically evaluated. A fusion protein of IFN-α-2 and human serum albumin, Albuferon, has entered clinical trials.[62] Albuferon has similar *in vitro* antiviral and antiproliferative activity to unmodified IFN-α-2, but with markedly improved pharmacokinetics. Several new techniques for selecting second-generation IFNs have produced molecules that are even more potent. Clinical trials of these molecules are either underway or planned. Lastly, additional naturally occurring IFN species, such as IFN-γ and IFN-ω, are being studied in clinical trials.

### *Oral IFN inducers*

Oral IFN inducers have the potential to generate an effective immune response by inducing or modulating cytokine responses at the site of infection, or by supplementing or replacing parenteral administration of IFN. Many have attempted to identify inducers of both IFN and other innate immune responses, but the central challenge in using such agents as oral therapy against chronic HCV infection has been delivery of effective doses to the liver. Agents known to induce IFN-α and other cytokines include (among many) relatively high molecular weight agents such as double-stranded RNA (poly I:C) and CpG oligonucleotide derivatives. Low molecular weight molecules may have useful immunomodulating properties but with a reasonable probability of oral absorption. Of the multiple candidate compounds, few have progressed to clinical trials for chronic viral hepatitis. Two chemical classes that are more advanced include the imidazoquinolones imiquimod and resiquimod[63] and the nucleoside analogues ANA245 and ANA971.

Imiquimod is approved for use as a topical agent in dermatology. Toxicity, probably resulting from cytokine induction, was reported in humans.[64,65] Resiquimod is currently in phase 2 studies for chronic viral hepatitis. ANA245 is a low molecular weight nucleoside analogue. There is preliminary evidence that ANA245 induces multiple cytokines, including IFN-α, and has immunologically mediated antiviral activity in a range of viral infection models. However, ANA245's oral bioavailability is limited at high doses. Preliminary evidence also indicates that ANA971, a novel molecule, efficiently delivers ANA245 to the plasma of various animals at concentrations associated with antiviral effects. As a component of the development programme for ANA971, studies in humans are underway to characterize the safety and pharmacokinetics of ANA245 after intravenous administration. If efficient, these drugs could be used in combination with other antiviral drugs to maximize durable responses.

### *Ribavirin-like molecules*

Ribavirin is a synthetic nucleoside resembling guano-

sine. As a monotherapy, ribavirin is ineffective in inducing sustained viral clearance, but it significantly enhances the sustained viral clearance rate after IFN therapy. The mechanism by which ribavirin enhances IFN efficacy is unknown, but four mechanisms have been proposed: immune-mediated activity on the host Th1/Th2 balance, inhibition of the inosine monophosphate dehydrogenase (IMPDH) activity, weak inhibition of the viral RdRp and induction of RNA mutagenesis.[66] Haemolytic anaemia is a frequent side-effect that limits ribavirin dosing and underscores the need for alternative molecules with similar mechanisms and efficacy but reduced toxicity.

Levovirin is the L-sugar analogue of ribavirin and has similar Th1/Th2 immunomodulatory activity. However, as an L-isomer, it does not inhibit IMPDH or accumulate in erythrocytes, the mechanism responsible for haemolytic anaemia.[67] In preclinical studies, levovirin was well tolerated by animals and did not have mutagenic effects in conventional short-term *in vitro* and *in vivo* assays. In humans, the drug is orally absorbed and well tolerated. Further development of this drug has been curtailed because of issues related to absorption and delivery of sufficient intracellular drug to hepatocytes.

Viramidine, a liver-targeted prodrug of ribavirin, is the amidine version of ribavirin, which is converted by adenosine deaminase (ADA) to ribavirin.[68] Oral dosing leads to preferential viramidine delivery to the liver because the liver is rich in deaminases. Viramidine is thus converted to ribavirin and its phosphorylated metabolites and preferentially retained in the liver rather than other tissues, including erythrocytes.[67] Rodent and chimpanzee experiments confirmed that viramidine targets the liver, and early studies suggest acceptable safety, pharmacology and toxicology profiles. In phase 1 studies, viramidine's profile of adverse events was similar to ribavirin's.[69] However, the extent of haemoglobin decline with the highest dose was lower than the haemoglobin drop with conventional combination therapy. A phase 2 proof-of-concept study of viramidine in combination with pegylated IFN-α (PEG-IFN-α) is underway. Preliminary results from this study in 180 patients indicate similar antiviral efficacy at week 24 of therapy compared with the PEG-IFN/ribavirin combination, and significantly less anaemia. Final results of this trial are now awaited, and a phase 3 programme is underway to evaluate this strategy.

IMPDH inhibitors (one of the putative mechanisms of action of ribavirin), such as mycophenolic acid (Cellcept) and VX-497,[70] are also currently being studied in patients with chronic hepatitis C. As with ribavirin, preliminary results with these agents have shown no direct antiviral efficacy in short-term studies. Recent results from a small European study suggest a higher on-treatment response rate with a triple therapy strategy including VX497, which was not associated with an enhanced sustained response rate.[71] Further combination therapy studies with PEG-IFN and ribavirin in combination with VX 497 therapy in non-responder populations are now planned.

### *Other immunomodulatory drugs*

Several parenterally administered immunomodulatory drugs are currently being used in combination with IFN or PEG-IFN in clinical trials. They include (1) histamine dihydrochloride,[72] which inhibits phagocyte-derived oxidative stress and inflammation and is currently being studied in phase 2 trials in combination with PEG-IFN and ribavirin and (2) thymosin α-1, which promotes T-cell maturation and natural killer (NK) cells and differentiation of pluripotent stem cells. Preliminary results with thymosin α-1 and IFN-α were inconclusive. Thymosin α-1 is currently being evaluated in large phase 2 and 3 trials in combination with PEG-IFN-α.[73]

In a randomized controlled trial, IL-10, an anti-inflammatory cytokine, did show an improvement in histologic inflammation and ALT levels that was associated with a proviral effect, despite a positive antifibrotic effect in an initial pilot study.[74,75] In a phase 2 study of IL-12, a different and proinflammatory drug, the results suggested that IL-12 was associated with additional toxicity and lacked efficacy.[76,77]

## Hyperimmune anti-HCV immunoglobulins

It has been hypothesized that multiple infusions of hyperimmune anti-HCV immunoglobulins (HCIg) may modify viral replication and the clinical course of HCV infection. Experiments with chimpanzees have provided compelling evidence that infection of susceptible animals can be prevented by neutralizing the epitopes located in the hypervariable region 1 of the HCV envelope gene.[78,79] Therapeutic HCIg could also be particularly valuable in preventing recurrent hepatitis C in HCV-infected liver transplant recipients. A polyclonal HCIg has been prepared from virus-inactivated, HCV RNA-negative, 5% IgG from 460 anti–HCV-positive plasma donors (Civacir™). In three experimentally and chronically infected chimpanzees, passive transfer of the HCIg decreased ALT concentrations. In two of the three it decreased HCV RNA concentrations once the level of passively transferred anti-HCV E2 reached a plateau. When HCIg infusions were stopped, both markers returned to baseline.[80] In three other HCV-inoculated chimpanzees, multiple infusions of HCIg prevented acute hepatitis and significantly shortened the length of HCV viraemia compared with animals treated with immunoglobulin preparations without anti-HCV. In two animals in the HCIg group, HCV RNA disappeared from serum after

a significantly shortened period of viraemia, but it recurred in one of the two when the level of anti-HCV E2 declined. Enzymatic and histopathological evidence of either acute or chronic hepatitis followed recurrent HCV viraemia.[80,81] The mechanisms by which HCIg may potentially affect the rate of HCV replication remain unclear.

### Therapeutic vaccines

A therapeutic vaccine capable of stimulating functional CD4+ and CD8+ T-cell responses in chronic carriers may also be beneficial. CD4+ and CD8+ T-cell responses are quantitatively weaker in the chronic phase of infection.[82] Recent qualitative data also suggest that HCV-specific CD8+ T cells lack effector function (secretion of antiviral cytokines and killing activity).[51,83] Various HCV recombinant polypeptide and plasmid DNA vaccines that have been tested in non-human primates can prime broad, functional CD4+ and CD8+ T-cell responses.

In a significant number of chimpanzees, a recombinant E1/E2 vaccine primed viral neutralizing antibodies and CD4+ T-cell responses and was more effective than a control in preventing infection or chronic illness.[84] Another vaccine, based on a yeast-derived fusion polyprotein comprising HCV genotype 1 NS3–NS4–NS5–core sequences combined with an immunostimulating complexes (ISCOMs) adjuvant, primed broad CD4+ and CD8+ T-cell responses in chimpanzees. This vaccine is now being tested for optimal formulations in early phase trials in chronic hepatitis C patients.

The cellular immune responses to the E1 envelope protein are frequently suppressed or absent in patients with chronic hepatitis C, but long-term responders to IFN-α therapy have on average higher levels of E1 antibodies.[85,86] A clinical grade HCV E1 protein produced and purified from mammalian cells has been evaluated in initial trials. In a phase 2a study, the vast majority of patients converted from a negative to a strong E1-specific T-helper response.[87] After the second course of E1 injections, levels of anti-E1 antibodies increased three- to fourfold, and the proportion of patients with a significant T-cell response to E1 increased from 9% to 91%. E1-treated patients also exhibited a decline in ALT relative to baseline, and in 38% of the patients liver fibrosis improved by one point or more. These changes were observed without any observed reduction in serum HCV RNA. Further clinical studies are now required to evaluate the long-term efficacy of this strategy.

## Progression of liver disease

If HCV cannot be eradicated, an alternative approach is to slow the progression of liver disease. Hepatic fibrosis is the major histological complication of chronic HCV infection, leading to cirrhosis within 10–50 years. During fibrosis progression, resident hepatic stellate cells are transformed from a quiescent to an activated state. This process is characterized by increased production of extracellular matrix, fibrogenesis and *de novo* expression of smooth muscle α-actin consistent with cellular transformation to myofibroblasts.[88–90]

Fibrogenesis after injury to the liver is characterized by significant increases in collagen (type I>III>IV) and other extracellular matrix constituents such as laminin, fibronectin and proteoglycans (dermatan sulfate, chondroitin sulfate, heparan sulfate).[88–90] This 'wounding' process involves matrix synthesis, deposition and degradation. Cross-talk between stellate cells and the extracellular matrix appears to play a critical role in fibrogenesis, as do a number of cytokines and small peptides, including transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), endothelin and angiotensin II.

Thus far, no drugs have been proven effective as hepatic antifibrotic agents in humans. The liver does, however, offer a unique advantage as a target for orally administered agents: those with efficient hepatic first-pass extraction will have inherent liver targeting, minimizing systemic distribution and non-liver adverse effects. It is uncertain whether antifibrotic therapies will require intermittent or continuous administration.

New antifibrotic therapies may be derived from at least three sources: (1) drugs indicated for other diseases; (2) drugs under development to treat other diseases; and (3) agents specifically developed for use in liver fibrosis. Unlike antiviral drugs, the efficacy of antifibrotics cannot be simply assessed. A clinical benefit may only appear after a prolonged treatment period. In addition, there are no established serum fibrosis markers that can substitute for percutaneous liver biopsies.

Nonetheless, several antifibrotic approaches are theoretically possible.

**Reduce** inflammation or the host response to avoid stellate cell activation. Ursodeoxycholic acid and antagonists to TNF-α may have some use in treating inflammatory liver disease.[91]

**Directly** downregulate stellate cell activation. The most practical approach is to reduce oxidative stress. Possible targets include anti-oxidants such as α-tocopherol (vitamin E);[92] the cytokines IFN-α and hepatocyte growth factor (HGF);[93,94] peroxisome proliferator activated nuclear receptors (PPAR), including PPARγ, through ligands (thiazolidinediones) that downregulate stellate cell activation.[95]

**Neutralize** proliferative, fibrogenic, contractile and/or proinflammatory responses of stellate cells. Many inhibitors of proliferative cytokines, including PDGF, FGF and TGF-α signalling through tyrosine kinase receptors, are undergoing clinical trials in other diseases. TGF-β

antagonists could have the dual effect of inhibiting matrix production and accelerating its degradation. Endothelin-1 antagonists have been tested as both antifibrotic and portal hypotensive agents.[96,97] Finally, halofuginone, an anticoccidial compound, has antifibrotic activity.[98]

**Stimulate** apoptosis of stellate cells. A recent study using gliotoxin reduced fibrosis in rats with liver injury due to $CCl_4$.[99] Additional targets may include TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) receptors.

**Increase** scar matrix degredation, either by stimulating cells that produce matrix proteases, downregulating their inhibitors, or directly administering matrix proteases. In addition to preventing new scarring, antifibrotic therapy will need to provoke resorption of existing matrix. Direct administration of metalloproteinase mRNA via gene therapy in animal models of hepatic fibrosis has confirmed that, in principle, matrix can be resorbed.[100]

## Conclusion

The World Health Organization has estimated that 3 to 4 million people worldwide are newly infected with HCV each year.[1] High quality, patient-centred research is necessary to develop improved therapeutics to treat the majority of hepatitis C patients effectively and economically, and to eventually develop preventative vaccines. To that end, many recent advances and insights into the molecular biology of HCV and the host immunopathogenesis of this disease will continue to help us understand the process of infection and to identify potential new targets for drug development. Such knowledge provides many opportunities to provide patients who have chronic HCV infection with newer, more effective and more individually targeted therapies.

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# Chapter 35
# Prevention

Sergio Abrignani, Grazia Galli, Michael Houghton

## Introduction

Although the transmission of hepatitis C virus (HCV) has declined substantially,[1] there is still a pressing need to develop an efficacious HCV vaccine. In fact, given that about 200 million persons worldwide harbour infection[2] and that certain modes of HCV transmission, such as injection drug use, will continue for the foreseeable future, the pool of asymptomatic chronic HCV carriers, who represent an infectious reservoir, will remain substantial. Furthermore, because of the high rate of chronic infection following acute HCV infection and the limited efficacy of the therapies currently available, prevention of new infections would be a cost-effective strategy for control of the disease.

An efficacious HCV vaccine would be of potential benefit to all persons at risk of coming in contact with contaminated blood. This includes health-care workers, haemodialysis patients, those with diseases requiring frequent blood products and intravenous drug users (IDUs). Considering intravenous drug use, an HCV vaccine might be broadly given to adolescents considered at risk for later drug use. Although the risk of sexual transmission of HCV is low, it may be reasonable to recommend vaccination to the sexual partners of infected individuals. Eventually, a safe and efficacious HCV vaccine could be recommended for widespread general use.

## What should an HCV vaccine protect against?

An HCV vaccine should be capable of either preventing infection altogether or preventing the development of chronic infection following acute infection. Although preventing initial infection by providing 'sterilizing immunity' would be ideal, this may be difficult to achieve. As the great majority of acute infections are asymptomatic and without clinical consequences, and the clinically relevant disease is due to the ensuing chronic HCV infection, a vaccine that allowed only a 'transient infection' (either subclinical or of limited acuity) while preventing the development of chronic HCV infection could be as beneficial as one that provided sterilizing immunity. Theoretically, a vaccine that did not accomplish either effect could still be beneficial if it prevented or delayed the development of progressive liver disease, such as cirrhosis.

An essential requirement for an HCV vaccine is that it must protect against the major circulating genotypes. An HCV vaccine that demonstrated narrow genotype-specific efficacy might require geography-specific limitations in its use and would be difficult to use in practice. Eventually, an efficacious HCV vaccine might exhibit a gradation of efficacy such that it might prevent infection by heterologous subtypes at a lower but still clinically significant level compared with more closely related subtypes.

## How difficult will it be to develop an HCV vaccine?

To frame and estimate problems in developing an HCV vaccine, it is interesting to compare the similarities and differences faced in developing an HIV (human immunodeficiency virus) vaccine. Both HCV and HIV infections exhibit a propensity towards chronic infection. However, while spontaneous resolution of HIV infection has not been found to occur, about 25% of HCV infections spontaneously resolve,[3] presumably due in part to activation of host immune defence mechanisms capable of clearing infection. Thus, it may be easier to protect against HCV if the types of immune responses occurring in natural infection that lead to clearance can be stimulated. Both viruses exhibit marked genetic and phenotypic heterogeneity with regions that are relatively conserved or hypervariable. Our current limited understanding of the critical correlates of protection for both HCV and HIV infection is a major challenge in vaccine development for both diseases. However, an HCV vaccine does not have to overcome additional problems faced in developing an HIV vaccine. Mucosal transmission via sexual activity predominates in HIV infection but is uncommon in HCV infection. HCV does not exist

in a latent integrated proviral state as is the case with HIV.

The problems that arise in designing an HCV vaccine because of the peculiarity of HCV are as follows. Firstly, HCV is a relatively low viraemic virus *in vivo*, and is only readily detected as RNA by polymerase chain reaction (PCR). Secondly, the only species that can be infected by HCV are humans and chimpanzees. Finally, the virus does not replicate efficiently *in vitro* and, therefore, little is known about the appearance of HCV.

## Virology of the hepatitis C virus

Hepatitis C virus (HCV) is classified as a member of the Hepacivirus genus within the Flaviviridae family[4] and is a positive-stranded RNA virus with a genome of approximately 10 000 nucleotides.[5] The genome encodes a polyprotein precursor of about 3000 amino acids (aa) that is cleaved co-translationally and post-translationally to yield a variety of structural and non-structural (NS) proteins (Fig. 35.1).[6]

Translation is mediated through an internal ribosome entry site (IRES) situated within the 5′ untranslated region (UTR) of the RNA genome.[7] Recently, a –2/+1 ribosomal frame shift has been observed to occur around aa 11 of the C protein, resulting in the synthesis of the F protein. The sequence of the F protein is partially conserved within the genus but its function is unknown.[8,9] However, the basic charged character of the F protein may also imply a nucleocapsid-like function.

As large-scale purification of the virus is not feasible, the information on the structure of the virion is largely derived from expression studies with cloned cDNAs. Such studies have revealed co-translational processing of the polyprotein precursor via host signalase to yield the presumptive structural proteins (the nucleocapsid (C) and envelope glycoproteins gpE1 and gpE2).[10–12]

The two structural proteins gpE1 and gpE2 have been shown to translocate into the lumen of the endoplasmic reticulum (ER), where they remain tightly anchored in the form of a non-covalently bound heterodimer.[13–15] Deletion of the C-terminal transmembrane anchor regions (TMR) of either gpE1 or gpE2 results in secretion of the respective ectodomain from cDNA-transfected cells via the Golgi apparatus, although this is an inefficient process in the case of gpE1.[11,16] The nucleocapsid is also cleaved by host signal peptide peptidase to yield a smaller species (p19 or p21), which appears to be the form found within virions circulating in the bloodstream.[17,18] The C protein has the ability to self-associate into core particles[19] and is phosphorylated.[20]

A large variety of NS proteins are thought to be processed downstream of the structural protein region (Fig. 35.1).[6] p7 is processed through the action of host signalase,[21] and in the related pestiviruses it has been shown to be required for virion assembly and/or secretion.[22] Further downstream proteins are processed from the precursor polyprotein through the action of viral-encoded proteases encoded by NS genes 2, 3 and 4a.[23–25] The NS proteins associate together on the ER, to form a viral replication complex, and various individual functions have been assigned (Fig. 35.1). The 3′ UTR region comprises a 3′ terminal 98 nt region that is highly conserved, which is located immediately downstream from a short pyrimidine-rich tract. This pyrimidine-rich tract is preceded by a more variable region, immediately downstream of the polyprotein precursor terminator codon.[26,27]

As RNA viruses encode error-prone RNA replicases that lack the proofreading abilities of host DNA polymerase, the HCV genomes and encoded proteins are highly

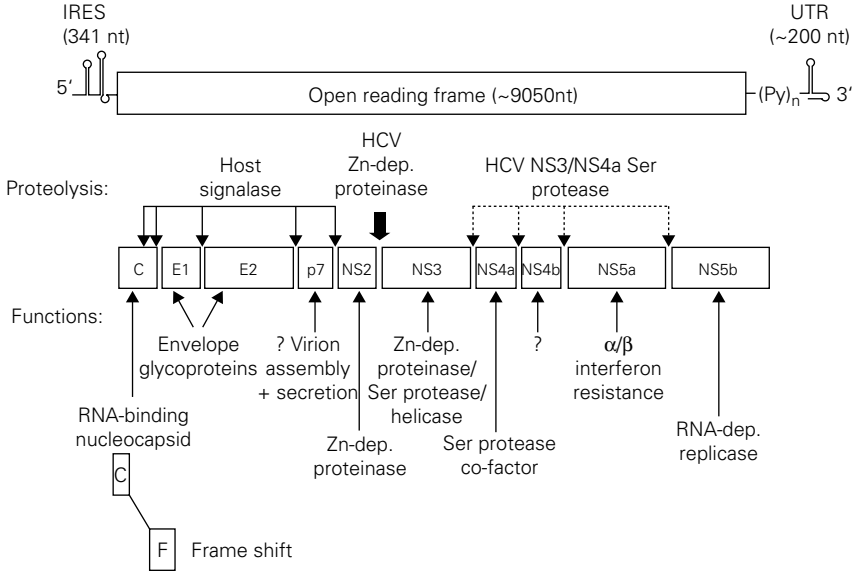


**Figure 35.1** Organization of the HCV genome and encoded proteins.

variable. The 5′ and 3′ UTRs are highly conserved, as is the C gene and encoded nucleocapsid protein, but the rest of the viral genes/proteins exhibit considerable heterogeneity.[28] In addition, the N-terminal 30 aa of gpE2 (E2HVR1) is hypervariable and, using the chimpanzee infection model, it has been shown to contain epitopes binding viral-neutralizing antibodies that are under considerable selective pressure.[29–34]

At least six different basic HCV genotypes have been distinguished phylogenetically along with numerous subtypes (Fig. 35.2),[35] with type 1 being the most common genotype in the United States, China, Japan and Europe (Fig. 35.3).[36] Unfortunately, due to the lack of an *in vitro* virus-neutralizing antibody assay, the number of serotypes is unknown. Recent progress in developing mouse infection models may facilitate further studies in this important area.[37]

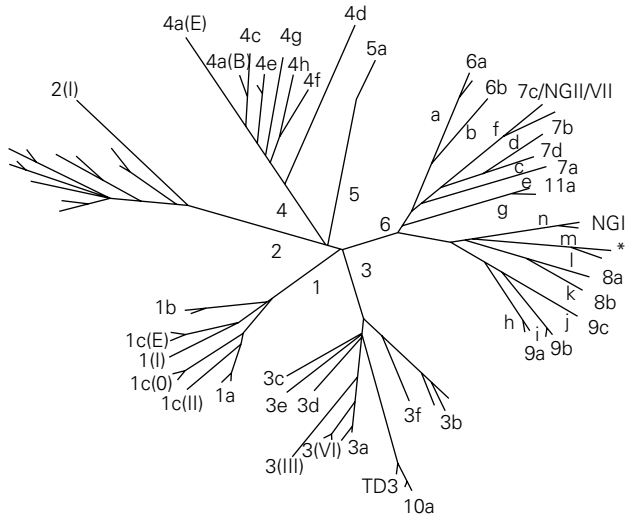


**Figure 35.2** Phylogenetic analysis of nucleotide sequences from part of the HCV NS5b region amplified from HCV-infected blood donors and patients from several countries (from Simmonds[35]).

## Immune correlates of protection

Recent studies in the chimpanzee challenge model and of multiply exposed humans have demonstrated that there is significant natural immunity against HCV. In one chimpanzee study,[30] an animal was infected by intrahepatic administration of an infectious RNA derived from HCV strain HCV-1 (of the 1a subtype). This subtype is the most common clade in the United States (Fig. 35.3). Following resolution of the ensuing acute infection and disappearance of viraemia, the animal was shown to be resistant to an intravenous rechallenge with homologous virus. No viraemia was observed following rechallenge, indicating that sterilizing immunity was generated by the original infection. When rechallenged subsequently with a heterologous 1a strain, the animal experienced very transient, minimal viraemia, while control naïve animals showed substantial viraemia. Resolution of the infection in the rechallenged animal was confirmed by showing the absence of viral RNA from the blood and liver. Furthermore, when rechallenged again with a heterologous 1b strain, the most common clade worldwide (Fig. 35.3), only a transient viraemia was observed prior to disappearance of the virus from plasma and the liver.[30] Very similar results were obtained in separate chimpanzee studies in which animals were challenged and then rechallenged intravenously with different infectious viral inocula. Only transient viraemia occurred when animals that recovered from a first 1a strain infection were rechallenged with either a heterologous 1a strain or a heterologous 1b strain.[38,39] Additional chimpanzee studies indicate the existence of cross-protective immunity between HCV types 1 and 3 (A.J. Weiner, unpublished

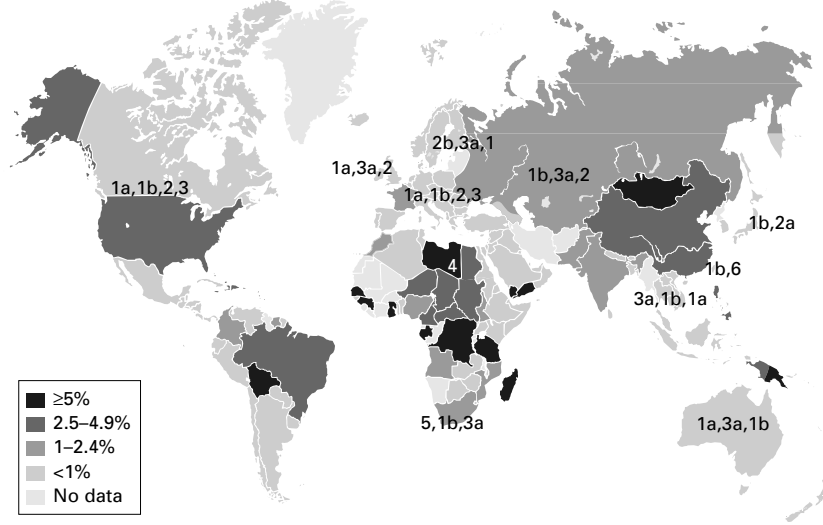


**Figure 35.3** Approximate HCV prevalence and genotype distribution (from Ebeling[36]).

observations, 2001). The latter HCV type is commonly found in IDUs.

Similar findings have also been reported in a prospective study of IDUs from the United States. Strikingly, the incidence of persistent viraemia in IDUs who had recovered from a previous infection was 12 times lower than that in IDUs who had not experienced a previous infection.[40] As seen in the chimpanzee studies, peak viral loads were substantially higher (by almost 2 logs) in the first-time infections as compared with the reinfections.[40] Also, HIV co-infection produced persistent HCV infection in all cases, indicating the role of the immune response in HCV recovery.[40] Intriguingly, it has now been shown that HIV patients co-infected with HCV have lowered peripheral immune responses to HCV as compared with patients with HCV mono-infections, even in the absence of severe CD4+ T-cell depletion.[41]

Collectively, these chimpanzee and human data provide evidence for the existence of significant immunity to HCV and, importantly, for the existence of cross-protective immunity within and between commonly occurring HCV clades. It is important to note, however, that not all reinfections in chimpanzees were resolved without progressing to chronicity (three of nine became chronically infected),[40] indicating that natural immunity to HCV is not complete and not as effective as for the hepatitis A and B viruses. It should also be mentioned that earlier studies in the chimpanzee model have concluded a lack of protective immunity to HCV.[42] This apparent contradiction may be due to the earlier studies measuring immunity more in terms of sterilizing immunity, rather than the ability to prevent the development of chronic infection, as in the more recent studies.

## Role of cell-mediated responses

It was first noted that early and broad major histocompatibility complex (MHC) class II-restricted CD4+ T-helper responses to HCV are associated with recovery from acute, symptomatic infections of man.[43,44] Subsequently, using the valuable chimpanzee model again, it was shown that recovery could occur in the absence of any antibody to the HCV envelope glycoproteins, but in the presence of an early and broad MHC I-restricted CD8+ cytotoxic T-lymphocyte (CTL) response to the virus.[45] This association of HCV-specific CD4+ T-helper and CD8+ CTL responses with resolution of acute infection has been confirmed in many human studies,[46–51] although the relative importance of each type of cellular immune response is unknown at present.

A recent study sheds further light on the potential mechanism of recovery from clinically asymptomatic, acute infections in man. Shortly after infection, activated HCV-specific CD8+ T cells appeared in the peripheral blood that were associated with an increase in serum alanine aminotransferease (ALT) levels (signifying liver damage), and with a small decrease in viral RNA levels. These activated CD8+ cells did not secrete gamma-interferon (IFN)-γ. Shortly afterwards, a 5 log reduction in viral load occurred commensurate with the appearance of HCV-specific, IFN-γ-secreting CD8+ cells with a non-activated phenotype (CD38-negative). This large reduction in viral load was not accompanied by an increase in serum ALT levels, suggesting that a non-cytolytic viral clearance mechanism mediated by IFN-γ might be operative (Fig. 35.4).[50,52] This conclusion is consistent with the demonstrated anti-HCV activity of IFN-γ in cell cultures containing HCV replicons.[53]

In contrast, individuals who develop chronic, persistent infection show weaker cellular immune responses to the viruses that are not maintained over time[46–50,54] and that have dysfunctional CD8+ T-cell effector functions.[55–57] The association of HCV-specific cellular immune responses with recovery is further reinforced by data from several studies showing that memory T-cell responses to the virus can be detected in long-term convalescent individuals,[54] in non-viraemic and HCV antibody-negative, healthy family members of HCV patients,[58] and in other individuals who lack HCV antibody and RNA but who may have been exposed to the virus earlier.[59]

The kinetics of induction of HCV-specific T-cell responses to multiple epitopes may be crucial in determining the outcome of infection, because the ability of the virus to mutate and thus evade CD8+ T-cell responses has been demonstrated convincingly in the chimpanzee model.[60] The latter study suggests that if a multi-specific T-cell response is made early in infection, then it is harder for the virus to mutate several epitopes simultaneously and, therefore, more likely to result in resolution of infection. Host and viral factors have been found to be involved in determining the breadth, strength, kinetics and decline[55,56] of HCV-specific cellular immune responses. In this regard, certain MHC class I and II alleles are associated with recovery in humans.[61–63]

## Role of the antibody response

The role of anti-envelope antibody in resolution of HCV infection is unclear at present. Initially, it was found that nearly all chronically infected humans have significant antibodies to gpE1 and gpE2, as measured in ELISA assays, and that there was not a clear relationship between the induction of these antibodies and resolution of acute infection.[64] These findings were extended in other studies showing that in man and chimpanzees, finding of anti-envelope antibody (as measured in ELISA assays) was more likely in chronically infected individuals than in those resolving the acute infection.[65–67] This lack of correlation between anti-envelope antibodies and immunity apparently contrasts with the situation found for many

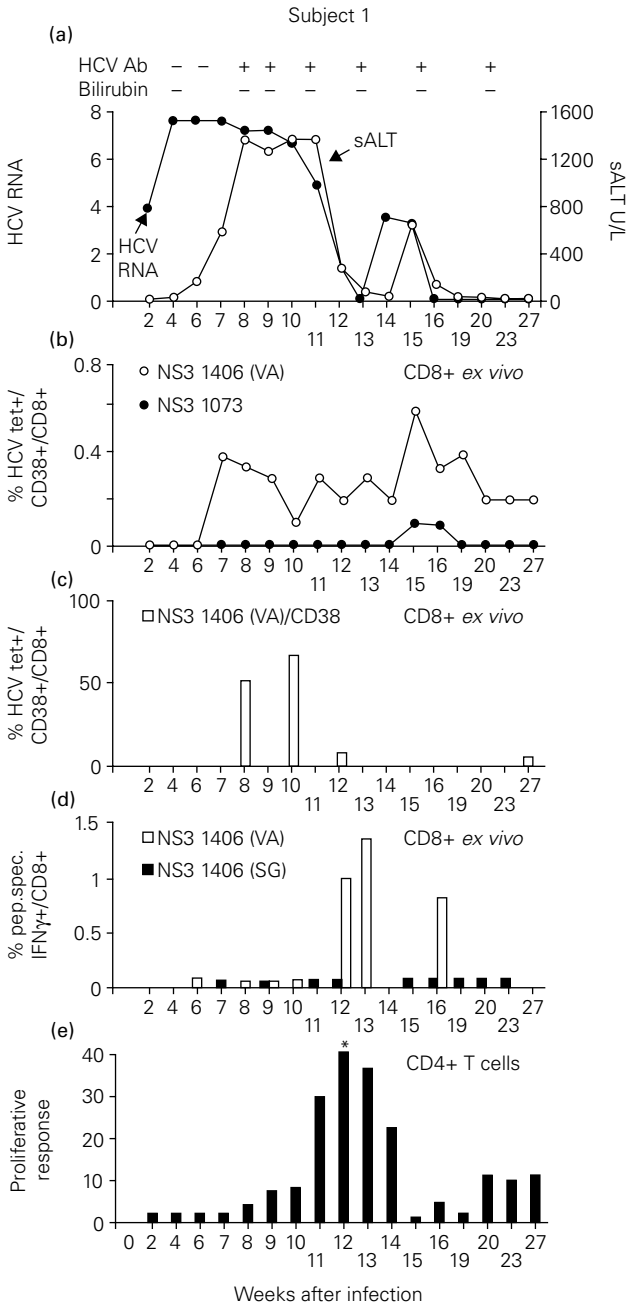


**Figure 35.4** HCV-specific T-cell responses in subject I during asymptomatic, resolving acute HCV infection. (a) Course of infection. (b) Percentage of CD8+ lymphocytes that were tetramer-positive at each time-point. CD8+ T-cell responses were tested directly *ex vivo* using HLA-A2 tetramers complexed with five different HLA-A2 restricted epitopes. CD8+ T-cell responses against two epitopes (NS3 1073 and NS3 1406 [VA]) were detectable. (c) Percentage of NS3 1406 (VA)-specific CD8+ T cells expressing the activation marker CD38. (d) Percentage of CD8+ T cells that produced IFN-γ in response to HCV NS3 1406 (VA) (black bars) and NS3 1406 (SG) (white bars). (e) Proliferative CD4+ T-cell responses against core, NS3, NS4 and NS5 are shown as the sum of all positive stimulation indices. *Sum of all specific stimulation indices is 56 (from Choo *et al*.[52]).

infectious agents, e.g. the hepatitis B virus (HBV).

It should be noted, however, that a true *in vitro*, viral-neutralizing antibody assay has not been developed due to the lack of a reliable cell culture system for HCV, and so the presence, level and affinity of neutralizing antibody cannot be easily monitored. However, the presence of neutralizing antibody has been demonstrated using the chimpanzee model. Serum derived from a patient was shown to be capable of neutralizing the infectivity of the virus taken from the same patient, 2 years earlier. Interestingly, the same viral inoculum could not be neutralized using serum from the patient obtained many years later.[68]

Additional evidence for the generation of neutralizing antibody comes from several studies showing the efficacy of human immunoglobulin preparations, derived from numerous donors, in preventing the transmission of HCV following blood transfusion[69] and liver transplantation,[70] and between sexual partners.[71] Chimpanzees that received human immunoglobulin prior to experimental viral challenge also showed a clear inhibition of acute hepatitis and viraemia throughout the lifetime of the antibodies.[72] Also, preliminary studies in humans have reported that the early induction of anti-E2HVR1 antibodies may correlate with resolution of acute infection.[73,74] Finally, it has been shown that antisera raised against the E2HVR1 can neutralize the infectivity of homologous virus in chimpanzees.[33] When combined with clinical evidence for worse HCV-associated disease in hypogammaglobulinaemic patients,[75,76] these data clearly suggest that HCV infection does induce neutralizing antibody.

Why, then, is there a lack of correlation between anti-envelope antibody and the outcome of infection? Several possibilities exist. Many studies have suggested that the E2HVR1 region mutates readily under the pressure of the specific humoral immune response, thus evading the binding of anti-E2HVR1 antibodies.[32,34,77] This might also explain the apparent protective efficacies of complex human immunoglobulin preparations, if they contain highly plural anti-E2HVR1 antibodies.

Another mechanism through which the HCV virus can escape the antibody response can result from its ability to bind low-density lipoproteins that can mask neutralizing epitopes.[78–80]

A third possibility is that HCV may be transmitted between adjacent hepatocytes because giant multinucleate hepatocytes are observed in the HCV-infected liver.

In conclusion, the role of anti-envelope antibody in the protective immune response is poorly defined at present, although it is most likely a complex situation. While it is well established that humans and experimentally infected chimpanzees can resolve acute infections in the absence of detectable anti-envelope antibodies, but in the presence of early and broad T-cell responses,

this does not rule out a role for neutralizing antibodies in the protective immune response in all individuals.

## Strategies for an HCV vaccine

An ideal HCV vaccine should prime cross-neutralizing anti-envelope antibodies and provide broad HCV-specific helper and inflammatory CD4+ T-cell responses, as well as HCV-specific CD8+ T-cell responses. Conventionally, it has proven difficult to achieve the latter through the use of soluble proteins combined with adjuvants, although there has been recent progress in this area using recombinant HCV proteins combined with the ISCOM adjuvant (a particulate adjuvant comprising cholesterol, phospholipid and naturally occurring saponins). Therefore, most activities designed to achieve the priming of CD8+ T cells to the virus have focused around the use of DNA vaccines, using either bacterial plasmids or various viral vectors. In this way, HCV proteins are synthesized endogenously within the cytosol, where they can then be processed within the proteosome and the resulting peptides are transported to the cell surface in association with MHC class I antigens, where they can stimulate CD8+ T-cell responses. In addition, some groups have also aimed at priming anti-envelope antibody responses via protein or DNA immunization involving either the gpE1/gpE2 heterodimer or either envelope glycoprotein alone. Other strategies have pursued the creation of a consensus E2HVR1 peptide that can prime broadly cross-reactive neutralizing antibody.

Due to the lack of a system for propagating HCV *in vitro*, the prospect of using a killed or attenuated HCV viral vaccine is not feasible at present.

### Vaccines based on adjuvanted, recombinant HCV proteins

A vaccine comprising the recombinant gpE1/gpE2 heterodimer combined with an oil/water microemulsified adjuvant has been tested for efficacy in the chimpanzee model.[5,52,81] The heterodimer was derived from HeLa cells infected with a recombinant vaccinia virus expressing a C–gpE1–gpE2–p7–NS2 gene cassette derived from the HCV-1 strain (1a clade). Generally, 30–40 μg of the subunits were administered intramuscularly (i.m.) in approximately months 0, 1 and 6, and a homologous viral challenge was given intravenously (i.v.) 2–3 weeks after the third immunization. Encouragingly, of seven animals receiving the vaccine, five were completely protected against the challenge, with no signs of viral infection in any of the assays, including sensitive RT-PCR assays for viral RNA (Fig. 35.5, middle panel).[52] In addition, the two chimpanzees that became infected following challenge eventually resolved the acute infection without becoming chronic carriers (Fig. 35.5, bottom panel).[52,81,82] In contrast, most control animals became chronically infected following viral challenge (Fig. 35.5, top panel).[52,81,82] The five apparently sterilized animals were the highest responders to the vaccine in terms of elicited titres of anti-gpE1/gpE2.[52] Sterilization did not correlate with antibody titres to E2HVR1 but did correlate with the titre of antibodies that block the binding of recombinant E2 to the CD81 ligand,[83] a tetraspanin that has been shown to act as *bona fide* HCV receptor.[84]

Given that the prime, practical goal of an HCV vaccine would be to prevent the development of chronic infection following exposure to the virus, this study provided much encouragement for the development of an effective vaccine.[52,81,82] This work has now been extended to address the key question of whether the vaccine protects against experimental challenge with a heterologous 1a viral strain. Initial data were derived from three vaccinees, previously protected against homologous viral challenge, that were then re-boosted with vaccine prior to rechallenge with the HCV-H strain (another member of the 1a clade common in the United States). While sterilization was not achieved, two of the three animals exhibited an amelioration of the acute infection followed by resolution.[81,82] Using additional chimpanzees, including five naïve animals that had not been challenged with HCV previously, it has now been shown that out of a cumulative total of 10 vaccinees challenged with the heterologous HCV-H strain, while all 10 animals experienced acute infections, only one developed chronic, persistent infection. In contrast, most control animals challenged with HCV-H became chronic carriers (seven of nine animals; $p = 0.005$; S. Abrignani and M. Houghton, unpublished observations, 2001).

Another group has also immunized a chimpanzee with insect cell-derived recombinant gpE1 and gpE2 (from HCV strain N2), in addition to a peptide spanning the E2HVR1 (from HCV strain 6). The two envelope proteins were each expressed and purified separately and lacked the C-terminal transmembrane anchors. The E2HVR1 peptide was conjugated to keyhole limpet haemocyanin and Freund's adjuvant was employed to augment immune responses. Their conclusion, from a series of immunizations and challenges with HCV strain 6 in the same animal, was that sterilization was dependent on high anti-E2HVR1 titres rather than anti-gpE2 or anti-gpE1 titres.[85,86] However, the outcome of challenging with other viral strains was not reported. It should also be noted that insect-derived gpE2 (truncated at the C-terminus) has been shown to bind poorly to a putative HCV receptor, CD81, as compared with gpE2 derived from mammalian cells.[83] However, the gpE1/gpE2 heterodimer is generally considered to be a more native reflection of the HCV virion than either ectodomain alone and, as mentioned above, has demonstrable prophylactic efficacy in

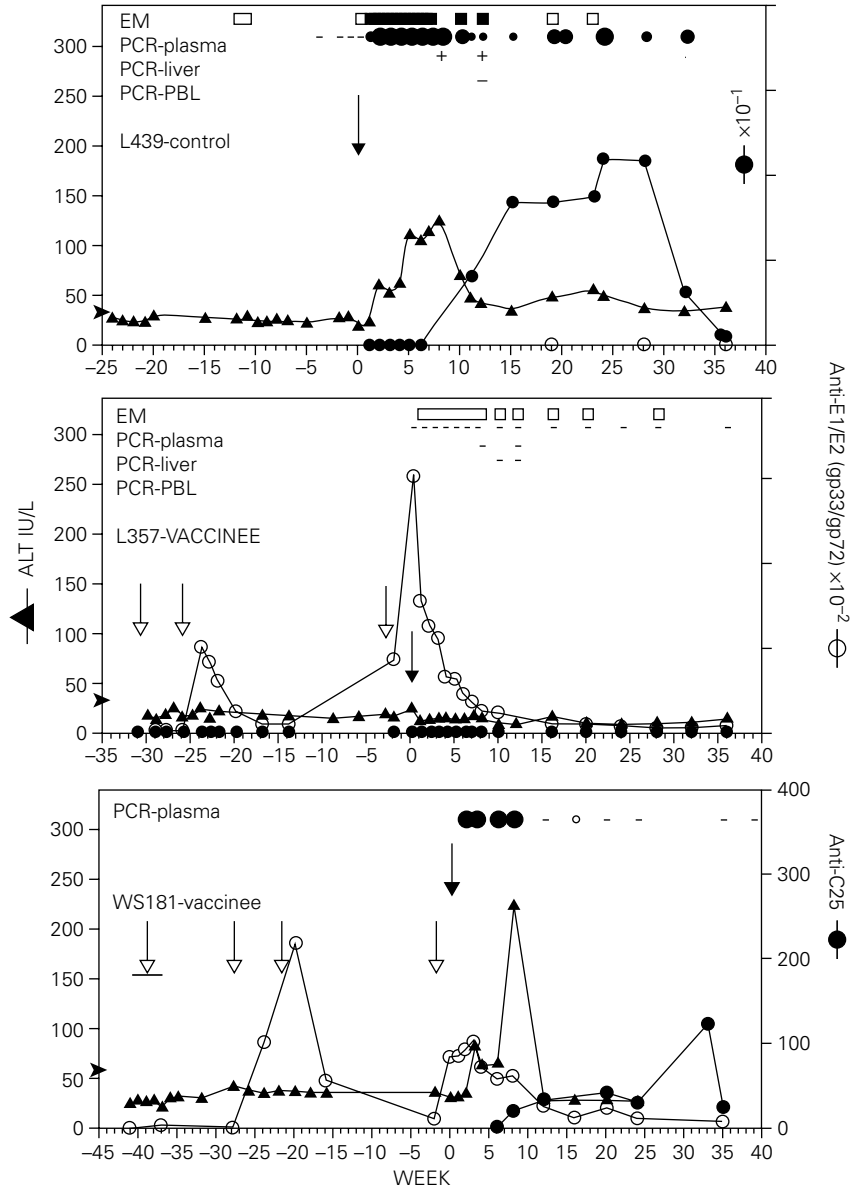


**Figure 35.5** HCV-1 challenge of vaccinated and control chimpanzees. Control and immunized chimpanzees (L, Laboratory for Experimental Medicine and Surgery in Primates; WS, White Sands) were challenged with about 10 $CID_{50}$ of HCV-1. The presence (solid boxes) or absence (open boxes) of hepatocyte ultrastructural changes observed with electron microscopy (EM) are indicated. The approximate relative levels of HCV-1 RNA detected in plasma using reverse transcriptase (RT)-PCR assays are reflected by the sizes of the shaded circles (open circles denote borderline positives; minus signs denote undetectable levels). The results of RT-PCR assays of liver and PBL (peripheral blood lymphocytes) extracts are recorded as either + or –. The arrows in the ALT axes indicate the mean + 3.75 SD of prechallenge values; open vertical arrows denote time of subunit vaccine administration, and the solid vertical arrow denotes viral challenge on week 0. IU/L, international units per litre (from Choo *et al*.[52]).

the chimpanzee. Therefore, recombinant gpE1/gpE2 remains an encouraging human vaccine candidate.

Some work has been directed to the synthesis of virus-like particles (VLPs) by expressing the structural genes in different cells. In insect cells, expression of the C–gpE1–gpE2 gene cassette has been reported to result in the generation of 40–60-nm VLPs within cytoplasmic cysternae.[87] Following partial purification, these VLPs appear to be immunogenic in small animals and, as such, represent a potential vaccine candidate for humans.[88] Similar-sized VLPs have been observed in the process of budding into the lumen of the ER, following expression of the same gene cassette in mammalian cells using a Semliki Forest viral vector.[89] HCV gene expression was observed to induce convoluted membranes or membranes similar to those seen in infected livers.[89] However, some viral or host factor is limiting the release of the VLPs into the lumen and from its secretion into the cell media.[89]

Because the E2HVR1 region of gpE2 has been shown to possess viral neutralizing epitopes and to be highly mutable, attempts have been made to select a cross-reacting version using phage display of mimotopes. Rabbit antiserum raised against one mimotype was highly cross-reactive with the E2HVR1 of many viral isolates and reacted to discontinuous epitopes.[90] Such mimotypes or consensus E2HVR1 peptides may be valuable components of an HCV vaccine. In this regard, a consensus E2HVR1 peptide sequence has been fused to the B subunit of cholera toxin and expressed in plants through the use of a tobacco mosaic viral vector. Crude extracts were administered intranasally to mice, which

then generated cross-reactive E2HVR1 antibodies that were capable of capturing virions.[91]

Recombinant C protein is also being studied as a potential component of an HCV vaccine for several reasons. Firstly, broad HCV-specific CD4+ and CD8+ T-cell responses, including those against the C protein, are associated with recovery from HCV infection.[45–48,50] Secondly, the C protein is the most conserved HCV polypeptide and contains CD4+ and CD8+ epitopes that are highly conserved among the different HCV genotypes, which should therefore facilitate the generation of cross-protective immunity.[92,93] Thirdly, recombinant C protein has been shown to self-assemble into particles[19] with concomitant high immunogenicity in animal models. Substantial CD4+ T-cell priming and high anti-C antibody titres have been obtained in mice and sheep.[93,94] Furthermore, through the use of the ISCOM adjuvant, it has been possible to prime strong CD8+ CTL activity (and CD4+ T-cell activity) in rhesus macaques, using a recombinant C antigen derived from *Escherichia coli* (Fig. 35.6).[95] Cytotoxic T-lymphocytes to at least two epitopes were identified. This approach is a promising and practical option for the development of an HCV vaccine capable of priming cross-reactive HCV-specific T-cell responses associated with protection.

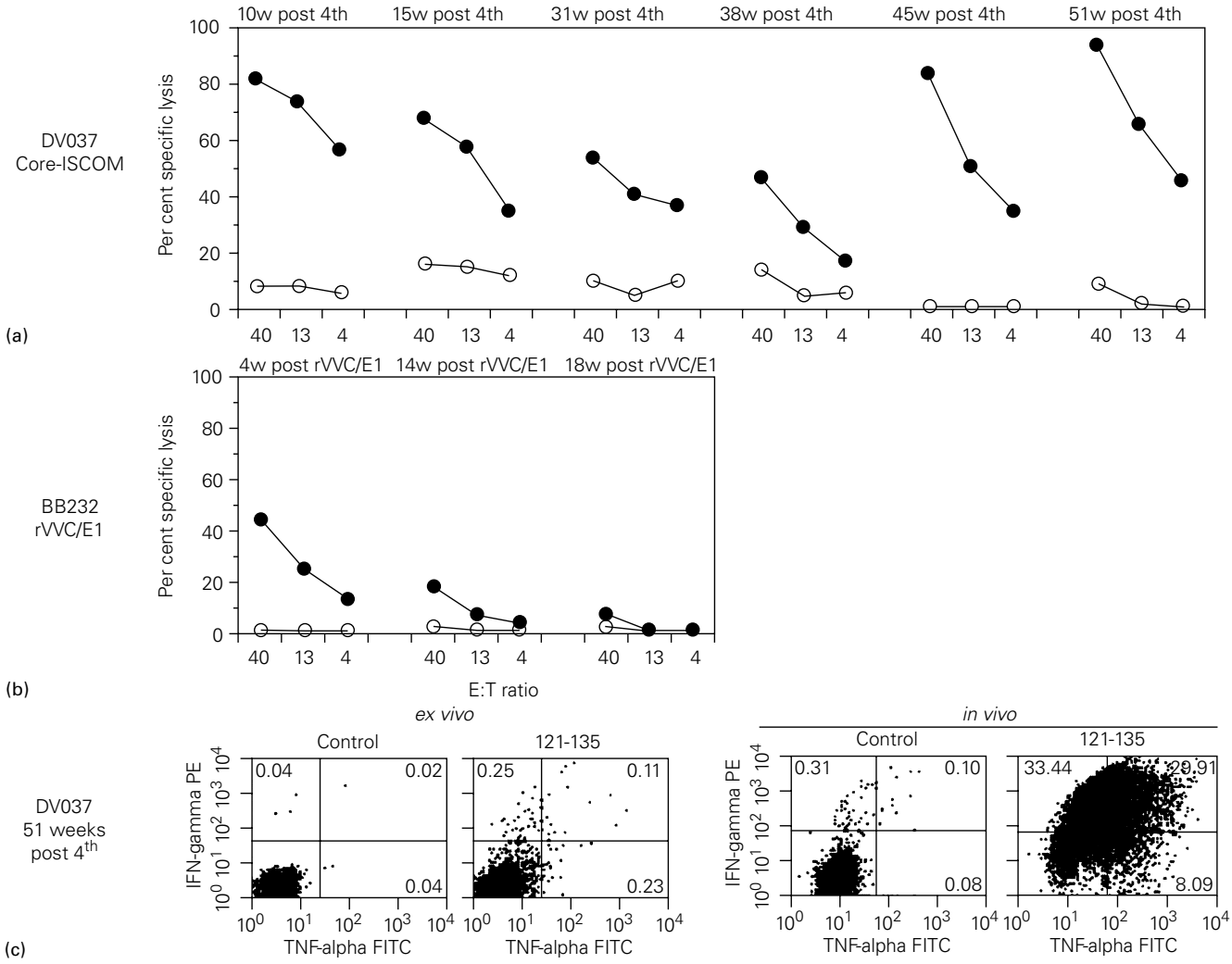


**Figure 35.6** Longevity of the CTL responses primed by vaccination with recombinant HCV core in ISCOMs adjuvant. Peripheral blood mononuclear cells (PBMCs) from rhesus macaques DV037 (a) and BB232 (b) were restimulated *in vitro* with the epitopic peptide 121–135. After CD8+ enrichment, cells were tested for cytotoxic activity against autologous B-LCLs (B-lymphoblastoid cell lines) sensitized with the epitopic peptide 121–135 (black circles) or an irrelevant peptide (open circles). (c) Freshly isolated PBMCs from DV037 51 weeks after its last immunization (two left panels) or *in vitro* restimulated PBMCs from the same time-point (two right panels) were restimulated for 12 hours with peptide 121–135 or a control peptide and stained for surface CD8 and intracellular IFN-γ and TNF-α. Lymphocytes were gated by side versus forward scatter light and then for CD8-PerCP. Plots show log fluorescence intensity for TNF-α-FITC and IFN-γ-PE (from Polakos *et al*.[95]).

There are numerous CD4+ and CD8+ epitopes that are conserved among the different HCV genotypes. These reside within the various HCV-encoded virion and non-structural proteins.[92,96,97] The inclusion of a multiplicity of these epitopes will facilitate the vaccine-mediated generation of broad cellular immune responses to the virus, which could then result in the elicitation of cross-protective immunity against different HCV genotypes. One such approach involves the assembly of 'HCV polytope vaccines' consisting of a consecutive sequence of these conserved HCV T-cell epitopes, in the form of a recombinant polypeptide or DNA vaccine. Focusing the immune response on a collection of highly conserved epitopes that can be presented by diverse human MHC class I and class II antigens may optimize the generation of strong, cross-protective immunity and possibly avoid the 'dilution' of the immune response to variable, more mutable HCV epitopes.

Other approaches being explored to generate cellular immune responses include the fusion of recombinant HCV proteins with bacterial pore-forming toxoids. Certain detoxified bacterial toxoids have the ability to induce pore-mediated endocytosis in antigen-presenting cells (APCs), thus resulting in the generation of strong CD8+ and CD4+ T-cell responses to the toxoid and fused HCV moiety.[98] Electrically mediated uptake of synthetic HCV peptides has also been reported to result in priming of reactive CTLs in electroporated BALB/c mice.[99] Host heat-shock proteins are known to be good mediators of CD8+ MHC class I-restricted immune responses to associated foreign protein sequences.[100] As such, this approach may be applicable to the development of HCV vaccines, provided that harmful, autoimmune responses are not generated. Another intriguing avenue of research involves the use of influenza-based virosomes to deposit recombinant HCV proteins or synthetic HCV peptides on the MHC class I pathway, resulting in the generation of viral-specific CD8+ CTLs. Comprising liposomes with influenza haemagglutinin and neuraminidase on the surface, these VLPs allow fusion with the membrane of APCs and the subsequent deposition of the HCV moiety in the cytosol. This then facilitates association of peptides with MHC class I molecules and TAP-mediated transfer to the surface of the cell where priming of reactive CTLs occurs.[101]

## Vaccines based on HCV DNA

The advantages of using a DNA vaccine relate to the ease and cost of manufacture, the gene-mediated synthesis *in vivo* of native and often complex protein structures (that would otherwise be difficult to produce in the form of recombinant proteins), good stability (that renders the use of DNA vaccines particularly suitable to the needs of developing countries) and the ready ability to employ multiple genes, gene cassettes or plasmids to elicit broad immune responses. Another important feature relates to the ability of DNA vaccines to stimulate CD8+ MHC class I-restricted CTL responses via the endogenous synthesis of proteins *de novo* in the cytosol. Disadvantages, however, can include a weaker potency as compared with other vaccine formulations as well as safety issues revolving around their potential to integrate into the host genome, thereby increasing the risks of mutagenesis and carcinogenesis.

In the case of HCV, relatively long-term expression of the C gene has been reported to exert multiple pathogenic effects in transfected cells and animals. Its role in inducing steatosis and hepatocellular carcinoma in transgenic mice has been documented,[102,103] as has its ability to co-promote cellular transformation *in vitro*.[104] There have been numerous other reports demonstrating the ability of the C gene product to bind to a member of the tumour necrosis factor (TNF)-α superfamily,[105] modulate apoptosis,[106] induce oxidative stress,[107,108] activate cellular and viral promoters,[109] and affect other regulatory functions.[110] While the pathogenic significance of these findings in infected humans, if any, remains to be determined, it may be prudent in the meantime to omit the C gene from a potential HCV DNA vaccine (although the intermittent use of a recombinant C polypeptide subunit vaccine is unlikely to pose such a safety risk). Similarly, the 5′ terminal region of the NS3 gene (encoding the protease domain) has been linked with transformation of cells and carcinogenesis in nude mice.[111]

Many studies have immunized mice with plasmids expressing the envelope genes of HCV, generally using the immediate-early CMV promoter and intron to achieve high expression levels. Accordingly, the generation of humoral and cellular immune responses to gpE2 has been widely reported, including cross-reactive anti-gpE2 antibodies between subtypes 1a and 1b.[112–118] The use of a DNA vaccine encoding intracellular forms of gpE2, rather than secreted forms, has been emphasized in order to elicit anti-gpE2 antibodies capable of preventing the interaction between gpE2 and CD81, the latter being a candidate HCV receptor.[119] Boosting DNA-primed mice with recombinant gpE2 has been reported to elicit higher anti-gpE2 titres than repeated DNA or protein immunizations.[120]

Immunogenicity of gpE2 DNA vaccines has also been demonstrated in rhesus macaques.[117] However, it must be underlined that small animals and even rhesus macaques are questionable models for immunogenicity of DNA vaccines in humans. Only one small HCV DNA vaccine study in the chimpanzee model has been reported so far. In an attempt to optimize immunogenicity, the ectodomain of gpE2 (aa 384–715) was fused to the CD4 C-terminal, transmembrane region (TMR) that facilitated sequestration of the encoded gpE2 glycopro-

tein to the outer cell surface, rather than being anchored in the lumen of the ER via the use of the homologous TMR. Ten milligrams of DNA were administered using a bioinjector into the quadriceps of two animals at weeks 0, 9 and 23, followed by experimental challenge with homologous, monoclonal virus 3 weeks later. Humoral and cellular immune responses to the vaccine were observed in only one animal but, following challenge, viraemia was lowered as compared with a control animal, and hepatitis occurred earlier, as a result of the primed immunity. Importantly, both vaccinees resolved their acute infections quickly, whereas the control unvaccinated animal became chronically infected following viral challenge. This result is promising, but further studies are warranted due to the small number of animals and because one of the vaccinees had already experienced an experimental HCV infection, prior to vaccination, that would have conferred immunity.[121]

In order to recapitulate the broad T-cell responses associated with protective immunity against HCV infection, many groups are investigating DNA vaccines capable of priming HCV-specific CD4+ and CD8+ T-cell responses to many HCV gene products. A DNA vaccine encoding NS3, NS4 and NS5 not only primed broad and specific antibodies, CD4+ T-helper and CD8+ CTL responses, but also conferred protection to the immunized BALB/c mice against challenge with syngeneic SP2/0 myeloma cells expressing NS5.[122] Co-expression of the GM-CSF cytokine gene has also been shown to augment cellular immune responses to these NS gene products when administered as a bicistronic plasmid to Buffalo rats.[123] Many studies have been conducted with DNA vaccines containing the HCV C gene, as this encodes the most conserved viral protein and is known to contain important T-cell epitopes. Immunogenicity in mice has been reported[124–127] and, in addition, the co-administration of either interleukin (IL)-2 or granulocyte macrophage colony-stimulating factor (GM-CSF) has been shown to augment humoral and cellular immune responses to C in mice.[127] On the contrary, co-immunization with a plasmid expressing IL-4 resulted in the elicitation of a Th0 phenotype and a concomitant suppression of C-specific CTLs.[127] The use of transgenic mice expressing human HLA-A2.1 has also shown the ability of NS3 DNA vaccines to induce specific CTLs to the same immunodominant epitope observed in humans.[128]

As with DNA vaccines targeting other infectious agents, the mode of injection drastically alters the immune response. Administration of a gpE2 DNA vaccine by gene gun, in which DNA is physically administered intra-epithelially on gold microparticles, resulted in anti-gpE2 titres that were 100-fold higher than if delivered by needle i.m.[115] This increase in vaccine potency should translate to the use of lower doses in humans, thus lessening safety concerns. Other methods to improve the potency of DNA vaccines include the application of an electric field at the site of DNA injection (so-called 'electroporation'). In the case of a gpE2 DNA vaccine, electroporation led to 10-fold increases in expression levels and in concomitant anti-gpE2 responses. The latter also included cross-reactive anti-E2HVR1 antibodies that were not obtained without the use of electroporation, and in significant increases in gpE2-specific CD4+ T-helper and CD8+ CTL responses.[129] Other improvements include the formulation of DNA vaccines into particles, thereby increasing the uptake by antigen-presenting dendritic cells with corresponding increases in immunogenicity.[130] Lipid formulations of DNA that result in improved transfection efficiency *in vivo*, as well as better uptake by dendritic cells, have been shown to be part of an encouraging immunization regimen for rhesus macaques against SHIV challenge and have applications to HCV and other infectious diseases.[131]

Finally, it is also noteworthy that defective RNA vaccines have been shown to be very effective at protecting against flaviviral infections.[132] Conferring good protective immunity in the absence of integration into the host genome, this approach has great promise for HCV and other infectious diseases.

## HCV vaccines based on disabled microbial vectors

The use of a defective or attenuated viral or bacterial vector to deliver vaccines has several potential advantages. Firstly, a wide tropism of the host vector leads to the efficient delivery of the vaccine genes and encoded antigens. Preferably, this tropism includes APCs, leading to a very effective priming of the immune response and thereby requiring only one immunization for long-lasting immunity. The use of a vector already used as a vaccine itself offers further obvious advantages with respect to manufacturing, distribution and user acceptance. Finally, many vectors allow the insertion of multiple genes, thus facilitating the induction of a broad, cross-protective immune response, particularly useful against heterogeneous agents such as HCV. One such promising approach for HCV has been the use of an attenuated rabies viral vector into which either the HCV gpE1–gpE2–p7 gene cassette was inserted, or just the ectodomain of gpE2 linked to the CD4, C-terminal TMR, and cytoplasmic domain. In the case of the latter construction, recombinant rabies virions were produced that actually contained the hybrid gpE2. Virions expressing gpE1–gpE2–p7 were immunogenic in mice, eliciting CTL responses to gpE2.[133] Similarly, defective Semliki Forest virions containing the HCV NS3 gene produced long-lasting NS3-specific CTLs after one immunization in mice transgenic for human HLA-A2.1.[134] As observed in HCV-infected patients, the immune re-

sponse was directed to one immunodominant epitope within NS3. Defective recombinant adenoviruses expressing the HCV C–gpE1–gpE2 gene cassette have also been shown to prime HCV-specific CTLs in mice immunized i.m., although the induction of anti-gpE1/gpE2 antibodies required further immunization with purified gpE1/gpE2 glycoproteins.[135] Replication-defective adenoviruses expressing C and gpE1 also primed long-lasting specific CTL responses in mice.[136] Recombinant canary pox viruses, expressing an HCV gene cassette containing C–gpE1–gpE2–p7–NS2–NS3, elicited HCV-specific humoral and cellular immune responses in mice, although the optimum immunization regimen required first priming with a plasmid DNA expressing the HCV genes prior to boosting with the recombinant canary pox virus.[137] Attenuated *Salmonella typhimurium* transformed with a plasmid expressing the HCV NS3 gene has also been shown to elicit NS3-specific CTLs in mice transgenic for human HLA-A2.1. The bacterium was administered orally and the resulting CTL responses persisted for at least 10 months.[138]

## Conclusions

Recent studies in man and in the chimpanzee model provide evidence for significant natural immunity to HCV infection, encouraging the efforts to develop a vaccine against HCV. Chimpanzee challenge studies, using recombinant envelope glycoprotein vaccines, have demonstrated prophylactic efficacy and the feasibility of protecting against the development of chronic infection following experimental challenge with both homologous and heterologous viral strains. The application of broad technologies to HCV vaccination should result in effective vaccines, which should be very useful in substantially lowering the current incidence of HCV infection with its ensuing clinical sequelae, although they may not be 100% effective.

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# Section VI
# Hepatitis D Virus

# Chapter 36
# Structure and molecular virology

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Hepatitis delta virus (HDV) was first detected as a new nuclear antigen in the hepatocytes of patients infected with hepatitis B virus (HBV) and was frequently associated with severe acute or chronic hepatitis.[1] The antigen, named delta antigen (HDAg), was initially thought to be a previously undescribed HBV-encoded antigen, but later was found to be associated with a new transmissible agent, an RNA-containing virus enveloped by the surface antigen of HBV, HBsAg.[2] HDAg was subsequently shown to be an internal component of the HDV virion particles, and antibodies against this viral structural protein were detected in patients during infection.[3]

HDV infection has been reported worldwide, with particularly high prevalence rates in the Mediterranean Basin, South America, Middle East, West Africa and certain South Pacific islands.[4,5] Transmission of HDV requires either co-infection with HBV or superinfection in individuals who are HBV carriers. Although HDV infection is closely associated with HBV, HDV clearly belongs to a distinct virus group. Currently, HDV is assigned a floating genus, Deltavirus.

## Virion structure

HDV is a small RNA virus consisting of spherical particles of about 36 nm in diameter and with a buoyant density of 1.25 g/cm$^3$ in caesium chloride.[3,6–8] The outer coat of HDV consists of hepatitis B surface antigen (HBsAg) and lipid, which envelopes the RNA genome and the delta antigen.[2,3] All three components (S, M, L) of HBsAg are present in the HDV virion particles. However, the ratio of the three (S:M:L = 95:5:1)[3] is different from that of the infectious HBV Dane particles, but more like that of the 22-nm non-infectious HBV particles. An internal, spherical nucleocapsid approximately 19 nm in diameter was released from the virus particle after HDV was treated with non-ionic detergents.[9] It consists of HDV RNA and the delta antigen, the latter of which consists of two protein species (27 and 24 kDa) of variable ratios. There are approximately 70 HDAg molecules per RNA molecule.[9] The precise structure of the nucleocapsid has not been determined.

## The genome

The RNA genome is approximately 1700 nucleotides long, single-stranded and circular, with a high (60%) G+C content and a high degree (up to 70%) of intramolecular base-pairing.[10–12] The last property was clearly demonstrated by electron microscopy, in which native HDV RNA molecules appear as compact rods, but convert to circular molecules under denaturing conditions.[13] Because of its double-strandedness under native conditions, HDV RNA is relatively stable under a variety of manipulations in the laboratory.

Many different HDV isolates have been sequenced. Based on sequence relationship, HDV isolates have been grouped into three genotypes.[14] Genotype I comprises most of the HDV isolates sequenced so far and includes isolates obtained from almost every part of the world. The pathogenic properties of this genotype also show great diversity. Genotype II represents isolates prevalent in some part of Asia, particularly Taiwan and Japan,[15–17] and may be associated with relatively milder diseases.[17] Genotype III represents several isolates from South America[14] and is associated with a particularly fulminant form of hepatitis.[18] These three genotypes can be differentiated based on the differences in the immunogenic properties of their delta antigen.[19] Recombination between different isolates has been reported.[20] HDV RNA in patients undergoes continuous evolution throughout the clinical course of disease, with an evolution rate as high as $3 \times 10^{-2}$ to $10^{-3}$ substitutions per nucleotide per year in some cases.[21,22] There are several highly conserved regions, which correspond to important functional domains of HDV RNA or its encoded protein, HDAg.[21,23]

For purposes of genomic sequence organization, a *Hin*dIII restriction site present in the cDNA of the prototype chimpanzee-passaged HDV RNA[12] was designated

nucleotide 0 (Fig. 36.1).[11] The region from approximately nucleotide 615 to 950 shows some sequence similarity with a group of plant pathogens, namely, viroids and virusoids,[24] and is called the viroid-like domain.[25] Similar to some virusoid RNAs, this domain exhibits ribozyme activities, i.e. autocatalytic cleavage activities, on both the genomic and its complementary antigenomic strand.[26–29] Cleavage occurs in the absence of protein and requires divalent cations such as $Mg^{2+}$. The cleavage site is located between the U and G at nucleotide 688/689 (in the Makino *et al*. 1987 sequence[11]).[26,29] RNA sequence required for the ribozyme activity resides in approximately 85 nucleotides at the 3′-side of the cleavage site. The sequence at its 5′-side is not critical but may affect the cleavage rate.[30–32] The HDV ribozyme can fold into five helical segments connected as a double pseudoknot.[33] The unique structural and sequence requirements of HDV RNA catalysis indicate that HDV RNA belongs to a novel class of ribozymes, distinct from other known ribozymes, such as the 'hammerhead' or 'hairpin' types.[34–36] Furthermore, the HDV ribozyme can undergo base catalysis in the absence of divalent ions, another novel property among ribozymes.[37] The catalytic activity on the antigenomic-sense RNA cleaves the HDV RNA at nucleotide 903/904,[26,38] which is complementary to the genomic cleavage site in the proposed HDV RNA rod structure. Both of the genomic and antigenomic ribozyme activities are required for HDV RNA replication.[39,40] The ribozyme activities *in vitro* can be inhibited by aminoglycoside, tetracycline or peptide antibiotics,[41,42] but the effects of these antibiotics on HDV ribozymes *in vivo* have not been demonstrated. HDV RNA also contains an autocatalytic ligation activity under an artificial condition, namely in the presence of a complementary cDNA fragment.[27] However, the functional role of this ligation activity has not been proven.

HDV RNA contains only one functional open reading frame (ORF), which is located on the antigenomic strand and encodes HDAg. This ORF exists in two sizes, a small and a large form, on different RNA molecules, with coding capacities for 195 and 214 amino acids, respectively. The difference between these two ORFs resides in a single base mutation at position 1015. This nucleotide on the antigenomic strand is either A, which leads to a termination codon UAG, or G, giving UGG (Trp), which allows for the read-through of 19 additional amino acids. Both forms of RNA exist in every delta hepatitis patient.[12,21,43] The generation of this mutation is the result of an RNA editing process and is closely associated with the replication process of HDV (see below). Based on these properties, HDV RNA can be divided into two structural domains.[25] One is the viroid-like domain, which contains the ribozyme activity on both genomic and antigenomic strands, and is evolutionarily related to viroids. The remaining RNA sequence contains a functional ORF that encodes the delta antigen.

## Hepatitis delta antigen (HDAg)

HDAg is the only known functional protein encoded by HDV RNA, and is an internal component of HDV virion particles.[2,3] HDAg is usually found in patients' serum or liver in two forms of 27 000 kDa and 24 000 kDa molecular weight,[6,44–46] designated large and small HDAg, respectively. These forms are not present in a fixed stoichiometric ratio as expected for structural proteins of a defined regular structure. As indicated above, these two HDAg forms are translated from two RNA species different by a single point mutation. The two protein forms are identical except that the large HDAg has 19 additional amino acids at the C-terminus. The N-terminal two-thirds of the protein are highly basic, while the C-

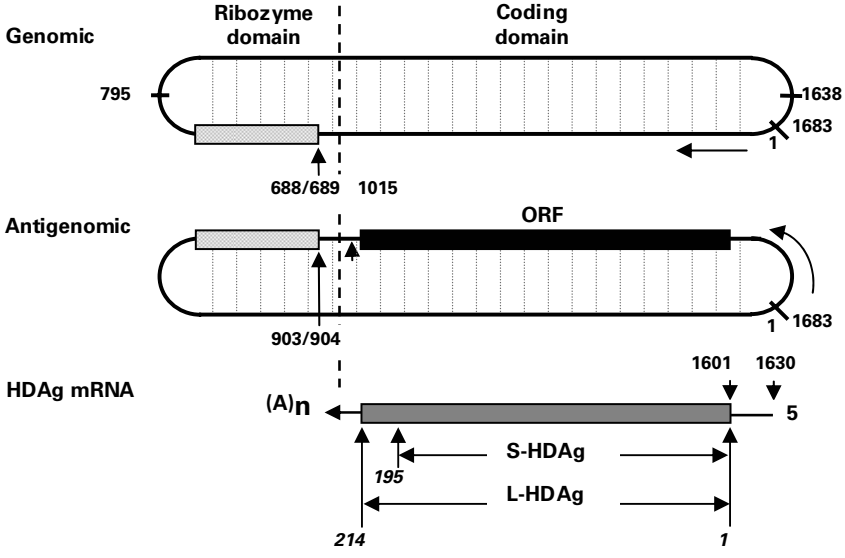


**Figure 36.1** Schematic diagrams of the structure of HDV RNA. The antigenomic (Ag) RNA and 0.8-kb mRNA are present only in the cells. The nucleotide numbers are according to Makino *et al*. (1987)[11] and represented in genomic orientation on both the genomic and antigenomic strands. The genomic RNA is represented in clockwise orientation, while antigenomic RNA is counterclockwise. Nucleotides 688/689 and 903/904 are ribozyme cleavage sites for genomic and antigenomic RNAs, respectively. The hatched boxes represent the ribozyme domain. Nucleotide 1015 denotes the RNA editing site. The amino acid residue numbers are in italics.

terminal one-third is relatively uncharged.[47] Both forms are phosphorylated, with phosphorylation occurring at serine residues.[47,48] Phosphorylation may be carried out by casein kinase II, protein kinase C or the double-stranded RNA-activated kinase PKR and is required for certain steps of HDV RNA replication.[49,50] In particular, phosphorylation at serine-177 appears to regulate the replication of different strands of HDV RNA.[51] HDAg is a general RNA-binding protein,[52–54] with particularly high binding affinity for HDV RNA. However, the binding specificity is not absolute. In the virion particles, HDAg is bound to HDV RNA.[54] This HDAg–RNA interaction probably plays a role not only in the structural organization of virion particles, but also in HDV RNA replication (see below). In virus-infected cells, HDAg appears to be localized exclusively in the nuclei, based on immunohistochemical staining.[1] However, it can be seen variably in the nucleoli, nucleoplasm or diffusely throughout the nuclei, depending on individual cells.[55] Recent biochemical fractionation and metabolic labelling studies showed that a substantial portion of HDAg is present in the cytoplasm[56,57] and that it can be shuttled between the cytoplasm and the nucleus.[58]

At least four functional domains have been identified in HDAg (Fig. 36.2). The first is the RNA-binding motif, which is located in the middle one-third of HDAg.[54] This domain consists of two stretches of arginine-rich motif (ARM),[59] which is characteristic of many RNA-binding proteins.[60] Both of the ARM sequences and a spacer sequence of appropriate length are required for RNA binding.[59] Another potential RNA-binding motif has been identified in the N-terminal region of the protein.[61] Curiously, an RNA chaperone activity, which requires RNA-binding activities, has been found in the N-terminal one-third of the protein.[62] This domain includes the N-terminal, but not the middle, RNA-binding motif. The second domain is a nuclear localization signal located in the N-terminal half of the protein.[55] This domain contains two stretches of basic amino acids, both of which are required for targeting HDAg to the nuclei. HDAg carries HDV RNA to the nucleus, where RNA replication occurs in the nucleus.[63] The third domain is a coiled-coil sequence, present in the N-terminal one-third of the protein.[55,64] This domain is responsible for oligomerization of the protein. The large and small HDAg can form either homo- or heterodimer.[55,65] The oligomerization of HDAg is required for the various functions of both the large and small HDAg (see below). The fourth domain is present only in the large HDAg, i.e. the C-terminal 19 amino acids of the large HDAg. This region is prenylated at cystine-211.[53,66–68] Prenylation alters the conformation of the HDAg, resulting in the masking of a C-terminal epitope (9E4),[69] so that it is shielded from being recognized by the humoral arm of the immune system.[70] It also enables the interaction between the large HDAg and HBsAg,[71,72] which is a critical step in HDV particle assembly. The 19 amino acids are necessary and sufficient as the signal for virion assembly.[73] Curiously, this 19 amino acid sequence is extremely divergent among different genotypes,[14] except for the last 4 amino acids, which is the prenylation motif (CXXC). Prenylation inhibitors inhibit the production of infectious virus particles.[74] Besides prenylation, HDAg is also modified by phosphorylation. The extent of phosphorylation is important for the regulation of the various functions of HDAg. In addition, it is also methylated at Arg-13 (Li and Lai, unpublished observations). Like phosphorylation, methylation is important for the functions of HDAg in HDV RNA replication.

Although the structure and biochemical properties of the large and small HDAgs are very similar, they have significantly different functions (Table 36.1). The small HDAg is absolutely required for HDV RNA replication *in vivo*.[75] It acts *in trans* and complements defects of the HDV RNA that do not synthesize a functional HDAg.[75] The ability of S-HDAg to promote HDV RNA replication appears to be genotype-specific, as S-HDAg of genotype I cannot support genotype III RNA replication and vice versa.[76] It has been suggested that S-HDAg can promote the elongation of RNA synthesis in DNA-dependent as well as RNA-dependent RNA synthesis carried out by RNA polymerase II *in vitro*.[77] Another possibility is that the small HDAg may merely serve as a carrier to transport HDV RNA into the nucleus, but may not participate in RNA replication directly.[78] In any case, the S-HDAg appears to be required for both the initiation and maintenance of continuing RNA synthesis. The large HDAg is required for the assembly and export of virion particles.[79–81] This function requires prenylation of the last 19 amino acids. Another function ascribed to L-HDAg

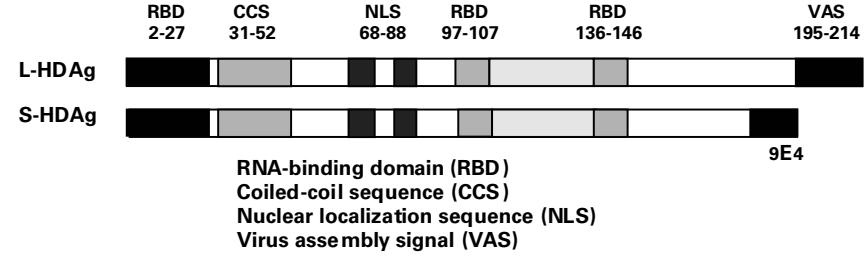


**Figure 36.2** Schematic diagram of the functional domains of the large and small hepatitis delta antigen.

**Table 36.1** Comparison of biochemical properties and biological activities of the large and small HDAg

| Biochemical properties and biological activities | Large HDAg | Small HDAg |
|---|---|---|
| Amino acid residues | 214 | 195 |
| Dimerization | + | + |
| RNA binding | + | + |
| Phosphorylation | + | + |
| Isoprenylation | + | – |
| C-terminus-specific epitope (9E4) | – | + |
| *Trans*-acting function for RNA synthesis | – | + |
| *Trans*-dominant suppression of RNA synthesis (*in vitro*) | + | – |
| Interaction with HBsAg (virion assembly) | + | – |
| RNA chaperone | + | + |
| Stabilization of HDV RNA | + | + |
| Enhancement of ribozyme activity | + | + |

is the suppression of HDV RNA replication. Thus, L-HDAg was thought to be critical for modulating the amount of HDV RNA in the viral life-cycle.[82] However, recent studies indicate that this interpretation is not correct. The inhibitory activity was noted only when L-HDAg was expressed artificially early in the replication cycle of HDV.[57] Even under this condition, it interferes with the synthesis of only genomic RNA from the antigenomic RNA template, but not vice versa.[83] Furthermore, it has been shown that the presence or absence of L-HDAg does not affect the final level of HDV RNA.[57] Despite these obvious differences, the S- and L-HDAg did share some common functions, such as stabilization of HDV RNA,[84] enhancement of ribozyme activity[85] and RNA chaperone activity[62] (Table 36.1). They were reported to have other activities, such as suppression of polyadenylation of mRNA,[86] which is likely an artefact of the experimental systems.

HDAg can induce humoral antibodies as well as T-cell responses in infected patients or experimental animals.[70,87,88]

## Replication

In natural infections, HDV infects only hepatocytes. No extrahepatic infection has been detected. The mode of HDV entry into target cells is currently unknown. As the HDV envelope consists of all three protein species (L, M and S) of HBsAg,[3,6] HDV probably utilizes the same cellular receptor as HBV. Once inside cells, HDV can replicate in the absence of HBV.[89,90]

Several experimental approaches are available for studying HDV replication. Besides the chimpanzee model, woodchucks provide the most convenient small animal model. However, the majority of our knowledge on HDV replication came from cell culture studies using HDV cDNA or RNA transfection approaches. In these approaches, the production of S-HDAg is the first required step for the initiation of HDV RNA replication. S-HDAg can be made *in vivo* from HDV RNA transcripts (from the transfected cDNA) or from a co-transfected mRNA (that encodes S-HDAg) in the transfected cells, or provided as a recombinant protein. In natural infection, S-HDAg is present inside the virus particles, probably complexed with HDV RNA. The presence of S-HDAg allows the initiation of HDV RNA replication.

Although HDV infects only hepatocytes *in vivo*, HDV RNA can replicate in a wide range of cell types, including hepatocytes, fibroblasts and monkey kidney cell lines, upon transfection of HDV cDNA or RNA into those cells. One of the exceptions is avian cells, which appear to lack certain host factors for HDV RNA replication,[91] although a different study claimed that the failure of avian cells to support HDV replication is due to killing of the infected cells.[92] Replication of HDV RNA is thought to occur via a rolling circle mechanism similar to that described for the replication of viroids.[93] In this model, antigenomic RNA of multiple genomic lengths is first synthesized, followed by its processing into monomer-size RNAs. The monomer antigenomic RNA is ligated intramolecularly to form a circular RNA, which is then used as the template for a second round of rolling circle replication to generate the monomer genomic-sense RNA, thus completing the replication cycle (Fig. 36.3). This model, called the 'double rolling circle mechanism', is supported by the kinetic studies of the *in vivo* labelled HDV RNA in the cells transfected with HDV RNA. In these cells, the oligomeric HDV RNA was detected before the monomer RNA appeared.[57] The processing of the multimeric RNA into monomer is mediated by ribozyme activities, which are present in both genomic and antigenomic RNA. Abolition of the ribozyme activity of either genomic or antigenomic strand inhibited HDV RNA replication, indicating that both genomic and antigenomic strands need to be processed before replication can proceed,[39] further supporting the double rolling circle replication model. The cleavage site specificity and RNA sequence and structural requirements for the HDV ribozyme activity in the cells are the same as those *in vitro*.[39,40] However, the HDV ribozyme activity

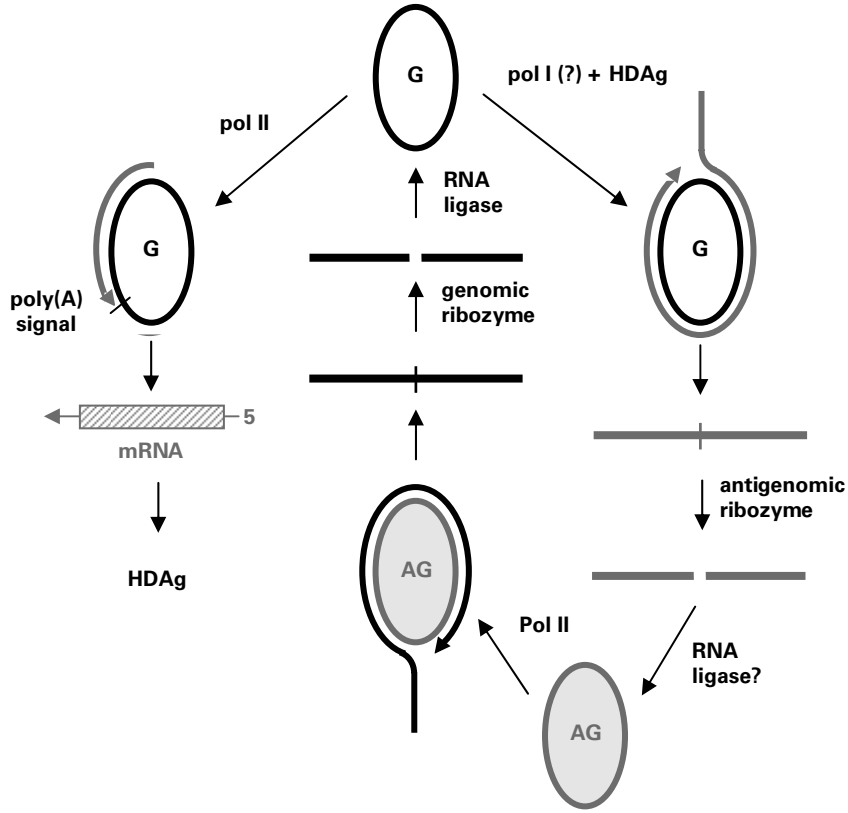


**Figure 36.3** The proposed replication and transcription models of HDV RNA. The incoming genomic RNA is transcribed into an antigenomic mRNA encoding the small HDAg, which together with pol I (or another cellular polymerase), is involved in the replication of the genomic RNA into the antigenomic RNA strand. The remaining chain of the replication is the 'double rolling circle' model, involving the cleavage of the RNA intermediates by antigenomic and genomic ribozymes and ligation by RNA ligases. The enzymes involved in all these processes have not been established.

requires a complex tertiary structure,[94–96] whereas HDV RNA naturally assumes a rod-like structure. Therefore, the conformation of HDV RNA is probably dynamic, and the conformational changes may be facilitated by cellular or viral factors. Indeed, RNA chaperone activity of HDAg can facilitate the HDV RNA ribozyme activity *in vitro* and *in vivo*.[62,85] The intermediates of HDV RNA replication exist as dimer, trimer and higher oligomers, indicating that the newly synthesized RNA is processed soon after synthesis. These intermediates eventually are processed into monomers. Thus, the processing of these RNA products probably involves both a rapid and a slow phase. The monomeric RNA and some of the dimeric RNA are ligated intramolecularly into circular RNA. This process was initially thought to be carried out by a self-ligating activity of HDV RNA but now appears to be carried out by a cellular enzyme, such as RNA ligase.[97] It is not known whether the reported self-ligating activity of HDV RNA *in vitro*[27] has any functional significance. The origins of replication on both genomic and antigenomic RNAs have not been unequivocally determined. *In vitro* studies suggested that the antigenomic-sense RNA synthesis may initiate from one end of the rod-like structure of HDV RNA.[98]

Replication of HDV RNA occurs in the nuclei of the cells as both HDAg, detected by immunofluorescence, and HDV RNA, detected by *in situ* hybridization, are localized exclusively in the nuclei.[99,100] Metabolic labelling of newly synthesized HDV RNA with $^{32}$P-orthophosphate showed that the radiolabelled RNA first appeared as the multimer form in the nuclei.[101] As the replication proceeded, the monomer form gradually accumulated and became the predominant RNA species, suggesting that the multimer form is the precursor of HDV monomer RNA.[101] Recent data further showed that HDAg and HDV RNA (as ribonucleoprotein complex) shuttled in and out of the nuclei.[58,63] HDV RNA has also been shown to be rapidly transported to the cytoplasm soon after its synthesis in the nuclei, probably after the RNA intermediates have been cleaved into monomer and ligated to the circular form.[56] The role of the cytoplasmic phase in HDV RNA replication is still not certain. It appears that a substantial portion of the genomic RNA is transported to the cytoplasm after its synthesis, whereas antigenomic RNA is retained mostly in the nucleus. The biological functions and mechanism of the differential transport of the genomic and antigenomic RNA in the life-cycle of HDV is not known. It should be noted that the cytoplasmic export of HDV genomic RNA is not only required for the eventual assembly of virus particles, but may also be part of the RNA replication process.

An antigenomic-sense 0.8-kb RNA has also been detected in the HDV RNA-replicating cells.[102,103] This RNA species contains a poly(A) sequence.[102] The 5′ end of

this transcript starts at position 1630, which is near the initiation codon of the ORF for HDAg.[104] The 3′ end of the transcript is 76 nucleotides downstream from the termination codon of HDAg and is preceded (15 nucleotides upstream) by an AAUAAA polyadenylation signal.[103] Thus, this RNA covers the entire ORF for HDAg and serves as the mRNA for encoding HDAg. This is the only HDV RNA species capable of synthesizing HDAg,[105] even though the full-length antigenomic RNA also contains the coding sequence for HDAg. Thus, the transcription of this mRNA is important for continuous RNA replication. It was thought that this mRNA was present in very low abundance (roughly 60 molecules per cell as compared with 6000 molecules of the antigenomic RNA and 300 000 molecules of the genomic RNA) and was synthesized only at the beginning of the HDV life-cycle.[102,103] However, recent studies showed that these views were not correct.[106] The original hypothesis was that the generation of the 0.8-kb mRNA is part of the replication process of antigenomic RNA. When sufficient HDAg accumulates, the HDAg serves as an antiterminator protein, allowing the subsequent rounds of RNA synthesis to pass beyond the polyadenylation signal.[86,107] As a result, a multimeric HDV RNA is synthesized. However, recent studies showed that the transcription of 0.8-kb mRNA and replication of HDV RNA are independent processes, and that the mRNA is synthesized throughout the viral life-cycles. Furthermore, it can be as abundant as the HDV antigenomic RNA.[106] As discussed below, the transcription of this mRNA and replication of genomic RNA are most likely mediated by different polymerases. Thus, conceivably, they are carried out in different subcellular compartments.

During HDV RNA replication, a specific nucleotide conversion (U→C) occurs at nucleotide 1015 of the genomic sense RNA, changing the termination codon for small HDAg (on the antigenomic strand) to a tryptophan codon and extending the ORF for an additional 19 amino acids.[108] This mutation results in the synthesis of the large HDAg, which initiates virus particle assembly.[79,80] This specific mutation, termed RNA editing, requires a rod-like RNA structure and specific nucleotide sequences around the editing site and its complementary region.[109] It occurs on the antigenomic-sense RNA, causing an A→G mutation, resulting in a U→C conversion in the genomic-sense RNA.[110,111] (Earlier reports reaching opposite conclusions were most likely incorrect.[109,112]) This RNA editing appears to be carried out by a double-stranded RNA-adenosine deaminase (ADAR).[113] More recent studies have further identified ADAR I, but not ADAR II, as the enzyme responsible for HDV RNA editing.[114,115] ADAR I is a nuclear protein. The efficiency of RNA editing *in vitro* appears to be very high. However, the extent of RNA editing in the cells is regulated, so that L-HDAg is not over-produced. Such a feedback inhibition probably is caused by the enhanced deleterious mutations occurring in the genome triggered by the edited sequence[116] or L-HDAg *per se*.[117] In a woodchuck model, the amount of the L-HDAg in hepatocytes increased during the course of acute HDV infection, but it decreased during chronic infection.[118] However, in human delta hepatitis patients, the relative ratio of the large and small HDAg appears to be variable, irrespective of the clinical stage of infection.[119] In every delta hepatitis patient examined, both RNA species containing a large and a small HDAg ORF are present.[43] However, RNA editing did not occur in some experimental systems, such as transgenic mice expressing HDV RNA.[120]

The enzymology of HDV RNA replication is not yet entirely clear. Significant progress has recently been made in this regard. It has been shown that HDV RNA replication is suppressed by a low concentration (1–5 μg/mL) of α-amanitin, suggesting the involvement of DNA-dependent RNA polymerase II (pol II) from the host cells.[78] Pol II can elongate RNA-dependent RNA synthesis *in vitro* using HDV RNA as a template, although the RNA product is different from the natural RNA species.[121] More recent studies showed differential sensitivity of the synthesis of the two strands of HDV RNA to α-amanitin. The genomic RNA synthesis (from the antigenomic RNA template) is sensitive to α-amanitin, whereas antigenomic RNA synthesis (from the genomic RNA template) is resistant (up to 100 μg/mL of α-amanitin).[101,122] The mRNA transcription is also sensitive to the low concentration of α-amanitin. These findings suggest that a different polymerase, in addition to pol II, is also involved in HDV RNA replication. Most likely, pol I is involved in the antigenomic RNA synthesis from the genomic RNA template. The dichotomy of genomic and antigenomic RNA synthesis in their metabolic requirement is consistent with other known properties of these two RNA species. For example, antigenomic RNA cannot initiate RNA replication when it is transfected together with the recombinant HDAg derived from *Escherichia coli*, whereas genomic RNA can.[123] Certain phosphorylation (at serine-177) and methylation of HDAg is required for antigenomic but not genomic RNA synthesis[51] (Li and Lai, unpublished observations). Genomic but not antigenomic RNA synthesis is inhibited by L-HDAg, when the latter is expressed early in viral replication.[83] Finally, the genomic and antigenomic RNA have different distribution patterns within the nucleus.[124] HDAg also has been reported to complex with a nucleolar protein B23 and HDV RNA synthesis is enhanced when B23 is overexpressed,[125] another indication that HDV RNA may be located in the nucleolus at certain stages of the HDV life-cycle.

The question remains as to how RNA polymerase II or other cellular polymerases can utilize an RNA template rather than their normal DNA template. Possibly, HDAg

may complex with cellular transcription factors and then interact directly or indirectly with pol II, thereby altering its template specificity. As HDAg binds to HDV RNA,[47,54] a transcription complex consisting of HDAg, pol II and cellular transcription factors could conceivably complex with HDV RNA and carry out RNA replication. However, HDV RNA synthesis did occur in the absence of HDAg in cell-free lysates,[78,121,126] although some of these studies have so far failed to be reproduced. Another study showed that HDAg can promote the elongation, but not initiation, of HDV RNA-dependent RNA synthesis carried out by pol II in a reconstituted pol II transcription reaction.[77] It is noteworthy that the rod-shaped structure of HDV RNA resembles double-stranded DNA. Therefore, it is conceivable that cellular RNA polymerases and transcription factors may recognize double-stranded RNA. Indeed, the double-stranded DNA counterpart of the region encompassing the replication origin for the antigenomic-strand HDV RNA has a promoter activity for transcription.[127,128] These questions remain the most critical issues as regards the HDV replication cycle.

## Virus assembly

Co-transfection studies using different forms of HBsAg gene and plasmids coding for either L- or S-HDAg and HDV cDNAs have shown that the large form, but not the small form, of HDAg is required for the assembly of HDV particles.[79–81] A virus-like particle can be formed even in the absence of HDV RNA, the minimum requirement being the large HDAg and HBsAg. It has been shown that the large HDAg by itself can bind to HBsAg *in vitro*.[69] This interaction requires prenylation and specific amino acids within the C-terminal 19 amino acids.[66,67] The 19 amino acids are necessary and sufficient for virus assembly.[69,73] On the other hand, the small HBsAg alone, but not other forms of HBsAg, is sufficient for HDV particle assembly,[80,81] but the presence of the large HBsAg (pre-S1) polypeptides in the assembled particles is required for the infectivity of the assembled HDV particles in primary primate hepatocyte cultures.[81] Although the small HDAg is not required for HDV particle assembly, it can be packaged into virus particles,[64] probably because of its interaction with the large HDAg.[129] The relative ratio of the large and small HDAg in HDV virus particles obtained from infected serum[46,130] varies from 0.5 to 10 or higher, suggesting that there is no strict requirement for a fixed ratio between the large and small forms of HDAg in virus particles. Alternatively, there may be a population of virus particles containing exclusively the large form or the small form, respectively.

The intracellular site of interaction between HDAg and HBsAg is not certain, inasmuch as HDAg is present mainly in the nuclei, while HBsAg is localized exclusively in the cytoplasm. Recent studies showed that HDAg can shuttle in and out of the nuclei.[58] It has been shown that an HDAg mutant with a deletion in the nuclear localization signal could still be packaged into virus particles,[131] suggesting that the interaction takes place in the cytoplasm. The prenylates of the large HDAg may allow it to interact with a cellular membrane component that harbours HBsAg. This interaction may be the nucleation point of virus assembly. Other virion components, such as the small HDAg and HDV RNA, are incorporated into virus particles through their interaction with the large HDAg. HDV RNA is packaged most likely by binding to the large or small HDAg. However, although the large HDAg is sufficient for packaging of HDV RNA into virion, efficient RNA packaging requires the small HDAg as well, suggesting that HDV RNA may bind to both the large and small HDAg in the virion.[132] Only the genomic sense RNA is packaged into virion, although the antigenomic sense RNA binds equally well to HDAg *in vitro*.[52,54] The basis for the selectivity of RNA packaging is not clear. In the assembled virus particles, HDAg has been shown to complex with HDV RNA.[54] So far, no distinct nucleocapsid structure has been observed within virus particles.

## Molecular basis of viral pathogenesis

Current knowledge on the molecular biology of HDV provides clues toward the understanding of the viral pathogenesis and clinical manifestation of HDV infection. Both viral and host factors probably play significant roles in this process. It has been recognized that genotype III isolates, which are detected only in South America, are associated with more severe, frequently fulminant, hepatitis.[14,133] In contrast, genotype II isolates, which are found mainly in Asia, are associated with milder hepatitis.[17] The virulence of genotype I viruses is more variable. The genetic basis of HDV RNA for such variance is not clear. Interestingly, the most divergent region among the three genotypes is the region encoding the C-terminal 19 amino acids of L-HDAg, which shows genotype-specific variations.[14] This region is known to be essential for virus assembly and for inhibition of viral replication early in the viral life-cycle. HDV RNA with genotype II-specific 19 amino acids has a poorer efficiency of RNA editing and virus assembly than genotype I RNA. Correspondingly, genotype II viruses had lower virus yields and poorer virus spread.[134] Type II-derived HDV cDNA clones appear to replicate less efficiently than type I cDNA clones.[135]

Another potential mechanism of HDV pathogenesis is that HDV RNA replication may disturb cellular functions. It has been noted that a stretch of HDV RNA sequence bears homology to the cellular 7S RNA,[136,137] which is involved in protein translation and transport. However, no evidence is available to suggest that trans-

lation of cellular proteins is affected by HDV infection. Furthermore, HDV RNA replication utilizes cellular transcriptional machinery. As a consequence, transcription of cellular genes may be inhibited. This possibility has been evidenced in the inhibition of HBV replication and gene expression by HDV co-infection.[138–142] Both the small and large HDAg have been shown to affect cellular pol II-mediated transcription, although the precise effects (stimulatory or inhibitory) are contradictory in two different studies.[143,144] In addition, HDAg, particularly the small HDAg, was shown to be cytotoxic when it was expressed at high levels in the cells.[145–148] However, no significant pathology can be seen in the HDAg-expressing transgenic mice,[120,149] HDAg-expressing cell lines, and HDV infection of primary hepatocyte cultures.[150–152] Interestingly, HDV reinfections of transplanted livers in patients very often remain asymptomatic until HBV reactivates,[153] suggesting that HDV by itself may not be pathogenic and requires HBV for the manifestation of the disease. Still another possibility is that pathogenesis of HDV is due to immunopathic attack by cytotoxic T lymphocytes.[100,154] This possibility is supported by the finding that experimental vaccination of chimpanzees or woodchucks with HDAg exacerbated the illness of subsequent HDV infection.[155] These possibilities have still to be investigated.

Although a double-stranded, rod-like HDV RNA could be expected to act as a powerful interferon (IFN) inducer, the ability of HDV to replicate actively in infected cells indicates that HDV RNA either fails as an IFN inducer or evades a response to IFN. In fact, administration of IFN-α or IFN-γ to HDV cDNA-transfected cells failed to inhibit HDV replication in the presence of otherwise functionally preserved IFN-activated pathways, which include the dsRNA activation of protein kinase (PKR).[156] This was confirmed in another study where PKR activation by HDV RNA was not associated with inhibition of protein synthesis.[157] Obviously, many viral and cellular factors may contribute to HDV-induced pathogenicity. The elucidation of these factors will require further understanding of the molecular biology of the virus.

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# Chapter 37
# Epidemiology and natural history

Floriano Rosina, Mario Rizzetto

## Epidemiology of delta hepatitis

The hepatitis delta virus (HDV) was identified in 1977 in Italy from a nuclear immunofluorescence staining in liver biopsies of patients infected with the hepatitis B virus (HBV).[1] The staining was recognized as an antigen (delta antigen, HDAg) of the new virus and antibodies directed against the HDAg (anti-HD) were identified in sera from HDV-infected patients. When an assay for anti-HD was developed, evidence of HDV infection was found all around the world.[2]

Although the use of an antibody test is not optimal for the correct interpretation of HDV epidemiology, direct markers of HDV replication have been rarely used in epidemiological studies. Radioimmunoassays and enzyme-linked immunoassays for detection of HDAg give positive results only in the early stages of acute HDV infection and molecular hybridization and PCR assays for the RNA genome of HDV (HDV RNA) were not available when most epidemiological studies were done or were deemed to be unreliable in frozen stored samples due the intrinsic frailty of RNA. Thus, epidemiological data rest primarily on indirect assays such as IgG and IgM anti-HD, both expressions of the host response to the virus.

In acute hepatitis D anti-HD appears late and may be missed if repeated testing is not performed.[3] Thus, the true incidence of acute hepatitis D may be underestimated. This is particularly true in immunodeficient individuals such as patients infected with human immunodeficiency virus (HIV), in whom the antibody response to HDV may be delayed or absent.[4,5] Furthermore, if HDV infection does not progress to chronicity, after resolution of acute hepatitis D anti-HD may disappear with time, making the diagnosis of past acute HDV infection impossible. In contrast, patients who develop chronic hepatitis D mount brisk antibody responses that persist in high titres throughout the course of HDV infections.[6] Data on HDV epidemiology have therefore mostly been gathered from chronic hepatitis B virus (HBV) carriers superinfected by HDV in whom HDV infection has progressed to chronicity.

Data collected in the 1980s indicated that no less than 5% of the HBsAg carriers in the world were infected by HDV. As the number of HBV carriers was then estimated to be around 300 000 000, no less than 15 000 000 persons were expected to be infected by HDV. The geographical distribution of HDV infection did not parallel that of HBV, as some endemic areas for HBV were almost HDV-free. Currently, HDV remains endemic in tropical and subtropical areas. In the 1970s to 1980s, HDV infection occurred sporadically in the general HBV population in the Western world, but was endemic in HBsAg subpopulations sharing risk factors predisposing to viral transmission.

Three epidemiological patterns of HDV infection have been described: the endemic and epidemic patterns and HDV infection of high-risk groups.

### Endemic HDV infection

HDV infection was endemic in populations living around the Mediterranean Sea up to the early 1990s. Infection tended to occur early in life, affecting mainly children and young adults. The main route of transmission was inapparent permucosal or percutaneous spread. Intrafamilial transmission was common and may have been aided by poor hygiene. A recent study conducted in Italy indicates that the prevalence of HDV infection diminished sharply in the country in the last two decades.[7] In 1997, anti-HD was detected in only 8.3% of 834 HBsAg-positive patients compared with a 23% and 14% prevalence reported in 1987 and 1992, respectively.

In areas that maintain endemicity, the diffusion of HDV is uneven at the regional level. In Brazil,[8] Kenya[9] and China[10,11] pockets of hyperendemic infection have been identified close to areas where the prevalence of HDV was negligible. In part, such discrepancies depend on the disparate sampling of the patients enrolled to extrapolate local rate of HDV infection. Because of

the association between HDV and liver disease, higher prevalences of HDV were reported when those tested were HBsAg carriers with abnormal liver function tests than when they were healthy carriers recruited at blood banks.

In endemic areas, HDV is a major cause of fulminant hepatitis, especially when an HBsAg carrier with ongoing liver disease is superinfected by HDV.[12–19]

### Epidemic HDV infection

HDV infection has been reported to occur in epidemics in some populations or in high-risk subjects. Separate epidemics have been described among the Yupca and the Yanomani Indians of Venezuela,[20,21] both associated with a high rate of fulminant hepatitis and with a peculiar liver histology characterized by foamy degeneration of hepatocytes due to microvesicular steatosis.[22] Small-scale outbreaks of HDV infections have been related to nosocomial transmission, either by haemodialysis[23] or cancer chemotherapy.[24]

### HDV infection in high-risk groups

In North America and northern Europe, HDV was largely confined to high-risk groups that share as predisposing factors the carriage of HBsAg and apparent or inapparent parenteral exposure to uncontrolled blood or promiscuity in non-hygienic and overcrowded conditions.

Intravenous drug addicts sharing needles have been reported as the major victims of HDV infection in the Western world, with prevalence in the 1980s varying from 17% to 98% and a consistent presence of anti-HDV also in anti-HBs-positive subjects.[25–30]

### Ongoing changes in HDV epidemiology

The epidemiology of HDV infection has changed in the last 10 years. Improvements in socio-economic conditions, increased awareness of the risk of transmitting infectious diseases fostered by AIDS prevention policies and vaccination campaigns against HBV have all contributed to a spectacular decline in the incidence of HBV and HDV infections, especially in areas such as Italy and Greece where HDV was endemic.[7,31,32] However, new migration patterns can be expected to further modify the epidemiology of HDV. People migrating from eastern countries such as Romania and Albania to Western European countries may bring with them HDV infection.[33] Southern Africa, where no HDV infection was identified,[34,35] has been relatively isolated from the rest of Africa until quite recently, but with increases in travelling and trade with other African countries the 15% of South African HBsAg-positive black individuals may now be at risk of becoming infected with HDV.

### Molecular epidemiology of HDV

Genetic analysis of HDV isolates from around the world has shown that there are at least three phylogenetically distinct genotypes clustered in different geographic areas.[36–39] The most widespread geographically is genotype I, which was identified in North America, Europe, East and West Asia and the South Pacific and is associated with a broad spectrum of chronic diseases. Genotype II was isolated only in East Asia, and may be responsible for some of the milder forms of HDV disease in that region. Genotype III is exclusively found in northern South America, where HDV infection occurs in epidemics among Indians and exhibits a particularly severe course. Superinfection or mixed infections with different genotypes can occur, particularly in patients who are at high risk for multiple exposures. In these patients, a single genotype usually predominates, with the minor genotype representing only 10% of the total viral population.[40] The association between genotypes and disease pattern may be explained by the high rate of sequence divergence among the genotypes: up to 40% for the entire RNA genome and 35% for the amino acid sequence of HDAg. Furthermore, recent studies of HDV RNA replication in transfected cell lines have indicated that the HDV genotypes differ functionally as well as genetically.

## Natural history

### Acute HDV infection and disease

Three patterns of acute HDV infection have been described: two – co-infection and superinfection – occurring in the general population, the third – HBV-independent latent HDV infection – which in humans apparently occurs only in the liver transplant setting.

#### *Co-infection*

Co-infection occurs through simultaneous infection with HBV and HDV of a subject who has not been previously infected by HBV.

Most clinically apparent co-infections present as an icteric hepatitis with clinical features similar to those of acute hepatitis B, running a benign self-limiting course and evolving to chronicity in <7% of cases.[41–43] Unlike uncomplicated hepatitis B, co-infection tends to run a biphasic course with two peaks of liver necrosis a few weeks apart, where the first episode of hepatitis is associated with HBV and the second with HDV or vice versa.[44] The average form of co-infection may lack the early antigenaemic phase and the diagnosis can be supported only by an increase of IgM and IgG anti-HD. The antibody response to HDV is relatively slow. The initial

IgM response may be delayed for days to weeks after the onset of hepatitis and the IgG response may first appear during the convalescence phase. Prolonged monitoring for antibodies to HDV is therefore required to exclude or confirm HDV co-infection in patients presenting with HBsAg hepatitis (Fig. 37.1).

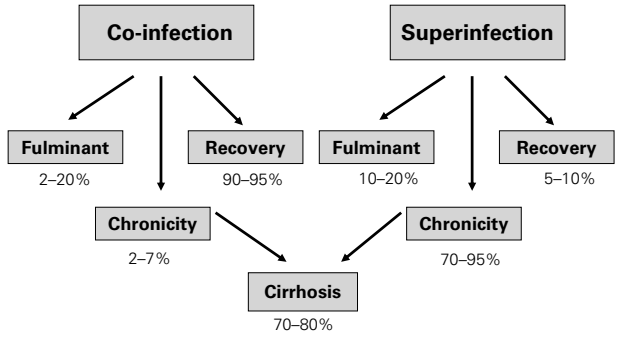


**Figure 37.1** Outcome of co-infection and superinfection with HDV with respect to the risk of developing fulminant hepatitis, chronicity and cirrhosis.

### *Superinfection*

Individuals chronically infected with HBV are prone to HDV infection. Because HBsAg expression is established prior to HDV exposure, in superinfection HDV finds a fertile ground for replication and for the full expression of its pathogenic potential.

The clinical presentation of HDV superinfection depends on whether the patient was a healthy carrier of HBsAg or whether clinical features of chronic hepatitis were present.

The acute phase is characterized by very high levels of HDV viraemia and expression of HDAg in >50% of hepatocytes. Severe acute hepatitis typically follows the peak of viraemia and coincides with the appearance of antibody to HDAg. The risk of fulminant hepatitis is high, especially in the setting of a pre-existing HBV-related liver disease[45–47] (Fig. 37.2). In an epidemic of HDV hepatitis in a community of intravenous drug users in the USA, 91% of patients with fulminant hepatitis had anti-HD in their serum versus 45% in the non-fulminant cases.[28] In tropical areas, fulminant hepatitis occurring during epidemics of HDV infection have a characteristic microscopic appearance of microvesicular steatosis and foamy degeneration,[22] features only occasionally observed in fulminant hepatitis D in developed countries.[48]

In superinfection, the antibody response is more uniform and consistent than in co-infection. There is a relatively brisk IgM and IgG anti-HD response, both antibody titres rise when superinfection progresses to chronicity. In at least 70% of cases, HDV superinfection does not resolve but becomes chronic, resulting in chronic type D hepatitis. In patients whose hepatitis D resolves, the IgM anti-HD disappears rapidly while the IgG declines slowly (Fig. 37.3).

### *Helper-independent latent HDV infection*

A third form of HDV infection was described in the transplant setting and is characterized by a helper-independent latent infection of the hepatocytes by HDV which can be rescued by the helper virus at a later time. Intranuclear HDAg can be detected in the grafted liver as early as a few hours after transplantation in the absence of both productive HDV infection (i.e. serum HDV RNA cannot be detected by hybridization assays) and HBV reinfection (i.e. undetectable HBsAg in serum). This situation is possibly due to reinfection of the

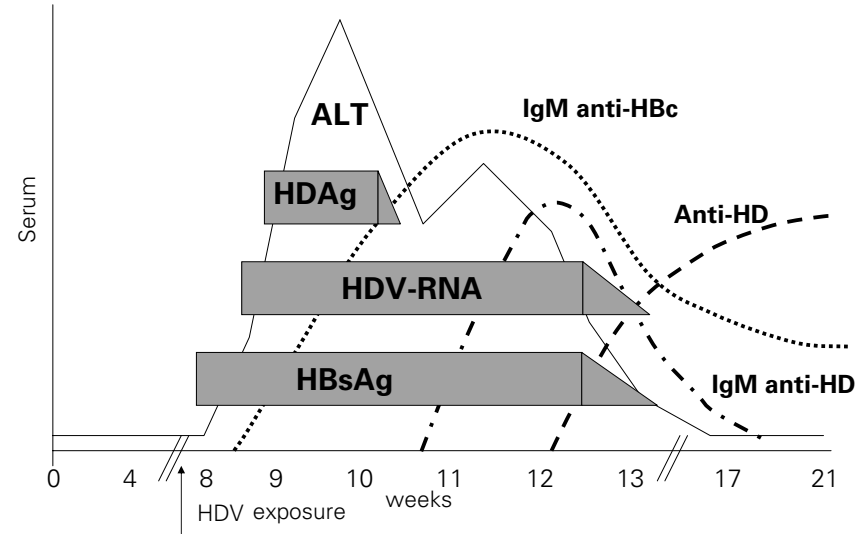


**Figure 37.2** Serological and serum biochemical changes associated with acute HDV co-infection. ALT, alanine aminotransferase; HDAg, hepatitis delta antigen; HDV-RNA, hepatitis delta virus RNA; anti-HD, antibody to HDAg; HBsAg, hepatitis B surface antigen; anti-HBc, antibody to hepatitis B core antigen.

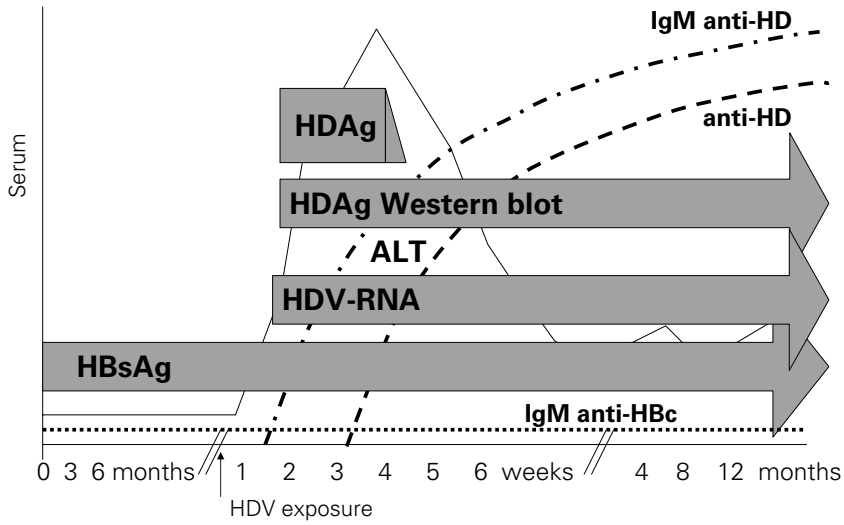


**Figure 37.3** Serological and serum biochemical changes associated with acute HDV superinfection. ALT, alanine aminotransferase; HDAg, hepatitis delta antigen; HDV-RNA, hepatitis delta virus RNA; anti-HD, antibody to HDAg; HBsAg, hepatitis B surface antigen; anti-HBc, antibody to hepatitis B core antigen

allograft by HDV alone, while simultaneous infection of the hepatocytes by HBV is prevented by hepatitis B immunoglobulins. During this phase there is no or limited evidence of liver disease. HD viraemia and hepatitis recur only when HBV evades neutralization, resulting in co-infection of the allograft and establishment of productive HDV infection.[49] According to our present understanding of the HDV life-cycle, during the non-productive phase, HDV can replicate its genome and produce HDAg inside the hepatocyte. Such incomplete virion lacking the HBsAg envelope cannot be exported out of the hepatocyte. When the hepatocyte is infected by HBV, the full HBsAg-coated virion of HDV can be assembled and exported out of the hepatocyte, and can infect contiguous hepatocytes or circulate.

This phenomenon has been reproduced *in vivo* in the woodchuck model of HDV infection. Woodchucks that have never been exposed to the woodchuck hepatitis virus (WHV) were inoculated with sera containing a high proportion of HDV versus WHV particles. HDV viraemia was undetectable despite the presence of intrahepatic HDAg. Productive HDV infection occurred only when the woodchucks were challenged – 1 month after the first inoculation – with a high infectious dose of WHV, which apparently rescued HDV replication.[50]

## Chronic HDV infection and disease

Throughout the 1980s, HDV infection was consistently associated with serious liver diseases spanning from fulminant hepatitis to chronic active hepatitis rapidly progressive to cirrhosis, liver failure and death or transplantation.[12] However, it is now clear that, at least in some areas, chronic infection can result in a relatively mild disease that is not significantly different from that due to HBV infection alone.

### *Cross-sectional studies*

In apparently asymptomatic HBsAg carriers with anti-HD the liver biopsy often shows occult but significant hepatic damage[51–57] (Table 37.1).[51,56–59]

In a study conducted in Italy, in 2487 individuals fortuitously found to have the HBsAg in the blood, 112 (5%) were anti-HD-positive. Liver function tests were impaired in 38% of the anti-HD-positive carriers but in only 9% of the 2375 anti-delta-negative subjects. Liver

**Table 37.1** Histological patterns in chronic HDV hepatitis

| Reference | Number of cases | NL/CPH/CLH | CAH | Cirrhosis |
|---|---|---|---|---|
| Rizzetto *et al.* 1983 | 137 | 12 (9%) | 93 (68%) | 32 (23%) |
| Colombo *et al.* 1983 | 50 | 7 (14%) | 17 (34%) | 26 (52%) |
| Govindarajan *et al.* 1983 | 19 | 1 (5%) | 1 (5%) | 17 (90%) |
| Aricò *et al.* 1985 | 31 | 12 (39%) | 7 (22%) | 12 (39%) |
| Sagnelli *et al.* 1989 | 64 | 23 (36%) | 28 (44%) | 13 (20%) |

NL, normal; CPH, chronic persistent hepatitis; CLH, chronic lobular hepatitis; CAH, chronic active hepatitis.

biopsy specimens obtained from 31 antibody-positive and 97 antibody-negative subjects showed important histological changes in 61% of the 31 antibody-positive carriers (seven chronic active hepatitis, four active cirrhosis, eight inactive cirrhosis) but in only 19% of the 97 antibody-negative carriers.[58]

Another study conducted in 203 Italian HBsAg carriers showed that the 64 patients with positive HDAg on liver biopsy more frequently had severe chronic active hepatitis (CAH) (30% versus 12%) and less frequently chronic persistent hepatitis (CPH) (11% versus 29%) than the 139 HDAg-negative patients. Patients with HDAg-positive cirrhosis were significantly younger than those with HDAg-negative cirrhosis.[59] Similar findings were obtained for British patients.[55]

As in adults, chronic HDV hepatitis in children was usually accompanied by serious liver disease, which was unresponsive to immunosuppressive treatment. Serological evidence of HDV infection was found in 34 of 270 Italian children with HBsAg-positive liver disease. The prevalence of HDV infection increased in parallel with the histological activity of the disease and was maximal in children with cirrhosis. During 2–7 years of follow-up, the hepatitis deteriorated in 38% of the patients with delta infection and ameliorated only in 9%. By contrast the disease usually ran a mild course in the 236 delta-negative carriers of HBsAg, with remission in 55% and deterioration in only 7%.[60]

### *Prospective and retrospective-prospective studies*

In 1983, our group described the natural history of 101 chronic HDV patients followed for 2–6 years. At enrolment, the predominant liver disease was chronic active hepatitis in 93 patients with cirrhosis in 32; minor forms of chronic persistent or lobular hepatitis were seen in 12 patients. Cirrhosis developed in 41% of patients without nodular regeneration seen in the first biopsy specimen; five of these patients died. Treatment with prednisone or azathioprine did not induce histologic amelioration of delta hepatitis or prevent cirrhosis.[57]

Fattovich and co-workers compared 18 chronic HDV patients with 128 patients with chronic hepatitis B. During 1–15 years of follow-up, histological deterioration was documented in 77% of anti-HDV-positive patients but only in 30% of HBsAg carriers without HDV infection. In seven of the 10 anti-HDV-positive patients who showed transition from chronic active hepatitis to cirrhosis, this event was observed within the first 2 years of follow-up.[53]

In recent years, due to the availability of longer follow-up periods, natural history studies have been more focused on clinical end-points of HDV disease and less on intermediate markers of disease progression such as liver histology deterioration. Furthermore, these studies have correlated distinct histories of disease with the way of exposure to HDV and with the presence or absence of independent modulators of disease progression (co-infection with HIV and/or HCV, drug and alcohol abuse, poor socio-economic conditions).

While studies conducted in communities with possible interfering factors (poor hygienic conditions, institutionalization) show an association between HDV infection and greater risk of liver-related mortality,[61,62] other studies (preferentially enrolling patients from endemic areas around the Mediterranean basin) could not establish for HDV alone an independent role in leading to liver-related death.[63–65]

Data collected in a closed Greek community suggest that HDV infection, if contracted in an endemic context without frequent high-risk exposures, may run a mild course without overt liver disease in a significant proportion of cases.[66]

In a study conducted on 159 patients from northern and southern Italy enrolled over a period of two decades and followed up for 78 ± 59 months, we characterized in a subgroup of 10% of the patients a non-progressive form of chronic hepatitis D resembling the mild course described as predominant among HDV-infected persons in the Greek community of Archangelos.[67] In the other 90% of the patients, baseline histology was marked by severe hepatitis and cirrhosis and the disease was progressive, the mean interval from primary infection to compensated histological cirrhosis spanning 9 years. Once this histological stage was reached, the clinical disease slowed down and the medical conditions remained stable and compatible with a good quality of life for an average of 13 years, with a total delay of 22 years from primary infection to clinical decompensation[32] (Fig. 37.4).

Thus, the chronic disease caused by HDV appears to run a peculiar biphasic course. During the initial florid phase of HDV infection, the disease rapidly progresses to liver failure, accounting for the steep decrease in survival curves observed in both severe hepatitis and histological cirrhosis. If the patient survives, the disease slows, delaying decompensation for more than a decade.

A formal comparison of 90 HDV/HBV patients versus 88 pure HBV-infected patients was conducted in Sicily. Subjects with HBV/HDV disease had more frequently active necroinflammation (65% versus 27%) and histological cirrhosis (73% versus 35%) at presentation. The more severe histology was associated with a trend – although not statistically significant – towards major disease events such as death, liver failure and development of hepatocellular carcinoma (HCC).[68] A prospective study conducted in European HBV patients with histologically confirmed cirrhosis at enrolment assigned to HDV infection a role in increasing the risk of

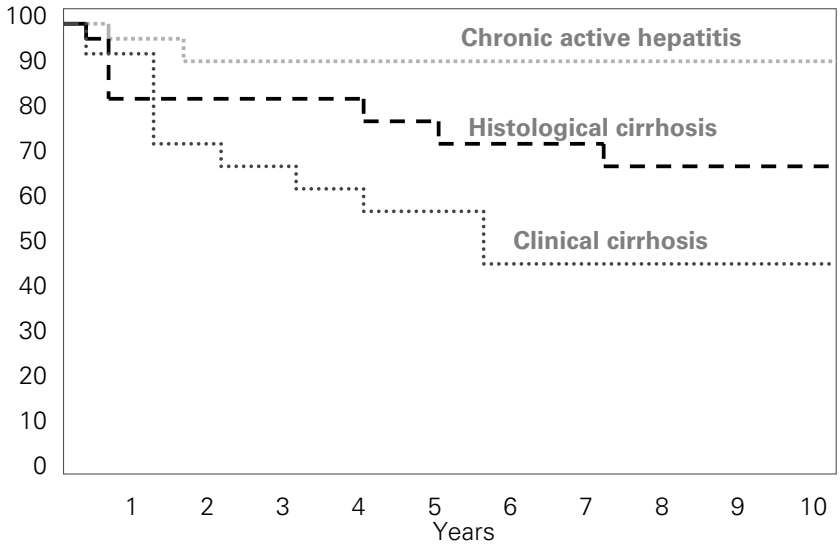


**Figure 37.4** Probability of survival of patients with chronic hepatitis D according to histology or clinical staging at enrolment.

liver decompensation and liver-related death (risk for decompensation and mortality increased by a factor of 2.2 and 2.0, respectively, in anti-HDV-positive relative to HDV-negative cirrhotic patients).[69] Such apparently limited influence of HDV as modifier of HBV infection natural history contrasts with the disproportionate impact of HDV in the liver transplant setting. In Italy, as elsewhere in Europe, the proportion of HDV patients receiving transplants in recent years amounted to 45% of HBsAg-positive cases versus a prevalence of HDV in the general HBsAg population of <10%. Furthermore, their age at the time of liver transplantation was significantly lower than that of cirrhotic patients transplanted for HBV infection alone.[70] Still follow-up periods that were too short and insufficient to detect divergencies in life expectancy between HDV/HBV and HBV patients, as well as a dilutional effect of the indolent non-progressive forms of hepatitis D, can account for such apparent discrepancies.

The relationship between HDV infection and the risk of HCC is controversial. In some parts of the world where HBV-related HCC is very common (such as Southern Africa and Taiwan), very few cases of HCC associated with HDV cirrhosis have been found.[71,72] Similar proportions of patients with cirrhosis with or without HCC were found to have serological markers of HDV infection in Italy,[68] but patients with HBV/HDV infection developed HCC earlier than patients with HBV infection alone.[73] Recently, Fattovich and co-workers have evaluated the impact of HDV infection on the clinical course of compensated cirrhosis type B in 200 European patients followed for a median period of 6.6 years. The median age at enrolment was lower (34 vs 48 years) in the 40 HBV/HDV-infected patients. The unadjusted Kaplan-Meier analysis of the cumulative incidence of HCC showed no significant difference between patients infected with HBV/HDV and HBV alone. However, after adjustment for age, clinical and serological differences at baseline, the risk for HCC was increased by a factor of 3.2 in anti-HDV-positive relative to HDV-negative cirrhotic patients. The adjusted estimated 5-year risk for HCC was 13%, 4% and 2% for anti-HDV-positive/HBeAg-negative, anti-HDV-negative/HBeAg-negative, and anti-HDV-negative/HBeAg-positive cirrhotic patients, respectively.[69]

## Factors influencing the outcome of delta hepatitis

The factors influencing the pathogenesis of HDV disease – HDV-related factors such as genotype, helper virus-related factors, such as levels of HBV replication, and host-related factors, such as immune response – may also influence its natural history.

### HDV-associated factors

Genotype II, which is predominant in the Far East, has been linked with a generally benign HDV disease.[74] Within this genotype an isolate (genotype IIb-M) associated with progressive disease has recently been separated.[75]

In the Mediterranean basin, genotype I represents the almost exclusive genotype and is associated with a broad spectrum of disease,[38,39] whereas in Taiwan and in Russia it appears to be responsible for fulminant hepatitis and rapidly progressive liver diseases.[37,76] On the other hand, all the cases of mild hepatitis D described

for the first time in the Archangelos community of Rhodes (Greece) are determined by genotype I but with an extraordinarily low sequence divergence.

HDV isolates responsible for the epidemics of fulminant hepatitis in Colombia, Venezuela and Peru all belong to a distinct viral genotype denoted genotype III, which is almost always joined with HBV genotype F.[36,77,78]

The mechanisms that link genotypes of HDV with clinical outcomes have not yet been fully elucidated. Genotypic variations are unevenly distributed along the sequences coding for HDAgs. Of these variations, the packaging signal at the C-terminus has a divergence of 74% between genotypes I and II. *In vitro* studies have shown that the package efficiency of genotype I HDV is higher than that of genotype II. Genotype II HDV therefore secretes fewer viral particles than genotype I HDV, which in turn may reduce the extent of infection of hepatocytes and result in less severe hepatic inflammation.[79]

An important factor modulating the speed of progression of HDV diseases is the replicative activity of HDV. Distinctly higher rates of serum HDV RNA have been reported in patients with severe hepatitis (76%) and with early florid histological cirrhosis (71%) than in those with late decompensated cirrhosis (33%).[32,74] This evidence suggests that HDV plays a role in driving severe hepatitis to cirrhosis, when cirrhosis is reached HDV replication abates, and this is likely to slow down further progression of the disease.

### Helper virus-associated factors

The presence and level of HBV replication play a role in determining the outcome of HDV infection. Patients with evidence of HBV replication – as detected by molecular hybridization methods – tend to have more severe liver disease and progress more rapidly to liver failure.[80] Detection of serum HBeAg and HBV DNA at baseline and the persistence of detetectable serum HBV DNA during follow-up have been recognized as significant risk factors for the development of end-stage liver disease (relative risk 9, 7 and 11, respectively).[32]

Spontaneous HBsAg clearance can occur over the years (10% during a 4-year follow-up), and in Italian patients has often been associated with improvement of the HDV disease.[81] Such a benefical effect has not been confirmed by Chen and co-workers in Taiwan, who showed progression to cirrhosis and HCC after HBsAg clearance only in patients with HDV or HCV co-infection.[82]

### Host-associated factors

At present, no data are available on the role of host genetic polymorphisms modulating immunologic-inflammatory response and fibrosis on the outcome of HDV disease.

In HIV-induced immunosuppression, simultaneous replication of both HBV and HDV is often detectable, leading to a rapidly advancing disease.[83] Progression to liver failure can be further accelerated if there is co-existent HCV infection.[84]

Cirrhosis seems to be more frequently observed in patients with multiple infections. The presence of a concomitant HCV infection induces a reciprocal inhibition between hepatitis B, D and C viruses. In 22 Spanish patients with HDV/HBV/HCV co-infection, HDV was the dominant virus and had a greater unfavourable influence on HCV than on HBV replication.[85] In French patients with triple infection, serum HCV RNA and markers of HBV replication were absent in 80%, suggesting that HDV acts as a dominant virus.[86] In contrast, of the three hepatotropic viruses HCV acted as the dominant one in co-infected patients studied in Taiwan.[87]

## Summary and conclusions

HDV was responsible for a high proportion of severe cases of acute and chronic liver disease all over the world during the 1970s and 1980s. Due to its dependence on HBV, in areas where vaccination against HBV is institutional and the risk of parenterally transmitted infection is perceived, its circulation is now sharply declining at a pace of approximately 1.5% per year. Such data would prelude the almost complete control of HDV infection in the near future.

Once considered almost invariably pathogenic and rapidly leading to liver failure and death, HDV is now associated with a broad spectrum of liver diseases which may or may not progress to cirrhosis and liver failure. HDV genotype seems to play a major role in determining the outcome of HDV disease, at least in some geographical areas.

The progressive variety of HDV hepatitis appears to run a peculiar biphasic course. During the initial florid phase, the disease can rapidly advance to cirrhosis and liver failure but, if the patient survives, disease activity slows down, delaying decompensation for more than a decade.

The diminishing epidemiological impact together with the disparate natural history has changed the medical scenario of chronic hepatitis D, which is now dominated by long-standing infections whose clinical expression is either an indolent non-progressive hepatitis that requires no therapy or cirrhosis for which ultimately only liver transplantation offers a therapeutic option.

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# Chapter 38
# Treatment

Grazia Anna Niro, Floriano Rosina, Mario Rizzetto

Delta hepatitis is the most uncommon and possibly the most severe form of chronic viral hepatitis in humans. Acute infection with the hepatitis delta virus (HDV) is often severe, with a fatality rate of approximately 5%. Chronic delta hepatitis leads to cirrhosis in at least two-thirds of persons and at a younger age than in patients with HBV infection alone. Hepatitis D is most resistant to current antiviral therapies.

Being linked to hepatitis B virus (HBV) and only occurring in persons who have hepatitis B surface antigen (HBsAg) in serum, HDV is spread in the same ways as hepatitis B, through parenteral or sexual exposure to blood or body fluids. Up to 5% of the world's population is infected with HBV, and probably 5% of the HBV carriers have HDV superinfection.[1] Prevention of hepatitis B will also prevent hepatitis D, and the implementation of vaccination for hepatitis B is contributing to the current decline in HDV incidence. Nevertheless, there are still several millions of persons with chronic HDV infection and, until hepatitis B is fully eradicated, new cases of delta hepatitis will occur.

Although many viruses encode the majority of their replicative and processing enzymes, HDV apparently does not. The sole enzymatic activity that HDV possesses is a ribozyme that autocleaves the circular RNA, producing a linear molecule.[2] Other enzyme activities are apparently provided by the host cell. These features make the HDV difficult to eradicate, as most of the possible therapeutic targets are normal cellular proteins.

## Treatment of acute hepatitis D

Acute hepatitis requires the monitoring of the clinical and biochemical parameters of liver function to allow the early detection of progression to fulminant hepatitis. If fulminant hepatitis occurs, orthotopic liver transplantation is the only treatment.

Trisodium phosphonoformate (foscarnet) was used to treat three patients with fulminant HBV/HDV hepatitis; all three recovered.[3] Foscarnet is an inhibitor of the DNA polymerase and is, therefore, capable of interfering with HBV replication. Paradoxically, *in vitro* studies showed that foscarnet enhances rather than inhibits HDV replication,[4] so it is conceivable that remission of fulminant hepatitis occurred by chance rather than from the effect of foscarnet administration. The drug has not been used further. Recombinant interferon (IFN)-α-2c was administered to nine patients with fulminant HDV hepatitis. Eight died and in the survivor treatment for 3 months did not prevent the development of chronic delta infection and liver cirrhosis. IFN-α-2c has no therapeutic value in fulminant HDV hepatitis.[5]

## Treatment of chronic hepatitis D

Concomitant infection with an RNA (HDV) and a DNA (HBV) virus makes chronic hepatitis D more difficult to treat than chronic hepatitis B alone. As with hepatitis B, poor results were obtained in the treatment of hepatitis D with immunosuppressive and immunostimulant drugs.[6,7] In the mid-1980s, pilot clinical trials suggested that IFN-α could inhibit HDV replication and control hepatitis D in some groups of patients. Usually, however, this effect has not outlived the end of short-term IFN therapy.[8–10]

### IFN

#### *Clinical trials with IFN*

On the assumption that prolonged treatment with IFN might induce a therapeutic effect, several clinical trials based on the long-term administration of IFN were undertaken in the late 1980s (Tables 38.1 and 38.2).[8–24] The response, assessed on the normalization of serum alanine aminotransferase (ALT) levels and the clearance of serum HDV RNA, varied widely and occurred at different times from the beginning of treatment, sometimes after discontinuation of IFN. The rate of response was proportional to the dose of IFN. Patients treated with 9 million units (MU) responded better than patients treated with 3 MU.[12] Virological and biochemical evidence of relapse was common when IFN was reduced to 3 MU/$m^2$ after a 4-month course with 5 MU/$m^2$.[8,15] Sustained

**Table 37.1** Controlled trials of treatment of chronic HDV hepatitis with IFN

| Reference | No. of patients | IFN schedule | End of treatment | | End of follow-up | |
|---|---|---|---|---|---|---|
| | | | HDV RNA-negative | ALT normal | HDV RNA-negative | ALT normal |
| Rosina *et al.*[10] | 12 | Alpha 2b recombinant t.i.w.<br>5 MU/m² × 3 months | 4 (33%) | 4 (33%) | 1 (8%) | 1 (8%) |
| | 12 | Untreated | 2 (17%) | 0 (0%) | 2 (17%) | 0 (0%) |
| Rosina *et al.*[11] | 31 | Alpha 2b recombinant t.i.w.<br>5 MU/m² × 4 months<br>3 MU/m² × 8 months | 14 (45%) | 8 (25%) | 14 (45%) | 1 (3%) |
| | 30 | Untreated | 8 (27%) | 0 (0%) | 10 (33%) | 0 (0%) |
| Farci *et al.*[12] | 14 | Alpha 2a recombinant t.i.w.<br>9 MU × 12 months | 10 (71%) | 10 (71%) | 0 (0%) | 5 (36%) |
| | 14 | Alpha 2a recombinant t.i.w.<br>3 MU × 12 months | 5 (36%) | 4 (28%) | 0 (0%) | 0 (0%) |
| | 14 | Untreated | 1 (7%) | 1 (7%) | 0 (0%) | 0 (0%) |
| Gaudin *et al.*[13] | 11 | Alpha 2b recombinant t.i.w.<br>5 MU/m² × 4 months<br>3 MU/m² × 8 months | 7 (66%) | 7 (66%) | 1 (9%) | 1 (9%) |
| | 11 | Untreated | 4 (36%) | 2 (18%) | NA | NA |
| Madejon *et al.*[14] | 12 | Alpha 2a recombinant t.i.w.<br>18 MU × 6 months<br>9 MU × 1 month<br>6 MU × 1 month<br>3 MU × 4 months | 6 (50%) | 4 (37%) | 0 (0%) | 0 (0%) |
| | 14 | Alpha 2a recombinant daily<br>3 MU × 12 months | 3 (19%) | 1 (7%) | 0 (0%) | 0 (0%) |
| Borghesio *et al.*[15] | 5 | Lymphoblastoid t.i.w.<br>10 MU up to normal ALT plus 12 months | NA | 0 (0%) | NA | NA |
| | 4 | Lymphoblastoid daily<br>5 MU up to normal ALT plus 12 months | NA | 2 (50%) | NA | NA |

t.i.w., three times a week; NA, data not available.

responses were unusual and often incomplete, with ALT persistently normal despite the recrudescence of HDV viraemia.[12] Concomitant sustained biochemical and virological responses were usually accompanied by the clearance of serum hepatitis B surface antigen (HBsAg) and seroconversion to anti-HBs.[22,25,26] Loss of HBsAg has occurred after up to 12 years of continuous IFN therapy and was associated with improvement of fibrosis at liver histology.[27] Histological examination revealed a significant decrease in hepatic injury and in hepatitis D antigen (HDAg) staining in patients responding to IFN.[12,28]

More complex schedules and combinations of IFN with other antiviral drugs (e.g. acyclovir) were not more successful and reactivation during the follow-up was the rule.[18,20]

### *Side-effects of IFN*

The side-effects are those typical of IFN treatment, with a trend towards more frequent and severe psychiatric side-effects. Flu-like symptoms, fatigue and weight loss were seen in almost all treated patients. The severity of reactions was usually proportional to the IFN dose and to the age of the patient. In drug abusers, compliance is often poor and the intermittent use of IFN increases the incidence and severity of side-effects. One such patient committed suicide in the 11th month of treatment, and another attempted suicide shortly after discontinuation of treatment.[13,22] Ordinary patients may also become deeply depressed if treated with high doses for prolonged periods. In an Italian multicentre trial comparing the long-term administration of lymphoblastoid IFN 10 MU three times a week and 5 MU daily, one patient in the 10-MU arm committed suicide 15 days after treatment discontinuation; three other patients, one in the 10-MU arm and two in the 5-MU arm, required treatment discontinuation because of severe depression.[15] Seven (29%) of the 25 patients treated by Rumi *et al*.[23] had to stop IFN (6 MU three times a week) because of depression, fatigue, myalgias and liver decompensation.

**Table 37.2** Uncontrolled trials of treatment of chronic HDV hepatitis with IFN

| Reference | No. of patients | IFN schedule | End of treatment | | End of follow-up | |
|---|---|---|---|---|---|---|
| | | | HDV RNA-negative | ALT normal | HDV RNA-negative | ALT normal |
| Farci *et al.*[9] | 5 | Lymphoblastoid t.i.w.<br>2.5–7.5 MU/m² × 2–12 weeks | 4 (80%) | 0 (0%) | 4 (80%) | 0 (0%) |
| Buti *et al.*[16] | 4 | Alpha 2a recombinant t.i.w.<br>9 MU × 6 months | 3 (75%) | 3 (75%) | NA | NA |
| Taillan *et al.*[17] | 8 | Alpha 2b recombinant t.i.w.<br>5 MU × 6 months | NA | 2 (25%) | NA | NA |
| Di Bisceglie *et al.*[8] | 12 | Alpha 2b recombinant daily<br>5 MU × 4–36 months | 5 (42%) | 0 (0%) | NA | NA |
| Marinucci *et al.*[18] | 8 | Alpha 2a recombinant t.i.w.<br>6 MU × 12 months<br>stop × 1 month<br>1 MU × 12 months | 3 (37%) | 1 (12%) | 1 (12%) | 1 (12%) |
| Craxi *et al.*[19] | 10 | Alpha 2b recombinant t.i.w.<br>5 MU/m² × 4 months<br>3 MU/m² × 8 months | 7 (70%) | NA | 2 (20%) | NA |
| Berk *et al.*[20] | 10 | Lymphoblastoid 5 MU/day<br>× 4 months<br>Acyclovir 2 g/day × 2 weeks<br>× 2 cycles | NA | 5 (50%) | NA | 2 (20%) |
| Marzano *et al.*[21] | 7 | Lymphoblastoid t.i.w.<br>5 MU/m² × 4 months<br>3 MU/m² × 8 months | 5 (71%) | 5 (71%) | 3 (43%) | 3 (43%) |
| Lau *et al.*[22] | 6 | Alpha 2b recombinant<br>10 MU × 5 days daily<br>10 MU × 1 month t.i.w.<br>5 MU × 2 months t.i.w.<br>3 MU × 9 months t.i.w. | NA | 4 (66%) | NA | NA |
| Rumi *et al.*[23] | 25 | Alpha 2b recombinant t.i.w.<br>6 MU × 24 months | NA | 6 (24%) | NA | 2 (8%) |
| Puoti *et al.*[24] | 16 HIV infected | Alpha 2b recombinant t.i.w. | NA | 2 (12%) | 1 (6%) | 1 (6%) |
| | 21 HIV uninfected | 10 MU × 6 months<br>6 MU × 6 months<br>3 MU × 12 months | NA | 2 (9%) | 2 (10%) | 2 (10%) |

t.i.w., three times a week; NA, data not available; HIV, human immunodeficiency virus.

Four patients had a severe hepatitic episode while on full dose IFN.[11,18] Liver kidney microsomal antibodies (anti-LKM) were present in one patient. However, in different trials 10 other anti-LKM-positive patients have completed cycles of IFN therapy without complications.[29,30]

IFN should be used cautiously in children with or cured of paediatric malignancies. Two of nine patients with compensated HDV cirrhosis developed a severe biochemical exacerbation during IFN therapy that led to fatal liver failure in one.[31]

### *Factors predictive of response to IFN*

Analysis of the pretreatment characteristics of patients has not identified differences between responders and non-responders to IFN with regard to demographic, clinical, serological, biochemical and histological findings.

Several adults and children with HDV infection and active HBV replication cleared HBV DNA and hepatitis B e antigen (HBeAg) from serum and seroconverted to anti-HBe during IFN therapy. This event usually occurred without relevant changes in liver chemistry and was not usually followed by return of ALT to normal levels.[13,19,26] In view of the poor prognosis of patients with active replication of both HBV and HDV,[32] therapeutic inhibition of HBV should nevertheless be considered a worthwhile therapeutic success.

Although disease of a short-standing nature may respond better,[21] young age may have an adverse in-

fluence. None of 10 children treated by Craxi *et al*.[19] improved clinically or histologically after treatment for a year. Among human immunodeficiency virus (HIV)-positive drug abusers, who had usually acquired HBV and HDV infection in the recent past, responses have occurred in those in whom immunocompetence was still preserved.[16,17,24]

No data are available as to whether HDV genotypes may influence the rate of response to IFN.

### *Mechanism of action of IFN*

The mechanism of action of IFN in chronic hepatitis D is poorly understood. In HDV transfected hepatoma cell lines, HDV replication is not affected by IFN.[33,34] *In vitro* experiments apparently contrast with the results observed in patients in whom response to IFN is often characterized by a concomitant reduction in HDV viraemia and ALT levels, suggesting a direct antiviral effect of the cytokine on HDV.

A major difference between these two models is that patients are always co-infected with HBV. It is conceivable, therefore, that in keeping with the frequent clearance of serum HBsAg in long-term responders, the IFN activity against HBV may play a crucial role. In HBsAg-negative long-term responders, HDV RNA and HDAg are no longer detectable in serum and liver.[22,25,26] Patients who do not clear HBsAg may enter a sustained biochemical and histological remission, despite persistence of active HDV replication.[12,35] IFN may be able to induce the emergence of less pathogenic or non-pathogenic mutants. Alternatively, it could accelerate the rate of mutation at the termination codon for delta antigen (codon 196), leading to overproduction of the non-cytotoxic large delta antigen (HDAg-p27).

In patients who respond to IFN, the inhibition of viral replication is associated with a high stability of the viral genome before and during treatment. A wide range of quasi-species before treatment and modifications of the viral genome during treatment might be responsible for the re-emergence of HDV during or after therapy.[36]

## Other treatments

Animal (woodchuck) and cell culture (woodchuck hepatocytes) systems are available to screen antiviral compounds for activity against HDV. So far, antiviral agents have not proven beneficial.

### *Suramin*

Suramin *in vitro* blocks entry of HDV virions into hepatocytes.[4] It is also known to act on hepadnaviruses and retroviruses, but the drug is too toxic for long-term use in humans.

### *Acyclovir*

Acyclovir, used previously and unsuccessfully as an adjuvant therapy in IFN-treated patients,[20] seems to enhance rather than inhibit HDV replication *in vitro*.[4]

### *Ribavirin*

Ribavirin, a nucleotide analogue, inhibits HDV replication in cell culture.[4] It was given at 15 mg/kg/day orally for 16 weeks to nine patients but had to be suspended in two of them after 2 weeks, in one because of haemolytic anaemia and in the other because of intractable itching. No significant virological or biochemical results were seen.[37] Negative results were also obtained in another small randomized series.[38]

### *THF γ-2*

THF γ-2, a synthetic thymus-derived octapeptide possessing a wide range of immunomodulatory effects, has been effective in chronic infection by HBV. The drug was used in 11 patients with chronic HDV disease without any consistent virological or biochemical effect.[39,40]

### *Deoxynucleotide analogues*

Although HBV is not involved in the replicative cycle of HDV, the HBsAg represents the envelope of HDV without which HDV cannot be secreted out of the hepatocyte. Repression of HBsAg production long enough for the infected hepatocytes to die could lead to eradication of HDV infection by preventing the spread of infectious virions to contiguous hepatocytes. This reasoning has provided the rationale for testing the therapeutic potential of deoxynucleotide analogues in hepatitis D (e.g. famciclovir, lamivudine, etc.).

In a pilot study, 15 adult patients (13 men, 2 women), aged 20–52 years, with chronic delta hepatitis were treated with famciclovir 500 mg three times a day for 6 months and were then followed up for 6 months post-treatment. HBV DNA levels decreased in 9 of the 15 patients but rose again after treatment. Famciclovir had no effect on ALT, HBsAg levels or serum HDV RNA. There was no improvement in liver histology.[41]

Five patients received lamivudine 100 mg orally daily for 12 months.[42] Despite a general reduction of HBV DNA so that viraemia was undetectable in four patients and decreased by 5 logs in the other, ALT remained abnormal, HDV RNA detectable and HBsAg positive in all. Liver biopsy showed no consistent improvement in

inflammatory or fibrotic score. Thus, the inhibitory effect of lamivudine on HBV was not sufficient over 1 year to produce a remission of hepatitis D. However, HDV disease remission was reported after 28 months of treatment with lamivudine 150 mg/day.[43]

A more extensive study has compared a 12-month versus a 24-month course of lamivudine 100 mg/day.[44] A total of 31 patients was randomized to treatment, 11 patients received placebo and 20 received lamivudine for 12 months. Thereafter, all were given lamivudine on an open-label basis for 12 months and followed up for a further 16 weeks. At the end of treatment, HDV RNA was negative and ALT normal in three patients. However, only two patients remained virus-free at the end of follow-up. Nineteen paired biopsies were available pre- and post-treatment. Five patients had a >2 point decrease in the Ishak score and were defined as responders. No significant differences in necroinflammation and fibrosis scores were observed in the two groups. One patient exhibited a complete response and became HBsAg-negative (normal ALT, negative HDV RNA, histological improvement). Lamivudine treatment for 12 or 24 months did not significantly affect biochemical, virological or histological parameters of chronic delta hepatitis in the others.

To evaluate the effect of the combination of lamivudine with high-dose IFN-α therapy, eight patients with chronic hepatitis D were treated with lamivudine for at least 24 weeks. Lamivudine was then combined with a high dose of IFN-α followed by a regular dose (9 MU three times per week) and the patients were followed up for 12 weeks post-therapy. The HBsAg concentration in serum decreased in two patients. The drop of HDV RNA in plasma from baseline during treatment was not significant. At the end of treatment, ALT had normalized in one patient and decreased in three other patients, but three of these four patients showed a biochemical rebound after withdrawal of therapy. The histology activity index (HAI) indicated a drop from a median score of 7 at baseline to 5 at the end of treatment. However, an increase in fibrosis from a median grade of 2 at baseline to 3 at the end of treatment was observed.[45]

Available evidence does not therefore support the use of deoxynucleotide analogues for the treatment of chronic hepatitis D.

## Current medical treatment

At present, IFN still represents the only treatment for chronic hepatitis D. Available data show that a proportion of patients respond to IFN if high doses (i.e. 9–10 MU three times a week) are administered for a sufficient length of time. Because HDV superinfection runs a chronic course in >90% of cases and early administration of IFN may improve the response rate, treatment should begin in patients with acute hepatitis progressing to chronicity as soon as the acute phase is over. Patients with a chronic history and without decompensated liver cirrhosis should be treated as soon as a diagnosis is made.

Parameters predictive of response are still unidentified. Whereas in chronic HBV and hepatitis C virus (HCV) infection response to IFN occurs within 3 months from the beginning of the treatment, in HDV infection response can take up to 10 months. Thus, IFN should be administered for at least a year before a patient is regarded as a non-responder.

A major challenge is when to stop therapy in a patient with a good initial response. Loss of serum HDV RNA and HDAg may not reflect the clearance of the virus, as patients who respond to treatment can lose the serum viral markers but may relapse once treatment is stopped. Treatment, however, can be safely interrupted if serum HBsAg has disappeared.

## Future perspectives

Interest in the therapy of chronic hepatitis D by pharmaceutical companies has much reduced in recent years. The dramatic reduction of HDV infection in Western countries accounts for the absence of interest in treatment of chronic delta hepatitis with new drugs such as pegylated IFN (PEG-IFN). Due to its weekly administration, PEG-IFN could nevertheless represent a reasonable therapeutic option for the long-term treatment required for chronic hepatitis D.

New treatment perspectives will probably rely on the knowledge of HDV structure, life-cycle and interaction with HBV.

The HDV RNA contains a ribozyme whose activities are important to the viral life-cycle and which depends on a unique pseudoknot structure. As is the case with other ribozymes, the autocleavage activity of the genomic HDV ribozyme is strongly inhibited *in vitro* in the presence of different aminoglycosides. Although none of the aminoglycosides that were effective *in vitro* exerted suppressive effects *in vivo* in cell lines, these observations suggest a potential strategy for anti-HDV therapy.[46]

### *Antisense oligonucleotides*

Antisense oligonucleotides hold considerable promise as therapeutic agents against several classes of viral infections. Recently, an analysis has begun of the effects of antisense oligonucleotides on HDV RNA replication in transfected cells in culture. Oligonucleotides directed against the self-cleavage domain were highly effective at reducing the level of replicating genomic HDV RNA (>90%). Transfection and treatment of chronically HBV-

producing cell lines are also under investigation to model the modulation of HDV replication under conditions that mimic *in vivo* conditions more closely.[47]

### *Prenylation inhibitors*

The essential role of prenylation in HDV assembly suggests a basis for a novel anti-HDV strategy. Mice were treated with prenylation inhibitors FTI-277 and FTI-2153. Both agents were effective in clearing HDV viraemia dependent on the duration of treatment.[48]

The HDV virion is composed of three general elements: an RNA genome, the delta antigen present in two isoforms and an envelope surrounding the other elements. Only the large delta antigen is capable of promoting particle formation with the HBsAg envelop protein. The molecular basis for this selective role in assembly lies within the 19 amino acids unique to delta antigen. In particular, the last four amino acids constitute a 'CXXX' box which is the substrate for a family of enzymes termed prenyltransferases.[48]

## Transplantation

Liver transplantation provides a valid treatment option for end-stage HDV liver disease. As with other viral disorders, transplantation is aggravated by the risk of reinfection, but this is significantly lower for HDV than for HBV and HCV, and the clinical course of recurrent hepatitis is milder than for HBV. Of 27 patients who underwent transplantation and who received no or only short-term prophylaxis with human immunoglobulins against the HBsAg, 22 (80%) became reinfected with HDV, but only 11 (40%) experienced a recurrence of hepatitis.[35] A high risk of reinfection (80%) was also reported in 14 untreated transplant recipients with HDV, but again the rate of hepatitis relapse was lower than for transplant recipients with hepatitis B.[49] Twenty-five of the 27 patients in the first study and all the patients in the second were alive 1–4 years after transplantation.

Of five transplant recipients with recurrent hepatitis D followed up long term (>8 years), three have a mild form of chronic hepatitis, one has a chronic active hepatitis and one developed a decompensated cirrhosis requiring retransplantation 5 years after the first graft. IFN (3–5 MU three times a week for up to 3 months) has not proved efficacious in these patients. In another seven HDV patients who underwent transplantation and received short-term prophylaxis, four became reinfected with HBV and HDV. Three cleared both viruses after an episode of acute hepatitis, whereas one developed cirrhosis 11 months after transplantation.[50]

The prospect of an uneventful clinical course after transplantation can be improved further by long-term administration of hyperimmune serum against HBsAg. The 5-year survival rate of 76 transplant recipients in Paris for terminal delta cirrhosis was 88%, with reappearance of HBsAg in only 9% under long-term anti-HBs prophylaxis.[51]

Apart from providing a valid therapeutic option, transplantation is a unique model for studying the biology and pathology of HDV infection. This model has shown that HDV can establish subclinical infections in the grafted liver independently of an overt HBV infection, but cooperation with HBV is necessary for disease expression. Disease recurrence is heralded by the exponential increase of HDV replication after full reactivation of HBV.[35]

Liver specimens from nine transplant recipients with relapsing hepatitis D, for whom an adequate histological follow-up was available, have been examined in detail.[52] Whereas in four patients a rise in aminotransferase heralding the return of hepatitis D corresponded with necroinflammation, in five patients the histology corresponded with a syndrome of degenerative lesions of the hepatocytes consisting of balloon-like degeneration, microsteatosis and macrosteatosis, and eosinophilic changes to the cytoplasm without inflammation. In contrast to the pattern of recurrent HBV in the former group, which comprised productive HBV infection accompanied by the full battery of HBV markers, the pattern of HBV reinfection in the latter patients was atypical. HBV DNA in the serum and hepatitis B core antigen (HBcAg) in the liver were absent. In four of these five patients, HBV infection converted to the typical productive pattern within a few weeks, and concurrently the histology changed to the classic necroinflammatory picture of viral hepatitis. These cytopathic changes, which may accompany non-viral acute hepatitis syndromes such as fatty liver of pregnancy,[53] Reye's syndrome,[54] and tetracycline and valproic acid toxicity,[55] appear also to be a feature of human HDV infection, causing fulminant hepatitis in tropical areas.[56] In transplant recipients, this unusual histological pattern was associated with incomplete HBV synthesis, which presumably resulted from some inhibitory interaction of the HDV on the HBV replicative cycle. We speculate that, as a result of this interaction, the expression of nucleocapsid HBV antigens is abrogated and the host immune reaction to the virus is abolished. The cytopathic changes could therefore be related to a direct effect of HDV. In most transplant recipients, however, HBV suppression was only transitory, infection of the graft rapidly resuming inflammatory virulence with expression of the HBcAg and the induction of the usual lymphocytotoxic reaction.

## Conclusion

The medical treatment of chronic hepatitis D rests at present on IFN, which should be administered at high

doses (9–10 MU three times a week) to patients with compensated liver disease and as soon as chronic HDV disease is diagnosed. Treatment should be prolonged for 12 months, as response – clearance of HDV RNA and normalization of ALT levels – can be delayed and sometimes occurs after the end of the treatment. A sustained response is accompanied by the clearance of the HBsAg from serum. Careful medical supervision of treated patients is mandatory for early detection of major medical and psychiatric complications.

Patients with decompensated HDV liver disease should be considered for liver transplantation. As in the case for other viral disorders, transplantation for HDV disease is associated with a risk of reinfection but this risk is much decreased if adequate protection with hyperimmune serum against HBsAg is given.

The strategy of antiviral treatment is changing, from the use of non-specific drugs, such as IFN, to molecularly tailored drugs able to interfere with crucial viral replicative processes. New insights on the replication cycle of HBV have identified potential targets for antiviral therapy, and now the nucleoside analogues are of proven value in the treatment of hepatitis B. These drugs have also been applied to the treatment of chronic hepatitis D, based on the concept that HBV provides the surface antigen (HBsAg) for the assembly of HDV and its propagation. These drugs therefore represent an indirect therapeutic target for hepatitis D. Finally, in recent years the basic knowledge on HDV pathobiology has been exploited, with the development of specific drugs on the basis of such knowledge. It is time to rethink the treatment of chronic delta hepatitis as the treatment of HDV infection and not as the treatment of HBV in order to suppress its helper function to the HDV, the latter approach having been, in the main, unsuccessful. In this regard, antisense oligonucleotides and prenylation inhibitors designed to interfere selectively with the life-cycle of HDV appear the best therapeutic perspective.

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# Section VII
# Hepatitis E Virus

# Chapter 39
# Structure and molecular virology

David A Anderson, R Holland Cheng

Hepatitis E virus (HEV) is a major health problem in many developing areas of the world due to its enteric mode of transmission. HEV infection of swine, and possibly other animals, is common throughout the world, and animal strains provide a reservoir for sporadic zoonotic infection in countries where human HEV is not considered endemic. Virions of HEV are naked, icosahedral particles of approximately 32–34 nm diameter (Fig. 39.1),[1] most likely comprising a single viral protein which encapsidates the single-stranded, positive-sense RNA genome of approximately 7200 nt.

HEV was originally classified within the Caliciviridae on the basis of capsid morphology, biophysical characteristics and overall genome organization, but more detailed sequence analysis[2] resulted in its removal from this family and HEV was considered as an unassigned genus, 'Hepatitis E-like viruses', in the 7th ICTV report.[3] HEV is now classified as the type species of the genus Hepevirus, family Hepeviridae in the 8th ICTV report.[4]

Extensive progress has been made in the development of HEV diagnostics and experimental vaccines based on heterologous expression systems for viral proteins, but the lack of reliable cell culture systems has precluded any detailed studies of HEV replication. The recent description of infectious cDNA clones of HEV[5,6] and the characterization of an HEV-like virus in chickens[7] provide new leads with which to study these areas.

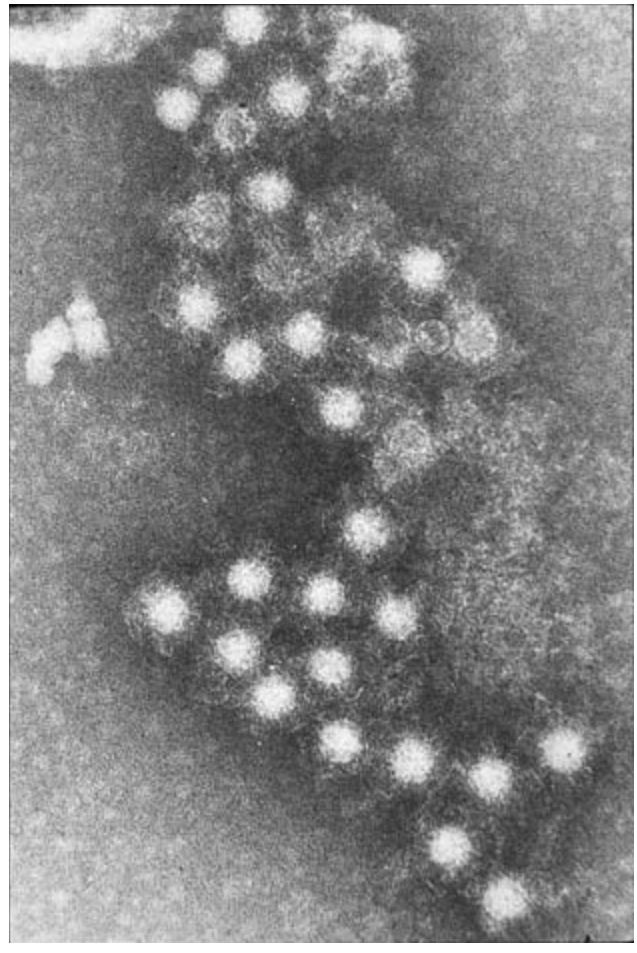

**Figure 39.1** Particles of HEV from the stools of an experimentally infected macaque, complexed with acute phase patient serum. (Photo courtesy of Prof. Zhuang Hui, Beijing Medical University; reproduced from Anderson and Shrestha,[28] with permission.)

## Physico-chemical properties

Virions of HEV are much less stable than those of hepatitis A virus (HAV), being sensitive to conditions such as CsCl and freeze-thawing that have no effect on HAV. This lower stability is likely to be a major factor in the low secondary attack rate for HEV. Virions have a sedimentation coefficient of 183S, and a buoyant density of 1.29 g/cm$^3$ (measured in potassium tartrate/glycerol).[8] The single capsid protein is encoded by open reading frame (ORF) 2, expected to yield a protein of 660 amino acids. However, the capsid protein of authentic viral particles has not been characterized.

## Genome organization

A schematic of the HEV genome and encoded proteins is shown in Fig. 39.2.[1] The 7204 nt infectious cDNA clone of the SAR-55 strain of HEV described by Emerson and colleagues[6] can be assumed to be full-length. Most isolates of HEV are close to this length, with the larger swine,[9] US-2[10] and T1 strains[11] having insertions of around 54 nt within ORF1, while the recently described avian HEV is substantially smaller at 6654 nt due to deletions in both ORF1 and ORF2.[7]

The 5′ end of the genome has a 7-methylguanosine cap,[12] as predicted on the basis of a putative methyltransferase in the viral polyprotein.[13] This cap is essential for infectivity of RNA transcribed from cDNA.[6] Notably, the first report of a putative infectious cDNA clone of HEV[14] did not include a 5′ cap, which would be expected to reduce its replicative capacity.

The viral RNA has short, highly conserved 5′ and 3′ untranslated regions (UTRs) of 25 and 68–75 nt, respectively (Fig. 39.2), which are likely to function in RNA replication and/or encapsidation as for many other RNA viruses. Three ORFs, organized as 5′-ORF1-ORF3-ORF2–3′ with ORF3 and ORF2 largely overlapping, encode the viral proteins (described here as PORFs): PORF1 (replicative polyprotein), PORF3 (unknown function) and PORF2 (capsid protein), respectively (Fig. 39.2). Subgenomic RNAs have been detected in the liver of HEV-infected macaques,[13] but nothing is known of the promoters for these putative subgenomic RNAs. Further studies are also required to determine which RNAs encode each of the viral proteins.

## Genotypes

Early studies identified the prototype Burmese[13,15] and divergent Mexican strains of HEV,[16] but more recent studies have greatly expanded the known diversity of HEV (see Huang FF *et al*.[7] and Schlauder GG, Mushahwar IK[17] for detailed reviews). This list now includes putative 'genotypes' (as defined by Wang and colleagues[18]). The Burmese and related strains (including most Chinese strains) are classified as genotype 1, the Mexican strain is classified as genotype 2, the swine HEV strain discovered in the USA[9] and closely related strains isolated from patients infected in the USA[10] are classi-

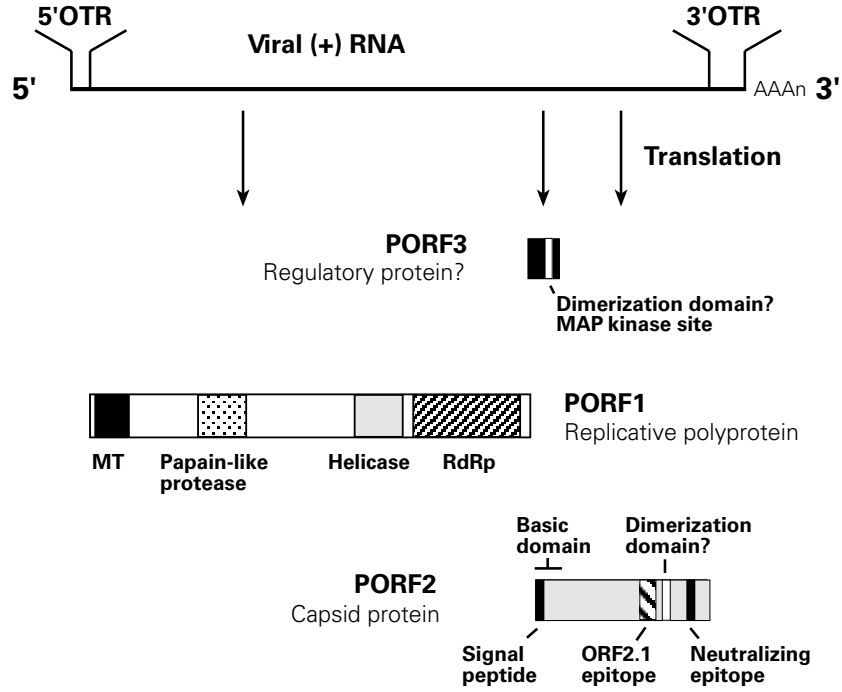


**Figure 39.2** Genome organization and protein domains of HEV. The genome of around 7200 nucleotides contains a 5′ 7-methylguanosine cap and highly conserved 5′ and 3′ untranslated regions (UTRs) of 25 and 68–75 nt, respectively. Three open reading frames (ORFs), organized as 5′-ORF1-ORF3-ORF2–3′, encode the viral proteins which may be translated from a set of subgenomic mRNA molecules. The PORF1 polyprotein contains protein domains consistent with replicative proteins (methyltransferase, protease, helicase, RNA-dependent RNA polymerase – RdRp), and PORF2 is the major capsid protein. The function of PORF3 is unknown, but it may have regulatory or replicative functions. Linear antigenic domains have been identified by peptide scanning throughout each of the three proteins, while within PORF2 a conformational, immunodominant epitope (the ORF2.1 epitope) is found between aa 394 and 457, and a conformational, neutralizing epitope is found between aa 578 and 607. (Modified from Anderson *et al*.,[28] with permission.)

fied as genotype 3, and distinct isolates from patients in China (T1 strain)[18] and both patients and swine in Taiwan[19,20] are classified as genotype 4. Until recently, the original Mexican strain was the only known representative of genotype 2. However, two reports have described strains isolated in Nigeria that appear to be genotype 2,[21,22] whereas other African strains are genotype 1.[23]

Analysis of shorter genome fragments, available from a much wider range of isolates, has led to the suggestion that there may be many more genotypes of HEV.[17] Formal classification of HEV genotypes, subgenotypes and strains will require consensus on the level of genetic heterogeneity for each classification, but should focus on utility for understanding HEV epidemiology and evolution.

Although Balayan and colleagues reported the experimental infection of pigs with HEV in 1990,[24] the isolation and subsequent characterization of swine strains of HEV endemic in the USA[9] provided the foundation for dramatic advances in our understanding of HEV. Recent descriptions of HEV-related viruses from chickens, causing big liver and spleen disease[25] and hepatitis-splenomegaly,[7,26] demonstrate that this viral family is more extensive than previously suspected, and may provide very useful experimental models for understanding HEV biology and replication. The avian HEV-like virus has been fully sequenced,[7] but due to its very low sequence homology with human and swine strains (around 50% at the nucleotide level, compared with a minimum of 75% between mammalian strains) it is uncertain whether this should be considered a strain of HEV, or rather the type species of a second genus within the Hepeviridae family.

Cross-species infection has been experimentally established for swine and human HEV,[27] although it is also likely that some strains are unable to cross species. The detection of endemic human cases of HEV corresponding to local swine HEV strains in many developed countries[10,19] demonstrates that cross-species infection does occur at significant rates in nature, and this poses a particular diagnostic challenge due to substantial variations in many epitopes. The sensitivity of diagnostic assays based on many HEV antigens is affected by amino acid variations in the encoded proteins. However, amino acid sequences are more highly conserved between the genotypes than are nucleotide sequences. The strongly immunodominant ORF2.1 epitope appears to be conserved between divergent genotypes[28,29] including swine.[30,31] ELISAs based on baculovirus-expressed, recombinant truncated PORF2 from both swine and human strains have been shown to have equivalent reactivity with sera against the full range of HEV genotypes, suggesting that there is no significant difference in the overall antigenic profile of the viruses.[32] Variable results are obtained using linear peptides and recombinant antigens that probably represent minor epitopes,[33] and these are less suitable for diagnostic use.

## Viral proteins

### ORF1

Translation of ORF1 yields a polyprotein (PORF1) of approximately 186 kDa[34] containing sequence motifs consistent with methyltransferase, papain-like protease, RNA helicase and RdRp activities[13] (Fig. 39.2). The sites of proteolytic cleavage and the sizes of mature proteins are unknown. However, functional methyltransferase activity of an approximately 110-kDa membrane-bound PORF1 product has been demonstrated in ORF1-transduced insect cells.[35]

Expression of PORF1 alone in HepG2 cells or in an *in vitro* translation system failed to demonstrate any proteolytic processing into mature products.[34] However, the same authors have subsequently used domain-specific antibodies to detect proteins of 35 kDa (methyltransferase), 38 kDa (helicase) and RdRp (36 kDa) in HepG2 cells transfected with a putative infectious cDNA clone of HEV,[14] leading to the conclusion that PORF1 processing can only occur in the context of the replication cycle. Further studies are clearly required to delineate the functional products of PORF1.

The organization of predicted enzyme domains within PORF1 is very different in HEV (Fig. 39.2) compared with Caliciviruses, with HEV PORF1 grouping instead with the non-structural polyprotein of rubella virus, a member of the enveloped Togaviridae family.[36] HEV is obviously very distinct from the togaviruses, justifying its revised taxonomic status as a member of the new family Hepeviridae.[4]

### ORF2

The capsid protein (PORF2) is translated as a 660 aa protein, including a predicted N-terminal signal peptide[13] (Fig. 39.2). Heterologous expression of PORF2 in mammalian cells indeed leads to some degree of translocation due to this signal peptide, marked by N-glycosylation of the protein[37,38] with asparagine 310 being the major glycosylation site.[39]

To date, the capsid protein of authentic HEV particles has not been characterized, and the role of glycosylation remains unresolved. Jameel and colleagues have demonstrated cell surface expression and export of PORF2 to the media,[37] although glycosylation is not required for export.[39] Deletion of the first 34 aa of PORF2 prevents translocation, glycosylation and surface expression,[39] consistent with the predicted role of aa 1–22 as a signal sequence.[13] In contrast, we have been unable to detect surface expression or export of PORF2,[38] but have

instead observed that translocated and glycosylated PORF2 is rapidly degraded within the cell.[40] Studies of HEV virions will be required to determine the nature of the mature capsid protein.

The region between aa 22 and 111 shows significant variation between HEV isolates, but always contains a high proportion of arginine residues. It appears likely that this basic region of the capsid protein is required for interaction with the negatively charged viral RNA.

PORF2 contains a number of linear B-cell epitopes identified by peptide scanning, but these most probably represent only a minor amount of total antibody reactivity. More importantly, there are significant neutralizing epitopes in the region 578–607[41] and a conformational, immunodominant epitope spanning 394–468[29] (Fig. 39.2). Individual monoclonal antibodies to this immunodominant ORF2.1 epitope can block around 60% of total convalescent antibody binding to HEV virus-like particles (VLPs).[29] However, there is no evidence that antibodies to this epitope are neutralizing.

When PORF2 is expressed in insect cells, it is cleaved at a predominant site between aa 111 and 112 and at various sites within the C-terminus of the protein. At least some of these truncated forms of PORF2 have the ability to self-assemble into VLPs or subviral particles (SVPs),[42–46] and cryoelectron microscopy has revealed structural details of such particles[47] (see below). One feature of such VLPs is prominent protrusions that suggest dimeric subunits.[47] Dimer formation has also been observed following expression of PORF2 in yeast systems.[48,49] Interestingly, we have found that the recombinant ORF2.1 protein expressed in *Escherichia coli* also forms dimers, with dimer formation dependent on a leucine zipper-like hydrophobic α-helix in the region 500–514 (Fig. 39.2). Ablation of dimer formation through mutagenesis of this region also destroys the distal, conformational epitope spanning 394–457 (Clements and Anderson, unpublished observations). As this epitope is immunodominant in both ORF2.1 and VLPs,[29] we conclude that there is an important role for dimer formation in the overall structure of the virus.

## ORF3

ORF3 partially overlaps both ORF1 and ORF2 (Fig. 39.2). PORF3 may be translated from the same subgenomic RNA as PORF2[11] or from a discrete subgenomic RNA. Alternatively, PORF3 could be translated from a 'bicistronic' subgenomic RNA, a mechanism which is used by feline calicivirus for its overlapping ORF2 and ORF3 proteins.[50]

PORF3, with a molecular mass of ≈ 13 kDa, is quite variable between HEV strains, but in all cases appears to be very basic (with an isoelectric point around 12.5). PORF3 is commonly reactive with patient antibody. However, it should be noted that reactivity is also seen to epitopes within the non-structural (PORF1) protein of HEV,[51] and PORF3 is not generally considered a structural protein.

Jameel and colleagues have extensively characterized the possible roles of this small protein. PORF3 is associated with the cytoskeleton via the N-terminal hydrophobic α-helix and is phosphorylated at serine residue 80 by mitogen-activated protein kinase (MAPK).[52,53] PORF3 also appears to form dimers via a second hydrophobic α-helical domain, overlapping the MAPK site. These properties are intriguing and suggest that PORF3 may have regulatory functions in virus replication or assembly, but the precise role of PORF3 remains unclear.

## Replication cycle

Our knowledge of HEV replication is poor, due largely to the lack of practicable cell culture systems for the virus and its genetic distance from well-studied viral families. Propogation of HEV has been clearly demonstrated in primary macaque hepatocytes,[54,55] but the level of replication is too low to allow detailed studies of replication. Two groups have reported the detection of cytopathic HEV replication in continuous cell cultures,[56–59] but there have been no independent confirmations of these reports, and contamination by adventitious agents cannot be excluded.

Emerson and colleagues have developed both infectious cDNA clones[6] and replicons[5] of HEV, and these are likely to provide the basis for greatly improved understanding of HEV replication. The development of subgenomic HEV replicons, in which PORF2 and PORF3 have been replaced with green fluorescent protein (GFP), has already demonstrated, perhaps surprisingly, that these proteins are dispensable for genome replication.[5] It can also be hoped that the avian HEV-like virus will prove less refractory to cell culture and may provide essential clues to the overall replication cycle of these viruses, in much the same way that duck hepatitis B virus played a central role in understanding the replication of hepadnaviruses.[60]

In the absence of specific data, the general outline of HEV replication can only be inferred (Fig. 39.3). HEV ingested in water or food must reach the liver via blood, either via direct uptake of the inoculum through the gastrointestinal mucosa into the circulation, or following one or more rounds of amplification in enterocytes, as appears to be the case for HAV.[61] Virus will then interact with one or more specific receptors/co-receptors on the basolateral domains of hepatocytes. Preliminary evidence suggests that HEV VLPs can bind specifically to the basolateral domains of polarized HepG2 cells in

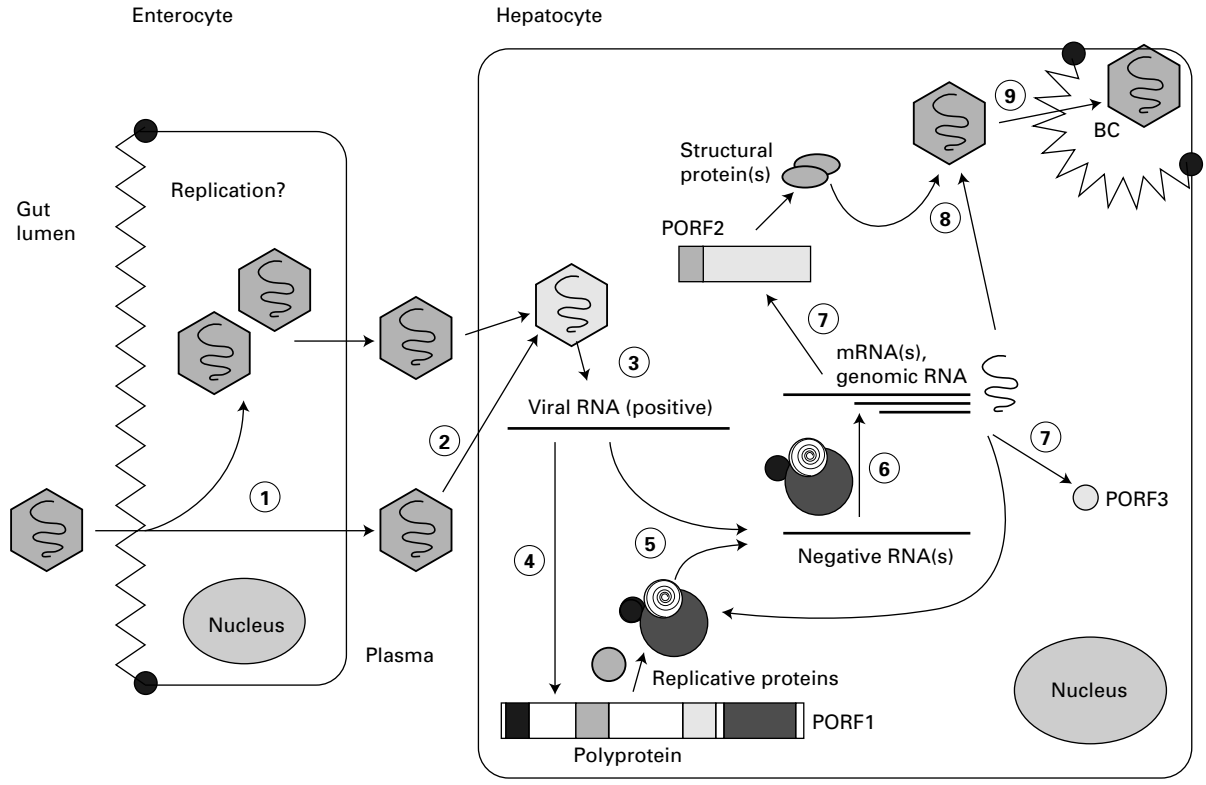


**Figure 39.3** Putative replication cycle of hepatitis E virus (HEV). From the gut lumen, HEV particles enter at the apical surface of the mucosal epithelia (1) where they may either replicate, or be transferred directly by transcytosis through the basolateral domains to the plasma. Virions reach the liver where they attach to an unidentified receptor on the basolateral surface of hepatocytes, leading to virus penetration (2) and uncoating of the genome (3) within the cell. Translation of the input genome (4) yields the PORF1 polyprotein which is cleaved at unknown sites to yield the replicative proteins, which copy the input genome to yield full-length negative-strand RNAs (5), followed by subgenomic positive-strand (messenger) RNAs and full-length positive-strand RNAs (new viral genomes) (6). These subgenomic RNAs are translated to yield further molecules of PORF1, PORF2 (capsid) and PORF3 (regulatory) proteins (7). PORF2 and new viral genomes assemble into virions (8) and progeny virus is released through the apical domain (9) to the bile canaliculi (BC) and ultimately the faeces.

culture (Snooks and Anderson, unpublished observations) (Plate 39.1, found between p. 786–7),[29,42] but the nature of this putative receptor is unknown.

Following penetration and uncoating of the virus, the input viral RNA serves as mRNA for the translation of PORF1 (and possibly PORF3), after which the PORF1 polyprotein is cleaved by viral proteases to yield the mature replicative proteins. The RDRP/helicase complex then copies the input viral genome to yield negative-strand RNA, which in turn serves as a template for the transcription of further positive-strand RNA molecules, including new genomes and subgenomic mRNAs for PORF2 and PORF3. There are some experimental data to support the production of subgenomic RNAs, as multiple RNA species have indeed been detected in the liver of HEV-infected primates.[13]

HEV genomic RNA then assembles together with PORF2, but the site of HEV assembly in the cell, and the role of proteins other than the capsid protein PORF2 in assembly, are unknown. At least two key questions remain unanswered. Firstly, are endoplasmic reticulum (ER) translocation and glycosylation involved in HEV assembly? A large proportion of PORF2 is membrane-associated and glycosylated following heterologous expression in mammalian cell culture,[37] but this glycosylated protein appears to be rapidly degraded, while around 5% of the newly synthesized protein fails to undergo translocation to the ER and remains in a stable, unglycosylated state in the cytosol.[40] Secondly, what is the size of the mature capsid protein? Expression of PORF2 in insect cells results in cleavage between aa 111 and 112, releasing the basic N-terminal domain and allowing the proper antigen folding of the downstream sequences.[42,46,62,63] As noted previously, the N-terminal domain may normally function in binding of the genomic RNA for packaging, and it is thus likely that this remains part of the viral particle during assembly, but it is equally possible that it may be cleaved at some stage

during viral assembly or maturation. Differences in antigen folding are also evident with a range of C-terminal truncations. Characterization of HEV virions should be a high priority.

Mature HEV is finally released from the cell. A small amount of HEV is found in plasma during infection, consistent with release of progeny virus through the basolateral domains of hepatocytes leading to spread through the liver, but the majority of virus appears to be excreted through the biliary system to complete the transmission cycle, corresponding with release of virus through the apical domain of hepatocytes. However, in the case of HAV, we have found that the majority of virus is initially released at the basolateral domain of polarized hepatocytes *in vitro* (Snooks and Anderson, unpublished observations), suggesting a more complex pathway for excretion of these enterically transmitted viruses.

## Structure and assembly

Virions of HEV isolated from the bile or faeces are non-enveloped, icosahedral particles of around 32 nm diameter. Naked particles demonstrate some limited surface morphology, but this is generally obscured by the use of antibody to complex virions for electron microscopy (Fig. 39.1).

Expression of full-length PORF2 in mammalian cells results in large amounts of protein degradation, with most intact PORF2 accumulating as insoluble aggregates,[40] or alternatively in PORF2 glycosylation and export.[37,39] The reasons for these differences are unclear, but neither of these pathways yields any particles resembling HEV virions in mammalian cells. In contrast, early reports of full-length PORF2 expression in insect cells included the detection of small numbers of VLPs,[45,64] later shown to be dependent on a fortuitous proteolytic cleavage between aa 111 and 112. Subsequent studies showed that the yield of VLPs was enhanced by expression of truncated (aa 112–660) rather than full-length PORF2.[42,65,66] However, the truncated product undergoes further proteolytic processing prior to assembly in some cases,[42,44–46,67] and the heterogeneity of PORF2 products in insect cells has made it difficult to interpret the precise relationships between processing and subviral particle assembly. From available studies, it appears that PORF2 fragments 112–660 aa (62 kDa),[46,65,67] 112–625, 636 or 640 aa (56.5–58.1 kDa)[43,62] and 112–578 aa (50 kDa)[42,47] assemble into VLPs or other subviral particles, whereas the intermediate fragment 112–609 aa (55 kDa) is not particulate.[44,68] Interestingly, the non-particulate 55-kDa antigen is the leading HEV vaccine candidate,[63] which suggests that higher-order structures are not essential for at least some protective epitopes.

HEV VLPs are smaller than the intact virus particle, but Cheng and colleagues have used cryoelectron microscopy to give the first indications of HEV structure[47] (Plate 39.2, between p.786–7). A most striking feature of the VLP structure is the presence of dimeric subunits (Plate 39.2b).[47] Results of the cryoelectron microscopy analysis suggest that HEV VLPs are assembled as a T = 1, icosahedral particle containing 30 dimeric subunits of 50-kDa PORF2, with the potential to form an intact virion of the correct size with a T = 3 arrangement of 90 dimeric subunits (Plate 39.2c and Plate 39.2d).[47] One candidate for PORF2 dimerization is a leucine zipper-like hydrophobic α-helix in the region of 500–514 aa (Clements and Anderson, unpublished observations).

While it must be noted again that these observations have been made in the context of heterologous expression of PORF2, such studies provide some hypotheses that should be testable using infectious cDNA clones. Native virions should be produced and further analysed by cryoelectron microscopy and other structural means, but in the interim it is likely that constructs of various capsid fragments can be used to functionally characterize the dimeric interaction domain of PORF2, the mapping of antigenic domains on the particle, and the polypeptide termini in the viral capsid assembly pathway.

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# Chapter 40
# Hepatitis E as a zoonotic disease

Xiang-Jin Meng

Accumulating evidence indicates that there are animal reservoirs for the hepatitis E virus (HEV) and that hepatitis E is a zoonotic disease (Table 40.1).[1–59]

## Swine hepatitis E virus (swine HEV)

### Discovery

The first evidence of HEV infection in pigs came from a study by Balayan *et al*.[31] who reported an experimental cross-species infection of domestic swine with an Asian strain of human HEV. Unfortunately, the virus used in that study was not molecularly characterized, and subsequent efforts to reproduce their results by two independent laboratories in the United States using well-characterized infectious stocks of an Asian strain (Sar-55) and a Mexican strain (Mex-14) of human HEV were unsuccessful.[60,61] Detection of HEV antibodies (anti-HEV) and RNA was also reported from pigs in the Kathmandu Valley of Nepal.[41] However, the identity of the virus infecting Nepalese pigs was not determined.

In 1997, Meng *et al*.[1] discovered and characterized the first animal strain of HEV, swine HEV, from pigs in the United States. Serological survey of pigs revealed that the majority of adult pigs in the midwestern United States were positive for anti-HEV, suggesting that pigs were exposed to an agent antigenically related to HEV.[1] In an attempt to identify the agent responsible for the seropositivity in pigs, a prospective study was conducted in a commercial swine farm in Illinois. Twenty piglets born to both anti-HEV seronegative and seropositive sows in a swine farm were closely monitored for more than 5 months for evidence of HEV infection. The first piglet seroconverted to anti-HEV at 14 weeks of age, followed within a few weeks by seroconversion of piglets in other pens housed in the same building (Fig. 40.1).[1] By 21 weeks of age, 16 of the 20 piglets monitored in this prospective study had seroconverted to anti-HEV. A novel virus ge-

**Table 40.1** Evidence for hepatitis E as a zoonotic disease

| Animal reservoir | Evidence | References |
|---|---|---|
| Swine | Discovery of swine hepatitis E virus (swine HEV) in pigs closely related to human HEV | 1 |
| | Identification of genotype 3 and 4 human HEV isolates genetically closely related, or identical, to swine HEV isolates in the same geographic regions | 2–30 |
| | Experimental cross-species infections of non-human primates by swine HEV and of pigs by human HEV | 31, 32 |
| | Significantly higher anti-HEV antibody prevalence in pig handlers than in age- and geography-matched control subjects | 9, 10, 33–35, 59 |
| | Epidemiological link of sporadic cases of acute hepatitis E to consumption of undercooked pork livers, and detection of identical or near identical HEV sequences from patients and packaged pig livers sold in local grocery stores | 28, 30 |
| Other species | Existence of numerous other animal species (chickens, monkeys, cats, dogs, cattle, goats, sheep and rodents) positive for anti-HEV antibodies | 36–52 |
| | Discovery of avian hepatitis E virus (avian HEV) in chickens that is antigenically and genetically related to human HEV | 48, 53–56 |
| | Experimental cross-species infections of lambs and rodents by human HEV | 37, 39, 42 |
| | Sporadic cases of acute hepatitis E linked to consumption of raw deer meat, and detection of identical or near identical HEV sequences from patients and leftover deer meat | 57 |
| | Significantly higher anti-HEV antibody prevalence in humans with occupational exposure to animals than in control subjects | 58 |

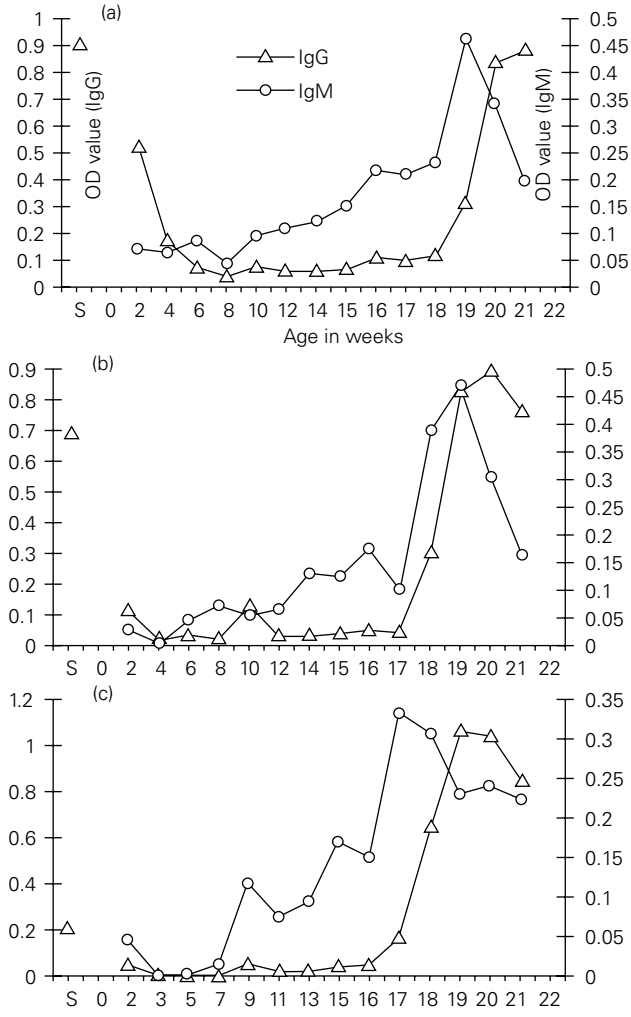



**Figure 40.1** Seroconversion to anti-HEV in piglets naturally infected by swine HEV in a commercial swine farm. Anti-HEV responses of three representative piglets in a prospective study are presented. (a) A piglet born to a seropositive sow with a high titre of anti-HEV. (b) A piglet born to a seropositive sow with a lower titre of anti-HEV. (c) A piglet born to a seronegative sow. The ELISA optical density value of IgG anti-HEV in sows is indicated (S). Reproduced with permission from the National Academy of Science from Meng *et al*.[1]

netically closely related to human HEV, designated swine HEV, was molecularly identified and characterized from the acute phase sera of the naturally infected piglets. Specific-pathogen-free (SPF) pigs were experimentally infected with the acute phase sera (containing swine HEV) of naturally infected pigs, and the same virus was recovered from experimentally infected SPF pigs.[61,62]

## Genomic organization and genotypes

The complete genomic sequence of swine HEV has been determined.[32,63,64] The genomic organization of swine HEV is very similar to that of human HEV, which contains a short 5′ non-coding region (NCR), followed by three partially overlapping open reading frames (ORFs), and a short 3′ NCR. ORF1 encodes a single polypeptide of 1709 amino acid residues. Several putative functional domains and motifs similar to human HEV have been assigned to the ORF1 of swine HEV: a methyltransferase domain at the 5′ end, a papain-like cysteine protease (PLP), a proline-rich domain that may provide flexibility, a hypervariable region (HVR), a helicase and an RNA-dependent RNA polymerase (RdRp).[32,63,64] ORF2 encodes an immunogenic capsid protein of 661 amino acid residues. The function of the small ORF3 of swine HEV (123 amino acid residues) is unknown, but it has been reported that the ORF3 of a human HEV is a cytoskeleton-associated phosphoprotein that may be involved in virus replication.[65]

At least four major genotypes of human HEV have been recognized worldwide:[66–69] genotype 1 (primarily Burmese-like Asian strains), genotype 2 (a single Mexican strain), genotype 3 (strains from rare endemic cases in the United States, Japan and Europe) and genotype 4 (variant strains from sporadic cases in Asia). Swine HEV isolates identified thus far from pigs in different countries all belong to either genotype 3 or 4, and shared significant sequence identity with and, in a few cases had identical sequence to, genotypes 3 and 4 of human HEV strains in the same geographic regions.[2–30,68] Genotype 3 and 4 strains are primarily responsible for sporadic cases of human hepatitis E. Genotype 1 or 2 strains, which are responsible for hepatitis E epidemics in many developing countries, have not been identified in pigs.

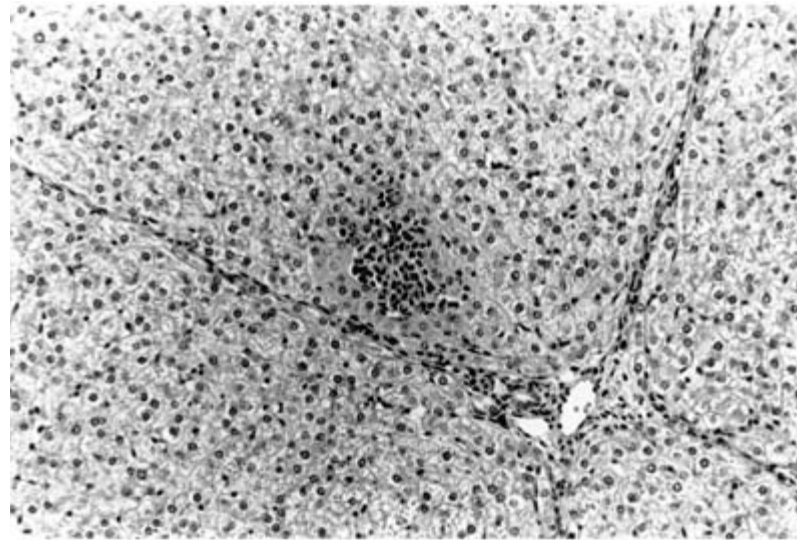
## Transmission and pathogenesis

Like human HEV, the transmission route for swine HEV is presumably faecal-oral. Faeces from infected pigs contain large amounts of viruses[61,62,70] and are probably the main source of virus for transmission. It has been demonstrated that, under experimental conditions, a sentinel uninoculated SPF pig housed in the same room with a swine HEV-inoculated pig became infected about 2 weeks after the experimentally inoculated pig had become infected.[32] It is thought that pigs acquire infection through direct contact with infected pigs or through ingestion of faeces-contaminated feed or water. However, experimental reproduction of swine HEV infection in pigs via the oral route of inoculation proved to be difficult,[71] even though pigs can be readily infected by swine HEV via the intravenous route of inoculation.[61,62] Other route(s) of transmission cannot be ruled out.

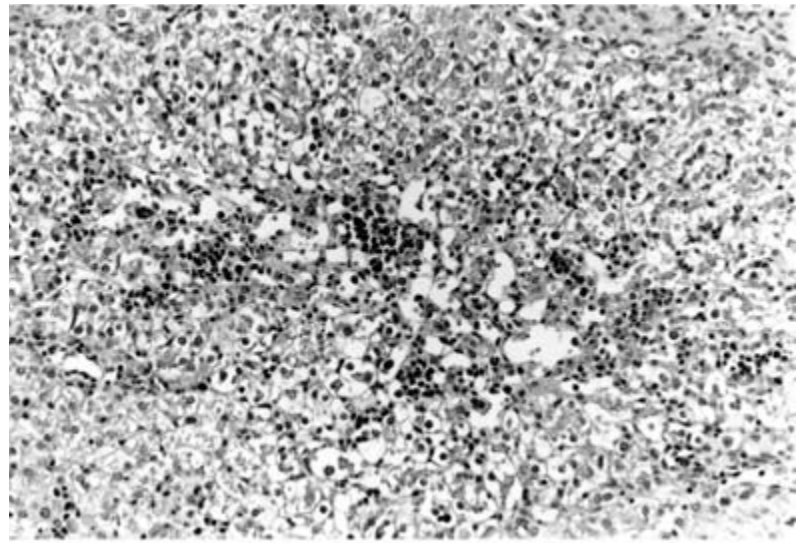
Little is known regarding the pathogenesis of swine HEV. Clinical disease was absent in naturally infected pigs.[1] In a prospective study, four piglets naturally infected with swine HEV were necropsied during the acute stage of infection. Gross lesions were not detected in the

liver or 18 other tissues and organs examined during necropsy. However, all four necropsied piglets had microscopic evidence of hepatitis characterized by mild to moderate multifocal and periportal lymphoplasmacytic hepatitis with mild focal hepatocellular necrosis. All four piglets also had lymphoplasmacytic enteritis, and three piglets had mild multifocal lymphoplasmacytic interstitial nephritis.[1] Under experimental conditions, SPF pigs experimentally infected with swine HEV or with a strain of human HEV remained clinically normal.[62] However, the infected pigs did have mildly to moderately enlarged hepatic and mesenteric lymph nodes from 7 to 55 days post-inoculation (DPI). Microscopic lesions characterized by mild-to-moderate multifocal lymphoplasmacytic hepatitis and focal hepatocellular necrosis were observed in experimentally infected pigs (Fig. 40.2).[62] Hepatic inflammation and hepatocellular necrosis peaked in severity at 20 DPI. HEV RNA was detected in faeces, liver tissue and bile of infected pigs from 3 to 27 DPI.[62]

The initial site of swine HEV replication in pigs is still not known. In primates and pigs experimentally infected with swine HEV, virus replication in the liver has been documented.[66] It is believed that, after replication in liver, swine HEV is released to the gallbladder from hepatocytes and then is excreted in faeces. However, it is not known how the virus reaches the liver. Williams *et al*.[70] studied the potential extrahepatic sites of HEV replication in pigs experimentally infected with swine HEV and the US-2 strain of human HEV. As a positive-sense RNA virus, HEV produces an intermediate negative-strand viral RNA during virus replication. By using a negative-strand-specific reverse transcriptase-polymerase chain reaction (RT-PCR), replicative negative-strand HEV RNA,



(a)



(b)

**Figure 40.2** Hepatitis lesions in pigs experimentally infected with swine HEV (a) and a genotype 3 human HEV (b). (a) Liver of a pig experimentally infected with swine HEV. There is mild focal infiltration of lymphocytes, plasma cells and macrophages and mild diffuse inflammation in hepatic sinusoids at 14 days post-inoculation (DPI). Hepatocytes are mildly swollen and vacuolated. (b) Liver of a pig experimentally infected with the US-2 strain of human HEV. There is severe lymphoplasmacytic and histiocytic hepatitis and severe vacuolar degeneration and swelling of hepatocytes at 14 DPI. Haematoxylin and eosin stain. Reproduced with permission from the American Society for Microbiology from Halbur *et al*.[62]

indicative of virus replication, was detected in the livers as well as several extrahepatic tissues and organs including small intestine, colon, hepatic and mesenteric lymph nodes from infected pigs.[70] By using *in situ* hybridization, Choi and Chae[72] also detected swine HEV RNA in hepatocytes and bile duct epithelium as well as in small and large intestines, lymph nodes, tonsil, spleen and kidney. The clinical and pathological significance of these extrahepatic sites of virus replication is not known.

## Incidence of infection in pig farms

Sero-epidemiological studies have demonstrated that swine HEV infection is ubiquitous in pigs worldwide in both developing and industrialized countries, regardless of whether HEV is endemic in the respective human population (Table 40.2).[1,9,15,27,33,41,46,47,73–79] Anti-HEV prevalence in pigs is age-dependent. Most pigs younger than 2 months of age are seronegative, whereas the majority of pigs over 3 months of age, when pigs are moved to pens where there is increased opportunity for environmental faecal contamination, are seropositive.[45,67] The seropositivity rates varied from herd to herd, and from region to region (Table 40.2). Sows with high anti-HEV titres can passively transfer anti-HEV to their piglets, and thus some piglets are born seropositive.[1] Maternal antibody usually waned by 8–9 weeks of age, at which point most piglets acquired infection. Some SPF pigs raised in good

**Table 40.2** Age-dependent anti-HEV prevalence in pigs in both developing and industrialized countries

| Country | Herd | Pig age | No. positive/no. tested (%) | Reference |
|---|---|---|---|---|
| USA | 1 herd | 6–12 weeks | 0/16 (0) | 1 |
| | | 5 months to adult | 27/41 (66) | |
| | 1 herd | 3–8 weeks | 0/24 (0) | |
| | | 3 months to adult | 34/37 (92) | |
| | 1 herd | 2 months | 1/8 (13) | |
| | | 3 months to adult | 28/32 (88) | |
| | 11 herds | Adult | 110/115 (96) | |
| Japan | 25 herds | 2 months | 37/500 (7) | 73 |
| | | 3 months | 301/750 (40) | |
| | | 4 months | 433/500 (87) | |
| | | 5–6 months | 677/750 (90) | |
| Spain | 6 herds | 3 weeks to 2 months | 2/10 (20) | 15 |
| | | 5 months to adult | 13/50 (26) | |
| | 3 herds | Mixed | 10/73 (14) | 27 |
| Australia | 2 herds | Mixed | 12/40 (30) | 74 |
| | Wild pigs | Unknown | 15/59 (17) | |
| Taiwan | 10 herds | Mixed | 102/275 (37) | 9 |
| Canada (Quebec) | 37 herds | Nursery | 82/310 (26) | 33 |
| | 16 herds | Adult | 34/90 (38) | |
| Canada (Ontario) | 10 herds | Nursery | 1/230 (<1) | 33 |
| | 4 herds | Adult | 12/82 (15) | |
| Canada | 80 herds | 6 months | 594/998 (60) | 75 |
| Korea | Multiple | 1–2 months | 6/40 (15) | 33 |
| | | 3–6 months | 39/80 (49) | |
| | | Adults | 12/20 (60) | |
| | 13 herds | 1–2 months | 3/90 (3) | 76 |
| | | 3 months | 5/50 (10) | |
| | | 4–7 months | 28/90 (31) | |
| | | Sows | 3/34 (9) | |
| New Zealand | 1 herd | Mixed | 54/72 (75) | 77 |
| Nepal | Multiple | Mixed | 18/55 (33) | 41 |
| China | 3 herds | Mixed | 22/72 (31) | 33 |
| | Multiple | <3 months | 1/10 (10) | 47 |
| | | Adults | 329/409 (80) | |
| Thailand | 1 herd | 1–2 months | 0/20 (0) | 33 |
| | | 3–4 months | 13/20 (65) | |
| | 3 herds | Adults | 10/35 (29) | |
| India | Multiple | Unknown | 155/234 (66) | 46 |
| | 7 herds | 2–24 weeks | 122/284 (43) | 78 |
| | Multiple | 3.2–6.4 months | 54/57 (95) | 79 |

biosecurity environments in the United States (Meng *et al*., unpublished data) and 17% of wild boars in Australia[74] were also tested positive for anti-HEV. The ubiquitous nature of swine HEV provides ample opportunities for potential zoonotic transmission.

Swine HEV RNA is frequently detected in the sera and faeces from pigs of 2–4 months of age, the period in which active swine HEV infections occur (Table 40.3).[3,9,10,20,21,27,41,47,73,75–81] Most adult pigs, although positive for IgG anti-HEV, are free of swine HEV RNA.[45,67] Faeces appear to be better than sera for the detection of swine HEV RNA, as faecal virus shedding of swine HEV lasts longer than viraemia, and there are more viruses in faeces than in sera.[61,62,70] Thus far, only genotypes 3 and 4 swine HEV strains have been identified from pigs (Table 40.3).

## Experimental cross-species infection by HEV

Under experimental conditions, swine HEV infected non-human primates (rhesus monkeys and a chimpanzee).[32] Two rhesus monkeys experimentally inoculated with a $10^{4.5}$ 50% pig infectious dose of swine HEV seroconverted to anti-HEV 4 weeks post-inoculation, developed viraemia and shed virus in faeces. Biochemical evidence of hepatitis was observed, as there was an elevation of serum liver enzymes, isocitrate dehydrogenase (ICD) and alanine aminotransferase (ALT), in infected primates (Fig. 40.3).[32] Mild acute viral hepatitis characterized by focal necroinflammatory changes was detected in liver biopsies near the time of serum liver enzyme elevations. The chimpanzee inoculated with swine HEV also became infected, as swine HEV RNA was detected in faeces of the inoculated chimpanzee, and seroconversion to anti-HEV occurred at 6 weeks post-inoculation.[32] Both rhesus monkeys and the chimpanzee infected with swine HEV remained clinically normal. Experimental infection of non-human primates, the surrogates of man, with swine HEV demonstrated that swine HEV can cross species barriers, therefore possibly infects humans.

A central Asian strain of human HEV was reportedly transmitted to domestic swine, and the infected pigs developed clinical hepatitis.[31] However, others failed to infect SPF pigs experimentally with human strains of

**Table 40.3** Detection of genotype 3 and 4 swine HEV RNA from faecal and serum samples of pigs in different geographic regions

| Country | Herd | Pig age | No. positive/ no. tested (%) | Swine HEV genotype | Human HEV genotype in the country | Reference |
|---|---|---|---|---|---|---|
| USA | 37 | 2–4 months | 34/96 (35) F | 3 | 3 | 80 |
| Japan | 25 herds | 2 months | 0/180 (0) S | 3, 4 | 1, 3, 4 | 73 |
| | | 3 months | 113/750 (15) S | | | |
| | | 4 months | 24/180 (13) S | | | |
| | | 6 months | 0/250 (0) S | | | |
| Spain | 1 herd | Mixed | 6/12 (50) F | 3 | 3 | 27 |
| Taiwan | 2 herds | Mixed | 1/56 (2) S | 4 | 4 | 3, 9 |
| | Multiple | <2 months | 0/11 (0) S | 3*, 4 | 4 | 10, 21 |
| | | 2 months | 3/67 (4) S | | | |
| | | 3–4 months | 3/255 (1) S | | | |
| | | 5–6 months | 2/112 (2) S | | | |
| | | Adult | 0/76 (0) S | | | |
| | Multiple | 1–7 weeks | 0/20 (0) F | 3*, 4 | 4 | 10, 21 |
| | | 2–4 months | 2/22 (9) F | | | |
| | | >5 months | 1/12 (8) F | | | |
| | Multiple | Mixed | 3/235 (1) S | 4 | 4 | 10 |
| Canada | 1 herd | 8 weeks | 3/6 (50) F | 3 | None | 75, 81 |
| Korea | 10 herds | 1–4 months | 3/128 (2) S | 3 | None | 76 |
| Netherlands | Multiple | 3–9 months | 25/115 (22) F | 3 | 3 | 20 |
| New Zealand | 2 herds | 7 weeks | 0/17 (0) F | 3 | 3 | 77 |
| | | 10 weeks | 10/21 (48) F | | | |
| | | 12 weeks | 7/7 (100) F | | | |
| China | Multiple | <3 months | 2/10 (20) S | 4 | 1, 4 | 47 |
| | | Adults | 3/253 (1) S | | | |
| Nepal | Multiple | Mixed | 3/47 (6) S/F | ND | 1 | 41 |
| India | 7 herds | 2–24 weeks | 13/284 (5) S | 4 | 1 | 78 |
| | Multiple | 3.2–6.4 months | 6/57 (11) S | 4 | 1 | 79 |

F, faecal samples; S, serum samples; ND, sequence not determined.

*The genotype 3 swine HEV isolates isolated in Taiwan were identified from breeding sows imported from the USA.

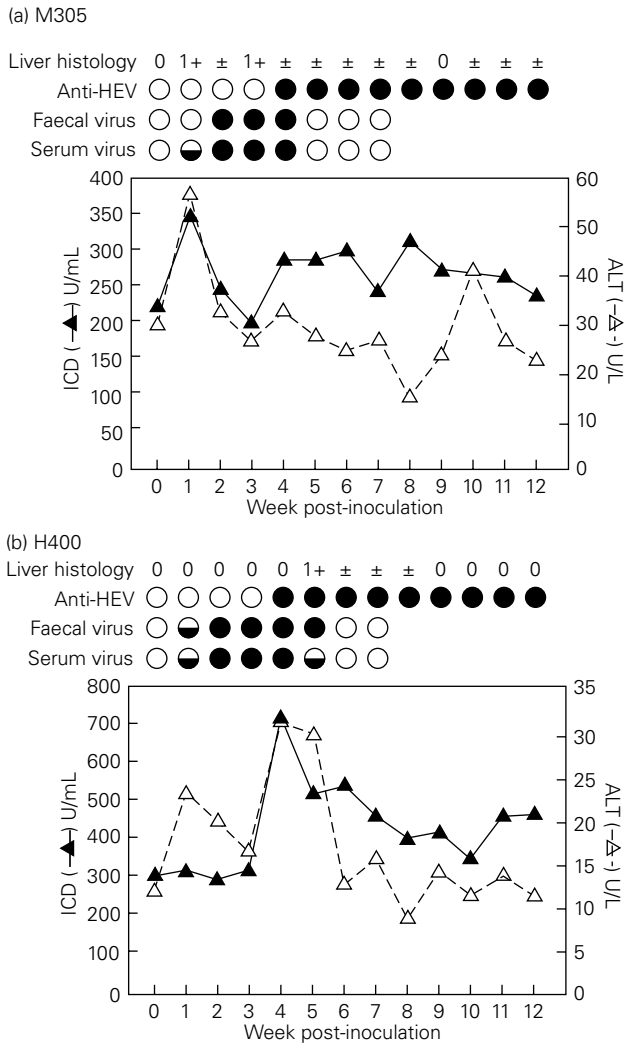



**Figure 40.3** Experimental cross-species infection of rhesus monkeys by swine HEV. Levels of serum liver enzymes (ALT and ICD) are plotted. Presence (filled circle) or absence (open circle) of anti-HEV and swine HEV RNA in serum and faeces is indicated. Half-filled circles indicate detection of swine HEV RNA only by a nested PCR. The degree of histological liver lesions observed in liver biopsies is indicated (scale of 0 to 4+). Modified with permission from the American Society for Microbiology from Meng *et al*.[32]

HEV. Meng *et al*.[61] were unable to infect crossbred SPF swine with an Asian strain (Sar-55, genotype 1) or a Mexican strain (Mex-14, genotype 2). Similarly, Platt *et al*.[60] could not infect SPF swine experimentally with the Mexican strain of human HEV. It is possible that these epidemic strains of HEV (genotypes 1 and 2) may have a more limited host range than swine HEV, which belongs to either genotype 3 or 4. In fact, when SPF swine were inoculated with a genotype 3 human HEV (strain US-2), the inoculated pigs rapidly became viraemic and seroconverted to anti-HEV within 2 weeks post-inoculation, suggesting that the US-2 strain of human HEV is already competent to replicate in swine and may be of swine origin. One infected pig even spread, through direct contact, the US-2 human virus to an uninoculated pig housed in the same room.[32] The virus recovered from infected pigs has been partially sequenced and confirmed to be the same virus used in the inoculum.

Experimental interspecies transmissions of HEV have also been reported in other animal species. Usmanov *et al*.[39] have reported experimental infections of lambs with human HEV isolates Osh-225 and Osh-228. The inoculated lambs developed clinical signs of acute hepatitis, and shed virus in faeces. It has been reported that rodents are susceptible to experimental infection by human HEV.[39] Wistar rats experimentally inoculated with a human stool suspension containing HEV became infected, as HEV RNA was detected in faeces and serum, and HEV antigen was detected in the liver and several other tissues of the inoculated rats.[39] However, others failed to confirm the rat transmission results with a genotype 1 human HEV, a genotype 3 swine HEV, a genotype 4 swine HEV, and an avian HEV (J.C. Wu, personal communication; Sun and Meng, unpublished data). However, it is important to note that serial passages in laboratory rats with a virus of rat origin are successful, but the rat virus has not yet been genetically characterized.[68]

## Novel strains of human HEV closely related to swine HEV

Hepatitis E was generally not considered to be endemic in industrialized countries. Most reported clinical cases in industrialized countries had a history of travelling to endemic regions,[66,68,82–85] although in some cases this risk factor was absent.[86–88] In the United States, two cases of acute hepatitis E have been reported, one in Minnesota and one in Tennessee.[2,8] The patient from Minnesota (US-1) had no history of travelling outside the USA, whereas the patient from Tennessee (US-2) had travelled to Mexico prior to the diagnosis of hepatitis E. The two US strains of human HEV (US-1 and US-2), which belong to genotype 3, are most closely related to the genotype 3 swine HEV recovered from a pig in Illinois (about 98% sequence identity in ORF1). Another unique HEV strain was identified from a US patient who had recently returned from vacation in Thailand, an HEV endemic country. Sequence analyses revealed that this HEV strain was most similar to the US swine and human HEV strains.[16]

Novel strains of human HEV closely related to swine HEV have also been reported in other countries. Numerous genotype 4 strains of human HEV have recently been identified from patients with sporadic cases of hepatitis E in Taiwan and China.[3,5,9,10,12,18,19,21,29,47] These genotype 4 isolates are genetically distinct from the genotype 1

epidemic strains in the region, but are very closely related to swine HEV isolates identified from pigs in Taiwan and China. Similarly, both genotype 3 and 4 strains of human HEV were recently identified from Japanese patients with sporadic cases of acute hepatitis E, and found to form phylogenetic clusters with the genotype 3 and 4 swine HEV isolates recovered from Japanese pigs[17,22–24,26,28,64,73] (Fig. 40.4). A genotype 4 Japanese human HEV isolate HE-JA1 shared 99% sequence identity over the entire genome with a Japanese swine HEV isolate, swJ13–1.[26] In India, the epidemic strains in humans belong to genotype 1, but the virus recovered from Indian pigs was found to be genotype 4, which has not yet been identified from humans in India.[78,79] A Spanish E11 strain of HEV of possible swine origin is closely related to two Spanish strains of human HEV but is less related to other known strains of HEV.[15] Similarly, novel genotype 3 strains of human HEV from patients with acute hepatitis E in the Netherlands and New Zealand are closely related to genotype 3 strains of swine HEV in the respective regions.[20,25,77] The fact that the genotype 3 or 4 swine virus is closely related to the viruses responsible for sporadic cases of human hepatitis E in the same geographic regions provides further supporting evidence that swine are reservoirs for HEV, at least for genotypes 3 and 4 strains.

## Anti-HEV prevalence in swine and other animal handlers

The demonstrated ability of swine HEV to infect across species raises concerns for potential zoonotic infection in high-risk groups such as pig handlers. In Taiwan where HEV was not considered to be endemic, Hsieh *et al*.[9] found that about 27% of the Taiwanese pig handlers were positive for anti-HEV compared with only about 8% of control subjects. Recently, a total of 465 swine veterinarians were tested for IgG anti-HEV using recombinant capsid antigens from swine HEV and a Pakistani Sar-55 strain of human HEV.[35] Among the 295 swine veterinarians from eight US states from which 400 normal

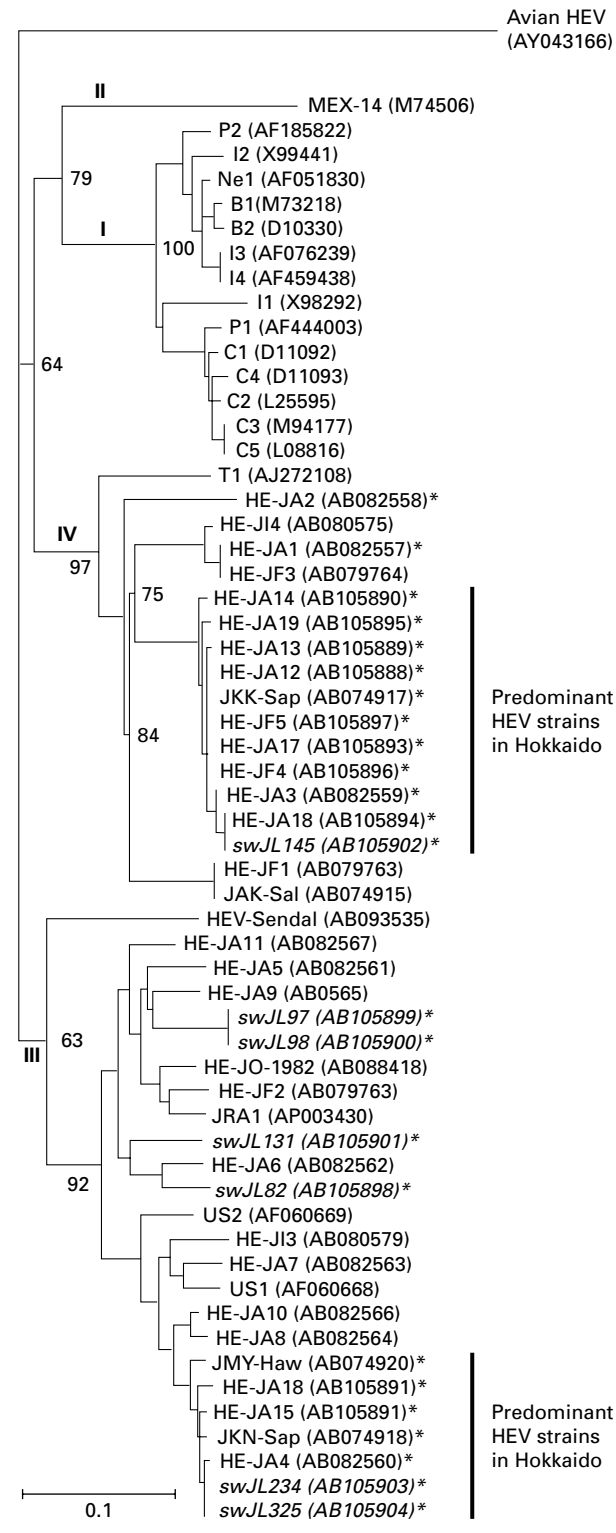



**Figure 40.4** Genotype 3 and 4 swine HEV strains are very closely related to, or in a few cases indistinguishable from, genotype 3 and 4 human HEV strains in Japan, and hepatitis E may be food-borne via consumption of undercooked pig livers in Japan.[28] The phylogenetic tree based on a 412-bp sequence of the ORF2 region of selected genotype 1–4 HEV strains (accession numbers in parentheses) was constructed by the neighbour-joining method with avian HEV from a chicken as an outgroup. The seven swine HEV isolates (swJL82, swJL97, swJL98, swJL131, swJL145, swJL234 and swJL325) identified from packaged pig livers sold in grocery stores in Hokkaido, Japan are shown in italics. Those isolates from Hokkaido, Japan are indicated with an asterisk. Bootstrap values obtained from 1000 resamplings are indicated at major branches. Reproduced with permission from the Society for General Microbiology from Yazaki *et al*.[28]

US blood donors were available, about 23% (swine HEV antigen) or 27% (Sar-55 antigen) of swine veterinarians were positive for anti-HEV compared with 17% (swine HEV antigen) or 18% (Sar-55 antigen) of normal blood donors. Swine veterinarians in the USA were 1.51 times (swine HEV antigen, $p = 0.03$) and 1.46 times (Sar-55 antigen, $p = 0.06$) more likely to be anti-HEV-positive than normal US blood donors. Veterinarians who reported having needle-stick injuries while performing procedures on pigs were about 1.9 times more likely to be seropositive than those who did not. Also, subjects from traditional major swine-rearing states appear to be more likely to be seropositive than those from traditionally non-swine-rearing states. For example, subjects from Minnesota, a major swine-rearing state, are about five to six times more likely to be seropositive than those from Alabama, which is traditionally not a major swine-rearing state.[35] Drobeniuc *et al*.[34] also assessed the anti-HEV prevalence and risk factors to HEV infection in 264 swine farmers and 255 control subjects in Moldova. About 51% of swine farmers were anti-HEV-positive, whereas only 25% of control subjects with no occupational exposure to swine were seropositive. Swine farmers who reported cleaning barns or assisting at pig births were 2.46 times more likely to be seropositive than controls.[34] In a cross-sectional serosurvey, Withers *et al*.[59] found that swine workers (n = 165) in North Carolina had a 4.5-fold higher anti-HEV prevalence (10.9%) than the control subjects (2.4%, n = 127). These data provided compelling evidence that hepatitis E is a zoonotic disease, and that pigs are reservoirs.

The existence of a population of anti-HEV-positive individuals in industrialized countries could be explained by subclinical infection of humans with swine HEV.[35,45,67,89] However, city dwellers in the USA and many other industrialized countries, who have no significant exposure to pigs except possibly as food, are also found to be positive for anti-HEV.[45,66,68] Therefore, other animal species may also serve as reservoirs for HEV. Karetnyi *et al*.[58] tested anti-HEV prevalence in selected populations of Iowa including 87 field staff members of the Department of Natural Resources (DNR) and 332 normal blood donors. The DNR field workers showed significantly higher anti-HEV prevalence than normal blood donors ($p < 0.05$), suggesting that human populations with occupational exposure to wild animals have increased anti-HEV prevalence. Therefore, the risk for zoonotic HEV infection is not limited to swine handlers, as many domestic and farm animals were also found to be positive for HEV antibodies.

## Serological evidence for other potential animal reservoirs

In addition to swine, anti-HEV has been detected in several other animal species including rodents, chickens, dogs, cats, sheep, goats, cattle and non-human primates, suggesting that these animals have been exposed to HEV (or a related agent) and thus might serve as reservoirs.[36,43,44,46,47,49–52,59]

Rodents are frequently found in both urban and rural environments and thus could potentially play an important role in HEV transmission. Kabrane-Lazizi *et al*.[43] reported that about 77% of the rats from Maryland, 90% from Hawaii and 44% from Louisiana were positive for IgG anti-HEV. Rats from urban as well as rural areas are seropositive. The anti-HEV prevalence rate increases in parallel with the estimated age of the rats. All three different species of wild rats (*Rattus norvegicus*, *R. rattus* and *R. exulans)* are strongly positive for anti-HEV. Favorov *et al*.[44] tested a total of 806 rodents of 26 different species caught in the USA for anti-HEV prevalence, and the highest prevalence rate was found in the genus *Rattus* (59.7%). Rodents from urban habitats had a significantly higher anti-HEV prevalence rate than rodents captured from rural areas.[44] In Japan, about 32% of Norway rats (*R. norvegicus*) and 13% of black rats (*R. rattus*) tested positive for IgG anti-HEV.[51] In contrast, none of the 32 *R. norvegicus* rats from India were anti-HEV-positive, whereas about 55% of *Bandicota bengalensis* rats and 16% of *R. rattus rufescens* rats from India were positive for IgG anti-HEV.[46] In Nepal, about 13% of *R. rattus brunneusculus* rats and about 21% of *B. bengalensis* rats were positive for anti-HEV.[52] Interestingly, anti-HEV was not detected in mice in several studies.[46,51,52,59] The results from these studies suggest that there is a widespread infection of rats by HEV (or a related agent) in different countries. He *et al*.[52] recently reported the detection of HEV sequences from rats caught in Nepal. The authors reportedly amplified a 405-bp HEV sequence from the serum samples of four rats, and showed that the HEV sequences from rats are most closely related to two Nepalese human HEV isolates with 95–96% nucleotide sequence identity. However, GenBank database Blast search revealed that the HEV sequence from the four Nepalese rats actually had 100% sequence identity to that of a Pakistani strain of human HEV (Abb-2B) isolated from a 1988 outbreak in Abbottabad, Pakistan by the same group.[90] Unfortunately, the authors failed to report or discuss this intriguing fact in the publication.[52] Therefore, it remains to be determined whether or not the HEV sequence amplified from the rats in the study is indeed of rat origin. Other groups could not amplify HEV sequences from infected rats with degenerate primers based on known HEV strains,[68] suggesting that the virus infecting rats is probably very divergent genetically from the known HEV strains.

Anti-HEV was also reportedly detected in 44% of chickens and 27% of dogs in Vietnam.[36] About 29–62% of cows from three HEV endemic countries (Somali,

Tajikistan and Turkmenistan), about 12% of cows from Ukraine, and about 42–67% of the sheep and goats from Turkmenistan have also tested positive for anti-HEV.[36] Arankalle *et al*.[46] reported that about 4–7% of cattle and 23% of dogs in India had antibodies to HEV. A variety of non-human primate species were also found positive for IgG anti-HEV.[38,40,49] However, the source of seropositivity in many of these animal species, with the exception of pigs and chickens, could not be definitively identified, as virus was either not recovered from these species or the recovered virus was not sequenced to confirm its identity. The specificity and sensitivity of these anti-HEV serological assays are also not known, although some anti-HEV assays are validated with serial serum samples from animals experimentally infected with different HEV strains.[45,91] Therefore, the interpretation of these serological data should be cautious. Nevertheless, the existence of a population of animal species positive for anti-HEV further supports the argument that animal reservoirs for HEV exist.

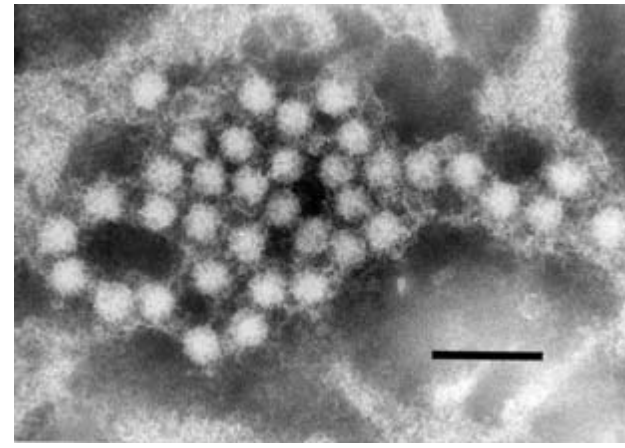


**Figure 40.5** Electron micrograph of avian hepatitis E virus (avian HEV) particles from a bile sample of a chicken with hepatitis-splenomegaly syndrome. Negative staining, bar = 100 nm. Reproduced with permission from the Society for General Microbiology from Haqshenas *et al*.[53]

## Discovery of avian HEV in chickens that is antigenically and genetically related to human HEV

Payne *et al*.[56] first reported the identification of a virus associated with big liver and spleen disease (BLS) of chickens in Australia, and determined the sequence of a 523-bp fragment of the BLS virus (BLSV). The BLS disease affects commercial broiler breeder flocks and causes decreased egg production and slight increase in mortality in broiler breeder flocks in Australia.[92] Analyses of this 523-bp sequence indicated that BLSV is genetically related to human HEV with about 62% nucleotide sequence identity.[56]

Haqshenas *et al*.[53] isolated a virus genetically related to human HEV from bile samples of chickens with hepatitis-splenomegaly (HS) syndrome in the USA. HS syndrome is an emerging disease in chickens in North America, but the cause was unknown. Based upon the similar genomic organization and significant sequence identity of this novel chicken virus with HEV, it is designated as avian HEV to distinguish it from human and swine HEV. Electron microscopic examination revealed that avian HEV, a non-enveloped virus particle of 30–35 nm in diameter, is morphologically similar to human HEV (Fig. 40.5).[53] The complete genomic sequence of avian HEV is about 6.6 kb in length, which is about 600 bp shorter than the genomes of human and swine HEV.[93] Like human and swine HEV, the avian HEV genome consists of a 5′ NCR, followed by three ORFs and a 3′ NCR. The nucleotide sequence identity in the entire genome between avian HEV and other HEV strains is only about 50%.[48,93] The helicase gene is the most conserved between avian HEV and other HEV strains, displaying 57–60% nucleotide sequence identities. The RdRp gene and the putative capsid gene (ORF 2) of avian HEV shares 52–53% and 48–51% nucleotide sequence identities with the corresponding regions of other known HEV strains, respectively. Avian HEV shares about 80% nucleotide sequence identity with the Australian BLSV, suggesting that BLS in Australia and HS syndrome in North America may be caused by variant strains of the same virus.[53,93] Among the four recognized genotypes of HEV, the inter-genotypic sequence difference is about 25–30%.[22,23,69,73] Therefore, avian HEV, with about 40–50% sequence difference with known HEV strains, is distinct. However, cross-antigenicity study revealed that avian HEV shares common antigenic epitopes in its putative capsid protein (ORF2) with swine and human HEVs as well as with BLSV and thus, avian HEV is not only genetically but antigenically related to human and swine HEV as well.[54] Phylogenetic analyses indicate that avian HEV may represent a fifth genotype of HEV or may belong to a separate genus.[48,93]

Like swine and human HEV, avian HEV isolates from chickens in different geographic regions are heterogeneic, and avian HEV infection is widespread in chicken flocks in the USA.[48] A total of 1276 chickens of different ages and breeds from 76 different flocks in five states (California, Colorado, Connecticut, Virginia and Wisconsin) were tested for the prevalence of avian HEV antibody using a recombinant avian HEV capsid protein as the antigen.[48] About 71% of chicken flocks and 30% of chickens were positive for antibodies to avian HEV. Like swine HEV, avian HEV antibody prevalence in chickens is also age-dependent: about 17% of chickens younger than 18 weeks were seropositive, whereas about 36% of adult chickens were seropositive. Avian HEV isolates re-

covered from chickens with HS syndrome in five states displayed 78–100% nucleotide sequence identities to each other, and 56–61% identities to known strains of human and swine HEV.

Cross-species infection by avian HEV has been demonstrated, as avian HEV recovered from a chicken with HS syndrome infected turkeys (Sun *et al*., unpublished data). However, an attempt to experimentally infect two rhesus monkeys with an infectious stock of avian HEV was unsuccessful.[93] Thus, it appears that, unlike swine HEV, avian HEV may not infect humans, but additional studies are warranted to further assess the potential zoonotic risk of avian HEV.

## Other potential zoonotic concerns

### Food and environmental safety

As a faecal-orally transmitted disease, waterborne epidemics are the characteristic of hepatitis E outbreaks.[66,82,83,85] The existence of a number of potential animal reservoirs for HEV raised concerns for food and environmental safety. Faeces from infected pigs are found to contain large amounts of virus[61,62,70] and thus, in areas where swine are raised, swine manure and faeces could be a source of contamination of irrigation water or coastal waters with concomitant contamination of produce or shellfish.[94] Swine HEV has been detected in swine manure and waste water associated with hog operations.[95,96] In addition, strains of HEV of both human and swine origins have been detected in sewage water.[4,15,27,97,98] Consumption of contaminated shellfish has been implicated in sporadic cases of acute hepatitis E.[99,100]

There is a potential risk of transmitting swine HEV via either drinking faeces-contaminated water or consuming contaminated food such as pork products. Yazaki *et al*.[28] recently reported that sporadic cases of acute hepatitis E in Hokkaido, Japan may be foodborne with a zoonotic origin. Nine of the 10 patients with sporadic acute hepatitis E in Hokkaido had a history of consuming grilled or undercooked pig livers at about 2–8 weeks prior to the onset of disease. About 2% of the packaged raw pig livers sold in local grocery stores were positive for swine HEV RNA,[28] although it is not known if the virus in the packaged pig livers is still infectious. Most importantly, the sequences of seven swine HEV isolates recovered from packaged pig livers in grocery stores are very closely related, or identical in a few cases, to the viruses recovered from human hepatitis E patients (Fig. 40.4). For example, swine HEV isolate swJL145 detected from a packaged pig liver had 100% sequence identity to the virus recovered from an 86-year-old hepatitis E patient in Hokkaido. Retrospective interviews were subsequently conducted for 22 randomly selected acute hepatitis patients of non-E aetiology in the same hospital. None reported having consumed pig livers before disease onset,[28] suggesting that consumption of undercooked pig livers is a potential risk factor for HEV infection ($p < 0.01$).

More recently, Tei *et al*.[57] reported a cluster of four cases of human hepatitis E in two families who shared and consumed raw deer meat 6–7 weeks before the onset of disease. An HEV sequence was detected from the leftover frozen deer meat, and found to have 99.7–100% nucleotide sequence identity to the viruses recovered from the four patients. Family members who ate none or little deer meat were not infected. Although these findings appear to provide direct evidence of zoonotic transmission of HEV, additional studies are warranted to more definitively assess the zoonotic risk of HEV in deer and other animal species. It is important to isolate and fully characterize HEV strains from naturally infected deer, and to determine the anti-HEV prevalence in deer populations.

### Xenozoonotic risk

Due to the shortage of human organ donors, xenotransplantation with pig organs is currently under intensive research and pigs may become the organ donors of choice.[67] Swine HEV is ubiquitous in pigs and has the ability to infect across species barriers. Therefore, xenozoonosis due to the transmission of swine HEV from pig xenografts to human recipients and the potential subsequent transmission of the virus to others are possible.[67] Although swine HEV appeared to cause only subclinical infection in pigs and non-human primates under experimental conditions, it may become pathogenic in immunosuppressed xenograft recipients, as a result of species-jumping or adaptation.[67] Unlike porcine endogenous retrovirus, xenograft donor pigs free of swine HEV can be generated through strict breeding procedures. Piglets negative for swine HEV have been successfully generated through segregated early weaning from pregnant SPF sows experimentally infected with swine HEV[101] (Brad Thacker, personal communication). Thus, the potential xenozoonotic risk of swine HEV in xenotransplantation is preventable through adequate screening and strict breeding procedures for donor pigs. Serological assays alone are inadequate in screening swine HEV infection in donor pigs, as anti-HEV generally appears at least 2 weeks after infection. Viraemia and faecal virus shedding occur in infected pigs earlier than anti-HEV and thus, seronegative pigs could still harbour swine HEV.[61,62,70] A sensitive RT-PCR assay should be used in combination with serological assays to screen xenograft donor pigs.

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# Chapter 41
# Epidemiology, clinical and pathologic features, diagnosis, and experimental models

Kris Krawczynski, Rakesh Aggarwal, Saleem Kamili

Hepatitis E, previously known as enterically transmitted non-A, non-B hepatitis, was first recognized as a distinct clinical entity in the 1980s, when sera from persons affected during a large waterborne epidemic of acute hepatitis during 1955–1956 in Delhi, India and another epidemic in Kashmir, India tested negative for serological markers of acute hepatitis A and B.[1–3] The occurrence of the first recorded epidemic of hepatitis E as late as 1955 and the infrequency of this disease in developed countries suggest that hepatitis E is a new, emerging infectious disease. However, several epidemics of enterically transmitted hepatitis with epidemiologic features resembling those of hepatitis E outbreaks occurred in Europe and the United States in the 18th and 19th Centuries.[4,5] It is conceivable, therefore, that hepatitis E virus (HEV) infection may have been more widespread in the past and has only recently become restricted to certain geographic regions, mostly underdeveloped with poor environmental sanitation.

**Table 41.1** Epidemiologic features of hepatitis E

| |
|---|
| Large outbreaks in developing countries affecting several thousand persons |
| Sporadic hepatitis cases frequent in disease-endemic areas |
| Sporadic hepatitis cases uncommon in non-endemic areas (occur mainly among travellers to disease-endemic areas) |
| Faecal-oral transmission (usually through contaminated water) |
| Highest attack rate among young adults aged 15–40 years, with relative sparing of children |
| Insignificant person-to-person transmission |
| No evidence of parenteral or sexual transmission |
| Mother-to-newborn (transplacental) transmission probable |
| High mortality rate (15–25%) among pregnant women, especially those in third trimester |

## Epidemiology

### Hepatitis E in disease-endemic regions

Regions of the world can be considered as hepatitis E disease-endemic or non-endemic based on the periodic occurrence of disease outbreaks. Several large epidemics of hepatitis E, characterized by the epidemiologic features summarized in Table 41.1, have been observed in the Indian subcontinent, and in developing countries of south-east and central Asia.[1,2,6,7] Outbreaks of hepatitis E have been observed in the Middle East, and northern and western parts of Africa. In North America (Mexico), two small outbreaks were reported in the year 1986–1987 (Fig. 41.1).[8,9] The hepatitis E outbreaks in HEV-endemic regions are large,[1,2,6,7] frequently affecting several hundred to several thousand persons. Their time-course varies from single-peaked outbreaks lasting a few weeks to prolonged, multi-peaked epidemics lasting for over a year. The outbreaks recur with a periodicity of 5–10 years. The exact reason for this phenomenon remains unknown. The outbreaks frequently follow heavy rainfall and floods, but sometimes may occur in hot and dry summer months.

In areas where hepatitis E outbreaks occur, HEV infection accounts for a substantial proportion of acute sporadic hepatitis in both children and adults. In India, HEV infection accounts for 50–70% of all patients with sporadic viral hepatitis.[10,11] Demographic and clinical features of patients with sporadic hepatitis E (age distribution, severity and duration of illness, worse prognosis among pregnant women and absence of chronic sequelae) closely resemble those of epidemic hepatitis E.[12–14]

#### *Transmission, routes of spread and attack rates in disease-endemic regions*

The faecal-oral route is the predominant mode of transmission of epidemic HEV infection. Most reported outbreaks have been related to consumption of fae-

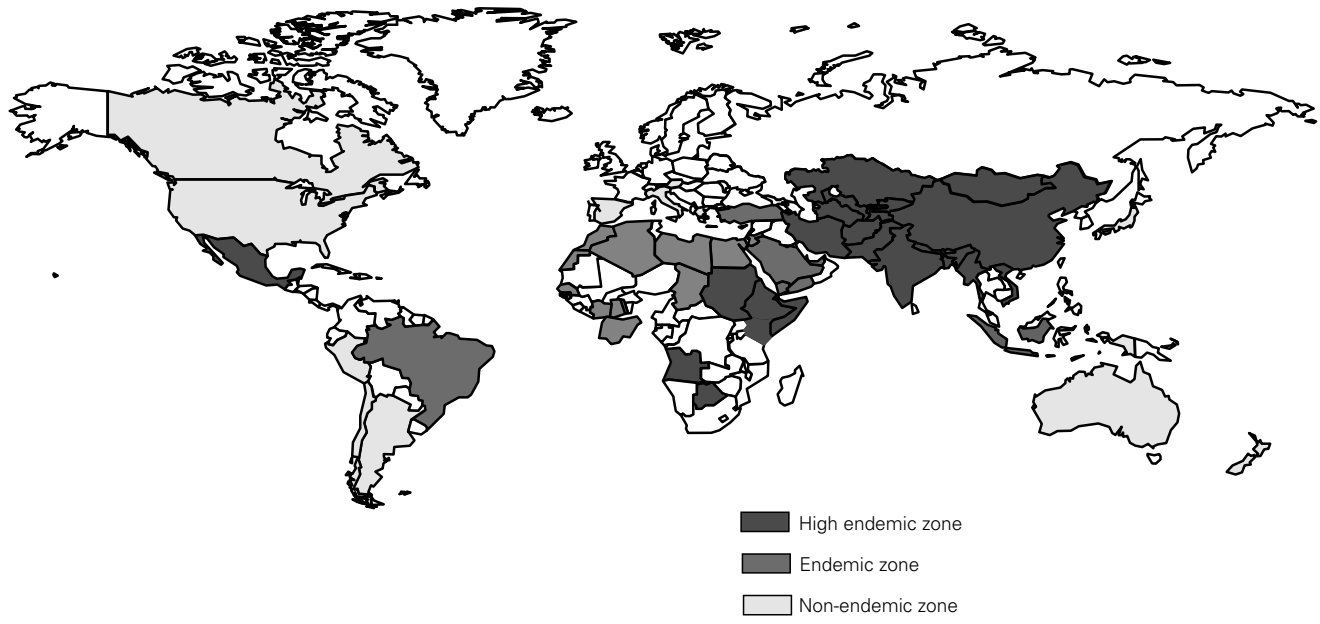


**Figure 41.1** Geographical distribution of hepatitis E.

cally contaminated drinking water.[1,2,6,15] The outbreaks frequently follow heavy rains and floods, when water sources become contaminated.[2,15] Some epidemics have occurred in hot summer months, when the diminution of water flow rate in rivers and streams may increase the concentration of contaminants, thereby increasing the risk of infection.[6,16] In some outbreaks, contamination of water occurred in leaky water pipes with intermittent water supply passing through areas contaminated with sewage, where a negative pressure in the pipes during periods of no flow leads to suction of contaminants. In south-east Asia, disposal of human excreta into rivers, and use of the same river water for drinking, cooking and personal hygiene has been shown to be associated with recurrent epidemics,[17] possibly through continuous existence of conditions that allow faecal contamination of water. In some small outbreaks of HEV infection in China that occurred after community feasts, food-borne transmission has been postulated.[7] However, these reports did not include any control data or results of serologic investigations.

During hepatitis E outbreaks, person-to-person transmission of HEV appears to be distinctly uncommon.[2,18] Such transmission is also uncommon from patients with sporadic HEV infection in disease-endemic regions.[19] The mode of transmission responsible for sporadic hepatitis E thus is unclear. Secondary attack rates among household contacts of patients with hepatitis E cases are only 0.7–2.2%[2,20] in contrast, 50–75% of susceptible household contacts of patients with hepatitis A become infected.[21] Even when multiple cases occur among members of a family, this is related to exposure to a common source of contaminated water rather than to person-to-person spread.[18] Presumed nosocomial spread of HEV has been reported in South Africa, where three health-care workers who treated a patient with fulminant hepatitis E developed acute hepatitis 6 weeks later.[22]

Vertical transmission of HEV infection from mother to infant has been reported. In one study, five of six babies born to mothers with either acute uncomplicated or fulminant hepatitis E in the third trimester of pregnancy had HEV RNA in their blood samples taken at birth, suggesting transplacental transmission of infection.[23] However, in an experimental study, HEV-infected pregnant rhesus monkeys failed to transmit the virus to their offspring.[24] Until recently, HEV was believed not to be transmitted through transfusion of blood or blood products because individuals with symptomatic HEV infection are unlikely to donate blood and HEV viraemia does not become chronic. Accordingly, anti-HEV antibody prevalence rates among patients with haemophilia and thalassaemia, who frequently receive transfusions, and among intravenous drug users are not higher than those in the general population.[25] However, a recent study has documented the presence of HEV viraemia among healthy blood donors and transmission of this infection to transfusion recipients in a disease-endemic region.[26]

During hepatitis E outbreaks, overall attack rates range from 1% to 15%, being much higher among adults (3–30%) than those among children below 14 years of age (0.2–10%).[1,6,15,27] In most reports, the male-to-female ratio among cases has varied from 1:1 to 4:1; it is unclear whether this reflects greater frequency of exposure among men or a true difference in susceptibility. The outbreaks are characterized by a particularly high attack

rate and mortality among pregnant women.[1,2,6,7] In an epidemic in Kashmir, India, attack rates among those in the first, second and third trimesters were 8.8%, 19.4% and 18.6%, respectively, as compared with 2.1% among non-pregnant women and 2.8% among men.[28]

## Hepatitis E in disease non-endemic regions

In non-endemic regions, where outbreaks have not been reported, the disease accounts for <1% of reported cases of acute viral hepatitis, and indigenous transmission of hepatitis E in these regions appears to be rather rare. A few isolated sporadic cases have been described from the USA, countries in Europe (including Austria, Spain, Italy, Greece and Turkey),[29–33] developed countries in Asia (Japan, Taiwan, Hong Kong),[34–36] South America (Argentina)[37] and Africa (Egypt, Senegal and Tunisia).[38,39] Most of the sporadic cases in non-endemic regions have been associated with travel to HEV-endemic regions,[40,41] although some cases, including two cases in the USA,[42–44] have been reported among persons with no history of travel to disease-endemic countries. Recent Japanese cases have been associated with consumption of inadequately cooked animal meat that contained HEV.[45]

## HEV molecular epidemiology and seroprevalence rates

Nucleotide sequences have been determined for HEV isolates from disease-endemic countries and from non-endemic regions. Four genotypes of HEV have been proposed: genotype 1 comprises south-east and central Asian isolates from Burma, Pakistan, India and China; genotype 2 comprises a single Mexican isolate; genotype 3 comprises US human and swine isolates; and genotype 4 comprises new Chinese isolates.[37,46–48] A variant strain of HEV has also been reported from tissue and faecal specimens of wild-trapped rodents from Nepal.[49] More recently, a novel virus related to HEV was identified in chickens with the hepatitis-hepatosplenomegaly syndrome in the United States. Genomic sequence analysis of the avian HEV in the helicase region reveals its partial identity (58–60% in amino acid and nucleotide sequences) to other HEV strains.[50] Comparison of nucleotide sequences in HEV isolates derived from patients affected during an outbreak in the disease-endemic region showed a high degree of identity.[51] Clinical and epidemiological differences have been observed between HEV strains within the same genotype.[52] It has been suggested that different strains may co-exist in a single population, some causing epidemic disease and others sporadic disease.[52]

Anti-HEV IgG antibody has been found in healthy subjects living in all geographical areas, although its prevalence differs significantly in various countries. In disease-endemic areas of Asia and Africa, the prevalence of antibody to HEV in documented endemic regions has been much lower than expected (3–26%), and the prevalence of such antibody in non-endemic regions has been much higher than anticipated (1–5%). In most disease-endemic areas, anti-HEV has been detected in up to 5% of children younger than 10 years of age. This proportion increases to 10–40% among adults older than 25 years of age.[53,54] However, in a report from India, anti-HEV antibodies were detected in >60% of children below the age of 5 years.[55] The differences between different disease-endemic areas may be related to varying epidemiological conditions in different geographic areas, differences in diagnostic techniques used, or both.

In developed countries of Europe and North America, 1–5% of the population has anti-HEV.[56,57] This range appears to be relatively high compared with the low rate of clinically evident hepatitis E disease in these areas. Several assays were applied to both acute hepatitis E and population studies for detection of IgG anti-HEV. In a direct comparison using a panel of coded sera,[58] sensitivity rates of these assays were found to vary widely from 17% to 100%, and concordance rates among reactive sera ranged from none to 89% (median 32%). In another study, in which two different serologic tests for anti-HEV were used, it was found that concordance between the two tests was only 27%.[59] It remains unclear whether the anti-HEV seroreactivity in non-endemic areas reflects subclinical and/or anicteric HEV infection, serologic cross-reactivity with other agents, false-positivity of serologic tests, transmission of infection by contact with various animal reservoirs (swine, rodents), or a combination of all these factors.

## Reservoirs of HEV

In disease-endemic areas, the source of HEV for maintaining the disease in a population has not been completely determined, and the existence of many reservoirs has been postulated. Based on data from a small group of patients, it has been suggested that protracted viraemia and prolonged faecal shedding of HEV may have a role in the continuous contamination of sewage.[60] However, recent data generated from studies on a larger number of patients showed that the period of viral shedding in the faeces is much shorter.[61] Subclinical HEV infections that maintain the presence of the virus in a population during inter-epidemic periods may be a potential reservoir of HEV in disease-endemic regions. In the experimental model of HEV infection in cynomolgus macaques, which faithfully reproduces HEV infection in humans, animals with HEV infection but without biochemical evidence of liver injury excreted large amounts of HEV infectious to HEV-naïve animals.[62] Shedding of large quantities of viable virus

during subclinical infection in humans may, therefore, represent a potential reservoir of HEV during inter-epidemic periods. Existence of a reservoir of HEV in the form of subclinical infection in a population could be similar to the continuous presence of polio virus in areas where that infection used to be endemic.[63] HEV shed by individuals with subclinical infection may contaminate drinking water supplies during periods of flooding of river systems, leading to large outbreaks. However, more epidemiologic and laboratory data are needed to confirm the existence of continuous subclinical circulation of HEV in disease-endemic areas.

A zoonotic reservoir of HEV has also been suggested, based primarily on anti-HEV reactivity of serum specimens obtained from various animal species and on fragmentary genomic sequence data. Anti-HEV antibodies have been detected in pigs in several endemic regions such as Nepal, China, India and Thailand, and in several non-endemic regions including the USA, Canada, Korea, Taiwan, Spain and Australia.[64–69] Anti-HEV has also been detected in chicken, cattle, sheep and rodents in several disease-endemic regions[64] and in 44–90% of rats in different parts of the USA.[70–72] The discovery of a swine HEV and the demonstration of a genetic relationship of animal HEV strains from the USA, Taiwan and Spain with human isolates from these respective geographical areas may support this hypothesis.[29,44,73] Nucleotide sequence homology between HEV RNA isolated from some Japanese hepatitis E patients and those from the undercooked animal meat that they had eaten may also support animal-to-human transmission.[45] Although the infrequent occurrence of clinically overt HEV infection in non-endemic areas with a high prevalence of anti-HEV among pigs and rats may depend on better sanitation, good water quality, and attention to personal hygiene, it is inconsistent with the hypothesis of universal zoonotic origin of hepatitis E. Moreover, Asian and Mexican strains of HEV associated with large outbreaks of hepatitis E have failed to induce infection in experimentally inoculated pigs.[42,74,75] More molecular data are needed before the zoonotic reservoir can be implicated as playing a major role in the epidemiology of hepatitis E, especially given that genomic sequences of HEV strains isolated from animals varied from those found in humans in a hepatitis E-endemic region in India.[76]

In summary, current epidemiologic data indicate that large outbreaks, sporadic hepatitis E cases and subclinical HEV infection in the disease-endemic regions originate from an environmental, human, or yet to be identified animal HEV reservoir. Poor general sanitation, contamination of drinking water supplies and lack of attention to personal hygiene are important factors contributing to the spread of infection in these areas.

## Clinical and pathological features

The incubation period of hepatitis E is variable and has ranged from 2 to 10 weeks during waterborne outbreaks with a short and well-defined period of water contamination.[2,77] Clinical manifestations of HEV infection are similar to those of acute infection with other hepatitis viruses and encompass a wide spectrum of symptoms (Table 41.2). Acute icteric hepatitis is the most common recognizable form of illness associated with HEV infection. This illness is usually insidious in onset and has an initial prodromal phase lasting about 1–4 days, with a variable combination of flu-like symptoms, fever, mild chills, abdominal pain, anorexia, nausea, aversion to smoking, vomiting, clay-coloured stools, dark or tea-coloured urine, diarrhoea, arthralgias, asthenia and a transient macular skin rash (Table 41.3).[1,7,8,78–81] These symptoms are followed in a few days by the appearance of jaundice. The onset of the icteric phase is frequently heralded by darkening of the urine and may be accompanied by lightening of stool colour or itching. Physical examination reveals jaundice and a mildly enlarged, soft and slightly tender liver. A soft splenomegaly is observed in nearly one-quarter of patients.[81]

Laboratory test abnormalities include bilirubinuria, variable degree of rise in serum bilirubin (predominantly conjugated), marked elevation in serum alanine aminotransferase (ALT), aspartate aminotransferase and gamma-glutamyl transferase activities, and a mild rise in serum alkaline phosphatase activity. An elevation of aminotransferase levels may precede the onset of symptoms by as long as 10 days and reaches a peak by the end of the first week. As the illness subsides, aminotransferase levels decrease significantly, followed by diminution in serum bilirubin level, and liver function test values usually return to normal by 6 weeks.[81] The magnitude of transaminase elevation does not correlate well with the severity of liver injury. Some patients show

**Table 41.2** Clinical features of hepatitis E

| |
|---|
| Incubation period 2–10 weeks |
| Variable clinical manifestations including: |
| icteric hepatitis |
| severe hepatitis leading to fulminant hepatic failure |
| anicteric hepatitis |
| inapparent, asymptomatic infection |
| Clinical illness similar to other viral hepatitis (except among pregnant women) |
| Milder illness in children |
| Low mortality rate (0.07–0.6%) |
| High attack rate in pregnant women, particularly those in second and third trimesters |
| High mortality (15–25%) among pregnant women |
| No relation with chronic hepatitis, cirrhosis or hepatocellular carcinoma |

**Table 41.3** Clinical findings in hepatitis E outbreaks

| Symptoms and signs | Delhi, India[80] 1956 (%) (n = 958) | Accra, Ghana[78] 1963 (%) (n = 136) | Kashmir, India[1] 1978 (%) (n = 275) | Ethiopia[79] 1989 (%) (n = 423) | Xinjing, China[81] 1986–88 (%) (n = 85) |
|---|---|---|---|---|---|
| Jaundice | 100 | 100 | 100 | 100 | 91 |
| Malaise | | 95 | | 100 | 95 |
| Anorexia | 66 | 95 | 79 | 100 | 69 |
| Abdominal pain | 63 | 37 | 41 | 82 | 55 |
| Hepatomegaly | 62 | 67 | 85 | 10 | 80 |
| Nausea, vomiting | 29 | 48 | 46 | 100 | 91 |
| Fever | 23 | 57 | 44 | 97 | 53 |
| Pruritus | 14 | 47 | 20 | 14 | 59 |

mild leukopenia and relative lymphocytosis. The illness is usually self-limiting and typically lasts 1–4 weeks.[80,81] Histopathological features of hepatitis E are similar to those of other forms of acute hepatitis, such as the presence of ballooned hepatocytes and acidophilic bodies, and focal and confluent hepatocyte necrosis with collapse and condensation of the underlying reticulum. Nearly half of hepatitis E patients have cholestatic hepatitis, which is characterized by canalicular bile stasis and gland-like transformation of parenchymal cells. In these patients, degenerative changes in hepatocytes are less marked and polymorphonuclear infiltration is prominent.[82] In both forms, lobules and enlarged portal tracts show inflammatory infiltration.

No evidence of chronic hepatitis or cirrhosis has been detected among patients followed up clinically and with liver biopsies after acute hepatitis E.[9,82,83] A few patients, however, have a prolonged course with marked cholestasis (cholestatic hepatitis), including persistent jaundice and prominent itching. In these cases, laboratory tests show elevations in alkaline phosphatase and bilirubin levels even after transaminase levels have returned to normal. The prognosis is good, as jaundice finally resolves spontaneously after 2–6 months.

HEV-infected individuals may develop non-specific symptoms, resembling those of an acute viral febrile illness without jaundice (anicteric hepatitis). In its most benign form, HEV infection is entirely inapparent and asymptomatic, and passes unnoticed. The exact frequencies of asymptomatic infection and of anicteric hepatitis are not known, although these probably far exceed that of icteric disease.

In a small proportion of patients, disease is more severe and is associated with subacute (or late-onset) or fulminant hepatic failure (FHF) that can be rapidly fatal. In patients with severe liver injury, a large proportion of hepatocytes are affected, leading to submassive or massive necrosis and collapse of liver parenchyma. In disease-endemic regions, hepatitis E is an important cause of FHF. HEV infection (alone or in combination with other hepatitis viruses) in India was responsible for 62% of adult patients and 40% of children with sporadic FHF.[14,84,85] It has also been suggested that serious forms of hepatitis such as FHF and subacute hepatic failure may result from a combined infection with hepatitis B virus (HBV) and HEV.[12,86] In a recent study, Hamid *et al*. showed that hepatitis E infection in patients with pre-existing liver cirrhosis may lead to worsening of their clinical state.[87]

The case-fatality rate in HEV infection has ranged from 0.5% to 4%.[20,88] However, these reports are based on hospital data and thus underestimate the total number of persons affected and may overestimate mortality. Population surveys during outbreaks report lower mortality rates varying from 0.07% to 0.6%.[6,81,88] In an epidemic in Ethiopia, none of the 423 soldiers with icteric hepatitis developed FHF or died.[79] Pregnant women, particularly those in the second and third trimesters, are more frequently affected during hepatitis E outbreaks and have a worse outcome. Mortality rates among pregnant women, especially those infected in the third trimester, have ranged between 5% and 25%.[15,20,27,28] FHF developed in 22.2% of the affected pregnant women, in comparison with 2.8% and 0% of affected men and non-pregnant women, respectively.[28] Frequency of abortions, stillbirths and neonatal deaths is also increased among pregnant women with HEV infection.[27] The reason for particularly severe liver damage in pregnant women with hepatitis E remains unknown.

## Diagnosis

### Detection of antibodies to HEV

Historically, the fluorescent antibody blocking assay (FA) was one of the earliest tests used for identification of antibodies that react with HEV antigen (HEVAg) identified in hepatocytes of experimentally infected macaques.[89] Although the FA assay enabled the serologic identification of HEV infection in various geographic regions of the world,[89] it does not distinguish between recent and past infections, and requires liver

tissue substrate that contains HEVAg from HEV-infected primates.

Enzyme immunoassays (EIAs) for the detection of IgM and IgG antibodies to HEV have been developed in several laboratories using recombinant HEV antigens expressed in *Escherichia coli* or insect cells, and synthetic peptides corresponding to immunogenic epitopes of HEV.[56,90–94] A synthetic gene encoding multiple linear immunodominant antigenic epitopes from ORF2 and ORF3 regions has been synthesized, expressed as a protein and used in a solid-phase EIA.[95] Recently, a capture EIA format was shown to be more sensitive in detecting IgM, especially in the presence of high concentrations of IgG antibodies.[96] Although commercial kits for the detection of IgM and IgG anti-HEV are available from vendors in various countries, there is no commercial test for the detection of anti-HEV currently licensed for clinical use in the United States. The commercial kits and many 'in-house' EIAs use epitopes from two geographically distinct HEV strains representing diverse antigenic domains as target antigens.[58] Antigenic domains that contain strong IgG and IgM antigenic epitopes have been identified at the amino- and carboxyl-terminus of the ORF2 encoded protein[97] and have been shown to be more sensitive than ORF3-derived antigens, when used for detection of IgM and/or IgG anti-HEV.[98] A highly conserved conformational epitope mapped to 267 amino acids at the carboxyl-terminus of HEV ORF2 protein has been used in a sensitive and specific EIA format for the detection and quantification of both acute and convalescent phase HEV-specific IgG.[99]

In clinical studies, determination of IgM anti-HEV is important for diagnosis of acute HEV infection, whereas the detection of IgG anti-HEV may be indicative of convalescent phase or past infection. During acute HEV infection, IgM anti-HEV appears in the early phase of clinical illness, preceding the IgG anti-HEV by a few days, and disappears over a 4–5-month period.[100] In one study, 100%, 50% and 40% of sera collected from patients during various hepatitis E outbreaks 1–40 days, 3–4 months and 6–12 months after the onset of jaundice, respectively, tested positive for IgM anti-HEV.[91] In outbreak settings, IgM anti-HEV has been detected in >90% of patient serum samples obtained within 1 week to 2 months after the onset of illness.[100] The IgG response appears shortly after the IgM response, and its titre increases throughout the acute phase into the convalescent phase, and remains high from 1 to 4.5 years after the acute phase of illness.[56,100] In one study, anti-HEV was detected in 47% of persons 14 years after acute HEV infection,[101] but the exact duration of persistence of anti-HEV is not known.

As EIAs are the most convenient, inexpensive and suitable assays for routine diagnosis of HEV infection as well as for sero-epidemiologic surveys, their sensitivity and specificity need to be improved. Currently used diagnostic assays for detection of anti-HEV have a wide range of sensitivity between 17% and 100%, when applied to specimens from non-endemic areas.[58] Further studies on standardization of diagnostic anti-HEV tests may benefit from the availability of a reference reagent for human anti-HEV serum developed by the Expert Committee on Biological Standardization of the World Health Organization.[102]

## Detection of HEV-like particles and HEV RNA

The identification of HEV in faeces, bile, blood and liver has been accomplished by the use of immune electron microscopy (IEM) with immunoglobulin preparations obtained from hepatitis E patients during early convalescence.[103] Serologic cross-reactivity was observed between virus-like particles (VLPs) from epidemics of hepatitis E in Mexico, the former Soviet Union, Burma, Nepal, Pakistan, Sudan and Somalia, suggesting the existence of a single viral serotype associated with these epidemics.[103,104] For routine diagnostic use, IEM is not practical because visualization and identification of the virus is limited to samples with an abundance of VLPs.

A more efficient and a highly sensitive method for detection of HEV is the polymerase chain reaction (PCR). Several reverse transcriptase PCR (RT-PCR) assays have been developed for detecting genomic sequences of HEV in clinical and environmental samples.[29,105–109] HEV RNA sequences have been identified in stool and serum samples from patients and experimentally infected primates during the acute phase of hepatitis E.[61,110] Although various regions of sequence variation and conservation have been identified in the HEV genome, there is no preferred region selected for amplification. Primers based on either the RNA-dependent RNA polymerase region in ORF1 or the ORF2 gene are most often used for amplification of HEV genomic fragments.

HEV RNA can be detected in faeces of most patients with acute hepatitis E by RT-PCR during the initial few weeks.[61,111] In some patients, persistence of positive RT-PCR results for as long as 52 days has been reported.[60] HEV RNA has regularly been found in serum by RT-PCR in virtually all patients in the first 2 weeks after the onset of illness.[111] Prolonged periods of HEV RNA positivity in serum ranging from 4 to 16 weeks have also been reported.[60,112] However, a recent, more extensive study did not find any evidence of prolonged viral excretion or viraemia,[61] and no studies reported the presence of HEV in body fluids other than serum. Generally, RT-PCR for HEV RNA is less suitable than serologic identification of IgM anti-HEV for routine diagnosis of HEV infection because HEV RNA may be degraded during faecal shedding of the virus, and the short viraemic phase usually occurs before the disease is clinically apparent.

## Experimental HEV infection

Experimental HEV infection in humans[112,113] and primates[89,114–117] provided the most informative data on the pathogenetic events of the infection resulting from intragastric or intravenous administration of the virus. In 1983, a human volunteer ingested acute phase stool suspension from a water-borne epidemic of non-A hepatitis in central Asia, and provided detailed clinical and biochemical data on the course of infection.[113] This experimental infection led to the identification of VLPs in stool specimens by immune electron microscopy with the use of anti-HEV antibodies obtained from the same individual during convalescence. The discovery of HEV allowed definition of the aetiological agent of the acute viral hepatitis transmitted by the faecal-oral route (hepatitis E). In 1993, another human volunteer provided a set of clinical, virological and serological data that further characterized hepatitis E.[112] The incubation period of the disease in the human volunteers ranged from 4 to 5 weeks, HEV VLPs were found in stools 4–5 weeks after exposure, and elevated enzyme activity reached the highest levels at 6–7 weeks after exposure. HEV RNA in serum was first detected on day 22 post-exposure, a week before onset of disease on day 30, and anti-HEV antibody was detected 7 weeks post-infection. HEV infection in both volunteers resolved clinically with a return of liver enzyme activity to the normal range.

Experimental infection has successfully been attempted in non-human primates, such as chimpanzees, macaques (cynomolgus, rhesus and pigtail monkeys) and African green monkeys among the Old World species, and marmosets (tamarins) and owl monkeys among the New World species.[118] The levels of virus excretion, liver enzyme elevations and histopathologic changes in liver varied significantly in these animals.[119] Experimental, direct intrahepatic inoculation of chimpanzees and rhesus monkeys with RNA transcripts from full-length functional clones of HEV cDNA induced virological, pathological and serological characteristics typical of hepatitis E, and established a direct aetiological link between the genetic material of HEV and the disease.[120]

In intravenously inoculated cynomolgus macaques, the average incubation period for acute hepatitis E measured by a significant increase of ALT activity values is about 21 days. HEVAg was detected in hepatocytes on about day 7 post-infection and was found in 70–90% of hepatocytes at the peak of viral replication (K. Krawczynski, unpublished data) that occurred before or concurrently with the onset of ALT elevation and histopathological changes in the liver.[115,116] Both IgM and IgG anti-HEV have been detected in serum in assays using immunoreactive epitopes of ORF2 and ORF3. The IgM antibody level decreases rather precipitously, reaching negligible levels in the early convalescent phase followed by high titres of IgG anti-HEV detected during convalescence. The antibodies have been observed as long as 10 years after onset of acute hepatitis E in chimpanzees experimentally infected with serum specimens derived from Tashkent, Pakistan and Mexico.[121]

The variety of patterns of experimental infection in cynomolgus monkeys and chimpanzees resembles clinically overt or subclinical HEV infection in humans. In experimentally infected chimpanzees, the course of infection and disease measured by virus replication, antiviral humoral immune response and ALT activity was significantly different among individual animals.[110] These differences in virological, immunological and pathological sequelae of infection may be explained by either individual host susceptibility to HEV infection or the size of the dose of infectious HEV. Inoculum titration experiments in cynomolgus macaques have shown that animals infected with decreasing infectious doses had less marked clinical and pathological evidence of liver disease. Inoculation of the end-point dilution of an infectious inoculum (Mexico strain) was marked by seroconversion to anti-HEV only. No evidence of the virus shedding in stools, HEVAg in the liver or liver pathology was found. These observations indicated that the clinical presentation of hepatitis E in a primate model depends on the size of the infectious dose, and the severity of infection seems to be directly related to the infectivity titre of the challenge inoculum.[62] The subclinical infection caused by the smaller infectious dose was associated with faecal viral excretion similar in magnitude to that observed in the clinical form of the disease. The virus shed during subclinical infection was viable and capable of transmitting HEV infection to naïve cynomolgus macaques.[62]

In summary, several elements of pathogenesis of HEV infection can be outlined based on data from human volunteers and patients, and those from experimentally infected animals (Fig. 41.2). The virus enters the host primarily through the oral route, but there have not been enough clinical and experimental data collected to document that HEV reaches the liver from the gastrointestinal tract through portal circulation. In experimentally infected primates, HEV RNA appears in serum, bile and faeces a few days before the onset of ALT rise.[110,114,116,122,123] HEVAg in hepatocytes has been detected simultaneously with HEV identified in bile and faeces during the second or third week after inoculation, before or concurrently with the onset of ALT elevation and morphological changes in the liver.[89] These findings suggest that HEV may be released from hepatocytes into bile during the initial, highly replicative phase of infection, before the occurrence of the most prominent histopathological changes in the liver. The onset of ALT elevation and the occurrence of pathological changes in the liver generally correspond to the detection of anti-HEV in serum and

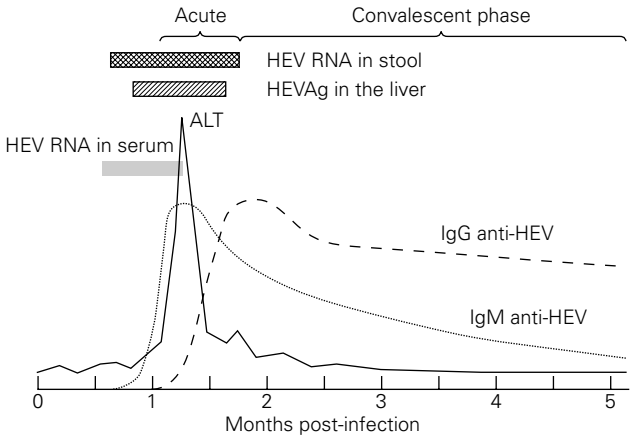


**Figure 41.2** Pathogenetic events in hepatitis E virus infection.

with decreasing levels of HEV antigen in hepatocytes. Lymphocytes infiltrating liver in experimentally infected monkeys have a cytotoxic/suppression immunophenotype.[124] Preliminary results of cellular immunity studies in hepatitis E patients indicate that lymphoproliferative responses to HEV peptides from ORF2 and ORF3 regions occur in patients with acute hepatitis E.[125] Concordance of pathological, virological and serological findings in hepatitis E suggests that the pathomechanism of the disease may be immune-mediated rather than related to cytopathic effect of HEV.

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# Chapter 42
# Prevention

Robert H Purcell, Suzanne U Emerson

## Introduction

Hepatitis E virus (HEV) is a small hepatatropic virus that is enterically transmitted and causes an acute, self-limiting hepatitis[1] (Chapter 39). Infection with HEV may be asymptomatic or may cause hepatitis ranging from mild to fulminant. Fulminant hepatitis E is most common in pregnant women. Hepatitis E is the most common form of sporadic and epidemic acute hepatitis in adults throughout most of Asia. At present, there are no effective means of prevention other than interdiction of transmission.

## Epidemiology

### History

Until the 1970s, all water-borne epidemics of hepatitis in Asia were thought to be caused by hepatitis A virus (HAV). However, with the development of sensitive assays for antibody to HAV, it became apparent that virtually everyone in developing countries was exposed to HAV by age 5 years.[2] Most of these infections went unrecognized, but they provided lifelong immunity against hepatitis A. Thus, the epidemics of viral hepatitis must have been caused by a previously unrecognized agent.[3,4] In 1983, Balayan *et al*. first visualized the virus, demonstrated by immune electron microscopy the development of antibody to it during convalescence and transmitted the agent to cynomolgus monkeys.[5] In 1990, Reyes *et al*. cloned and sequenced genomic RNA from the virus and it was subsequently named hepatitis E virus.[6–8]

In retrospect, epidemics of hepatitis E were probably misdiagnosed long before the 1970s. Outbreaks of hepatitis occurring in previous centuries and the early 20th Century were known variously as 'campaign jaundice' and 'infective hepatitis'.[9] Before the discovery of hepatitis E, these historical outbreaks of hepatitis were thought to have been caused by HAV. However, the epidemiological descriptions of such diseases resemble those of hepatitis E, not hepatitis A.[10]

### Geographic distribution and genetic variation

Clinical hepatitis E is found principally in developing countries with tropical and subtropical climates. The disease is largely limited to those regions with substandard water and sewage systems, in keeping with the faecal-oral transmission of this virus. In regions of high endemicity, transmission via blood transfusion is a rare but possible occurrence because of the transient viraemia associated with infection.[11,12]

Hepatitis E is clinically important primarily in developing countries of south-east and central Asia, the Middle East and North Africa.[13] Epidemic hepatitis E has also been reported in Mexico.[14] Individual cases of hepatitis E have been reported occasionally in industrialized countries, including the United States and several European countries.[15,16] Strains of HEV recovered from within one geographic region generally are genetically similar and characteristic of that region, and differ from strains indigenous to other regions. However, the overall heterogeneity of HEV strains is not great, and all strains recovered to date appear to belong to the same serotype. On the basis of analyses of completely or partially sequenced strains, HEV can be classified into four major genotypes: a heterogeneous group of Asian and African strains (genotype 1), strains recovered from Mexico and Nigeria (genotype 2), strains recovered from cases in North America (United States), South America (Argentina), several European countries (Italy, Greece, Spain, Austria, the Netherlands) and Asia (Japan) (genotype 3), and strains isolated from cases in China, Taiwan and Japan (genotype 4).[1]

### Host range and animal models

Chimpanzees, Old World monkeys (rhesus, cynomolgus, pigtail macaques, African green monkeys) and New World monkeys (owl monkeys, squirrel monkeys, tamarins) have been reported to be susceptible to experimental infection with HEV.[17] Extensive studies in primates have confirmed the utility of these animals, especially chimpanzees, rhesus monkeys and cynomol-

gus monkeys, for experimental transmission of human strains of HEV. In addition, wild rhesus monkeys in India, wild cynomolgus monkeys from Asia and captive rhesus monkeys within North American breeding colonies have been demonstrated to acquire antibody to HEV in a pattern strongly suggestive of endemic infection. However, a virus has not yet been recovered from these animals, and it is not clear whether rhesus monkeys are infected with a unique simian HEV or whether human strains of HEV are circulating in monkey populations. As in humans, hepatitis E in non-human primates is acute and self-limiting. Unlike experimental hepatitis caused by the other human hepatitis viruses, experimental hepatitis E is dose-dependent. High doses of virus are associated with histological and biochemical evidence of hepatitis, but lower doses of virus are more likely to be associated with normal liver histology and normal serum liver enzyme values.[18–20] It is not known whether this is true for hepatitis E in humans, but epidemiological patterns suggest that it is. The immune response to HEV infection in non-human primates is similar to that in humans. Chimpanzees, rhesus monkeys and cynomolgus monkeys have been very useful for evaluating approaches to passive and active immunoprophylaxis against hepatitis E.

Although there were early reports of transmission of HEV to swine, sheep and rats, it was molecular and serological studies that provided enough information for these studies to be evaluated, confirmed and extended.[21–24] In 1997 Meng *et al*. recovered a unique HEV strain from domestic swine in the United States.[25] The virus was the first documented genotype 3 isolate, and it was subsequently shown that two human isolates in the United States were closely related to the swine isolate.[15] Genotype 3 strains of HEV have been recovered from swine throughout the United States, Canada and Mexico, and in Europe, New Zealand and Japan. Genotype 4 strains of HEV have been recovered from swine in Taiwan, Japan and India. These are closely related to genotype 4 isolates from humans. Attempts to confirm early reports of transmission of HEV from humans to swine could not be confirmed with genotype 1 and genotype 2 isolates.[26] However, human genotype 3 isolates have been transmitted to swine.[27] A retrospective analysis of the original report of transmission of a human HEV isolate to swine has revealed that the human virus was genotype 1 but the virus recovered from the swine was genotype 3, indicating that the inoculated piglets were probably undergoing an inter-current infection with swine HEV at the time of the experiment.[28] This is consistent with observations that most swine are infected within the first few months of life.[25] In addition, genotype 3 and possibly genotype 4 HEV strains recovered from swine have been experimentally transmitted to rhesus monkeys, a surrogate of man (V. Arankalle, personal communication).[27] This suggests that genotypes 3 and 4, at least, are spread zoonotically to man, and this was confirmed recently in reports of the transmission to humans of genotypes 3 and 4 HEV by ingestion of raw boar liver or undercooked commercial pig liver, and of a family outbreak of hepatitis E following the ingestion of raw Sika deer venison in Japan.[29–31] In the latter study, HEV genomic sequences were recovered both from patients and from frozen stored venison and the sequences were identical.

As with swine, attempts to confirm earlier reports of the transmission of human strains of HEV to rats were not successful: strains of genotypes 1, 2 and 3 of known infectivity titre could not be transmitted to laboratory rats (S.U. Emerson, unpublished data).[32] However, a very high proportion of sera from urban rats as well as indigenous species of rats in the United States were positive for anti-HEV, and this was confirmed in Indian rats and other related species.[32–34] A transmissible agent was recovered from two of over eighty sera collected from urban rats in the United States.[35] The agents were difficult to transmit, replicated to relatively low titre ($10^3$–$10^5$ rat infectious doses) and were avirulent, but infected rats regularly developed anti-HEV that could be detected with an ELISA that utilized antigen from a human genotype 1 HEV strain. Genomic RNA from the rat HEV strains could not be amplified by reverse transcription-polymerase chain reaction (RT-PCR) with primers that could amplify low titres of genotypes 1–4, suggesting that the rat virus was genetically different from the human and swine strains of HEV. The rat virus could not be transmitted to rhesus monkeys, suggesting that it is not zoonotically spread to humans (Purcell RH, unpublished data).

Antibody to HEV has been found in the sera of a number of other species of animals, including sheep and cattle. Nothing is known about the viruses that infect these and other species but, because sheep and cattle are such an important source of food worldwide, zoonotic spread via beef and lamb could be important.

Recently, HEV strains were found to be the cause of a disease of chickens called 'big liver and spleen disease' in Australia and 'hepatitis-splenomegaly syndrome' in the United States.[36,37] Although serologically related to human and swine strains of HEV, the avian HEV strains were genetically quite different from the mammalian strains. Avian HEV was not transmissible to rhesus monkeys and is therefore not likely to be a threat to humans.[38]

## Cell culture

Attempts to replicate HEV in cell culture have generally been unsuccessful or difficult to reproduce. Early reports of success utilized cells not readily available to others.[39]

More recently, Meng *et al*. reported limited replication of one HEV strain in PLC/PRF/5 cells, as measured by RT-PCR.[40] The cells were used for preliminary evaluation of neutralizing antibody to HEV. Recently, after the development of an infectious cDNA clone of HEV, several primate cell lines, including human hepatoma cell lines, were shown to support the replication of HEV following transfection of the cells with RNA transcripts of the cDNA clone.[41] However, the virus did not spread in the cells. More recently, HepG2 human hepatoma cells have been infected with wild-type HEV. However, the sensitivity of the cells for direct infection was low. Nevertheless, they have been used for studies of virus inactivation and neutralization (S.U. Emerson, unpublished data).

## Epidemiologic perspective

HEV is the single most important cause of acute clinical hepatitis in adults in south-east and central Asia and the second most important cause of such disease in the Middle East and North and West Africa[1] (see Chapter 41). The more spectacular form of the disease, epidemic hepatitis E, is actually a relatively uncommon occurrence, and by far the majority of the cases occur as an endemic or sporadic disease. Most of the cases reported in industrialized countries occur in travellers recently returned from an endemic area, but rare cases of hepatitis E that appear to have been contracted locally do occur in industrialized countries.[16,42]

Questions remain about the relative sensitivity and specificity of serological tests for anti-HEV.[43] However, with more sensitive tests, a picture of the worldwide distribution and seroprevalence of HEV infection is emerging. Surprisingly, the prevalence of antibody to HEV in documented endemic regions is much lower than expected when compared with the seroprevalence of another enterically transmitted hepatitis virus, HAV, and the prevalence of anti-HEV in non-endemic regions, assayed by the same ELISA, has been much higher than anticipated. In fact, it has been comparable to that of HAV in some studies,[44–46] although there have been exceptions to this rule.[47] The age-specific acquisition of anti-HEV is also quite different from that of anti-HAV. The seroprevalence of the former peaks in older children and young adults and plateaus thereafter, whereas anti-HAV peaks in younger children in developing countries. In industrialized countries, both antibodies increase in prevalence with age. This pattern of antibody acquisition has been shown to represent the 'cohort effect' (e.g. a high prevalence of infection in early times and a lower prevalence of infection in recent times) for hepatitis A, but the significance of the pattern is not known for hepatitis E, as the pattern is similar in developing and industrialized countries. Evidence that such antibody patterns accurately reflect the existence of HEV in industrialized countries comes from studies of viruses in sewage from such countries. Clemente-Casaresa *et al*. recovered HEV strains from sewage collected in Spain, France and the United States.[48] All of the HEV sequences recovered were genotype 3, the most common genotype in all three countries. The prevalence of HEV in sewage samples was surprisingly high, comparable to that of HEV in sewage samples collected in India, a country in which HEV is highly endemic.[49,50]

Differences in the epidemiology of hepatitis A and hepatitis E may be explained by the following: (1) HEV of genotypes 1 and 2 may grow to higher titre and/or may be more virulent than genotype 3 and 4 viruses. Indeed, genotype 3 viruses of swine and human origin cause little or no disease in experimentally infected swine, and the same appears to be true for experimentally infected rhesus monkeys.[27,51] However, neither virus grows to high titre and the relative attenuation may be the result of the second characteristic of HEV; (2) the clinical expression of HEV infection is dose-dependent and hepatitis is not regularly observed in experimentally infected animals until the challenge titre reaches or exceeds 10 000 infectious doses[20] (R.H. Purcell, unpublished data). This would suggest that both the strain of HEV and the level of contamination of water and food define the incidence of disease more than the incidence of infection, and disease might occur more readily in the immunologically or physiologically compromised individual in industrialized countries. In fact, that is what is seen: the mean age of cases of hepatitis E caused by genotype 1 or 2 in developing countries is in the mid-20s, whereas the mean age of hepatitis E cases caused by genotype 3 or 4 is almost 60 years of age in industrialized countries (R.H. Purcell, unpublished data).

## Populations at risk

Based on seroprevalence studies, it has been estimated that a third of the world's population has been infected with HEV. The case-to-infected ratio for HEV was reported to be between 1:4 and 1:3 for young adults in Nepal.[52,53] Such figures suggest that about 500 million of the world's population have been clinically ill from hepatitis E, a very large disease burden for populations whose health is already compromised. Thus, the general populations, and especially the poorer rural populations of developing countries of Asia, the Middle East and Africa, are at risk of contracting hepatitis E and would be candidates for a hepatitis E vaccine.

Other groups at special risk of contracting hepatitis E may be recipients of blood transfusions and patients on maintenance haemodialysis in regions where HEV is highly endemic.[11,12,54] Groups at special risk of severe hepatitis E include pregnant women and patients with

chronic liver disease. Groups in industrialized countries at higher than average risk of acquiring hepatitis E are the military and other visitors to regions where hepatitis E is highly endemic. In addition, swine handlers in several countries have been shown to have a higher prevalence of anti-HEV than matched controls.[45,55] Finally, those who eat raw or uncooked food that may be contaminated (pork, shellfish, etc.) may be at higher risk of infection.[29–31,56,57]

### The virus genomic organization, antigenic composition and epitopes

Hepatitis E virus is the first member of a newly proposed virus family, Hepeviridae and the only member of the only genus, Hepevirus.[58] Its genome is single-stranded positive-sense RNA. It is capped at its 5′ end and polyadenylated at its 3′ end. It contains three open reading frames (ORFs): ORF1 encodes the non-structural proteins, ORF2 encodes the capsid protein and ORF3 encodes a small protein of unknown function. Proteins encoded by all three ORFs are immunogenic but the ORF2 protein is the most immunogenic, the ORF3 protein is intermediate in immunogenicity and the ORF1 proteins are the least immunogenic. On the basis of antibody mapping with synthetic peptides, a number of linear epitopes have been identified on HEV-encoded proteins. At least 12 such epitopes have been identified among the non-structural proteins, especially in the protein believed to encode the viral RNA polymerase.[59] Three major epitopes found, respectively, in the amino-terminal, central and carboxy-terminal regions of the capsid protein have been reported.[59,60] Similarly, a major epitope is found in the carboxy-terminal region of the ORF3 gene product.[60] The most highly conserved epitopes are those found in the capsid protein and, thus, this protein has been of most interest for the development of diagnostic assays and for vaccine development. Although originally thought to be linear in nature, the epitopes associated with the capsid protein have been shown to be conformational. The principal neutralization epitope was originally localized to the amino acid sequence between amino acid 578 and 607, but the smallest peptide retaining the conformation of this epitope was shown to span the amino acids from 459 to 607.[61,62] This peptide is encompassed within the smallest peptide that stimulated neutralizing antibody (aa 452–617) described by Meng *et al*.[63] and within the peptide said to form a homodimer that reacts with convalescent sera (aa 394–607) described by Zhang *et al*.[64] The peptide described by Zhou *et al*. also reacted with two neutralizing monoclonal antibodies, detected convalescent antibodies in rhesus monkeys infected with each of the four major genotypes of HEV, and the ELISA results obtained with this peptide correlated with protection against challenge with HEV in monkeys that had been vaccinated with recombinant hepatitis E vaccine.[62]

### Immune response and serologic assays

Little is known about the cellular immune response to HEV, but both IgM anti-HEV and IgG anti-HEV appear late in the acute phase of the infection and correlate with diminishing viraemia but also with increasing signs and symptoms of hepatitis, consistent with the belief that hepatitis E is an immunity-mediated disease.[65] IgM antibodies disappear after several months but IgG antibodies persist, although not at the levels observed in early convalescence. Antibodies to the ORF3 protein diminish in titre more rapidly than antibodies to the ORF2 protein. Consequently, antibodies to the ORF3 protein are useful for diagnostic purposes, but not for epidemiological studies of antibodies acquired in the past. In contrast, antibodies to the ORF2 protein can be detected decades after the primary infection and are useful for epidemiological studies. Antigens that contain the conformational neutralization epitope in the carboxy portion of the ORF2 protein are the most useful for serological studies.[62,66,67] This epitope is masked in the full-length ORF2 protein and is best displayed on the more soluble truncated forms of the antigen (see below).

## Prevention

### Sanitation

The role of improved sanitation and public hygiene in the control of hepatitis E is somewhat difficult to assess. Industrialized countries with a high level of public sanitation do not experience epidemics of water-borne hepatitis E or significant endemic or sporadic disease, although hepatitis A continues to be an important cause of clinical disease in some of these countries, for example, the United States.[68] In developing countries, even where the prevalence of antibody to HAV is extremely high, the prevalence of antibody to HEV is much lower. These observations suggest that HEV is either less readily spread or less stable in the environment than HAV or that other factors are involved. It is difficult at present to assess the importance of animal reservoirs for the maintenance of HEV in the environment but this clearly needs to be studied. Judged by the prevalence of HEV in sewage samples, HEV is present in the environment of industrialized as well as developing countries.[48,50] This suggests that factors other than sanitation and public hygiene are important. Regardless of mechanisms of transmission, however, improved sanitation is important in controlling all infectious diseases

that have faecal-oral transmission as a prominent part of their epidemiology.

## Passive immunoprophylaxis

Evidence that prior exposure to HEV, as measured by pre-existing anti-HEV, is associated with protection against subsequent exposure to the virus has come from epidemiological studies of water-borne epidemics of hepatitis E in Pakistan.[69,70] In these epidemics, which occurred among members of the military, the clinical attack rate (as measured by hospitalization) among the cohort of 63 individuals who lacked anti-HEV at the beginning of the epidemics was 37%. In contrast, not a single one of the 46 individuals who were positive for IgG anti-HEV at the beginning of the epidemics was hospitalized. Evidence that this protection was probably antibody-based comes from other epidemiological and laboratory-based studies. Immunoprophylaxis trials of normal immune globulin in countries where HEV is endemic have not demonstrated statistically significant protection, even when the globulin was manufactured within the country, but they have provided suggestive evidence.[14,71–75] The failure to demonstrate significant protection in such studies probably resulted from the relatively low titres of anti-HEV found in such globulin preparations. Also, the immune globulin was usually administered late in the epidemic, thereby diminishing the chances of administering it early enough to protect before exposure to the virus. However, direct demonstration of the protective efficacy of anti-HEV has come from studies in which convalescent plasma or serum obtained from naturally infected patients or experimentally infected non-human primates was infused into naïve non-human primates, which were then challenged with HEV.[76,77] The immune globulin protected the animals against hepatitis. In contrast, serum or plasma from non-infected humans or non-human primates, when infused into naïve animals, failed to protect against subsequent challenge with virulent HEV. Thus, if an immune globulin preparation with a sufficiently high titre of anti-HEV could be prepared, it probably would be efficacious in preventing HEV when administered before exposure. Other evidence that antibodies protect against hepatitis E comes from studies of monoclonal antibodies that can neutralize HEV.[78] These monoclonal antibodies, which were derived from a combinatorial library obtained from the bone marrow of an experimentally infected chimpanzee, are virtually identical to globulins of human origin. They were capable of neutralizing HEV in a model system in which the monoclonal antibody was incubated overnight with HEV and then the mixture was inoculated intravenously into naïve rhesus monkeys. The neutralizing monoclonal antibodies protected the rhesus monkeys not only from hepatitis but also from infection, whereas rhesus monkeys inoculated with HEV and an irrelevant monoclonal antibody were infected and developed hepatitis.

## Active immunoprophylaxis

### *Historical aspects*

Early studies of recurring epidemics of hepatitis E in the same communities revealed that second cases of hepatitis E did not occur in individuals who had developed hepatitis in a previous outbreak. Similarly, non-human primates that were infected with hepatitis E and rechallenged subsequently were protected against a second case of hepatitis E, even when the rechallenge occurred several years later.[79–82] These observations, coupled with the observation that antibody to HEV correlated with protection against hepatitis E in humans, as well as monkeys,[69,70,83] strongly suggested that vaccines would protect.

### *Candidate HEV proteins for vaccine development*

Among the HEV proteins, those encoded by ORF1 are non-structural and therefore not accessible to antibody. A vaccine prepared from ORF1 proteins would have to protect solely through cellular immune mechanisms. Furthermore, these proteins are among the least immunogenic of the virally encoded proteins of HEV. It is not known whether the protein encoded by ORF3 is structural or non-structural. It is immunogenic but, as noted, antibody to it is relatively short-lived and relatively genotype-specific. Furthermore, antibody to the ORF3 protein does not neutralize HEV.[84] Thus, the protein encoded by ORF2, the capsid protein of the virus, is the best candidate for a hepatitis E vaccine. Its sequence is highly conserved and antibody to it is long-lived and cross-reactive among diverse strains.[43] Finally, antibody to the ORF2 protein neutralizes HEV *in vitro* and protects non-human primates against HEV following challenge with virulent virus.[63,77,78,84,85]

### *Recombinant proteins expressed from the HEV ORF2*

As HEV grows poorly in cell culture, proteins for diagnostic use and vaccine development have been expressed as recombinant proteins in a variety of systems, but principally from *Escherichia coli* as fusion proteins or in insect cells from baculovirus vectors (Table 42.1). Although the first candidate HEV vaccine was expressed in *E. coli*, the proteins derived from bacteria have been used principally as antigens for diagnostic purposes.[86–94] Neither the least truncated nor the most truncated of these proteins expressed in *E. coli* has been widely used because truncated proteins of intermediate size have provided better sensitivity for detecting anti-HEV in ELISA and Western

**Table 42.1** HEV ORF2 antigenic peptides

| HEV (origin) | Designation | Amino acids | | VLPs‖ | Refs |
|---|---|---|---|---|---|
| | | N | C | | |
| Expressed in *E. coli** | | | | | |
| China | ORF2 | 1 | 660 | ? | 89 |
| Burma | TrpE-C2 | 221 | 660 | ? | 110 |
| Burma | SG 3 | 328 | 654† | ? | 94 |
| China | ORF2.1 | 394 | 660 | ? | 88 |
| China | pE2 | 394 | 607‡ | ? | 95 |
| Mexico, Burma | 3.2 | 612 | 654† | ? | 60 |
| Expressed in insect cells§ | | | | | |
| Burma | 72 kDa | 1 | 660 | ? | 96 |
| Pakistan | 63 kDa | 112 | 660 | ? | 101 |
| Burma | 62 kDa (56 549) ¶ | 112 | 636 | ± | 100 |
| Pakistan | 55 kDa (56 144) ¶ | 112 | 607 | ± | 101 |
| Burma | 54 kDa | 112 | 608 | ++ | 116 |
| Pakistan | 53 kDa (53 872) ¶ | 112 | 578 | ++ | 101 |
| DNA vaccine | | | | | |
| Burma | pJHEV | 1 | 660 | ? | 119 |
| Burma | pcHEVORF2 | 1 | 660 | ? | 123 |

*As gluthathione S transferase (GST) fusion protein.
†In commercial diagnostic tests.
‡Fusion protein component removed; forms homodimers.
§From baculovirus vector.
‖Assembles into virus-like particles (VLPs).
¶Molecular weight in (Da) as determined by mass spectrometry.
HEV, hepatitis E virus; ORF, open reading frame.
Modified from reference 66.

blot assays.[43] Their increased sensitivity appears to result from their ability to fold into native configurations that display conformational epitopes.[89,90]

One truncated ORF2 protein expressed in *E. coli* has proven to be useful both for serodiagnosis and as a candidate vaccine.[64,95] This protein, truncated to amino acid 394 at its amino-terminus and to amino acid 607 at its carboxy-terminus, is said to form homodimers that express a conformational epitope, but only in its dimerized form.

Similarly, proteins expressed in insect cells have been useful for detecting anti-HEV, but full-length ORF2 proteins are relatively insoluble and relatively insensitive for use as an antigen in serological tests.[96–104] However, proteins expressed in certain insect cell systems are post-translationally processed in several ways. First, at least some of the proteins are larger than their amino acid sequence would predict. Proteins with calculated molecular masses of 50 and 53 kDa were actually 53.8 and 56.1 kDa, respectively, when measured by mass spectrometry.[101] The ORF2 protein contains three putative glycosylation sites and seven sites for myristylation, but it is not clear whether the extra mass is the result of glycosylation, myristylation, or some other form of protein modification.[105,106] Second, proteinases, which are probably encoded by the baculovirus vector, cleave the ORF2 protein between amino acids 111 and 112, thereby removing the hydrophobic signal sequence of the protein and rendering it more soluble.[99,107] Third, proteinases successively truncate the protein from the carboxy end, yielding a series of proteins of different sizes and with different characteristics. For example, proteins that are approximately 55 kDa and larger are retained within the insect cell and, following cell lysis, they can be purified to a high degree of homogeneity as monomers.[99–101] In contrast, some proteins of approximately 53 kDa and smaller are secreted from insect cells and form virus-like particles (VLPs) that are smaller than the intact virion of HEV,[97,101] although the exact size of one of these proteins[97] has been the subject of speculation. The VLPs have been shown to be highly ordered icosahedrons with two-, three- and fivefold symmetry.[103] VLPs have also been reported in preparations of the larger proteins, but these are either formed inefficiently, require specific conditions to form or they may be the products of smaller forms of the protein that exist as minor populations in the preparations.[102,107–109] More importantly, the larger and smaller proteins differ in another important respect. Those proteins described in Table 42.1 that are approximately 54–56 kDa or larger contain the most important

conformational neutralization epitope found to date in the ORF2 protein. This epitope requires the carboxy portion of the peptide that extends to amino acid 607.[61–63]

### *Protein-based vaccines*

The first reported candidate vaccine for hepatitis E was a fusion protein expressed in *E. coli* from a truncated ORF2 gene.[110] The 440 amino acid protein, designated trip E-C2, was administered intramuscularly in two 80-μg doses with alum adjuvant 1 month apart to four cynomolgus monkeys. Two of the monkeys were given a third dose of vaccine without alum 7 weeks later. All the animals were challenged either with the homologous Burma strain of HEV (genotype 1) or with a Mexican strain of the virus (genotype 2). Neither challenge virus had been titred for infectivity, but both had been shown to be infectious in previous studies of non-human primates. Neither of the monkeys that received only two doses of vaccine was protected against hepatitis. Only the monkey that received three doses of vaccine and was challenged with the homologous Burmese strain of HEV appeared to be protected from infection as well as hepatitis.

In another study, a 214 amino acid peptide spanning amino acids 394–607 was expressed as a GST fusion protein in *E. coli* from a truncated ORF2 gene of a Chinese strain of HEV (genotype 1).[95] After purification of the fusion protein, the GST portion was removed from the ORF2 protein and the latter was used to immunize three rhesus monkeys; 100 μg of the protein was administered in four weekly intramuscular doses. The first contained complete Freund's adjuvant and the remaining three contained incomplete Freund's adjuvant. All three animals developed antibody during the course of immunization. The animals were challenged 2 weeks after the last immunization with an untitred pool of the homologous Chinese HEV strain. Following challenge, neither vaccinated nor negative control monkeys developed hepatitis, but all three control monkeys shed HEV, whereas the vaccinated monkeys did not shed virus following exposure to this apparently attenuated challenge virus.

Several candidate vaccines expressed from baculovirus vectors in insect cells have been studied. These vaccine candidates represent the various cleavage products derived from the 72-kDa full-length ORF2 protein when expressed from baculovirus in insect cells.

A 62-kDa protein, encompassing amino acids 112–660 (the carboxy-terminus of the protein), was purified and used to immunize three cynomolgus monkeys.[100,111] The protein was alum-precipitated as a 20-μg formulation and administered intramuscularly twice, with a 4-week interval. The three vaccinated monkeys and three control monkeys were challenged intravenously with 1000 50% monkey infectious doses ($MID_{50}$) of the heterologous Mexican strain (genotype 2) of HEV 6 weeks after the last vaccination. All three vaccinated monkeys were protected against hepatitis and two of the three were protected against infection. All three control monkeys developed hepatitis.

In another study, a 55–56-kDa protein, spanning amino acids 112–607 of the ORF2 protein derived from a Pakistani strain (genotype 1) of HEV, was expressed from baculovirus in insect cells, purified and used to immunize six cynomolgus monkeys.[77,107] Four monkeys received a single 50-μg dose with alum intramuscularly and two monkeys received a second dose of vaccine 1 month after the first dose. One month after the final dose of vaccine, the six vaccinated monkeys and five control monkeys were challenged with 1000 or 10 000 $MID_{50}$ of the homologous Pakistani HEV strain. All vaccinated monkeys were protected against hepatitis after challenge, but only the monkeys that received two doses of vaccine were protected against infection. In contrast, all five control monkeys developed hepatitis.

In a subsequent study, four groups of four rhesus monkeys each were vaccinated twice with graded doses of vaccine consisting of 50 μg, 10 μg, 2 μg or 0.4 μg in alum adjuvant.[107,112] All the animals developed antibody after the first dose and high levels of antibody after the second dose. All the animals were protected against hepatitis after challenge with 300 000 $MID_{50}$ of the homologous Pakistani strain of HEV (genotype 1) administered intravenously. However, all of the animals were infected, probably because of the very high challenge dose. Four additional monkeys that had been immunized with the highest dose of vaccine were challenged with 100 000 $MID_{50}$ of the heterologous Mexican strain (genotype 2) of HEV. All were protected against hepatitis but not infection. In contrast, eight monkeys that had received placebo all developed hepatitis following challenge with the Pakistani or the Mexican strain of virulent HEV. In addition, four rhesus monkeys were challenged with the homologous virulent Pakistani virus and vaccinated with a 50-μg dose of vaccine 48 hours after challenge, followed by a booster dose 1 month later. The purpose of this study was to evaluate the feasibility of post-exposure vaccination against hepatitis E. All four animals developed hepatitis as well as infection, and the only difference between this group and the placebo group was that the duration of viraemia and viral excretion was reduced slightly in the vaccinated group. Thus, unlike post-exposure vaccination for hepatitis A, post-exposure vaccination for hepatitis E was not protective.

In an extension of this study, the duration of protection and the effect of a third dose of vaccine were examined.[114] Sixteen seronegative rhesus monkeys were randomly assigned to six groups. Two groups each of four monkeys were inoculated intramuscularly at 0 and 1 month with 50-μg doses of vaccine and chal-

lenged with a homologous strain of HEV 6 or 12 months after the second vaccination. Two groups, each of two monkeys, were similarly immunized and given a third dose of vaccine at 5 or 11 months after the second vaccination. They were challenged 1 month after the third vaccination. Two other groups, each of two monkeys, were inoculated with placebo at 0 and 1 month and challenged at 6 or 12 months after the second inoculation. The challenge dose in this experiment was approximately 300 000 $MID_{50}$ of the virulent Pakistani Sar-55 strain of HEV. Six of eight monkeys challenged 6 or 12 months later were protected against viral hepatitis (one in each group developed hepatitis), whereas all of the monkeys that received a third dose of vaccine at 6 or 12 months after initial vaccination were protected against hepatitis. In contrast, all animals receiving the placebo developed hepatitis. The third dose of vaccine elicited a brisk and anamnestic antibody response. The anti-HEV titre of monkeys receiving the third dose of vaccine was approximately 30–60-fold higher than that of monkeys receiving two doses, at the time of challenge. The data suggest that anti-HEV would fall to below protective levels by approximately 2 years after vaccination if a third dose of vaccine was not administered.

A recombinant 53-kDa truncated form of ORF2 protein has also been tested for protective efficacy in rhesus monkeys.[114] The only neutralization epitope identified to date is located at least partially between amino acids 578 (the carboxy end of the 53-kDa protein) and 607 (the carboxy-terminus of the 56-kDa protein). Six rhesus monkeys were immunized twice with 385 ng of alum-precipitated 53-kDa protein, the molar equivalent of the smallest dose (400 ng) of 56-kDa protein tested previously and shown to be protective. Monkeys were challenged with 10 000 or 100 $MID_{50}$ of the homologous Pakistani virus 1 month after the second dose of vaccine. Monkeys receiving a placebo were similarly challenged. Antibody titres were similar to those observed previously following vaccination with the 56-kDa protein. Vaccination with the 53-kDa protein greatly reduced virus shedding but did not protect against hepatitis following the high-dose challenge. Neither hepatitis nor virus replication was detected in vaccinated monkeys receiving the low-dose challenge, whereas one of two placebo recipients developed hepatitis and both were infected. Thus, surprisingly, the 53-kDa protein was at least partially protective, suggesting that one or more additional neutralization epitopes are present in the truncated ORF2 proteins or that small amounts of larger forms of the protein were present in the vaccine preparation.

VLPs that spontaneously form from truncated ORF2 proteins expressed from baculovirus in insect cells have also been used for vaccine development. Originally detected in preparations of ORF2 protein expressed from a full-length ORF2 sequence that was spontaneously truncated to smaller forms, they were subsequently associated predominantly with 53-kDa and smaller forms of the protein.[101,102] VLPs were independently identified in preparations of expressed and secreted truncated ORF2 protein that was said to be 50 kDa in size,[103] but subsequent analyses put its size at approximately 54 kDa.[115] Surprisingly, the amino and carboxy ends of this protein were mapped to amino acids 112 and 608, respectively.[116] Thus, this protein appears to be virtually identical to the 56-kDa protein described previously.[101] The latter VLPs have been shown to have icosahedral symmetry, to be approximately 24 nm in diameter, and to induce both systemic and intestinal antibodies when administered orally as a vaccine to mice.[115]

Cynomolgus monkeys were similarly inoculated orally with purified recombinant HEV VLPs without adjuvant.[82] Two monkeys were administered 10 mg of VLPs on days 0, 7, 21, 36 and 80. Two additional monkeys were given saline orally according to the same schedule. The four monkeys were challenged on day 100 with HEV obtained from an Indian patient. The infectivity titre of the challenge inoculum was not fully determined but was said to be '$>5 \times 10^3$ monkey infectious dose'. Two cynomolgus monkeys that had been experimentally infected with HEV previously were also challenged and served as protected controls. Both monkeys that were vaccinated with saline were infected and developed hepatitis. Neither animal that had been infected previously was infected or developed hepatitis. Neither animal that was vaccinated with VLPs developed hepatitis and one was protected from infection. The other had transient and low-level shedding of the virus into the faeces. Thus, the vaccinated monkeys were completely protected against hepatitis and partially protected against infection. However, the cost of such a vaccine may make it prohibitive if comparable or larger doses of vaccine and regimens of administration are required for human vaccination, as one oral dose of this vaccine was equivalent to approximately 25 000 doses of a potent parenterally administered vaccine and one protective oral immunization regimen required vaccine sufficient for over 60 000 protective immunizations with the parenterally administered vaccine.[112]

VLPs have been further modified to bear antigenic epitopes of other agents. These have also been shown to elicit intestinal antibodies but not serum antibodies.[116] VLPs have even been used as vehicles for delivering heterologous DNA vaccine in this novel combination of two vaccination approaches.[108] Oral administration to mice of HEV VLPs loaded with HIV envelope cDNA resulted in significant levels of specific IgG and IgA antibody to the HIV envelope in faecal extracts and sera of the mice. Cytotoxic T-lymphocyte responses were also elicited. Finally, a truncated HEV ORF2 protein has been expressed in tomatoes.[117] Approximately 60 ng of ORF2 protein was

detected per gram of fresh tomatoes.[117] However, based on the oral vaccinations described above, one would need to feed a primate almost one metric ton of fresh tomatoes in order to provide protection against hepatitis E. Some approaches to vaccine development are more clever than practical.

### *DNA vaccines*

Significant progress has been made in the development of DNA vaccines to HEV. Early studies demonstrated an immune response in mice to vaccination with ORF2 cDNA and evidence of immunological memory in mice that had lost demonstrable antibody over time.[118,119] Better immune responses to HEV DNA vaccine were obtained when the DNA was co-delivered with cytokine genes (interleukin-2, granulocyte-macrophage colony-stimulating factor) or cytotoxic T-lymphocyte antigen 4, which targets the DNA to antigen-presenting cells.[120,121]

HEV DNA vaccines have also been tested in cynomolgus macaques, which permit evaluation of efficacy.[123] When four macaques were intramuscularly immunized with four sequential 100-μg doses of HEV DNA vaccine derived from the sequence of a Burmese strain, all four animals developed anti-HEV. Following challenge with 1000 $MID_{50}$ of a heterologous (Mexican) HEV strain, two of the four were protected against infection, as well as hepatitis, whereas the other two developed both infection and hepatitis. A single placebo control monkey was infected but did not develop hepatitis following challenge.

In a subsequent study, seven cynomolgus monkeys were immunized with four 25-μg doses of HEV cDNA vaccine, three monkeys by gene gun application and two monkeys by intradermal inoculation.[124] Two cynomolgus monkeys received placebo by gene gun. All three monkeys that received vaccine by gene gun had progressively higher titres of anti-HEV following each administration of vaccine. In contrast, anti-HEV was not detected in the serum of the two monkeys that received vaccine intradermally. Following challenge with 10 000 $MID_{50}$ of the heterologous Mexican strain of HEV, all three monkeys vaccinated by gene gun were protected from infection, although one had repeated liver enzyme elevations following challenge. Both monkeys that were vaccinated intradermally were infected with HEV following challenge, but neither had significant liver enzyme elevations. Both monkeys that received placebo were infected and one had liver enzyme elevations. Thus, administration of DNA vaccine by gene gun yielded significantly better results than administration of the same vaccine intradermally or than administration of a four times higher dose of vaccine delivered intramuscularly.

### *Preclinical and clinical trials*

Although hepatitis E vaccines consisting of 62-kDa (Genelabs) or 56-kDa (NIH) ORF2 proteins were both immunogenic and protective, the superior stability of the 56-kDa protein vaccine resulted in its selection by GlaxoSmithKline for clinical evaluation. A lot of vaccine suitable for administration to humans was prepared by Novavax, Inc., under the sponsorship of GlaxoSmithKline. The preparation of the vaccine was essentially as described previously.[101,112] The vaccine preparation contained purified 56-kDa capsid antigen derived from the HEV strain Sar-55 and expressed from baculovirus as recombinant protein in vaccine-acceptable SF9 insect cells. The formulations of the vaccine were 1 μg, 5 μg, 10 μg, 20 μg and 40 μg plus alum adjuvant in a volume of 0.5 mL.

Preclinical testing was carried out in 44 rhesus monkeys.[124] Two formulations of the vaccine, 1 μg and 10 μg, and two vaccination schedules (two doses of 1 μg or 10 μg at weeks 0 and 4, one dose of 10 μg at week 0) were compared in 12 monkeys for each regimen. GlaxoSmithKline's Havrix hepatitis A vaccine served as the placebo and was administered to eight monkeys. Both vaccine formulations were highly immunogenic, and there was little difference in the level of antibody following two doses of vaccine, regardless of the formulation. The antibody response to the single 10-μg dose was only approximately one-tenth of that of the two-dose regimens.

Following vaccination, the groups of monkeys were further divided and challenged at week 8 with one of three HEV strains: the Sar-55 strain from Pakistan (genotype 1), the Mex-14 strain from Mexico (genotype 2) or the US-2 strain from the United States (genotype 3). HEV genotype 4 had not been discovered at the time of the study.

Each challenge virus was diluted to deliver 10 000 $MID_{50}$ intravenously per monkey. None of the animals that received two doses of vaccine developed hepatitis, regardless of the challenge virus, whereas 17% of the monkeys that had received a single dose of vaccine developed hepatitis. In contrast, 75% of the monkeys receiving placebo developed hepatitis. Similarly, only 26% of monkeys that received two doses of vaccine were infected, whereas 75% of those that received a single dose of vaccine were infected. All eight of the placebo recipients became infected following challenge. Thus, the two-dose regimens had a protective efficacy of 100% (confidence intervals: 65–100%), whereas the single-dose regimen had a protective efficacy of 78% (confidence intervals of 29–94%). Protection against infection and against hepatitis was inversely related to the titre of anti-HEV at the time of challenge. Thus, as in previous studies, protection correlated with levels of antibody.[113]

### *Clinical trials*

Phase I clinical trials of the vaccine were carried out by the US Army at the Walter Reed Army Institute of Research.[125] The vaccine was found to be safe and immunogenic in 88 American volunteers, aged 18–50 years. It was administered in 1-, 5-, 20- or 40-μg formulations: each formulation was administered intramuscularly to 22 subjects at 0, 1 and 6 months. The seroconversion rate was 67% for the 1-μg formulation and 89–95% for those receiving the other formulations, but the serological test for measuring such antibody was different from that used in the preclinical trials, and a direct comparison cannot be made.

Based on these results, a further phase I evaluation was performed in Nepal, where hepatitis E is endemic. Three doses of formulations of 5 μg or 20 μg were injected intramuscularly into 22 Nepalese volunteers each at 0, 1 and 6 months. By the second month, 43 of the 44 volunteers had seroconverted, and by the seventh month the remaining volunteer had also developed antibody to HEV. Thus, the vaccine was well tolerated and immunogenic in the Nepalese volunteers.

A phase II/phase III trial of the vaccine was carried out by the US Army in members of the Nepalese military (Robert Kuschner, personal communication). Two thousand volunteers stationed in the Kathmandu valley of Nepal received 20 μg of the vaccine or a placebo at 0, 1 and 6 months and were followed for an additional 2 years. Although there was some initial concern that there would not be sufficient cases of hepatitis E to provide a statistically significant outcome, in fact, massive flooding in Nepal resulted in multiple epidemics of hepatitis E in the monitored population. Consequently, there were many more cases of hepatitis E than the minimum number initially predicted for statistical significance. To date, the code has not been broken for this field trial of hepatitis E vaccine, so its protective efficacy cannot be ascertained.

Other events almost derailed the study: most of the royal family was massacred by another family member and a Maoist uprising in the western frontier required shifting troops, including vaccinees, to the war zone during the course of the trial. At least one vaccinee was killed by enemy fire, an unusual untoward reaction in a vaccinee.

### *Prospects for the future*

Thus, a hepatitis E vaccine appears to be feasible. Although experience with recombinant vaccines produced in insect cells is limited, they appear to be safe and well tolerated, and purification of candidate vaccine is straightforward, yielding a preparation that is relatively free of insect proteins.[101] Based on *in vivo* and *in vitro* studies, the vaccine appears to protect against all four recognized genotypes of human HEV. Thus, only one vaccine will be needed for worldwide protection. It appears that three doses of vaccine will be necessary for optimum protection and, as the half-life of anti-HEV appears to be shorter than that of antibodies against other viruses, such as HAV, periodic booster immunizations may be necessary. Nevertheless, it should be possible to manufacture and market an economically viable hepatitis E vaccine.

A larger problem is the market for such a vaccine. The greatest populations at risk reside in developing countries where the cost of even the most essential vaccines can be prohibitive. The market for a hepatitis E vaccine in industrialized countries is relatively small and consists of military personnel and civilian travellers to developing countries. However, one industrialized country, Japan, has discovered that hepatitis E is more common than previously suspected, and that one source of such infections is the ingestion of raw or undercooked pork and venison. Whether the incidence of hepatitis E in Japan is sufficiently high to warrant vaccination programmes is yet to be determined.

Finally, if vaccination programmes are to be implemented, especially in developing countries, it will be necessary to consider universal vaccination, preferably of children between birth and adolescence, as most disease occurs in older children and young adults and as the most severe disease occurs in pregnant women. Attempts to halt epidemics, once they occur, by mass vaccination is unlikely to be effective because most exposures to the virus will probably have occurred by the time the epidemic is recognized, and because studies in monkeys have questioned the efficacy of post-exposure vaccination. Thus, a universal vaccination programme that would fit into WHO vaccination schedules, preferably with a vaccine that combines a hepatitis E vaccine with other vaccines in the Expanded Programme on Immunization, would appear to be the most feasible. However, it remains to be seen if any manufacturer will deem it in their interest to accept the challenge of manufacturing a hepatitis E vaccine.

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# Section VIII
# Clinical Aspects of Viral Liver Disease

# Chapter 43
# Aetiology of fulminant hepatitis

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## Introduction

Acute liver failure (ALF) or fulminant hepatic failure is characterized by severe and sudden onset of liver injury leading to coagulopathy and hepatic encephalopathy in previously healthy persons with no known underlying liver disease.[1] It can rapidly progress to coma and death from cerebral oedema and multi-organ dysfunction. There are an estimated 2000 cases of ALF in the United States yearly, representing 6% of liver-related deaths.[2] ALF is a clinical syndrome that represents a final common pathway for a wide variety of diseases that rapidly produce severe liver injury. There are numerous causes of ALF including drugs, viruses, toxins and miscellaneous disorders.[3] Patients with ALF can be classified into hyperacute, acute or subacute liver failure depending on the rapidity of development of encephalopathy from the first appearance of jaundice or symptoms.[4] Studies have shown that patients who had a more rapid onset of hepatic coma had a paradoxically better chance of spontaneous recovery. The mortality of ALF remains high, and the overall transplant-free survival rate varies greatly from 5% to 70% depending on the aetiology of liver injury.[5] In a recent large series of ALF cases from the USA, patients with acetaminophen overdose, hepatitis A virus (HAV) infection, shock liver or pregnancy-related acute liver failure, short-term survival without transplantation was 50% or greater,[6] while patients whose liver disease was of indeterminate cause or was presumed to be related to drugs other than acetaminophen, hepatitis B virus (HBV) infection, autoimmune hepatitis, Wilson's disease, Budd-Chiari syndrome or cancer had lower rates of short-term transplant-free survival (<25%).[6] Medical therapy of ALF is critical in management of the complications of ALF including infections, haemodynamic abnormalities, brain oedema, renal and pulmonary failure, and coagulopathy. Liver transplantation has been used increasingly with reasonably good outcome in the management of patients for whom spontaneous recovery is deemed to be unlikely.[2]

The aetiology of ALF varies greatly worldwide (Tables 43.1 and 43.2). Currently, the most common cause of ALF in the United States[6] and the UK[7] is drug-induced liver injury (most commonly acetaminophen toxicity). Viral hepatitis, once the most common cause of ALF in the USA,[8,9] now comprises only one-eighth of all ALF cases in a recent multi-centre study.[6,10] Similarly, there has been a decline in the proportion of ALF cases from viral hepatitis in the UK from 19% (1973–1990) to only 5% in a more recent study (1991–1997).[7] In developing countries, however, viral hepatitis remains the most common cause of ALF.[11,12] HAV infection is seen sporadically worldwide. In areas where HBV infection is endemic, exacerbation of chronic hepatitis B, either spontaneously or on withdrawal of immunosuppressive therapy, is the major cause of ALF.[11] Hepatitis E virus (HEV) infection is the main causative agent of ALF in the Indian subcontinent, especially among pregnant

**Table 43.1** Aetiology of acute liver failure in the USA (%)

| Study | Hepatitis A virus | Hepatitis B virus | Acetaminophen | Drug | Indeterminate |
|---|---|---|---|---|---|
| Rakela *et al.*[9] (n = 64) 1975–1978 | 2 | 34 | 0 | 17 | 34 |
| Shakil *et al.*[16] (n = 177) 1983–1995 | 7 | 19 | 19 | 12 | 28 |
| University of California at San Francisco[2] (n = 60) 1989–1992 | 8 | 15 | 18 | 15 | 38 |
| Schiodt *et al.*[15] (n = 295) 1994–1996 | 7 | 10 | 20 | 12 | 30 |
| Ostapowicz *et al.*[6] (n = 308) 1998–2001 | 4 | 7 | 39 | 13 | 17 |

**Table 43.2** Worldwide aetiology of acute liver failure (%)

| Study | Hepatitis A virus | Hepatitis B virus | Hepatitis E virus | Acetaminophen | Drug | Indeterminate |
|---|---|---|---|---|---|---|
| UK[19] (n = 943) 1973–1990 | 5 | 9 | 0 | 54 | 7 | 17 |
| UK[7] (n = 999) 1991–1997 | <5 | <5 | NA | 70 | 5 | 7 |
| France[3] (n = 330) 1972–1984 | 4 | 47 | NA | 2 | 15 | 17 |
| India[208] (n = 458) 1992–1998 | 4 | 11 | 23 | 0 | 5 | 47 |
| India[13] (n = 423) 1987–1993 | 2 | 28 | ~39 | 0 | 5 | NA |
| Japan[209] (n = 236) 1983–1987 | 8 | 48 | NA | NA | NA | 45 |

NA, not available.

women.[13,14] Less frequent viral causes of ALF include hepatitis C virus (HCV), hepatitis D virus (HDV), cytomegalovirus (CMV), herpes simplex virus (HSV) and Epstein-Barr virus (EBV) infections. In most series, a significant proportion of patients (10–44%) were classified as indeterminate or cryptogenic despite extensive history taking and serological evaluation.[6,15,16] Some may represent suicidal or toxin ingestions that are not reported because of fear of discovery or obtundation on admission to hospital. Others may represent environmental or bacterial toxins or viruses not yet identified.

In this chapter, viral causes of ALF (fulminant hepatitis) will be discussed (Table 43.3).

**Table 43.3** Causes of fulminant hepatitis

| |
|---|
| Hepatotropic viruses |
| Hepatitis A virus (HAV) |
| Hepatitis B virus (HBV) |
| Hepatitis C virus (HCV) |
| Hepatitis D virus (HDV) |
| Hepatitis E virus (HEV) |
| Putative new hepatitis viruses |
| GB virus C/hepatitis G virus (HGV) |
| TT virus (TTV) |
| SEN virus (SENV) |
| Non-hepatotropic viruses |
| Herpes simplex virus (HSV) |
| Cytomegalovirus (CMV) |
| Epstein-Barr virus (EBV) |
| Varicella zoster virus (VZV) |
| Parvovirus B19 |
| Adenovirus |
| Enterovirus (Coxsackie virus, echovirus) |
| Human herpes virus-6 (HHV-6) |
| Toga virus-like particles |
| Paramyxovirus |
| Other flaviviruses (yellow fever virus, dengue virus) |

## Hepatitis A virus (HAV)

HAV is a positive-strand RNA virus classified within the family of Picornaviridae. Its genomic organization is similar to that of other picornaviruses, including important human pathogens in the genera Enterovirus (e.g. poliovirus) and Rhinovirus. HAV infection causes an acute, usually self-limited necroinflammatory disease of the liver. The infection is spread mainly by faecal-oral transmission and remains a public health problem throughout the world. In the USA, the incidence of hepatitis A has declined steadily since the introduction of HAV immunization, and in 2001 (the most recent available data) it was the lowest ever recorded (4.0/100 000).[17] The decline in rates has been greater among children and in the states where routine childhood vaccination is recommended, suggesting an impact of childhood vaccination.[17] However, large community outbreaks of hepatitis A associated with mortality have been observed sporadically throughout the USA.[18]

Fulminant hepatitis is an uncommon but severe complication of hepatitis A, causing about 100 deaths annually in the USA.[2] The case-fatality rate in acute symptomatic hepatitis A is low and probably is <0.1% overall.[2] HAV is not a common aetiology of acute liver failure in developed countries. In the USA, the proportion of ALF cases from HAV infection has been relatively small (0–7%) and has not changed over the past 25 years despite a declining overall incidence of hepatitis A.[9,10,15,16] In the observation period from 1998 to 2001, 16 (4.5%) of 354 patients with ALF admitted to 19 academic medical centres in the USA had hepatitis A infection as the aetiological factor.[10] Similarly, hepatitis A was implicated in 46 (4.9%) of 943 cases of ALF seen at the Liver Failure Unit at King's College Hospital in UK from 1973 to 1990.[19] Even in developing countries where hepatitis A is endemic, the observed rates of HAV infection as

the cause of ALF have been relatively modest and have varied from 1.7 to 10.6%.[13,20] In Pune, India, there has been an increase in the prevalence of hepatitis A among adults who developed ALF (from 3.5% in 1978–1981 to 10.6% in 1994–1997).[20] This may be due to a change in epidemiology of hepatitis A rendering more individuals susceptible to infection in early adulthood. Hepatitis A remains a frequent cause of ALF requiring liver transplantation among children in endemic areas.[21–23]

In general, most cases of fulminant hepatitis A have a favourable outcome in comparison with other types of fulminant viral hepatitis.[6,15,19] Most series have documented a higher rate of spontaneous recovery and lower rate of liver transplantation among hepatitis A patients in comparison with patients with hepatitis B-related ALF.[10] Data from the Acute Liver Failure Study Group showed a 69% spontaneous survival rate among patients with hepatitis A, while only 19% of patients with hepatitis B had spontaneous recovery.[10] Liver transplantation rate was 62% among patients with hepatitis B in comparison to 19% among patients with hepatitis A.[10] The reason for these differences does not appear to be the severity of hepatic dysfunction at the time of clinical presentation but seems to be an inherent feature of the disease. An alternative explanation for higher transplant rate among patients with HBV-related ALF is a bias among physicians towards transplanting patients with hepatitis B because of reports of poorer survival among these patients.

The risk of ALF due to HAV infection increases markedly among persons older than 40 years[24,25] and patients with pre-existing chronic liver disease.[26] In a study from Italy,[26] patients with chronic hepatitis C with HAV superinfection had exceedingly high rates of ALF (41%) and death (35%). Interestingly, in this study such a severe manifestation of HAV superinfection was not observed in patients with underlying chronic hepatitis B.[26] Previous studies, however, have shown an increased mortality rate among hepatitis B surface antigen (HBsAg)-positive individuals in comparison with HBsAg-negative individuals during outbreaks of hepatitis A.[27,28] Therefore, all patients with chronic liver disease should be considered for immunization against hepatitis A. The cost-effectiveness of this practice remains controversial.[29,30]

The diagnosis of acute hepatitis A is made on the basis of the detection of HAV IgM antibody in the serum. During HAV-related ALF, the HAV RNA level may be very low or undetectable, suggestive of excessive host immune-mediated lysis of virally infected hepatocytes similar to what has been observed in HBV-related ALF.[31] Interestingly, low or undetectable HAV viral load has been shown to be independently associated with fulminant course and death or transplantation in patients with acute hepatitis A.[31] To date, few studies have been published on viral factors and clinical severity of hepatitis A.[32,33] Investigators from Japan sequenced the 5′ non-translated region (5′ NTR) of the HAV genome, which has an internal ribosomal entry site and is important in cap-independent translation of the viral message, from patients with fulminant hepatitis A.[33] Fewer nucleotide substitutions in the central portion of the 5′ NTR were noted among patients with a fulminant course. The authors concluded that nucleotide variations in the central portion of the 5′ NTR of HAV might influence the severity of hepatitis A. Thus far, no apparent associations between disease severity and HAV genotype have been documented.[31,34] A case of recurrent hepatitis A after orthotopic liver transplantation for fulminant hepatitis A has been reported.[35]

## Hepatitis B virus (HBV)

HBV is a partially double-stranded DNA virus of approximately 3.2 kilobase pairs that is classified within the family of Hepadnaviridae. HBV infection is a major public health problem worldwide. Approximately 2 billion people or one-third of the world's population have serological evidence of past or present HBV infection, and 350 million people are chronically infected. HBV is transmitted by percutaneous and mucous membrane exposure to infectious blood or body fluids. Hepatitis B infection can lead to a variety of clinical presentations including acute self-limited hepatitis, fulminant hepatitis, chronic hepatitis and hepatocellular carcinoma (HCC). In the USA and Western Europe, there has been a significant decline in the incidence of acute hepatitis B during the past decade since the implementation of a routine immunization programme.[36] In HBV endemic areas such as Taiwan, the universal hepatitis B vaccination programme has been shown to be highly beneficial. The incidence rates of chronic HBV infection and HCC among children younger than 15 years have been markedly reduced as a result of this mass vaccination programme.[37]

Fulminant hepatitis is an uncommon but potentially fatal clinical manifestation of acute hepatitis B. The case-fatality rate of acute hepatitis B is approximately 0.4–1.0%.[2,38] In the USA, the proportion of cases of ALF from hepatitis B has declined significantly over the past three decades, from 34% in the 1970s,[9] and 18–19% in the 1980s,[16,39] to 10% in the early 1990s[15] and to 7% in the most recent series from 1998 to 2001.[6] A similar trend has been observed in the UK.[7] This decline may simply reflect the reduction in the overall incidence of acute hepatitis B in these countries. Another contributing factor may be the increase in other aetiologies of ALF, particularly acetaminophen toxicity, which is now the most common cause of ALF in Western countries.[5,7] However, HBV remains a common cause of ALF in endemic areas.

Infection with HBV is the most prevalent cause of ALF in the Far East, France and many other southern European countries. For instance, HBV infection accounted for 80% and 63% of the cases of ALF in Hong Kong and Taiwan, respectively.[40,41] In India, HBV was found to be an aetiological agent in 28% of ALF cases, the second most common after HEV.[13] The importance of HBV infection in ALF in endemic areas is further illustrated by the decline in the mortality associated with fulminant hepatitis in infants in Taiwan after the implementation of the mass HBV immunization programme.[42]

The diagnosis of fulminant hepatitis B can be difficult. Typical serological markers of acute hepatitis B may not be measurable by the time of clinical presentation of acute liver failure.[3,43] One-half or two-thirds of patients have lost HBsAg by or within a few days of presentation. This relatively short course of hepatitis B surface antigenaemia is thought to be due to intense immune-mediated clearance of infected hepatocytes in an attempt to clear the virus. In some cases, the diagnosis of HBV-related ALF may depend solely on the presence of serum anti-hepatitis B core immunoglobulin M (anti-HBc IgM). However, anti-HBc IgM can also be detected during spontaneous flares of chronic hepatitis B,[44] thus the presence of anti-HBc IgM in patients with fulminant hepatitis does not exclude an underlying chronic HBV infection. Several studies reported that some patients with HBV-related ALF lacked serological evidence of HBV infection (negative for both HBsAg and anti-HBc IgM) but had detectable HBV DNA in the serum or liver using polymerase chain reaction (PCR) assays.[45–47] The prevalence of this occult form of hepatitis B infection in patients with ALF with no apparent cause has been reported to vary from 0–4%[48–52] to as high as 50%[46,47] (Table 43.4). The currently available data on the prevalence of occult HBV infection among patients with ALF are conflicting and based on studies with a relatively small number of patients.[45–54] It appears that occult hepatitis B infection associated with fulminant hepatitis is more prevalent in geographic locations where hepatitis B is endemic such as the Far East (Japan, Taiwan). Whether occult HBV infection plays a direct pathogenic role in causing ALF in these cases remains to be proven. A more recent study from a large series of ALF patients in the USA found no evidence of occult HBV infection among ALF patients with indeterminate aetiology.[55] However, it will be important to revisit these cases with ultrasensitive PCR-based diagnostic techniques that identify <10 viral particles/mL of blood and liver tissue before firm conclusions may be reached.

Fulminant hepatitis B can occur in the setting of acute *de novo* infection or in the setting of reactivation of chronic infection either as a spontaneous flare or after withdrawal of immunosuppressive therapy. This latter presentation appears to be a more common form of HBV-related ALF in the Far East (Hong Kong and Taiwan).[11] Spontaneous flares most frequently occur during the immune clearance phase of chronic HBV infection and precede clearance of the virus and hepatitis B e antigen seroconversion to anti-HBe.[56] Thus, severe flares leading to hepatic compensation may be observed in previously asymptomatic inactive HBV carriers. Withdrawal of immunosuppressive therapy also could lead to fulminant hepatitis in patients with chronic HBV infection.[57,58] Most reported cases occur after withdrawal of cytotoxic immunosuppressive chemotherapy for haematological malignancy or solid tumour.[59–61] Another well-described and increasingly recognized condition associated with reactivation of HBV is engraftment of bone marrow transplantation.[62–64] The mechanism of severe liver injury associated with withdrawal of immunosuppression or engraftment of bone marrow transplantation is thought to be a two-stage process.[11] The first stage occurs during intense immunosuppressive therapy, which leads to enhance viral replication and widespread infection of hepatocytes. The second stage develops during or soon after the withdrawal of immunosuppressive therapy, in which the recovery of

**Table 43.4** Prevalence of occult HBV infection in fulminant non-A, non-B hepatitis*

| Study | n | Hepatitis B virus DNA (%) | Comment |
|---|---|---|---|
| Wright *et al.*[45] (1992) | 12 | 50 | Serum and liver tissue |
| Hytiroglou *et al.*[53] (1995) | 9 | 33 | Liver tissue |
| Mason *et al.*[54] (1996) | 10 | 30 | Liver tissue, HBV DNA detected in 5% of controls |
| Fukai *et al.*[47] (1998) | 20 | 45 | Serum and liver tissue |
| Sallie *et al.*[48] (1993) | 45 | 0 | Liver tissue |
| Laskus *et al.*[51] (1994) | 8 | 0 | Liver tissue |
| Feray *et al.*[49] (1993) | 23 | 4 | Serum and liver tissue |
| Fagan and Harrison.[50] (1994) | 7 | 0 | Liver tissue |
| Mutimer *et al.*[52] (1995) | 23 | 0 | Serum and liver tissue |
| Teo *et al.*[55] (2001) | 22 | 0 | Serum and liver tissue (only eight available) |

*Cases of fulminant hepatitis with no serological evidence of HAV or HBV infection.

the immune system produces an intense immune-mediated destruction of the HBV-infected hepatocytes. A high pretransplant HBV DNA level has been shown to be a predictive factor of hepatitis B exacerbation in bone marrow transplant recipients.[65] Pre-emptive therapy with a nucleoside analogue such as lamivudine in bone marrow[66] or renal allograft[67] transplant recipients or patients undergoing chemotherapy[68,69] with underlying HBV infection has been shown to significantly reduce the incidence of exacerbation of hepatitis B.

Various mutations of HBV genome have been implicated in the aetiology and pathogenesis of fulminant hepatitis B. In the majority of cases, HBV mutations that affect HBeAg expression have been reported.[70–73] To date, mutations in the precore and core promoter regions have been most extensively evaluated. HBV variant with a mutation in the precore region of the HBV genome (G to A change at nucleotide 1896, G1896A) has been detected in several case series and outbreaks of fulminant hepatitis B from Israel,[71] Japan[72–75] and southern Europe.[76] This mutation creates a stop codon at codon 28 and leads to premature termination of the precore/core protein, thus preventing the production of HBeAg.[72,74] The precore stop codon mutation is much more prevalent in specific HBV genotypes (D>A) because genotype-specific nucleotide difference upstream of the stop codon mutation is involved in critical base-pairing with the mutation in the secondary structure of the HBV encapsidation signal.[77] Therefore, in areas where HBV genotype A is predominant such as the USA and France, the precore stop codon variant has been found at lower frequency in patients with fulminant hepatitis B.[78–80] Subsequent studies have identified additional mutations in the core promoter region in patients with fulminant hepatitis B.[81,82] Two mutations in the core promoter region have been noted in most studies: an A to T change at nucleotide 1762 (A1762T) and a G to A change at nucleotide 1764 (G1764A). Unlike precore variants, core promoter variants can be detected in both HBeAg-negative and HBeAg-positive individuals.[83] An association between core promoter variants and fulminant hepatitis B has been observed in Japan[82] and Germany[84] but not in the United States.[85] The pathogenesis of fulminant hepatitis associated with HBeAg-negative HBV mutants may involve viral factors that lead to enhanced viral replication and widespread infection of hepatocytes during acute infection. In support of this view, an HBV strain implicated in an outbreak of fulminant hepatitis B was shown to exhibit the phenotype of enhanced replication in tissue culture.[86] Specific naturally occurring mutations in the HBV core promoter (C1766T and T1768A) were found to be responsible for the enhanced replication of this particular variant via increased viral encapsidation of pregenomic RNA into the core particles.[87,88] A more common core promoter variant (A1762T/G1764A) has also been demonstrated by many investigators to moderately enhance viral replication.[89] Interestingly, additional mutations in the neighbouring nucleotides of the core promoter region (C1766T, T1768A, T1753C) have been shown to further enhance viral replicative capacity and alter HBeAg expression.[90] Taken together, these data suggest that multiple mutations may contribute to an increase in viral replication fitness implicated in the pathogenesis of fulminant course of hepatitis B.[91,92] In addition to viral factors that enhance viral replication, the lack of HBeAg production may also play an important role in the immunopathogenesis of fulminant hepatitis B. The absence of HBeAg, which has immunoregulatory property, may result in an exclusively Th1-like, proinflammatory response in the context of acute infection.[93,94] Furthermore, by sharing antigenic epitopes with the core protein, secreted HBeAg may downregulate the inflammatory response directed at the HBcAg by serving as a decoy to buffer anti-core protein immune response. In summary, a vigorous and extensive immune response in conjunction with enhanced viral replication may then lead to massive liver injury and ultimately fulminant hepatic failure.[93]

In addition to mutations affecting HBeAg production, other mutations in the HBV genome associated with fulminant hepatitis B have included HBV variants encoding changes in the core protein,[95] the pre-S2 gene[96] and S gene.[97,98] The role of HBV genotypes in fulminant hepatitis has recently been investigated. A study from Japan found an increase in frequency of HBV genotype B among patients with acute hepatitis B with a fulminant course.[99] Preliminary data from the US Acute Liver Failure Study group noted a higher prevalence of HBV genotype D among patients with HBV-related acute liver failure in comparison with the background distribution (32% vs 16%).[100] These viral factors, either alone or in various combinations ,and the interaction between the virus and host immune response are probably important in the development of fulminant hepatitis B.

## Hepatitis C virus (HCV)

HCV is an RNA virus of the Flaviviridae family and is the sole member of the genus Hepacivirus. It has a single-stranded RNA genome that is approximately 9.6 kilobases in length and encodes a single, large polyprotein of about 3000 amino acids. Hepatitis C infection is the most common chronic blood-borne infection in the USA with an estimated prevalence of 1.8%. HCV transmission occurs primarily through exposure to infected blood (injection drug use, blood transfusion before 1992, transplantation of solid organs from infected donors, unsafe medical practices, birth to an infected mother, sex with an infected partner). Transmission from blood products and organ transplants was virtually eliminated by the introduction of a more sensitive test for antibody to HCV (anti-HCV) in mid-1992. The incidence of

HCV infections declined sharply in the late 1980s. Currently, approximately 35 000 new HCV infections are estimated to occur each year. Most (68%) newly acquired cases of hepatitis C are related to injection drug use.[101] Only about one-third of adults with acute HCV infection develop clinical symptoms and jaundice.[102] Acute hepatitis C can be severe and prolonged but is rarely fulminant. Two deaths (0.1%) were observed among the 1536 cases of acute hepatitis C from 1995 to 2000 in Italy.[38] In a study involving 308 consecutive patients with ALF admitted to 17 tertiary care liver centres in the USA from 1998 to 2001, no cases of fulminant hepatitis C were identified.[6] In addition, HCV infection does not appear to play an important causative role in patients with fulminant hepatitis of indeterminate cause in the USA and Europe.[49,103–107] In several case series from Europe and the USA, HCV RNA was rarely detected in serum or hepatic tissue of patients with ALF of presumed viral origin but with no markers of HAV or HBV (non-A, non-B fulminant hepatitis). The prevalence of anti-HCV or serum HCV RNA among cases of non-A, non-B fulminant hepatitis from the USA and Europe is approximately 2% (range 0–12%)[49,103–107] with one exception: a study conducted in California reported a prevalence of 60% associated with low socio-economic status and Hispanic ethnicity.[108] In contrast, HCV infection appears to play a more prominent role in fulminant hepatitis in the Far East. Antibodies against HCV (anti-HCV) or serum HCV RNA were found in 40–60% of patients with non-A, non-B fulminant hepatitis in Japan and Taiwan.[109–111] Whether these discrepancies reflect geographic differences in the epidemiology of HCV infection or the pathogenicity of the prevalent viral strains is not known.

Dual infection with HBV and HCV has been observed in patients with fulminant hepatitis. A study from France[49] found that eight of seventeen (47%) patients with HBV-related fulminant hepatitis also had evidence of HCV infection. Some patients appeared to have acute HBV and HCV co-infection, while others were chronic HBV carriers with HCV superinfection. The findings of HCV RNA in the serum and liver tissue with negative results for anti-HCV among cases of HBV-related fulminant hepatitis indicated the presence of acute HCV co-infection or superinfection. A study from Taiwan also noted HCV superinfection in nine of forty-six (20%) chronic HBV carriers who presented with fulminant hepatitis.[111] Fulminant hepatitis due to reactivation of hepatitis C after withdrawal of chemotherapy has been reported among patients with underlying chronic HCV infection in Italy.[112] This presentation has been observed rarely and is far less common than reactivation of hepatitis B after withdrawal of chemotherapy.

Detection of serum HCV RNA is the earliest marker for the diagnosis of fulminant hepatitis C. In acute hepatitis C, HCV RNA can be detected in the serum in most cases within 1–2 weeks after exposure.[102] Anti-HCV is not a reliable test in the diagnosis of acute hepatitis C and is often negative in fulminant hepatitis C, possibly due to the extremely rapid course of the disease. In contrast to fulminant hepatitis B where HBV replication is barely detectable, fulminant hepatitis C is characterized by high levels of viraemia.[113] Viral factors and host immune response may play an important role in the pathogenesis of fulminant hepatitis C. Analysis of HCV isolates from patients with fulminant hepatitis C showed a remarkably low degree of genetic diversity, whereas in those who evolved into chronic hepatitis C, there was considerable diversity.[114] These data suggest that in fulminant hepatitis C, there is a trend to preserve the unique fitness of a particular viral variant, which supports the hypothesis that the inherent virulence of a specific HCV strain may lead to massive hepatocellular injury.[114] A case report of an unusual HCV strain causing two episodes of fulminant hepatitis in a single patient and severe acute hepatitis in a chimpanzee after inoculation by serum from the index patient also supports this view.[115]

## Hepatitis delta virus (HDV)

HDV is a negative-strand RNA virus that depends on a DNA virus, HBV, for propagation. It is the sole member of the genus Deltavirus of the Deltaviridae family. The HDV virion particle is a unique subviral pathogen made of circular HDV RNA and hepatitis D antigen (HDAg) coated by HBsAg. Three major genotypes have been characterized and associated with different geographic locations and clinical phenotypes.[116] HDV genotype III is associated with a severe and fulminant course of hepatitis D in countries in northern South America (Columbia, Venezuela, Peru and Ecuador).[117,118] HDV is spread in the same ways as hepatitis B, through parenteral or sexual exposure to blood or body fluids. Delta hepatitis has a worldwide distribution, but areas of dramatically high endemicity associated with severe clinical outcomes have been identified in the Amazon basin, Central Africa, and parts of Eastern Europe.[119–122] Most cases of acute icteric HDV infection among indigenous people in the Amazon basin of South America appear to be HDV superinfection in chronic HBV carriers rather than HDV and HBV co-infection. Prevention of hepatitis B will also prevent hepatitis D. In a triumph of public health, increasing vaccination rates for hepatitis B are contributing to the decline in HDV incidence in developed countries. In Italy, the prevalence of anti-HDV among HBsAg carriers has declined steadily from 23% in 1987 to 14% in 1992 and 8.3% in 1997.[123] Consequently, cases of fulminant hepatitis from HDV infection are becoming less common in southern Europe. The clinical presentation of severe acute hepatitis D that rapidly progresses

to liver failure commonly observed in southern Europe in the 1970s appears to be extremely rare in recent years. The majority of current HDV cases are those who were infected years ago and are now left with chronic mild liver disease, or those who rapidly advanced to cirrhosis but subsided with stable clinical conditions for more than a decade.[124]

Both co-infection with HBV and HDV and superinfection with HDV in individuals with pre-existing HBV infection have been associated with development of fulminant hepatitis.[125] The diagnosis of HBV/HDV co-infection relies on the presence of HBsAg, anti-HBc IgM and antibody to HDV IgM. HDV RNA can be detected in serum very early in the course of infection and quickly disappears with resolution of disease. Superinfection usually results in overt hepatitis in inactive HBsAg carriers and may rapidly lead to liver failure in carriers with pre-existing chronic hepatitis B.[43] The diagnosis is based on the early presence of HDV viraemia and presence of both IgG and IgM forms of antibody to HDV, while anti-HBc IgM is usually absent or present in low titres.[126] In acute HDV superinfection in tropical countries, hepatic histology may include intense microvesicular steatosis associated with conspicuous eosinophilic necrosis of the hepatocytes.[127,128] This pattern of liver injury has been rarely observed in Western countries.[129]

## Hepatitis E virus (HEV)

Hepatitis E, previously known as enterically transmitted non-A, non-B hepatitis, is a distinct clinical entity that was first recognized in the 1980s. HEV is a non-enveloped, 32–34-nm diameter RNA virus with a single-stranded, 7.5-kb RNA. HEV is responsible for large epidemics of acute hepatitis and a proportion of cases of sporadic hepatitis in the Indian subcontinent, south-east and central Asia, the Middle East, parts of Africa and Mexico. The virus is excreted in faeces and is transmitted by faecal-oral route. Most reported outbreaks have been related to the consumption of faecally contaminated drinking water.

The clinical manifestations of acute hepatitis E are similar to those of acute infection with other hepatitis viruses. In a small proportion of cases, disease is more severe and can rapidly progress to fulminant hepatic failure and death. The case-fatality rate of acute hepatitis E has ranged from 0.5% to 4%.[130] In endemic areas, HEV infection is an important cause of fulminant hepatitis. HEV infection is the most common aetiological agent in sporadic cases of ALF in India.[14,20] In a series of 180 patients with ALF from Kashmir during an observation period from 1989 to 1996, hepatitis E virus was found to be the causative agent in 79 (43.9%) patients.[14] In another series from a different region in India, among 264 patients with ALF who had no serological evidence of HAV or HBV infection (non-A, non-B fulminant hepatitis), HEV could be implicated as the causative agent in 62%.[13] HEV infection either alone or in combination with HAV is also an important cause of ALF among children in India and accounts for 60–90% of fulminant hepatitis cases in some areas.[20,131] In Western countries, HEV has occasionally been implicated in a relatively small number of sporadic cases of non-A, non-B fulminant hepatitis.[107,132] In some cases, no risk factor such as recent travel to endemic area could be identified.

Interestingly, pregnant women, particularly those in the second and third trimesters, are more frequently affected during hepatitis E outbreaks and have a worse outcome. This similar pattern has also been observed in sporadic cases of HEV infection.[133] A significant proportion of pregnant women with acute hepatitis E (up to 70%) progress to ALF with a short pre-encephalopathy period, rapid development of cerebral oedema and high occurrence of disseminated intravascular coagulation.[133] The mortality rate for acute HEV infection among pregnant women is high and has ranged from 15% to 25%.[134–137] The reasons for the high prevalence rate of HEV among pregnant women in endemic areas with particularly rapid and fulminant course remain largely unknown.

## Putative new hepatitis viruses

### GB virus C/hepatitis G virus

GBV-C/HGV belongs to the family of Flaviviridae and consists of a linear, positive, single-stranded RNA molecule comprising about 9500 nucleotides. GBV-C/HGV is globally distributed and the presence of viral RNA in healthy blood donors varies widely by geographic region, ranging from 0.6% to 11%. The prevalence is 1–2% among US blood donors. GBV-C/HGV is transmitted through blood transfusion. An association between HGV and fulminant hepatitis has been reported in earlier studies.[138–140] A study from Germany noted a specific strain of GBV-C/HGV associated with fulminant hepatitis.[140] A correlation between serum levels of GBV-C RNA and the serum ALT levels in three patients with fulminant hepatitis has also been documented in a study from Japan.[141] However, despite these intriguing observations, most subsequent studies have failed to demonstrate an association between GBV-C/HGV infection and fulminant hepatitis.[142–146] It has been shown in several studies that the finding of HGV RNA in serum from these patients is likely related to the administration of blood product transfusion after the onset of fulminant hepatitis.[146] Therefore, GBV-C/HGV does not appear to be causally related to non-A to E fulminant hepatitis.

### TT virus (TTV)

The TT virus subfamily consists of small, non-enveloped, single-stranded, circular DNA viruses in the Circoviridae family. TTV was discovered in 1997 by representational difference analysis performed in Japan on samples obtained before and after hepatitis from five patients with transfusion-associated hepatitis.[147] TTV can be transmitted by both parenteral and faecal-oral routes. It is now known that TT viraemia is very common in apparently healthy persons throughout the world. To date, no convincing evidence has been found that TTV plays a pathogenic role in humans. TTV does not appear to cause acute hepatitis or fulminant hepatitis.[148–152]

### SEN virus (SENV)

SENV is a small, non-enveloped, single-stranded, circular DNA virus, which belongs to the Circoviridae family and appears to be related to the TTV family. Two SENV variants (SENV-D and SENV-H) have been studied most extensively. In the National Institutes of Health (NIH) prospective study of transfusion-associated hepatitis,[153] SENV-D and -H were found in nearly 2% of US blood donors and were unequivocally transmitted by transfusion and frequently persisted. SENV was demonstrated in 11 (92%) of 12 cases of acute transfusion-transmitted non-A to E hepatitis. However, the vast majority of SENV-infected recipients did not develop hepatitis. Whether SENV plays a direct causal role in post-transfusion hepatitis remains uncertain. In a recent study,[154] two SENV variants (SENV-D and SENV-H) were detected by PCR amplification in 1706 patients and control subjects from different geographic locations (the USA, Europe and Asia). SENV was detected in 54 (22%) of 248 patients with acute or chronic non-A to E hepatitis, 9 (35%) of 26 patients with hepatitis-associated aplastic anaemia, and none of 17 patients with cryptogenic acute liver failure, compared with 150 (24%) of 621 control subjects with liver disease and 76 (10%) of 794 healthy control subjects. When controlling for geographic region, the prevalence of SENV among case and control subjects was not significantly different. Therefore, currently there is no evidence to indicate that SENV is a cause of fulminant hepatitis, hepatitis-associated aplastic anaemia, or cryptogenic chronic liver disease.

## Non-hepatotropic viruses

### Herpes simplex virus (HSV)

Hepatitis secondary to infection with HSV is a rare, frequently fatal disease with less than 100 reported cases in the literature.[155] HSV hepatitis can present during primary or recurrent HSV infection. Either type 1 or type 2 HSV can cause fulminant hepatitis, usually as part of disseminated infection. Most adults with fulminant HSV hepatitis are either immunosuppressed or pregnant, although cases of HSV hepatitis in immunocompetent adults have been reported.[155,156] Underlying conditions typically associated with fulminant HSV hepatitis include renal or bone marrow transplantation, use of corticosteroids, autoimmune disorders or pregnancy, usually in the second or third trimester.[157–161] The clinical features of HSV hepatitis are non-specific and the diagnosis is often delayed.[155–157] The diagnosis of HSV hepatitis was made *ante mortem* in fewer than 25% of published cases. Fever is present in 75% of cases. Other symptoms include anorexia, nausea, vomiting and abdominal pain. Typical mucocutaneous lesions may be absent in 31–67% of patients. Leukopenia is present in 43–67% and thrombocytopenia in 45–90%. Disseminated intravascular coagulopathy is frequent. Despite markedly elevated serum aminotransferase levels, most patients (up to 90%) are anicteric.[155–157] A diagnosis of HSV hepatitis should be considered in patients with ALF who present with fever, leukopenia and marked elevation of serum aminotransferase levels with minimal hyperbilirubinaemia in the absence of any other obvious cause of fulminant hepatitis.[155–157]

The definitive diagnosis of HSV hepatitis is based on liver biopsy. Histological findings include irregular zones of confluent haemorrhagic necrosis and characteristic viral inclusions and nuclear changes in hepatocytes surrounding the necrotic areas.[155–157] HSV can be demonstrated in the liver tissue by immunohistochemical staining with antibody against HSV protein.[160] HSV may also be identified in other sites (blood and mucocutaneous lesions) by a PCR assay and viral culture. As tests for IgM antibody against HSV may be negative in patients with reactivated infection and early primary infection, serological findings may be used to support the diagnosis but cannot be used to exclude it. Thorough examination of mucocutaneous tissues and testing of suspicious-appearing lesions and use of liver biopsy will likely lead to early diagnosis and increase the likelihood of survival. In most case series, a high index of suspicion and early use of antiviral therapy (acyclovir) have been shown to improve survival.[161,162] Without antiviral therapy, the mortality rate from fulminant HSV hepatitis exceeds 80%. Successful orthotopic liver transplantation in cases of fulminant HSV hepatitis has been reported.[163,164]

### Cytomegalovirus (CMV)

CMV hepatitis occurs as part of disseminated CMV infection. It occurs mainly among liver or kidney transplant recipients or immunosuppressed persons,[165,166] although cases of CMV hepatitis in immunocompetent hosts have

been reported.[167,168] It is the most frequent manifestation of CMV tissue invasive disease after liver transplantation.[165,169] The incidence of CMV hepatitis after liver transplantation is approximately 2%,[169] much lower than in an earlier report.[165] The frequency of systemic CMV infection is higher in CMV-seronegative recipients of CMV-seropositive grafts, after OKT3 treatment and HLA-DR matched livers.[169] Liver biopsy is required to establish the diagnosis of CMV hepatitis.[170] Antiviral treatment with intravenous gancyclovir is an effective therapy for disseminated CMV infection.[165,168,169] CMV has not been found to be a common aetiological agent in cases of fulminant hepatic failure of indeterminate cause.[54,171]

## Epstein-Barr Virus (EBV)

Liver function test abnormality is common in infectious mononucleosis. Serum aminotransferase values are usually mildly elevated in the range of two to three times the upper limit of normal.[172] Mild elevation of bilirubin level is noted in approximately 45% of cases, although frank jaundice occurs in only about 5%.[172] Severe or fulminant EBV hepatitis is rare and can occur in both immunocompromised and immunocompetent hosts.[173–176] Clinical manifestations include high fever, sore throat, lymphadenopathy, malaise and atypical lymphocytosis, which is usually more pronounced than in other types of viral hepatitis. The diagnosis can be established by a positive heterophile antibody test (Monospot test) or demonstration of EBV-specific antibodies. Detection of EBV DNA in liver tissue of patients with non-A, non-B fulminant hepatitis has been reported. However, the prevalence is not different to control samples from patients undergoing transplantation for other reasons.[54]

## Varicella zoster virus (VZV)

Varicella, usually a benign infection of childhood, is known to be associated with more serious complications, especially in adults and immunocompromised patients. Of these, varicella pneumonitis is the most common. Elevation of serum aminotransferases without clinical hepatitis is commonly observed in children with uncomplicated varicella.[177] However, clinical hepatitis is uncommon and has been infrequently reported among adults with primary varicella and pneumonitis. All cases of fatal varicella hepatitis have been described in immunocompromised patients[178–180] with only one reported case in an immunocompetent individual.[181] Most fatal cases of varicella hepatitis presented with typical generalized maculopapulovesicular rash with other visceral organ involvement.[178–181] Liver histology revealed multiple coagulative necrosis with haemorrhage. Intranuclear viral inclusions and multinucleate giant cells may be seen around necrotic areas.[181] Successful therapy with acyclovir in a patient with fulminant varicella hepatitis has been reported.[182] An unusual case of fulminant varicella hepatitis caused by reactivation of VZV infection has been described.[183]

## Parvovirus B19

Parvovirus B19 has been suggested as a possible cause of fulminant hepatitis[184,185] and hepatitis-associated aplastic anaemia.[186,187] Aplastic anaemia is a rare but well-recognized complication of acute hepatitis and ALF, especially among children who undergo liver transplantation for fulminant non-A, non-B hepatitis. In a study from the USA,[188] aplastic anaemia developed in six of eighteen (33%) children and one of nineteen (5%) adults who underwent liver transplantation for fulminant non-A, non-B hepatitis. In another study from the UK,[189] bone marrow failure occurred in 10.7% of children with acute liver failure. Among eight patients who had evidence of bone marrow failure, two had parvovirus B19 and six had non-A, non-B, non-C hepatitis. The role of parvovirus B19 in the pathogenesis of fulminant hepatitis and hepatitis-associated aplastic anaemia remains controversial. In a more recent study,[190] parvovirus B19 was detected in some liver tissue samples from patients with fulminant hepatitis (four [27%] of fifteen patients), but not at a significantly higher prevalence than in hepatic tissue from patients with non-viral liver disease (three [14%] of twenty-two patients). A confirmatory test was performed in a second group of liver tissues. Parvovirus B19 was present in eight (35%) of 23 liver tissue samples from patients with fulminant hepatitis and in ten (33%) of thirty livers from patients with known HBV and/or HCV infections. Importantly, parvovirus B19 viral transcripts were not detected in any of the parvovirus B19-positive liver tissues, arguing against the presence of active viral replication. Therefore, parvovirus B19 may be an innocent bystander and play no direct causal role in the development of fulminant hepatitis.[190]

## Other viruses

Fulminant hepatitis due to *adenovirus* has been reported as part of disseminated infection in bone marrow or liver transplant recipients.[191,192] As there is no specific antiviral therapy, mortality rate remains high.[193] Histologically, adenovirus hepatitis is characterized by small or large areas of coagulative necrosis with no particular zonal distribution. Inflammatory response is usually sparse or absent. Intranuclear viral inclusions with smudgy appearance and chromatin margination are characteristic.[194] Immunohistochemical examination of a liver biopsy and culture of liver tissue and blood can help confirm the diagnosis.[194] *Enterovirus* (Coxsackie vi-

rus, echo virus) infection can cause fulminant hepatitis in neonates.[195,196] It is associated with coagulopathy and in some cases, myocarditis[197] and carries high morbidity and mortality. *Human herpes virus-6* (*HHV-6*)-associated fulminant hepatitis has been described.[198,199] Some cases of fulminant non-A, non-B, non-C hepatitis may be caused by HHV-6. In a study from Finland, HHV-6 was found in 12 (80%) of 15 explanted livers of patients with ALF of unknown cause. Most of these patients (10/12) also demonstrated HHV-6 antigenaemia.[200] In another study, HHV-6 was detected in 7 (64%) of 11 Japanese paediatric patients with fulminant non-A, non-B, non-C hepatitis.[171] However, HHV-6 was not detected in any of the liver tissue samples from liver transplant recipients with fulminant non-A, non-B, non-C hepatitis from North America.[54] Other viruses implicated as aetiological agents in fulminant hepatitis include *toga virus-like particles*,[201,202] *paramyxoviruses*[203–205] and other *flaviviruses* (*yellow fever virus*, *dengue virus*).[206,207]

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# Chapter 44
# Treatment of fulminant hepatitis

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## Definition of fulminant hepatitis

Fulminant hepatic failure (or acute liver failure) is a rare, but dramatic clinical syndrome associated with a high mortality. Diagnosis of fulminant hepatic failure is based on three criteria that have to be met to fulfil its definition.[1,2]

**1** Acute disturbance of liver function with coagulopathy and jaundice.
**2** Exclusion of chronic liver disease.
**3** Presence of hepatic encephalopathy.

Hepatic encephalopathy distinguishes fulminant hepatic failure from *fulminant hepatitis* which is defined as a severe form of acute hepatitis. In the past, several authors used the term fulminant hepatitis as a synonym for fulminant hepatic failure. However, the distinct definitions are relevant for the spontaneous prognosis that is excellent in severe acute hepatitis and unfavourable in most cases of fulminant hepatic failure.

Exclusion of chronic liver disease is important, as three principal differences in the management of fulminant hepatic failure and acute-on-chronic liver failure exist.

**1** A different diagnostic approach, as acute decompensation of chronic liver disease can be triggered by other factors (e.g. spontaneous bacterial peritonitis).
**2** A different monitoring, as patients with fulminant hepatic failure develop other complications than those with acute-on-chronic liver failure (e.g. cerebral oedema).
**3** A different therapeutic approach, as only patients with fulminant hepatic failure are eligible for emergency liver transplantation (depending on the allocation system).

Depending on the interval between onset of jaundice and onset of hepatic encephalopathy, various definitions of liver failure with a different prognosis are used. In general, a shorter interval is associated with a better prognosis for the patient. According to the most widely used definition, *fulminant hepatic failure* has an interval of <2 weeks between onset of jaundice and encephalopathy.[3,4] *Subfulminant hepatic failure* is present if hepatic encephalopathy develops >2 weeks after the appearance of jaundice.

Another definition was introduced by O´Grady *et al*. and differentiates more precisely between *hyperacute liver failure* (interval jaundice to encephalopathy <7 days), *acute liver failure* (interval 7–28 days) and *subacute liver failure* (interval >4 weeks).[5] Recently, the International Association for the Study of the Liver (IASL) recommended another modified definition differentiating between *acute* (interval <4 weeks) and *subacute hepatic failure* (interval 4 weeks to 6 months).[5a]

Table 44.1 gives an overview of the different types of liver failure with their main clinical characteristics.

## Aetiology of fulminant hepatitis

Different aetiologies for fulminant hepatic failure are described in more detail in Chapter 43. They deserve to be mentioned briefly because specific treatments are discussed for certain aetiologies (see Table 44.2).

Several viruses are capable of inducing a fulminant liver disease, especially the hepatitis viruses A, B, D and E. There have been reports of fulminant hepatitis in relation to hepatitis C virus (HCV) infection. In recent times, non-viral factors have become more frequent while the incidence of viral fulminant hepatitis has decreased due to a wider distribution of vaccinations and other preemptive measures like the use of condoms.

### Hepatitis A virus

Hepatitis A virus (HAV) infection is mostly a harmless infection when acquired in childhood, with mortality increasing with patient's age and leading to death in approximately 2.5% of patients aged over 49 years.[6] However, vaccination against HAV could prevent both infection and disease.

### Hepatitis B virus

Although hepatitis B virus (HBV) infection is on the de-

**Table 44.1** Clinical characteristics of different types of liver failure

| Clinical charcteristics | Fulminant hepatitic failure | Subfulminant hepatitic failure | Fulminant hepatitis | Acute-on-chronic liver failure |
|---|---|---|---|---|
| Chronic liver disease | – | (+) | (+) | +++ |
| Hepatic encephalopathy | +++ | ++ | – | ++ |
| Interval from jaundice until HE | <2 weeks | >2 weeks | No encephalopathy | Different |
| Cerebral oedema | +++ | ++ | – | + |
| Infections | +++ | ++ | (+) | ++ |
| Renal failure | ++ | +++ | (+) | ++ |
| Circulatory failure | +++ | +++ | – | ++ |
| Respiratory failure | ++ | +++ | – | ++ |
| Hypoglycaemia | +++ | ++ | + | ++ |
| Survival without OLT | Bad | Very bad | Very good | Bad |

HE, hepatic encephalopathy; OLT, orthotopic liver transplantation.

cline, it is still a major factor in the aetiology of fulminant hepatitis.[7] Infection with HBV could be prevented by active immunization, which should be available to everyone. However, active vaccination programmes vary geographically.

## Hepatitis C virus

The hepatitis C virus (HCV) is found in most cases of non-A, non-B acute or chronic hepatitis. However, its role in fulminant hepatitis is still controversial (see Chapter 25). Cases have been reported from Japan[8] but very rarely from Western countries. In >300 patients treated for fulminant hepatitis in northern Germany, no HCV infections were observed.

## Hepatitis delta virus

Hepatitis delta virus (HDV) infection requires ongoing HBV infection. Co-infection and superinfection with HDV can both lead to fulminant hepatic failure.[9]

## Hepatitis E virus

Apart from rare cases, hepatitis E virus (HEV) infection on its own is not widespread in the Western world, nor is HEV-induced liver failure. However, it always has to be kept in mind for differential diagnosis in hepatitis A–D. A particular problem is the high rate of fulminant hepatitis in cases of HEV infection during pregnancy.[10,11]

## Other viral infections reported in relation to fulminant hepatitis

Herpesviridae have been associated with hepatitis, but are rarely found in fulminant hepatitis.[12] However, herpes virus simplex I and II (HSV-1, HSV-2) as well as cases of Epstein-Barr virus (EBV), varicella zoster virus (VZV) and human herpes virus 6 (HHV-6) infection have been described.[13–16] Additionally, adenovirus infections have been attributed to fulminant hepatitis, especially in immunosuppressed or paediatric patients.[17,18] In neonates,

**Table 44.2** Types of viral infections in fulminant hepatitis

| Types of virus | Main characteristics |
|---|---|
| Hepatitis A | More frequent with higher age |
| Hepatitis B | Still the most frequent viral cause of fulminant hepatitis |
| Hepatitis C | More frequent in Asia than in Europe and North America |
| Hepatitis D | Requires HBV co-infection |
| Hepatitis E | Especially severe in pregnancy |
| Herpesviridae | Rare |
| Coxsackie virus | Rare, in neonates |
| Adenovirus | Rare, in immunosuppressed patients |
| Parvovirus B19 | Might be associated with aplastic anaemia |
| Haemorrhagic fever viruses | Rare |

Coxsackie virus B infection may present itself as fulminant hepatitis.[19] The role of parvovirus B19, previously reported in relation to fulminant hepatitis,[20] has recently been questioned.[21] Finally, the haemorrhagic fever viruses such as dengue, Lassa, Ebola and yellow fever may also induce fulminant hepatitis. However, fulminant hepatitis is usually not the most important clinical factor in these diseases.

Two viruses presumed to be associated with viral hepatitis, the TTV (transfusion-transmissible virus) and the GB virus C (also known as hepatitis G virus) are not associated with fulminant hepatitis or with hepatitis at all.[22] Even though initial studies suggested a role of GB virus C in fulminant hepatitis,[23,24] this was not confirmed in subsequent larger studies.[25–27]

There is no available evidence reporting cases of acute liver failure in cytomegalovirus (CMV) infection, even though it is a well-known hepatotropic virus. On the other hand, other unknown viruses might be involved in cases of cryptogenic fulminant hepatitis when no known virus can be detected.[22]

### Non-viral factors

Non-viral factors contributing to fulminant hepatitis are mainly paracetamol (acetaminophen) intoxications due to intentional but also unintentional overdosing. It is now the most frequent cause of fulminant hepatitis in developed countries. Furthermore, Wilson's disease, intoxication of *Amanita phalloides* as a result of mushroom poisoning, fatty liver during pregnancy, acute Budd-Chiari syndrome, ischaemic liver failure, autoimmune hepatitis and a number of drugs such as halothane and isoniazid have been implicated in the aetiology of fulminant hepatitis. Finally, general sepsis, hyperthermia, malignant infiltration of the liver and Reye's syndrome may, in rare cases, resemble the clinical spectrum of fulminant hepatitis.

## Complications in fulminant hepatitis

Basically, prognosis of fulminant hepatitis depends on halting further cell damage and on the replication capacity of hepatocytes. However, extrahepatic complications frequently determine the prognosis of patients with fulminant hepatitis.[28] Organ involvement depends on the dynamics and aetiology of liver failure. Host defences are compromised as a consequence of increased toxic substances in the blood due to liver dysfunction and hepatocyte necrosis. This enables infections with activation of immune mechanisms (e.g. cytokine release) further worsening tissue hypoxia. The consequence is damage to a number of organs resulting in multi-organ failure.

Strategies to avoid complications include close monitoring and prophylactic treatment as well as unspecific and, if feasible, specific therapeutic interventions. An overview of the main complications with their pathophysiological basis, the recommended monitoring and potentially beneficial therapy is given in Table 44.3.

### Hepatic encephalopathy and cerebral oedema

Cerebral oedema is the main cause of death in fulminant

**Table 44.3** Complications in fulminant hepatitis

| Complication | Pathophysiology | Monitoring | Therapeutic possibilities |
|---|---|---|---|
| Hepatic encephalopathy, cerebral oedema | Circulating toxins, blood-brain barrier dysfunction, loss of autoregulation of cerebral blood flow | Clinical grading, EEG, cranial CT scan, epidural ICP catheters | Mannitol, hyperventilation, hypothermia, lactulose, gut lavage, ornithine-aspartate, barbiturates, flumazenil, ranitidine, phenytoin |
| Coagulopathy | Reduced hepatic biosynthesis, platelet dysfunction | Coagulation profiles | Fresh frozen plasma, AT III, acid suppression |
| Infections | Reduced hepatic clearance, fibronectin deficiency, use of synthetic catheters | Microbiological surveillance | Intestinal decontamination, broad-spectrum antibiotic and antimycotic prophylaxis |
| Renal failure | Hypovolaemia, parenchymal ischaemia, direct nephrotoxicity, acid-base disturbances | CV catheter, electrolytes, blood pH, base excess | Volume control, electrolytes, renal replacement therapy, bicarbonate infusion |
| Circulatory failure | Splanchnic vasodilatation, low systemic blood pressure | CV catheter, PAC or PiCCO | Volume control, catecholamines |
| Respiratory failure | Pulmonary shunting, oedema, ARDS, pneumonia, aspiration | X-ray, blood gas analyses | Oxygen, antibiotics, volume control, mechanical ventilation |
| Metabolic abnormalities | Hypercatabolism, reduced hepatic metabolism | Blood glucose test, serum triglycerides | I.v. glucose, parenteral and enteral nutrition |

ICP, intracranial pressure; CV, central-venous; PAC, pulmonary artery catheter; PiCCO, pulse-induced contour cardiac output system; ARDS, adult respiratory distress syndrome; AT III, antithrombin III; EEG, electroencephalogram; CT, computed tomography.

hepatic failure. It can be found in 80% of patients with advanced hepatic encephalopathy. Encephalopathy differentiates fulminant hepatic failure from severe acute hepatitis. The pathophysiological basis is multifactorial and includes various circulating toxins that impair neuronal function and damage the blood-brain barrier.[29,30] Among them, ammonia plays a crucial role. In fulminant hepatic failure, increased ammonia levels induce astrocyte swelling by glutamine accumulation which cannot be compensated as in chronic liver failure.[31] Only at the initial stage, astrocyte swelling is partially balanced by a reduction of intracranial cerebrospinal fluid. Later on, cerebral perfusion is impaired by a loss of its autoregulation[32] and by progressive cerebral oedema with increasing intracranial hypertension. Finally, brainstem herniation and death of the patient may follow.

Hepatic encephalopathy is graded according to the West Haven criteria shown in Table 44.4.[33] Clinical signs of cerebral oedema are systemic hypertension, increased muscle tone which may progress to decerebrate postures, pupillary abnormalities and seizures. In fulminant hepatic failure, subclinical seizure activity is increased.[34] Therefore, systematic electroencephalography (EEG) controls are recommended for all patients, not only in those presenting seizures or requiring mechanical ventilation.

To exclude cerebral bleeding, a cranial computed tomgraphy (CT) scan may be necessary. Even though it is helpful to detect cerebral oedema, indication for a CT scan should be carefully evaluated. It has to be considered that any procedure that includes moving the patient could potentially increase intracranial pressure.

Direct quantification of intracranial pressure (ICP) can be best performed by epidural ICP monitoring catheters.[35] However, their use is associated with specific risks such as bacterial infection (especially gram-positive strains) or intracranial bleeding.[36] To date, no controlled clinical trials have been published demonstrating a beneficial effect of epidural catheters. Therefore, they are only recommended for patients waiting for liver transplantation with hepatic encephalopathy of at least grade III. In addition, cerebral oxygen supply can be estimated by jugular bulb oxygen saturation measurement.[37]

Therapy of hepatic encephalopathy aims to ensure a sufficient cerebral perfusion and avoid brainstem herniation. Cerebral perfusion pressure (CPP) should be kept above 50–60 mmHg and is calculated by subtracting intracranial pressure (ICP) from mean arterial pressure (MAP). Therapeutic measures to decrease intracranial pressure are indicated at an ICP >20 mmHg. A poor neurological outcome is associated with a sustained ICP of >40 mmHg.[36,38]

Basic measures involve establishing a quiet environment with minimal interventions. Degree of head elevation is controversially discussed. Some authors recommend only up to 20°, others found a 30° posture preferable.[39,40]

The osmotic diuretic mannitol is the first-line treatment (1–2 g per kg body weight i.v.). It can be given as repeated boluses as long as serum osmolality does not exceed 320 mosmol/L.[41] In patients with renal failure, mannitol should be used with caution and sufficiently haemofiltrated.

Therapeutic hyperventilation leads to cerebral vasoconstriction in ventilated patients. It can be used in incipient cerebral oedema if CPP is maintained at sufficient levels. In advanced cerebral oedema, hyperventilation improves acute intracranial hypertension only for a short period of time.[42,43] Prolonged hyperventilation can aggravate cerebral hypoxia and should be avoided.

Mild hypothermia (body temperature of 32–33 °C) has recently shown positive effects on cerebral oedema that need further confirmation in larger patient series.[44,45]

Lactulose or retention enemas are commonly used to minimize bacterial translocation, but lack supporting controlled data. L-Ornithine-L-aspartate can decrease circulating ammonia levels, but prevention of brain oedema has not been proven.[46,47] Other therapeutic measures without sufficient evidence comprise thiopental that reduces cerebral metabolism and flumazenil that antagonizes endogenous GABA-like toxins.[48,49] If seizure activity is increased, therapy with phenytoin is indicated. Its prophylactic use in encephalopathy of grade III–IV is under discussion, as one study has shown a reduction of cerebral oedema.[34]

Hepatectomy is the *ultima ratio* in desperate situations of patients awaiting liver transplantation. Removal of the 'necrotic liver' can improve cerebral oedema and circulatory parameters.[50,51] However, total loss of liver function after hepatectomy is fatal if liver transplanta-

**Table 44.4** Grades of hepatic encephalopathy

| Grade | Symptoms | Survival |
|---|---|---|
| I | Disturbed sleep, altered personality, reduced concentration capability, asterixis | 70% |
| II | Drowsiness, inappropriate behaviour, dysarthria, temporal and spatial disorientation | 60% |
| III | Arousable stupor, agitation and aggression possible | 40% |
| IV | Coma without response to painful stimuli | 20% |

tion is not performed in time (i.e. during the next few hours to days).

## Coagulopathy

Disturbed hepatic protein biosysnthesis of the majority of coagulation factors and platelet dysfunction cause a severe deterioration of blood coagulation with significant risk of bleedings. In addition, reduced levels of coagulation inhibitors result in an imbalance of coagulation profiles that resembles disseminated intravascular coagulation.[52,53] Up to one-third of patients without substitution of coagulation factors develop spontaneous bleedings. Intracerebral and intra-abdominal bleedings are particularly feared. Even though no supporting data have been published to date, many centres perform a prophylactic substitution of coagulation factors.[54] In every case, correction becomes necessary at the time when overt bleeding develops or before invasive procedures. To avoid a dysequilibrium of the different factors, fresh frozen plasma (FFP) should be used instead of factor concentrates. Only antithrombin III (AT III) is selectively administered to disrupt disseminated intravascular coagulation.[55,56] For prevention of upper gastrointestinal bleeding, consequent acid suppression therapy is recommended.[57]

## Infections

Infections contribute greatly to mortality in fulminant hepatic failure. These patients have an increased infection rate as a consequence of different pathophysiological changes that impair cell-mediated and humoral immunity. The hepatic barrier and clearance function are disturbed, resulting in increased bacterial translocation. Reduced complement levels and especially reduced opsonization caused by a deficiency of fibronectin predispose to infections as well.[58] Furthermore, monitoring and therapy of fulminant hepatic failure require the use of synthetic catheters that are a well-known source of infections.

Bacterial infections can be found in up to 80% of patients with fulminant hepatic failure, in most cases gram-positive strains.[59] As a consequence, systemic inflammatory response syndrome (SIRS) may develop and contribute to intracranial hypertension.[60] The probability of infection is dependent on the length of stay in the intensive care unit.[61] Fungal infections typically appear late in the clinical course, but can be found in up to 30% of patients (especially *Candida* species).[62] They are associated with a high mortality (about 50%). Most infections affect the lungs (50%), other manifestations are solitary bacteraemia, urinary infections and colonization of catheters.[59] Even after liver transplantation, the infection rate in patients transplanted for fulminant hepatic failure is higher than in those transplanted for chronic liver failure.[63]

In many patients, infectious complications remain asymptomatic (30%), i.e. they develop neither fever nor leucocytosis.[59] Therefore, systematic microbiological surveillance is mandatory including chest X-rays and cultures taken from all available body compartments (e.g. blood, urine, sputum).

Because of the high incidence of infections in fulminant hepatic failure, different prophylactic antibiotic combinations have been evaluated in the past. A selective intestinal decontamination (e.g. with paromomycin) can reduce the frequency of infections by bacteria of the intestinal flora.[64] The additional benefit of a selective intestinal decontamination is questionable if systemic antibiotics are administered in parallel.[61] An early broad-spectrum antibiotic prophylaxis reduced the incidence of infections to 20%, and progression of hepatic encephalopathy to advanced cerebral oedema was also significantly reduced.[59] Enteral administration of amphotericin B resulted in reduction of fungal infections to 4%.[61] Prophylactic administration of fluconazole has proven to be effective in the prevention of mycosis.[65]

Even though no survival benefit could be demonstrated to date, antibiotic prophylaxis for all patients with fulminant hepatic failure is recommended. The specific regime should be chosen according to the local microorganism spectrum and resistances.

## Renal failure

Acute renal failure can be observed in nearly half of the patients with fulminant hepatic failure.[66] Those with additional intoxification of acetaminophen (paracetamol) are particularly at risk. The main causes are relative hypovolaemia, parenchymal ischaemia and acute tubular necrosis by direct nephrotoxicity. Metabolic acidosis can be aggravated by acetaminophen toxicity or lactic acidosis resulting from tissue hypoxia and reduced hepatic clearance.

Volume status should be controlled by continuous monitoring with central venous catheters to avoid an increase of intracranial pressure due to volume overload. Electrolyte levels have to be carefully controlled and corrected as well. Especially in case of hypophosphataemia, rapid parenteral substitution is needed. Hyponatraemia is likely to play a role in the development of cerebral oedema and should be avoided.

In case of incipient renal failure, early renal replacement therapy is indicated. Continuous veno-venous haemofiltration (CVVH) seems to be better tolerated and should therefore be preferably used.[67,68] Anticoagulation with heparin should be administered with caution because of the increased risk of bleeding.[69] Novel

dialysis procedures are described in more detail below in the section on extracorporeal liver support devices.

### Circulatory failure

Splanchnic vasodilatation is characteristic of fulminant hepatic failure. As a result, septicaemia is mimicked by increased cardiac output, reduced systemic blood pressure, reduced systemic vascular resistance and reduced vascular filling pressures such as central venous pressure.[70,71] Relative hypovolaemia plays a pathophysiological role in the aetiology of other complications like renal failure. On the other hand, intracranial hypertension can cause an increase of systemic blood pressure.

There are no published data regarding optimal management of volume status in patients with fulminant hepatic failure. Still, monitoring of central venous pressure is mandatory to manage volume replacement therapy. In case of progressive circulatory failure, extended monitoring is provided by pulmonary artery catheters or PiCCO systems (pulse-induced contour cardiac output).

Beside volume replacement therapy with electrolyte solutions, colloid fluids can be administered. They can sometimes be spared in patients receiving fresh frozen plasma to stabilize coagulation. Therapy of peripheral vasodilatation essentially consists of catecholamines. Usually, dopamine is the preferred catecholamine. If it is not sufficient to maintain an acceptable systemic blood pressure, noradrenaline must be administered even though it can further compromise tissue oxygenation.[72]

### Respiratory failure

In patients with fulminant hepatic failure, respiratory failure is often part of multi-organ failure. Usually, it does not develop prior to advanced hepatic encephalopathy. Respiratory failure is associated with a very high subsequent mortality.[73] Arterial hypoxaemia can be caused by intrapulmonary shunting, adult respiratory distress syndrome (ARDS), pneumonia, aspiration, intrapulmonary haemorrhage and pulmonary oedema.[74]

The only therapeutic option is artificial ventilatory support. Elective intubation to prevent aspiration is recommended for patients with hepatic encephalopathy grade III–IV.[71] In case of mechanical ventilation, sedation should be performed with propofol, as it seems to reduce intracranial pressure itself.[75]

### Metabolic derangements

Few controlled data are available regarding nutritional support in fulminant hepatitis. Therefore, it should be performed according to pathophysiological concepts. In contrast to patients with chronic liver diseases, only a minority of patients with fulminant hepatitis do initially present signs of malnutrition. As a result of intracranial hypertension, increased production of endogenous corticosteroids and catecholamines induces a hypercatabolic state with an increased resting energy expenditure.[76] In addition, impaired liver function limits intrahepatic substrate metabolism.[77] On the other hand, reduced gluconeogenesis and depletion of glycogen stores can result in hypoglycaemia. Therefore, repeated tests of blood glucose levels are mandatory.

Blood glucose should be maintained at levels of about 5–10 mmol/L by continuous infusion of 10% or 20% glucose. Lipids and amino acids should additionally be given if parenteral nutrition is necessary for >3 days. Energy supply is recommended for around 30 kcal per kg body weight per day. As a consequence of encephalopathy, amino acids should be administered with caution (0.4–1.0 g/kg/day). The rest of the calculated energy expenditure should be equally divided between glucose and lipids.[78] Lipids can be administered as long as the triglyceride levels remain below 4.0 mmol/L. Hypertriglyceridaemia can also result from overdosing of glucose because of impaired lipid utilization. This may induce acute hepatic steatosis, further worsening disturbed liver function.

Adaptive amino acid solutions were introduced to improve the disturbed amino acid patterns in fulminant hepatitis. They contain a higher proportion of branched chain amino acids and a reduced proportion of aromatic amino acids. Even though successful tests were accomplished in animal models,[79] no controlled studies on the effects of adaptive amino acids in patients with fulminant hepatitis exist.[80]

Enteral nutrition to prevent villous atrophy and to reduce the risk of bacterial translocation is recommended as long as paralytic ileus does not develop. Positive effects of immunonutrition (e.g. with glutamine) are not proven.

## Specific therapy in fulminant hepatitis

Whenever there is evidence for a specific therapy for fulminant hepatitis (see also Table 44.5), this should be initiated as early as possible in addition to standard treatment options for fulminant hepatitis.

A main problem in establishing any specific treatment is the rarity of certain aetiologies of fulminant hepatitis on one hand and the severity of the disease on the other hand. Once there is evidence for a potentially life-saving treatment option with little or no toxicity, a placebo-controlled trial might not be feasible owing to ethical constraints.

**Table 44.5** Specific therapy of viral infections in fulminant hepatitis

| Type of virus | Specific therapy |
|---|---|
| Hepatitis A | No specific therapy available; in case of liver transplantation HAV immunoglobulins should be given to prevent recurrence of hepatitis |
| Hepatitis B | Lamivudine likely to be effective; in case of liver transplantation combination prophylaxis of HBIg and lamivudine should be administered to prevent recurrence of hepatitis |
| Hepatitis C | No specific therapy available |
| Hepatitis D | No specific therapy available |
| Hepatitis E | No specific therapy available |
| Herpesviridae | Acyclovir |
| Coxsackie virus B | No specific therapy available |
| Adenovirus | No specific therapy available |
| Parvovirus B19 | No specific therapy available |
| Haemorrhagic fever viruses | Ribavirin |

## HAV

No specific therapy has been established for HAV infection. The outcome of fulminant hepatitis A was reported to be related to HAV viral load.[81] Therefore, antiviral therapy might be an option in the future, when an effective antiviral against HAV will be available. As there have been reports of persistence of hepatitis A after liver transplantation,[82,83] these patients could benefit from HAV immunoglobulins.

## HBV

For chronic hepatitis B infection, a number of different therapies have been established. The first was interferon (IFN)-α (see Chapter 20), followed by lamivudine and more recently adefovir dipivoxil and entecavir. There is good evidence for an overwhelming immune response in the pathogenesis of fulminant hepatitis B.[84–86] Thus, IFN-α can be dangerous in fulminant hepatitis B due to its immune-stimulating properties.

In contrast, the oral nucleoside analogue lamivudine inhibits hepatitis B viral replication with an immediate decline of serum HBV DNA. Lamivudine has been first reported to successfully prevent death in a few case reports of patients who developed acute decompensation of chronic hepatitis B, and thus were not eligible for high urgency transplantation.[87–91] In addition, one patient series in renal transplant recipients has been published.[92] Even though death was not universally prevented, mortality was lower in treated than in untreated patients. Importantly, these series also indicate that early start of lamivudine therapy appears to be essential.

Based on these reports and on the favourable safety profile of lamivudine, treatment with lamivudine was started in patients presenting severe acute hepatitis B at risk of developing hepatic failure. Treatment was initially aimed at preventing HBV reinfection after liver transplantation. However, only one of nine patients required liver transplantation compared with 16 of 20 from an historic control ($p = 0.002$).[93] All patients cleared HBsAg and normalized liver enzymes. In addition, a recent report showed prevention of further deterioration of liver impairment in three patients with severe acute hepatitis.[94]

In summary, lamivudine appears to be safe in patients with severe acute or fulminant hepatitis B, and may have the potential to prevent liver failure when administered early. It is therefore recommended to start lamivudine within prospective studies in these patients once prothrombin time deteriorates. No data on adefovir dipoxivil or entecavir are available yet.

## HCV

In contrast to acute hepatitis C infection, there are currently no data on any specific treatment option in fulminant hepatitis C. While IFN-α leads to a transaminase peak in hepatitis B, this is rarely seen in HCV infection. Thus, IFN-α might be a theoretical option in fulminant hepatitis C. However, data in relation to immune activation in fulminant hepatitis C are currently lacking. In the future, potent antiviral agents such as proteinase inhibitors or polymerase inhibitors are likely going to play a role. If they could drop the hepatitis C viral load within days, this could be an attractive option. Ribavirin, which is helpful in increasing the response to IFN in chronic infection, has shown little effect when given alone (see Chapter 34). Thus, ribavirin probably would be of very limited help, if any, in case of fulminant HCV infection.

## HDV

There is currently no highly efficient therapy available for chronic hepatitis delta. The only option with limited effect is high dose IFN (see Chapter 38), which again appears to be dangerous in cases of fulminant hepatitis D. Thus, in this disease, supportive care appears to be the

only treatment option beside liver transplantation. Adding lamivudine is not likely to cause any adverse event, and might have a positive effect in increasing clearance of HBsAg, but no such data exist at present.

### HEV

As for HAV infection, there is no established therapy for HEV infection (see Chapter 42). Whether emergency delivery is of any benefit, has not been investigated. Effective antiviral agents are currently unknown.

### Herpesviridae

In case of HSV infections, early diagnosis is required, as in any other cause of fulminant hepatitis. Probably only early intervention with acyclovir holds some promise to alter the course of herpesviridae-associated disease.[16] Otherwise, the diagnosis can often be made only after the patient has died.[95] Likewise, in case of EBV, VZV or HHV-6 infection, therapy with acyclovir probably is a theoretical option, even though clear data are missing to date.

### Specific therapy for non-viral causes of fulminant hepatitis

#### *Paracetamol intoxication*

N-acetylcysteine (NAC) for treatment of paracetamol (acetaminophen) intoxication was the first drug to show a beneficial effect in any cause of fulminant hepatitis.[96] Importantly, it has been shown that early administration is crucial.[97] Currently a dose of 140 mg/kg is given i.v. within 1 hour followed by 70 mg/kg/h.[98]

In addition, paracetamol may contribute to fulminant hepatitis in other causes of acute hepatitis, as patients frequently take paracetamol for their unspecific complaints prior to the clear onset of fulminant hepatitis. Thus, addition of NAC might improve patient survival in general. But prior to a final recommendation, a currently ongoing multicentre trial in the US should be awaited. Nevertheless, most centres currently administer NAC to patients with fulminant hepatitis, especially when there is anamnestic evidence for paracetamol intake prior to the onset of liver disease.

#### *Amanita intoxication*

Therapy with silibinin and penicillin has been established as an antidote for *Amanita phalloides* intoxication. If given in time, silibinin (20–50 mg/kg/day i.v.) and penicillin (300 000–1 000 000 IU/kg/day i.v.) can be curative treatment options to prevent the need for liver transplantation.[99,100]

#### *Wilson's disease*

Wilson's disease is a hereditary failure of copper excretion through the bile, leading to copper overload with subsequent damage to hepatocytes resulting in either chronic liver disease (cirrhosis) or acute liver failure. For patients with chronic Wilson's disease, treatment with d-penicillamine has been proven effective in halting disease progression. One recent report suggests that this medication might also ameliorate the course of fulminant Wilson's disease, circumventing the need for liver transplantation.[101] This has not been confirmed in any other study so far.

#### *Autoimmune hepatitis*

In patients with presumed autoimmune genesis of fulminant hepatitis, steroids may lead to a dramatic improvement, circumventing the need for liver transplantation .[102]

#### *Budd-Chiari syndrome*

Patients with Budd-Chiari syndrome may require treatment with portal decompressive surgery or liver transplantation. Transjugular intrahepatic portosystemic shunt (TIPS) represents a new treatment alternative. Early reports indicate that the procedure can help at least to bridge until liver transplantation.[103,104] In the future, it could help to circumvent the need for transplantation. However, anticoagulation is also required in these patients.

### Specific therapies for rare causes of fulminant hepatitis

Steroids may be helpful in giant cell hepatitis. When malaria falciparum leads to fulminant hepatitis, antimalarials are required. Immediate delivery ameliorates the course of disease in fatty liver of pregnancy. If heart failure leads to ischaemia, recompensation of cardiac function may also restore liver function. In case of malignant infiltration of the liver, chemotherapy is the only causal option.

## Prognosis, liver transplantation and alternative procedures

### Prognosis

Fulminant hepatic failure has a high spontaneous mortality. The likelihood of recovery depends on many factors such as aetiology, age, complications and duration of illness. To estimate survival, two main prognosis

**Table 44.6** Scoring systems for estimation of prognosis in fulminant hepatitis

| Criteria of King's College London | |
|---|---|
| *Paracetamol intoxication* | *Other aetiologies* |
| Arterial pH <7.3 | PTT >100 s (INR >6.7) |
| or all of the following: | Or three of the four following criteria: |
| PTT >100 s | age <10 or >40 years |
| creatinine >3.4 mg/dL | jaundice >7 days before onset of encephalopathy |
| hepatic encephalopathy 3–4° | bilirubin >17.4 mg/dL |
| | unfavourable aetiology (non-A-non-B-hepatitis, drug-induced) |
| French criteria (Clichy)[105] | |
| Factor V <20% and patient age <30 years | |
| Or | |
| Factor V <30% and hepatic encephalopathy 3–4° | |

PTT, prothrombin time; INR, international normalized ratio.

scores were introduced: the King's College Criteria[28] and the French Criteria[105] (detailed in Table 44.6). Both scoring systems aim to predict patients with an estimated mortality of >80%. In these patients, emergency liver transplantation becomes necessary. Specificity of both scores is good, but clinical evaluations have shown a low sensitivity. As a result, many patients who do not fulfil transplant criteria take a fatal course.[106–109] Therefore, more accurate indicators for assessing the prognosis are necessary and several others have already been studied. For instance, unfavourable outcome is indicated by an APACHE II score >15 points or an arterial lactate level of >3 mmol/L.[110,111]

The role of liver biopsy is still controversial.[112–114] As a consequence of coagulopathy, it has to be performed via the more complicated transjugular access. Histological assessment of parenchymal necrosis can be helpful for predicting prognosis and for decisions as to indications for transplantation. Additionally, liver biopsy may be necessary to exclude chronic liver disease or to clarify the aetiology of fulminant hepatitis in unknown cases.

## Orthotopic liver transplantation

Conventional orthotopic liver transplantation (OLT) is the only proven therapy for patients with a severe course of fulminant hepatitis.[115–118] According to the European Liver Transplant Registry (ELTR; http://www.eltr.org), 9% of all liver transplantations in Europe are performed for acute liver failure. This proportion doubles in patients with an age below 45 years.

Correct timing is crucial for indication of liver transplantation in fulminant hepatitis. On one hand, transplantation should be avoided in patients with a realistic possibility of hepatic regeneration. On the other hand, transplantation has to take place before irreversible complications like brainstem herniation develop. To prevent unnecessary loss of time, every patient with fulminant hepatitis should be discussed with a specialized centre for liver transplantation. As mentioned above, transplantation is indicated in patients with an estimated mortality of >80%. Contraindications are irreversible brain damage, fulminant sepsis, AIDS, advanced co-morbidities or a malignant disease. Patients with fulminant hepatitis have a higher priority for liver transplantation than those with chronic liver disease, resulting in short waiting periods of just a few days.

Outcome after transplantation is fair (ELTR: 5-year survival 60%) and depends greatly on pre-transplant

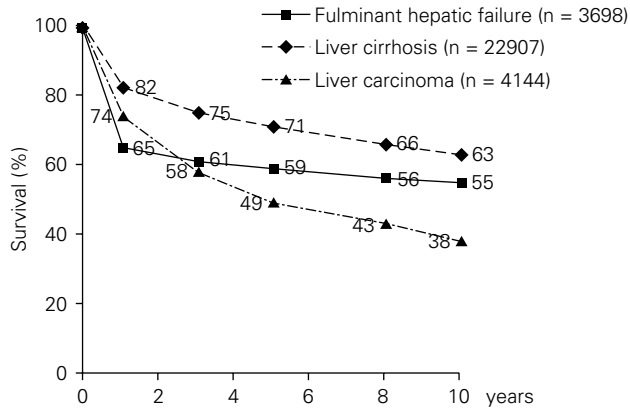


**Figure 44.1** Patient survival after orthotopic liver transplantation for fulminant hepatitis. Survival rates after liver transplantation of patients with fulminant hepatic failure (n = 3698) in comparison with patients transplanted for liver cirrhosis (n = 22 907) or carcinoma (n = 4144). All data were collected from the European Liver Transplant Registry from January 1988 to December 2001 (http://www.eltr.org). Survival in patients with fulminant hepatic failure was significantly worse in the first year after liver transplantation followed by a nearly stable survival curve in the long-term follow-up.

morbidity. A high mortality early after transplantation is characteristic and can be mainly explained by the sequelae of cerebral oedema and the increased infection rate. If the patient is able to survive the first months after OLT, the long-term prognosis is comparable to or even better than in patients transplanted for chronic liver disease.[119] Fig. 44.1 depicts patient survival after liver transplantation for fulminant hepatitis in comparison with other indications.

## Split liver transplantation

Conventional cadaveric transplantation is limited by the shortage of available organs. As a result, other options besides whole organ transplantation are being developed. Grafting only part of the liver is one of these options.[120] To provide enough functional liver parenchyma, the weight of the graft should be >1% of the recipient´s body weight. Therefore, it is usually necessary to transplant the right hepatic lobe in adults. The procedure is associated with a higher risk of complications such as biliary leakage.

## Living related donor transplantation

The concept of split liver transplantation was adopted to transplantation from living related donors. Apart from ethical aspects, the donor must be aware of the risk of morbidity and even mortality. In practice, it can be difficult to find and evaluate a suitable living donor in the short period of time available before severe complications develop.[121,122]

## Auxilliary liver transplantation

In fulminant hepatitis, chronic liver disease is absent by definition. Even in the case of total parenchymal necrosis, the patient's liver has the potential for full recovery. Therefore, technical variants of liver transplantation were introduced, allowing the patient´s own liver to remain in place. In auxilliary partial orthotopic liver transplantation (APOLT), the patient´s left hepatic lobe is resected and replaced by a split liver graft to bridge the period of liver dysfunction. After regeneration of the patient´s own liver, the procedure is completed by surgical graft removal or by withdrawal of immunosuppression resulting in graft atrophy.[123] Controlled data have shown a comparable survival to conventional liver transplantation even though APOLT seems to have an increased complication rate.[124,125] The proportion of patients who achieve successful withdrawal of immunosuppression is between 30% and 70%.[126,127] In practice, APOLT is rarely used, as only a few patients and grafts present optimal conditions with prospect of hepatic recovery.

## Hepatocyte transplantation

The transplantation of hepatocytes is an experimental procedure. Administration of freshly isolated, cryopreserved or cultivated hepatocytes has been performed in animal models and in a few human patients.[128–131] The necessary amount of hepatocytes seems to be much higher in patients with fulminant hepatitis than in therapy of hereditary metabolic diseases. Unsolved principal problems are the extraction of a sufficient amount of hepatocytes, establishing an optimal route of application, avoiding possible complications (e.g. pulmonary embolism) and evaluating the extent and type of immunosuppression.[132] In animal experiments, promising results were found with encapsulated hepatocytes that need confirmation in humans. Furthermore, studies in rabbits suggest a positive influence of induced transient ischaemia on efficacy of hepatocyte transplantation.[133]

## Xenotransplantation

After the initial euphoria about transplantation of xenogenous organs, progression in this field is much slower than expected. Immunological risks of complement-mediated, hyperacute rejection seem to be controllable by genetic engineering (e.g. removal of GAL-epitope on porcine endothelial cells). But the variety of donor species is limited, as organ size, metabolic pathways and export proteins must resemble the human model as much as possible. Therefore, primates and pigs are the main candidates for potential donor species. In addition, the possibility of transmission of zoonoses (e.g. porcine endogenous retrovirus; PERV) is controversially discussed.[134] To date, only three xenotransplantations in patients with acute or chronic liver failure have been performed and these took place several years ago. Maximum patient survival was 70 days.[135]

## Extracorporeal liver support devices

Three main types of extracorporeal liver support devices exist: bioartificial liver systems containing hepatocytes in bioreactors, mechanical detoxification systems (e.g. albumin dialysis) and extracorporeal liver perfusion. Recent meta-analyses could not find any survival benefit in patients with fulminant hepatitis regardless of the device.[136–138] Therefore, no general recommendation for the use of extracorporeal liver support devices can be given outside of clinical trials. An overview of all extracorporeal systems is given in Table 44.7.[139–161]

### *Bioartificial livers*

These systems ('bio livers') aim at providing enough functional liver cell mass to bridge the critical period

**Table 44.7** Extracorporeal liver support systems

| Procedure | Principle | References |
|---|---|---|
| Extracorporeal liver perfusion (ECLP) | | 103, 138 |
| Blood from the patient is purified while being guided through an explanted liver (human or xenogenous) in a sterile chamber | | |
| Bioartificial devices | | |
| Modular Extracorporeal Liver Support (MELS) | Human hepatocytes | 139–141 |
| HepatAssist | Porcine hepatocytes | 142–144 |
| Bioartificial Liver (BAL) | Porcine hepatocytes | 145 |
| Bioartificial Liver Support System (BLSS) | Porcine hepatocytes | 146 |
| Extracorporeal Liver Assist Device (ELAD) | Hep G2 cell line | 147 |
| Mechanical procedures ('liver dialysis') | | |
| Haemodialysis, plasmapheresis, haemadsorption | Classic dialysis | 148–152 |
| Biologic-DT | Classic dialysis | 153,154 |
| Single-Pass Albumin Dialysis (SPAD) | Albumin dialysis | 155 |
| Molecular Adsorbent Recycling System (MARS) | Albumin dialysis | 156–159 |
| Prometheus | Albumin dialysis | 160, 161 |

of liver dysfunction until transplantation takes place. Hepatocyte cultures are kept in a special matrix in a bioreactor with capillaries that contain the circulating blood of the patient. A functioning bioreactor requires the capability to maintain hepatocyte cell function as well as an effective bidirectional transport of nutrients, metabolites and toxins. Depending on their origin, three principal types of hepatocytes can be differentiated: hepatocytes from humans (allogenous) or animals (xenogenous) or cell culture lines (immortalized cells or tumour cell lines). Different bioreactors exist for all cell types, and all are associated with distinct management problems, as described below.

**1** Human hepatocytes. The only published system is the Molecular Extracorporeal Liver Support System (MELS; formerly called BELS on the basis of porcine hepatocytes).[139,140] It includes a cell module with human hepatocytes, a detox module for Single-Pass Albumin Dialysis (SPAD; see below) and a dialysis module for continuous veno-venous haemofiltration (CVVH). The required amount of human hepatocytes for support of one patient is comparable to the size of an entire liver.[141]

**2** Xenogenous hepatocytes. These have the same risks as xenotransplantation such as transmitting a zoonosis or creating immunological problems. Furthermore, biochemical compatibility must be sufficient. Examples with porcine hepatocytes are HepatAssist,[142–144] the Bioartificial Liver (BAL)[145] or the Bioartificial Liver Support System (BLSS).[146]

**3** Cell culture lines. The functional capacity of immortalized cell lines is impaired. Theoretically, tumour cell lines could enter the patient's circulation and induce neoplasia. A clone of C3A cells of the HepG2 cell line is used for the Extracorporeal Liver Assist Device (ELAD).[147]

A promising perspective is the use of human stem cells that are currently not available. Providing some kind of biliary efflux and the composition of the extracellular matrix are other important problems of the bioreactors. At present, hepatocyte function is limited outside the complex system of liver architecture and its adoption into clinical practice has therefore not been completely successful. Available data show a good biocompatibility and an improvement of detoxification, but no replacement of hepatic synthetic function and, especially, no survival benefit. The future of bioartificial livers will depend on the availability of functional and safe hepatocytes in a system that keeps the cells alive without losing clinical practicability.

### *Mechanical devices*

These devices use filtration techniques to support hepatic detoxification without substitution of liver synthesis ('liver dialysis'). Established nephrological procedures mainly remove water-soluble substances. Haemodialysis, exchange transfusion, plasmapheresis and haemadsorption with charcoal, alone or in combination, have brought about some biochemical and clinical improvements in fulminant hepatitis without affecting survival.[148–152] BiologicDT is a system that combines haemodialysis and haemadsorption.[153,154]

For effective detoxification, additional removal of protein-bound substances is inevitable. Therefore, novel concepts aim to remove both types of substances, water-soluble and protein-bound ('albumin dialysis'). The technical realization of albumin dialysis is quite similar in the Molecular Adsorbent Recycling System (MARS) and Single-Pass Albumin Dialysis (SPAD). The blood of the patient is purified over an asymmetric, non-al-

bumin-permeable membrane against albumin in a secondary circuit. In contrast to SPAD,[155] the MARS device recycles the 'loaded' albumin by two adsorbers and low-flux dialysis.[159] Controlled data regarding survival in patients with fulminant hepatitis are not available.[156,157] However, haemodynamic parameters improved in eight patients with fulminant hepatitis and MARS therapy in comparison with five controls.[158]

The Prometheus device is based on the concept of fractionated plasma separation and adsorption (FPSA).[160] In contrast to MARS, the plasma is separated through an albumin-permeable filter into the secondary circuit where it is directly purified by adsorber. This is followed by conventional high-flux haemodialysis. For the moment, the only available data are from patients with acute-on-chronic liver failure, showing a significant improvement of several biochemical parameters.[161]

### *Extracorporeal liver perfusion*

In extracorporeal liver perfusion (ECLP), the patient's blood is guided through a freshly explanted liver that is kept in sterile chambers outside the body. This organ is of xenogenous nature or a human liver that is otherwise not suitable for transplantation. First trials >40 years ago were not continued because of the increasing success of conventional liver transplantation. Recently, ECLP has regained interest as a result of progress in xenotransplantation and the increasing shortage of available human organs. If xenogenous organs are used, the immunological and infectious risks equal those in xenotransplantation, even though contact in ECLP is only temporary. Until now, neither hyperacute rejections nor xenogenous infections have been reported, and anaphylactic reactions were sporadic. In fulminant hepatitis, extracorporal xenoliver perfusion was feasible for up to 36 hours, indicating that hyperacute rejection might be limited due to impairment of the complement cascade in fulminant hepatitis.[162] However, two recently published meta-analyses could not demonstrate an improvement in patient survival or hepatic encephalopathy. All systems seemed to be safe, but use of human or baboon livers was associated with significantly better results than use of porcine livers.[137,138]

## Conclusion

Fulminant hepatic failure is an acute severe impairment of liver function including hepatic encephalopathy and jaundice in the absence of chronic liver damage.

The main viral agents are the classical hepatitis viruses (A to E). There are no specific treatments currently established for any of these viruses. However, early lamivudine therapy seems to ameliorate the course of fulminant hepatitis B. If there is evidence for herpesviridae as a cause of fulminant hepatitis, acyclovir should be given. In case of haemorrhagic fever virus infection, ribavirin is recommended.

Extrahepatic complications include cerebral oedema, coagulopathy with risk of bleeding, bacterial and fungal infections, changes in metabolism and multi-organ failure (especially renal failure). Because these complications highly determine survival, therapy should begin early and should be aggressive. However, despite significant improvements in intensive care medicine, spontaneous mortality still remains high. The patient's prognosis can be predicted by clinical scores. However, the scores have a low sensitivity. In patients with an estimated fatal course, conventional liver transplantation still represents the treatment of choice. Therefore, early contact with a transplantation centre is mandatory. In selected cases, living-related transplantation or auxilliary partial transplantation can be useful. Hepatocyte transplantation and xenotransplantation are experimental procedures that could become more important in the future. Likewise, controlled trials of extracorporeal liver support with bioartificial or mechanical devices that show an improvement of survival are lacking to date. Any therapeutic approach aimed at circumventing the need for liver transplantation should be initiated as early as possible.

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# Chapter 45
# Hepatitis and haemophilia

Christine A Lee

Two types of haemophilia, A and B, are identical in their clinical manifestations and sex-linked recessive inheritance patterns. In haemophilia A, the common variety, bleeding is caused by a deficiency of factor VIII in the blood. In haemophilia B, factor IX is deficient. Patients with severe disease (<1 U/dL) suffer repeated, spontaneous and painful bleeding into muscles and joints, which if untreated, rapidly leads to crippling. Uncontrolled bleeding in other sites, such as the brain, causes premature death. Patients with haemophilia have developed hepatitis as a result of treatment with blood and blood products. Thus, hepatitis may also occur as a result of transfusion treatment in the less common clotting factor deficiencies (e.g. factors XI, VII, V and X deficiencies, as well as von Willebrand's disease).

Although the first report of blood transfusion to treat haemophilia was as early as 1840,[1] effective therapy for the condition has become widely available only during the last 25 years. As recently as 1937, Birch[2] described 113 patients with haemophilia, of whom 82 died before the age of 15 years, often as a result of trivial injury. During the 1950s and 1960s, fresh frozen plasma improved the management of the condition and was the main treatment.[3,4] The introduction of clotting factor concentrates in the late 1960s revolutionized the treatment of haemophilia, making possible a near-normal life for many patients.

The discovery that cryoprecipitate was rich in factor VIII was made in 1955 by Remigy,[5] and the description of a method of production of a high-potency concentrate of anti-haemophilic globulin in a closed bag system (cryoprecipitate)[6] made home treatment possible. Although cryoprecipitate has the advantage of simplicity of manufacture and therefore remains the mainstay of treatment for the less economically developed parts of the world, it has been superseded in most Western countries by lyophilized clotting factor concentrates and increasingly by recombinant products which are now recommended for treatment in economically well resourced countries.[7]

Large-pool clotting factor concentrates are manufactured on an industrial scale, from plasma pools to which many thousands of donors have contributed.

Patients with haemophilia were at high risk of contracting hepatitis because of both a large donor exposure from a single pool of blood products and the use of unsterilized lyophilized blood products from many thousands of donors. The development of virucidal methods and their application since 1985 to clotting factor concentrates have largely, but not completely, stopped transmission of viruses causing hepatitis.[8]

Transfusion-associated jaundice was first described in the 1940s,[9,10] and became increasingly recognized as a complication of haemophilia treatment following the introduction of plasma product therapy in subsequent years.[11,12] By the 1970s, overall estimates of the rate of symptomatic acute hepatitis with jaundice in haemophilic individuals ranged from 2% to 6% of treated patients per year, being particularly recognized in those patients who had previously received little or no treatment with blood products.[11,13]

## Hepatitis A

Hepatitis A virus (HAV) is only rarely transmitted by transfusion[14,15] and has formerly not been recognized as a problem for the recipients of blood product therapy. However, in 1992–1993 a number of outbreaks of HAV were reported from Italy,[16] Germany,[17] the Republic of Ireland[18] and Belgium.[19] All cases were associated with the use of a high purity product, factor VIII sterilized by solvent detergent (Octa VI, Octapharma). In the German outbreak between 1988 and 1992, 17 patients demonstrated HAV seroconversions, 13 had high HAV IgM seroconversions and 10 had clinical symptoms.[20]

In the Italian outbreak, 52 cases were identified during the years 1989–1992. Most cases were identified because jaundice developed in 42 of 52 patients. It was possible to identify HAV sequences in five of twelve lots of factor VIII tested, indicating that viral contamination occurred relatively frequently. A case-control study found a strong correlation between the occurrence of hepatitis and the use of a high purity factor VIII concentrate, manufactured in Italy, which was similar to concentrates produced in Austria and Germany and was

administered to patients with haemophilia in Germany, Ireland and Belgium in whom hepatitis A developed.[21] All concentrates were produced by ion-exchange chromatography and contained little immunoglobulin and were virally inactivated by solvent detergent. The products differed in that the Italian product was manufactured from plasma obtained in the United States from paid plasmapharesis donors, whereas the German, Irish and Belgian products were manufactured from plasma of local unpaid donors. The epidemiological association of the factor VIII lots with the transmission of HAV together with the detection of the identical sequences in the factor VIII and the corresponding patients' serum samples in two cases strongly supported the conclusion that HAV was acquired from the factor VIII.

It is likely that HAV-infected plasma units contaminated the pools used to prepare the concentrates. The attack rate of HAV infection is decreasing in all Western countries.[22,23] Therefore, more susceptible patients with haemophilia now comprise the population of clotting factor recipients. Also, individuals susceptible to HAV infection are more likely to reach the age at which they might donate plasma during the asymptomatic viraemic phase of HAV infection. Furthermore, if a contaminated plasmapharesis unit entered a pool for fractionation, the content of specific neutralizing antibodies (which is decreasing because of the changing epidemiology of HAV infection) may not be sufficient to neutralize HAV. It is also possible that virus–antibody complexes might dissociate during the manufacture of the concentrate as an effect of the solvent detergent.

A further outbreak of HAV has been associated with solvent detergent-treated factor VIII (Alphanate) among patients with haemophilia in the United States.[24] As a result of this outbreak, which also involved a factor IX concentrate (AlphaNine), the manufacturers decided to add another virucidal step: heating at 80 °C for 72 hours.

Because HAV is non-enveloped, it is not inactivated by solvent detergent alone. Therefore, a double inactivation process is necessary.

It is also possible to protect susceptible patients against hepatitis A. There is accumulating evidence that haemophilic patients respond well despite the need to give the vaccine by the subcutaneous route, apart from those who are immunocompromised with human immunodeficiency virus (HIV) infection.[25,26]

## Hepatitis B and delta infection

The discovery in the mid-1960s of the Australia antigen, against which multi-transfused patients with haemophilia developed precipitating antibodies, led to the identification of several specific markers that are now available for the characterization of hepatitis B virus (HBV) infection and the recognition that HBV infection was a major cause of transfusion-transmitted disease. The majority of intensively treated older patients with haemophilia have serological evidence of previous HBV infection.[27] Advances made in blood donor screening and concentrate sterilization have largely stopped HBV transmission to haemophilic patients. However, because such patients are at higher risk of receiving unsterilized whole blood, hepatitis B vaccination is mandatory.

Hepatitis D virus (HDV, delta agent) requires the presence of HBV for its propagation. Superinfection with HDV can occur in chronic HBV infection and co-infection in acute hepatitis B. It has been shown that among individuals who receive multiple transfusions of blood and blood products, haemophilic patients have the greatest risk of acquiring infection with HDV.[28,29] Anti-HDV prevalence of 34% among 79 hepatitis B surface antigen (HBsAg)-positive haemophilic patients who had received commercial clotting factor concentrates in western Europe and the United States was reported.[29] However, there was no evidence of HDV infection among 24 HBsAg-positive haemophilic patients from Brazil, Germany (former GDR) and Australia who had received factor concentrate prepared locally.[29] In another study, anti-HDV was detected in 35 (9.1%) of the HIV-positive patients, with 10/35 (29%) also having IgM anti-HDV indicating active infection.[27] In the HIV-negative patients, 13 (4%) had anti-HDV and only one patient had IgM anti-HDV.

It was found that HDV markers were most prevalent in type B haemophilia after controlling for disease severity ($p$ <0.02.) These data suggest that in the past, factor IX concentrates have been more heavily contaminated than factor VIII concentrates. It could be that the different manufacturing techniques could have resulted in a higher level of factor IX contamination in the past by HBV and HDV.

An interesting phenomenon of suppression of hepatitis C virus (HCV) by replication of HDV has been demonstrated.[30] Eight chronic carriers of HBsAg from a cohort of 99 patients with haemophilia co-infected with HIV and HCV were tested for antibody to HDV and quantitatively for HIV RNA, HCV RNA and HDV RNA. HCV RNA was detected in only one of five patients with HDV infection. In contrast, all three without HDV had high levels of HCV RNA. This represents viral interference, a well-known, but poorly understood, phenomenon in hepatitis infections.

## Hepatitis C

The natural history of HCV in haemophilia has been reviewed.[31,32] People with haemophilia who received non-virucidally treated large-pool clotting factor before 1986 were infected with HCV, previously referred to as non-

A, non-B hepatitis. A few patients were infected from single donor cryoprecipitate, particularly those receiving regular treatment with this product. Thus, on average people with haemophilia have been infected with HCV for more than two decades and also in many cases have received a large viral burden.

The large-pool clotting factor used to treat patients was obtained from plasma donors in northern Europe and the United States, where the prevalent genotypes were 1, 2 and 3. The most common genotype to be found in haemophilic patients is genotype 1. It is not clear why a particular genotype becomes the dominant genotype in any one haemophilic patient. The prevalence of type 1 may reflect the immune response at the time of infection, perhaps the greater replication competence of type 1 or the prevalence of this genotype among blood donors at the time of infection.

In haemophilic patients in Edinburgh, the distribution of genotypes was similar to that found in Scottish blood donors[33] (Fig 45.1). These investigators found that over 10 years, there was a change in genotype and serotype in nine of 29 patients.[34] The distribution of genotypes in haemophilic patients compared with the US population is shown in Table 45.1 and there was a much higher proportion of genotype 3 in the haemophilic individuals.[35] Furthermore, changes in genotype over time occurred in 58%, and this was more common in the presence of HIV.

In a study in Belgium, HCV genotype 1b was found to be predominant in both haemophilia patients and the general population, but in the haemophilic patients double infection with two genotypes occurred, as well as a relatively high infection with types 2 and 3.[36]

It is interesting that in Japan, where patients with haemophilia have been treated with concentrates derived from US donors, the predominant genotype in patients with haemophilia was la, whereas for the general population of Japanese patients type 1b was the most common.[37]

## Natural clearance

Approximately one-tenth of patients with haemophilia who were infected with HCV have been shown to clear the infection naturally as demonstrated by the presence of antibody to HCV in the face of persistent negative HCV RNA.[38–40] The majority of these patients had non-severe haemophilia and therefore infrequent treatment with clotting factor concentrate. Patients who cleared HCV were also young at first exposure, and therefore it is possible that their immune response at the time of infection was more effective. Of patients who became infected with HIV from clotting factor concentrate, most would also have been infected with HCV either at the same time or earlier. Thus, it is remarkable that there are reports of individuals co-infected with HIV and HCV, and that there has been clearance of HCV naturally.[40]

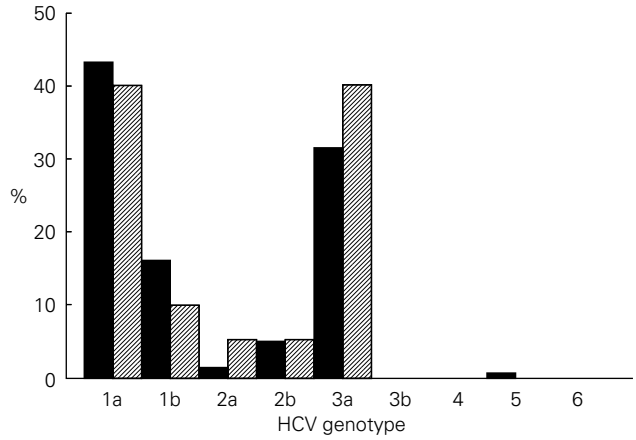


**Figure 45.1** HCV genotype in blood donors and haemophilia recipients. From Jarvis *et al*.[33]

## HCV mono-infection

Studies of the natural history of HCV in haemophilia are few in number. It is important to review the natural history of singly infected individuals separately from those co-infected with HIV. A cohort of 102 HCV-infected haemophilic patients who had been exposed to the virus for 15–34 years from three centres in Italy showed that 14% had cleared the virus naturally; 86% were HCV RNA-

**Table 45.1** Chronic HCV patients from Florida, Missouri and California

| | Prevalence of genotypes | | | |
|---|---|---|---|---|
| Results | HIV-positive (n = 17) | HIV-negative (n = 14) | Total (n = 31) | US population estimates (n = 139) |
| 1a | 6 (35%) | 4 (29%) | 10 (32%) | 37.4% |
| 1b | 2 (12%) | 2 (14%) | 4 (13%) | 37.4% |
| 2 | 1 (6%) | 1 (7) | 2 (6.5%) | 10.8% |
| 3 | 6 (35%) | 7 (50%) | 13 (42%) | 5.8% |
| 4a | 1 (6%) | 0 | 1 (3.2%) | Not detected |
| Mixed | 1 (6%) | 0 | 1 (3.2%) | 0.7% |

From Eyster and Hatzakis.[35]

positive and of these 69% had non-progressive liver disease, and only 7% showed cirrhosis.[41] The authors conclude that that there is slow progression of HCV infection in HIV-negative haemophilic patients. In a study of 4865 men and boys with haemophilia in the UK, it was shown that mortality from liver disease was 16.7 times higher than in the general population. However, the cumulative risks for those infected with HCV alone was relatively lower at 1.4% (all ages) compared with 6.5% for those co-infected with HIV.[42] A further study also has demonstrated that HCV singly infected individuals show only slow progression.[40] Thus, after all patients in a cohort of 310 would have been infected with HIV (125) the Kaplan-Meier progression rate to death from liver failure was 3% after 25 years of infection with HCV (Fig. 45.2). Overall, the time course of HCV mono-infection appears to be very slow.

## Age

Many studies have reported an influence of age on progression. Thus, in a Cox proportional hazards model the relative hazard of death for individuals co-infected, compared to infection with HCV alone, was 0.9, 3.5 and 9.7 for the age groups at infection of 10–19, 20–29 and >30 years, respectively.[40] A large study from the UK has also shown a strong influence of the age at infection and progression of liver disease. However, the influence of age may be reflecting time since infection.[42]

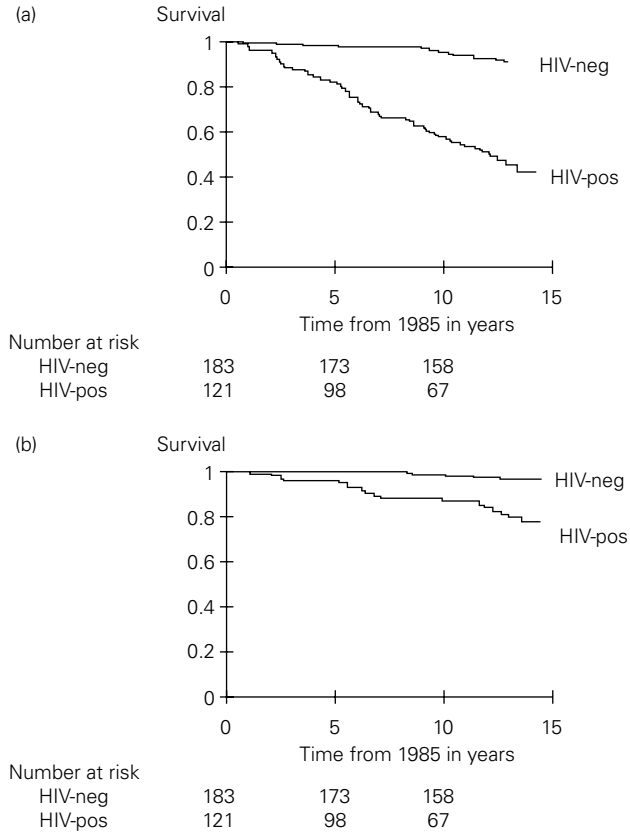


**Figure 45.2** Kaplan-Meier progression rate to death in HIV-positive and -negative patients: (a) all cause death, (b) liver death. From Yee *et al*.[40]

## Co-infection with HIV and HCV

Co-infection in haemophilia has been reviewed recently.[43] There have been several studies in haemophilia which have considered co-infection with HCV and HIV. Among 151 patients with haemophilia (85 HIV-positive and 72 HIV-negative) the rate of end-stage liver disease was greater among HIV-positive than HIV-negative individuals (relative risk 3.72).[44] In the study of 310 patients from London,[40] 125 were co-infected with HIV and of the patients who died 77% (20/26) were also infected with HIV. The Kaplan-Meier rates to death and liver-related death were increased in co-infected individuals (Fig. 45.2). Furthermore, using Cox proportional hazard models, the adjusted relative hazard of death for individuals co-infected with HIV compared with those infected with HCV alone was 19.47.[40] The study amongst haemophilic patients infected with HCV in the UK also showed higher mortality from liver-related disease in co-infected patients than those infected with HCV alone.[42]

More recently, a study in a cohort of 383 co-infected Spanish haemophilic patients showed that 18 (5%) died of end-stage liver disease – this represented 19% deaths before 1988, 4% during 1988–89, in 1990–1991 and 1992–1993, 2% in 1994–1995, 10% in 1996–1997 and rising to 33% in 1998–2001. Thus, the reduction in death attributable to highly active retroviral therapy for HIV has accounted for an increasing proportion of deaths from end-stage liver disease in this cohort in recent years[45] (Fig. 45.3).

The way in which HIV infection, which occurred 10 years after HCV infection in the haemophilic population, influences the progression of HCV is unclear. HCV viral loads have been shown to be higher in HIV-infected individuals.[46,47] The viral load is a reflection of the rate of viral replication and the rate of viral clearance in the infected host. HCV is an RNA virus with a high mutation rate leading to the generation of quasi-species. The high viral loads found in the face of HIV infection suggest that there is difficulty in containing the virus in the face of immunosuppression. It has been postulated that the progressive fall in CD4 counts, defective CD4 proliferation and CD4 apoptosis may weaken the lim-

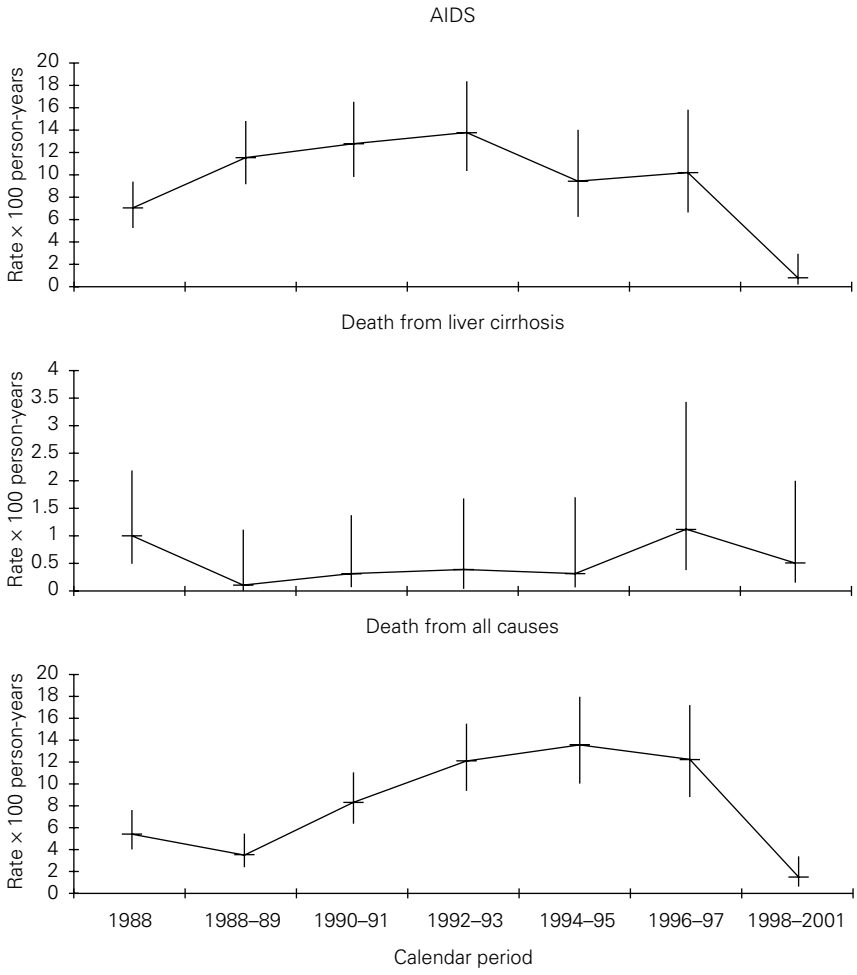


**Figure 45.3** Crude incidence rates per 100 person-years by calender period in HIV-positive haemophilic individuals. From Quintana *et al*.[45]

ited immune response to HCV.[48] HIV is also a potent activator of the immune response through the production of cytokines which are known to promote liver fibrosis.

HCV genotype 1 has been shown to be associated with more rapid progression to cirrhosis than other genotypes.[40,41] In one study, the adjusted relative hazard of death was 2.7 for genotype 1 when compared with other genotypes,[40] and in another study genotype 1 was associated with more advanced liver disease.[41] Interestingly, genotype 1 has also been shown to hasten the progression of HIV disease.[49]

## Cryoglobulinaemia

An association between HCV and cryoglobulins has been shown in both haemophilic and non-haemophilic patients with HCV. Serum cryoglobulins have been detected in 19–55% of patients. In the study reported from Italy, 24% of patients developed cryoglobulins after an average 14 years with clinical symptoms in 17%. Type 1 HCV was more commonly associated with cryoglobulins.[41] An anecdotal case report showed symptomatic cryoglobulinaemia with vasculitis, nephritis and arthritis at the time of HCV serocoversion in a patient with haemophilia.[50]

## Hepatocellular carcinoma

HCV infection is recognized as a risk factor for the development of hepatocellular carcinoma (HCC). This was first described in haemophilic patients in 1991 when a worldwide survey showed the incidence to be 30 times greater than the normal population.[51] Two cases were reported in a population of 102 who were infected on average for 25 years.[41] Five deaths were reported in the UK haemophilic population occurring over a period from 1969 to 1985. This represented a mortality from liver cancer 5.6 times greater than for the normal population.[42] In the study from London, 3 of 310 patients had developed HCC a median 22 years from time of HCV infection.[40] It is clear that the risk increases with the time from HCV infection.[42] It is likely that with the widespread use of antiviral agents for the treatment of HCV this end-stage complication will be overcome.

### Sexual transmission of HCV

Sexual transmission of HCV is an important issue for haemophilic patients, their families and those involved in their health care. Studies on HCV-infected patients and their partners indicate that transmission has been uncommon (Table 45.2).[52–59] Although 13 partners showed infection, seven of 13 had other risk factors. The transmission of HIV was also higher. There was an increased risk of transmission of HCV if the index case was infected with HIV. HIV has been shown to increase the risk of HCV transmission in non-haemophilic individuals.[60] Thus, the present evidence suggests that the transmission of HCV does occur between partners but is a rare event, compared with the transmission of HIV. The sexual route is the most likely route, although this has not been proven. Transmission is much more likely in prolonged relationships and in the presence of advanced liver disease and HIV infection where the viral load is often high. Many haemophilic couples have used barrier methods of contraception for years because of fear of HIV and, therefore, this may have also limited the transmission of HCV.

## Multiviral infections and interference

It is clear from many international studies that the likelihood of multiple infection depends on a number of factors, including national transfusion and treatment programmes, prevalence of infection among donor populations, when donor screening and exclusion policies were introduced, the use of virus attenuated blood products, and the vaccination of recipients. This was illustrated in a multicentre study in which 74% of HIV-infected patients with haemophilia had serological evidence of HBV and HCV infection, whereas only 31% of HIV-negative patients were similarly affected. The former group included patients who were older and who were exposed to untreated concentrate.[27]

Multiple infection by hepatotropic viruses may lead to complex viral interactions and has the capacity to complicate the diagnosis and management of chronic hepatitis in patients with haemophilia. Data suggest that some viruses may inhibit the replication of another virus and ameliorate the severity of the hepatitis. This is the phenomenon of interference and it has been reviewed in the context of haemophilia.[61] An anecdotal report described viral interference in a haemophilic patient co-infected with HCV, HBV and HDV. The initial infection with HCV resulted in a delay in the appearance of markers of HBV (Fig. 45.4).[61a]

In a further study of 60 haemophilic patients: 42 had anti-HCV by enzyme-linked immunosorbent assay (ELISA); 33 of 42 were positive for HCV RNA. In the

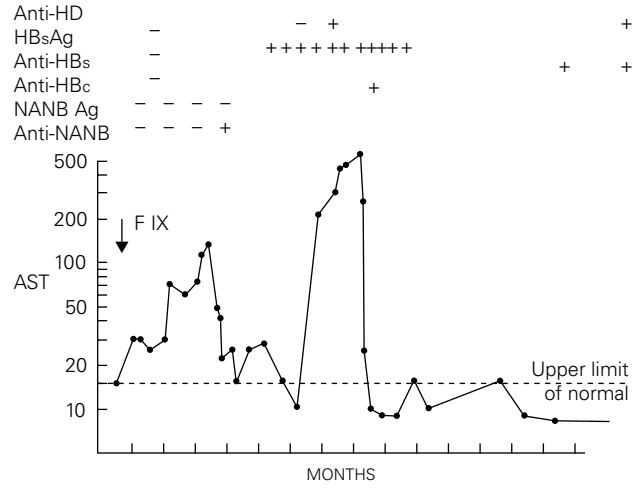


**Figure 45.4** Non-A, non-B (NANB) hepatitis and hepatitis B after exposure to factor IX concentrate. AST, aspartate aminotransferase. From Lee *et al*.[61a]

**Table 45.2** Anti-HCV and anti-HIV seroprevalence in partners of haemophilic patients

| | Patients | | Partners | |
|---|---|---|---|---|
| Reference | HCV-positive index cases | HIV-positive index cases | Partners HCV-positive (%) | Partners HIV-negative (%) |
| Kolho *et al*. (1991)[52] | 30 | NK | 0 | NK |
| Eyster *et al*. (1991)[53] | 192 | 162 | 5 (2.6) | 25 (12.8) |
| Brettler *et al*. (1992)[54] | 106 | 66 | 3 (2.7) | 4 (6.1) |
| Brackmann *et al*. (1993)[55] | 141 | 79 | 1 (1) | 5 (11) |
| Bresters *et al*. (1993)[56] | 33 | 0 | 0 (0) | 0 (0) |
| Scaraggi *et al*. (1993)[57] | 40 | 7 | 0 (0) | 1 (25)* |
| Telfer *et al*. (1992)[58] | 68 | 48 | 1 (2) | 4 (8) |
| Hallam *et al*. (1993)[59] | 104 | 58 | 3 (2.9) | 4 (7) |

NK, not known. From Telfer, [60] with permission.
*Four partners tested.

nine patients who were HCV RNA-negative, there were five individuals with HBsAg. In contrast, none of the patients who were positive for HCV RNA showed HBsAg. These studies in a small population suggest that active replication of HBV may suppress HCV.[62]

## Liver biopsy in haemophilic patients

As in other patient groups, a liver biopsy is indicated where there is doubt regarding the aetiology of the liver disease. However, the decision to perform a liver biopsy should be balanced against the cost and side-effects of the procedure. Transjugular liver biopsy using ultrasound by an experienced operator is the safest route, but this must be supported by treatment for the coagulation disorder, as shown in Table 45.3. Treatment by continuous infusion is also an option.[63]

The role of liver biopsy has been reviewed recently.[64] Since the publication of a large series of 126 biopsies in 1985,[65] where there were two deaths, this recent review summarizes the details of a number of small case series (range 6–103) representing a total of 387 biopsies. There were no fatalities and the calculated complication rate was 0.33% – similar to that in non-haemophilic patients. However, the numbers biopsied in haemophilia remain small in comparison to non-haemophilic individuals.

In the UK, national guidance has been issued on the use of ribavirin and interferon (IFN) by NICE (National Institute for Clinical Excellence). This now includes the statement 'those in whom liver biopsy poses a substantially increased risk (such as patients with haemophilia) may be treated on clinical grounds without histology'.[66]

Clearly, for individuals with type 2/3 HCV where there is >90% chance of cure with pegylated IFN and ribavarin, biopsy is redundant. It could also be argued that for type 1 patients, a trial of treatment could be initiated without the need for prior liver biopsy. Failure of response at 3–6 months using monitoring with transaminases and HCV PCR would require cessation of treatment.

Thus, although liver biopsy can be performed safely in haemophilic patients, where necessary, for diagnostic purposes, the vast majority of patients can be treated and monitored effectively without biopsy.

## Liver transplantation

The ultimate treatment for liver failure is liver transplantation, and for patients with haemophilia this results in cure of the inherited coagulopathy. The Pittsburgh group performed the first liver transplantation in a patient with haemophilia A and end-stage liver disease in 1985.[67] Post-transplant normal circulating factor VIII levels were sustained following discontinuation of FVIII concentrate supplementation at 18 hours. This was followed in 1987 by successful transplantation in a patient with haemophilia B.[68] In 1988, details of 26 haemophilic patients who were known to have been transplanted in Europe and the United States between 1985 and 1996 were reported.[69] The 3-year survival in HIV-negative and HIV-positive patients was 83% and 33%, respectively. At the time of this report, overall five of twenty HIV-negative and five of the six HIV-positive patients had died.

More recently, liver transplantation has been reported in 11 haemophilic patients treated at a single UK centre.[25] This report describes in detail 11 of 16 patients referred between 1990 and 2001 (Table 45.4). The mean age of the patients was 46, nine had haemophilia A and two had haemophilia B. Factor concentrate was administered using a continuous infusion regimen following initial bolus dosing. Concentrate infusion was stopped at a median of 36 hours (range 24–72 hours) post-transplant. Nine patients survived for a median of 5 years (6 months to 11 years). One patient died 6 years post-transplant of myocardial infarction and another of liver failure secondary to HCV infection following a second transplant because of HCV cirrhosis. Overall, five further patients developed HCV post-transplant.

It is significant that the HIV-positive patient who was transplanted was well with continued suppression of circulating HIV load on anti-retroviral triple therapy.[70] The authors conclude that the outcome of liver transplantation in haemophilic patients is good and is associated with relatively little morbidity.

## Prevention of hepatitis

Selection of low-risk donors and the screening of blood

**Table 45.3** Treatment regimen for liver biopsy in haemophilic patients

| | |
|---|---|
| Day 0 | |
| Pre-biopsy dose | Give calculated dose of VIII/IX to increase factor VIII/IX to 1.0 U/mL (100%) |
| Dose 2 – pm | (Factor VIII.) Further infusion to increase factor VIII to 1.0 U/mL (100%) |
| Day 1 | |
| Assay factor VIII/IX | Give calculated dose of VIII/IX to increase factor VIII/IX to 1.0 U/mL (100%) |
| Day 2 | |
| Assay factor VIII/IX | Give calculated dose of VIII/IX to increase factor VIII/IX to 0.5 U/mL (50%) |

From Makris *et al.*[63]

donations are important measures to minimize the risk of blood-borne viruses and must be implemented and continually improved. However, these measures are not sufficient to abolish viral transmission because a few infected donations will escape the screening process. Even though the fractionation procedures currently used to produce high purity coagulation factor concentrates improve safety by mechanically removing some of the viral burden, they are not sufficient to abolish transmission. Virucidal methods are now obligatory for the licence of concentrates in many countries. There are three main virucidal methods: (1) terminal heating of the lyophilized products at 80 °C (dry-heating), (2) heating in solution at 60 °C (pasteurization) in the presence of stabilizers or in moisture with hot vapour under high pressure, and (3) adding a solvent/detergent mixture during the manufacturing process. The risk of transmission of the hepatitis viruses has been markedly lowered but not completely eliminated. These methods are quite effective against HBV and HCV, but HAV can be transmitted rarely because solvent detergent is not effective against this virus (Tables 45.5 and 45.6).[8]

## Vaccination

The immunization of patients with bleeding disorders differs from that of the normal population with respect to the risk of haematoma formation at the vaccination site and the unusual risks associated with the potential and past exposure to blood products. Thus, it is recommended that vaccinations should be given subcutaneously.[71] Individuals should be tested for hepatitis immunity prior to vaccination due to previous community-acquired hepatitis A or hepatitis B infection through the use of non-virus-inactivated clotting factor concentrates.

### Hepatitis A vaccination

It is important to protect recipients of plasma-derived coagulation factor concentrates, because there have been several outbreaks of hepatitis A among recipients of

**Table 45.4** Liver transplantation in haemophilia: patient characteristics

| Patient | Haemophilia diagnosis | Age at transplant (years) | Date of transplant | Child Pugh status at transplant | HCV genotype | Acute rejection | Comments/post-transplant progress |
|---|---|---|---|---|---|---|---|
| 1 | Mild A | 46 | 20/03/1990 | B | – | Yes<br>No treatment | Died of myocardial infarction09/09/96 |
| 2 | Mild A | 57 | 23/10/1990 | A | 2a/2c | No | HCC pre-transplant/ cyclosporine-induced CRF |
| 3 | Mild A | 43 | 28/05/1992 | B | 3a | Yes<br>Treated Pred ×1 | Burkitt's of terminal ileum 1995 |
| 4 | Severe B | 52 | 06/04/1996 | C | 3b | Yes<br>Treated Pred ×1 + switch CyA-Tacro | 1997 central retinal vein thrombosis |
| 5 | Moderate A | 40 | 16/06/1996 | B | – | No | MRSA chest infection. Delayed graft function |
| 6 | Mild A | 52 | 24/09/1996 | B | 1b | Yes<br>Treated Pred ×1 | – |
| 7 | Mild A | 44 | 19/11/1998 | B | 2b | No | – |
| 8 | Moderate B | 59 | 05/01/1999 | B | 1a | Yes<br>No treatment | Acute renal failure, anastomotic biliary stricture. CMV infection at 7 weeks. Incisional hernia |
| 9 | Severe A | 43 | 02/07/1999 | B | 3a | Yes<br>No treatment | HIV-positive. Pneumonia. Postop incisional hernia |
| 10 | Severe A | 37 | 29/03/2000 | C | – | No | HCC pre-transplant. Retratransplanted May 2001. Died at 3 months |
| 11 | Mild A | 46 | 24/03/2001 | B | – | Yes<br>No treatment | HCC pre-transplant |

HCC, hepatocellular carconoma; MRSA, methicillin-resistant *Staphylocccus aureus*; CMV, cytomegalovirus; HIV, human immunodeficiency virus.
From Wilde *et al.*[25]

**Table 45.5** Main blood-borne viruses transmitted by coagulation factor concentrates

| Virus | Genome | Lipid-enveloped | Size (nm) | Solvent/detergent-resistant | Heat-resistant |
|---|---|---|---|---|---|
| Human immunodeficiency virus type 1 (HIV1) | RNA | Yes | 80–100 | No | No |
| Hepatitis A virus (HAV) | RNA | No | 27 | Yes | No |
| Hepatitis B virus (HBV) | DNA | Yes | 42 | No | No |
| Hepatitis C virus (HCV) | RNA | Yes | 35–65 | No | No |
| Hepatitis D virus (HDV) | RNA | Yes | 35 | No | No |
| Parvovirus B19 | DNA | No | 20 | Yes | Yes |

From Mannucci.[8]

**Table 45.6** Cumulative results of HIV safety studies carried out in anti-HIV negative haemophilic patients

| Number of patients studied | Virucidal method | Number of seroconverters | Confidence intervals of the risk of seroconversion (%)* |
|---|---|---|---|
| 210 | Pasteurization | 0/210 | 0–1.5 |
| 81 | Vapour heating | 0/81 | 0–3.7 |
| 245 | Solvent/detergent | 0/245 | 0–1.2 |

*Expressed as one-sided 95% confidence intervals around the true risk of anti-HIV seroconversion. From Mannucci.[8]

contaminated factor VIII.[16–19] Also patients with chronic hepatitis C may develop severe (decompensated) liver disease if they are co-infected with hepatitis A.[72] The hepatitis A vaccine is only licensed for use in patients over 1 year of age, and as hepatitis A is rarely symptomatic below the age of 8–10 years it is advised to wait for the patient's first birthday. It has been shown that children and adults react well to vaccine given subcutaneously.[26,73] However, in a study which included HIV-infected individuals the immune response was related to CD4 count.[74]

## Hepatitis B vaccination

Despite the advances that have been made in blood donor screening and concentrate sterilization, haemophilic patients continue to be at higher risk of acquiring HBV infection. For this reason, HBV vaccination is recommended. Hepatitis B vaccine is conventionally given intramuscularly, but subcutaneous vaccination has been shown to be effective in haemophilic patients to avoid the risk of haemorrhage.[75–77] Most vaccines use recombinant technology. An accelerated vaccination schedule can be used at 1, 2 and 6 weeks with good effect.[73]

Those infected with HIV respond to vaccine with a lower titre of antibody and may lose antibody much earlier.[77–79]

There is currently debate on the need to monitor anti-HBs levels and give booster hepatitis B vaccinations.[71] It is likely that once a person has been vaccinated and documented to have a good response (i.e. anti-HBs >100 IU/dL) a booster should be given every 5 years.

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# Chapter 46
# Occupational aspects of hepatitis

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## Compensation for occupationally acquired infection

Virus infections may be transmitted in the workplace through precisely the same routes of infection as occur in the wider community at large. However, recognition that a virus (or indeed any other) infection is a particular risk of certain occupational groups raises the issue of compensation due to workers who acquire infections through exposure at work. The Industrial Injuries Scheme in the UK provides a benefit that can be paid to an employed earner because of an industrial accident or prescribed disease contracted through work. The benefit is non-contributory and 'no-fault', and is administered by the Department for Work and Pensions: the Department is advised in these matters by the Industrial Injuries Advisory Council (IIAC). The IIAC was established in 1948 under the Industrial Injuries Act 1946 and has advised the government ever since.

The most recent legislation, the Social Security Contributions and Benefits Act 1992, states that the Secretary of State may prescribe a disease where he is satisfied that the disease:

**1** ought to be treated, having regard to its causes and incidence and any other relevant considerations, as a risk of the occupation and not as a risk common to all persons; and

**2** is such that, in the absence of special circumstances, the attribution of particular cases to the nature of the employment can be established or presumed with reasonable certainty.

In other words, a disease may only be prescribed if there is a recognized risk to workers in a particular occupation, and the link between disease and occupation can be established or reasonably presumed in individual cases. In determining whether a particular disease should be prescribed, the IIAC considers whether the risk for a particular occupational group has been doubled or whether there are other factors which allow attribution in particular cases to be made.

Viral hepatitis was first prescribed in 1975 and the arrangements were revised in 1984. The current list defines Prescribed Industrial Disease (PID) 8 as 'viral hepatitis' linked to an occupation that involves contact with either (1) human blood or human blood products, or (2) a source of viral hepatitis. This would clearly apply to most health-care workers (HCWs) but does require that the infected person demonstrates that their work does involve exposure to blood or blood products or some other source of the virus.

In 2003, the IIAC recommended to the Secretary of State that the description of viral hepatitis should be amended, by dividing PID8 into two and by making the illnesses more specific.[1] Hence, PID8a would be hepatitis A linked to jobs involving exposure to raw sewage, whilst PID8b would be hepatitis B or C linked to work involving contact with human blood or blood products, or a source of hepatitis B or C virus. Other hepatitis viruses would not be considered to be prescribed for the purposes of access to state benefit. These proposed changes simply bring up to date the details of the prescribed industrial disease to match what is known about occupational transmission.

## Hepatitis A

Hepatitis A virus (HAV) is transmitted by the faecal-oral route. Person-to-person spread is the most common method of transmission, although consumption of contaminated food or water will also result in infection. The introduction of a safe and effective vaccine in the 1990s has raised the question of who should be vaccinated, and in the context of occupationally acquired infection, this has resulted in a number of studies looking at seroprevalence rates in different occupational groups. Perhaps the most obvious group of workers with potentially increased risk of acquisition of HAV infection are those who come into contact with raw sewage. Studies in Singapore, France and the USA have reported seroprevalence rates in sewage or wastewater workers more than double those of non-occupationally exposed popu-

lation groups,[2–4] and outbreaks of HAV infection have been reported in sewage workers.[5]

HCWs are another potential risk group. Nosocomial outbreaks of HAV infection involving HCWs have been reported in neonatal and paediatric intensive care units, and other ward settings.[6–9] However, seroprevalence studies in HCWs have generated conflicting data. Increased rates were reported in general HCWs,[10] physicians and dentists,[11] dental workers,[12] and nurse aides.[13] However, others found no increase for physicians[13] or nurses.[11] Staff who work with children, e.g. in day-care centres and kindergartens, may also be at risk.[11]

It is relatively straightforward to protect individuals from HAV infection by vaccination. HAV vaccine is offered selectively to those subgroups of the population at risk. However, the UK guidelines for HAV vaccination[14] for occupationally at-risk workers recommend vaccination for very few occupational groups, reflecting the uncertainties as to who is or is not at increased risk of infection indicated by the seroprevalence data discussed above. Immunization is recommended only for laboratory workers who are working directly with the virus. Routine immunization of HCWs in general is not recommended. Immunization may be indicated for staff and residents of institutions for people with learning disabilities dependent on a local risk assessment. Within child day-care centres, immunization is only recommended in the specific instance of a local community outbreak of HAV infection. Whilst immunization is not recommended for all sanitation workers, it should be considered for those who come into contact with raw untreated sewage.

## Hepatitis B and C viruses

In the particular context of viral hepatitis within occupational settings, most attention has been focused on the potential spread of the blood-borne members of the group – hepatitis B and C viruses (HBV and HCV). It is not difficult to construct a list of occupations where there is at least a theoretical risk of acquisition of one or both infections. This will comprise those whose work practices bring them into contact with blood, tissues and bodily fluids, or with inanimate objects contaminated with the same. The most obvious and largest occupational group at risk are therefore HCWs, but there are others, including paramedics, emergency responders, police and fire-fighters. Much effort has therefore been expended to devise appropriate strategies to protect such workers from occupational exposure to, and acquisition of, HBV and HCV. However, in the heath-care setting, just as it is possible for HCWs to acquire infection from their patients, there has been an increasing realization that virus may travel in the opposite direction, i.e. patients are at risk of acquiring infection from their HCWs. The remainder of this chapter will therefore deal with the issues of prevention of spread of HBV and HCV from patients to HCWs, and also from HCWs to patients.

## Occupational acquisition of HBV by workers

### Evidence of risk in different occupational groups

A number of serosurveys performed prior to the widespread adoption of HBV vaccination demonstrated convincingly an increased prevalence of markers of HBV infection in workers exposed to blood and bodily fluids. In the USA, an overview of published data concluded that the overall risk to persons employed in health-related fields was four times that of the general adult population. In particular subgroups of such workers, the risk was even higher – five to ten times for physicians and dentists, and more than 10-fold for surgeons and laboratory workers having frequent contact with blood samples.[15] Similar findings were reported for emergency physicians[16] and oral surgeons.[17]

In 1990, the Centers for Disease Control and Prevention in the United States estimated that 6500–9000 new HBV infections occurred among HCWs. Extrapolating from the known natural history of infection, this equates to the generation of 350–950 chronic carriers, with a prospective 100–150 deaths from cirrhosis and 25–40 from hepatocellular carcinoma.[18]

Surveillance of acute hepatitis B infections in the UK reveals a similar story. Whilst the average annual rate of infection in men in the whole population between 1975 and 1979 was 4 per 100 000, this rose to up to 36 per 100 000 for HCWs. Comparative rates in the years 1980–1984 were 6 and 37 per 100 000, respectively.[19,20] Again, surgeons and laboratory workers were the subgroups with the highest rates of infection.

Occupations allied to health care may also carry a risk of HBV infection. Embalmers, who commonly give a history of needle-stick injuries at work, were reported to have twice the frequency of markers of HBV infection as blood donors.[21] Other groups include staff and specialized teachers in schools for those with learning disabilities,[22] and emergency ambulance staff and paramedics.[23–25] Studies of police officers, prison officers and firemen on the whole have failed to find a significant increased risk.[26–29] Amongst professionals unrelated to health-care or emergency service workers, outbreaks of infection have been reported in butcher's shops, presumably arising through spread from an infected employee to colleagues from cuts sustained at work,[30,31] and inapparent parenteral infection arising from dermatitis and small cuts from scissors was suggested to account for an excess of HBV markers in hairdressers in Scotland.[32]

### Modes of occupational transmission of HBV to workers

The most obvious potential route of occupational transmission of HBV is via inoculation of infected blood either by needle-stick injuries (stab injuries with blood-contaminated needles), or sharps injuries (cuts with blood-contaminated scalpel or other sharp instruments). A number of studies have documented the risk of HBV transmission after accidental inoculation of infected blood. All find a significantly higher risk of clinical hepatitis (19–31%) if the source patient was hepatitis B 'e' antigen (HBeAg)-positive compared with 1–6% for HBeAg-negative sources.[33–36] Data to allow estimates of the risk of HBV transmission after other types of exposure are not available. By analogy with HIV, there must also be at least a theoretical risk of transmission by exposure of mucous membranes to blood splashes, although it is likely that this will be lower than for inoculation injuries. There are no reports of transmission of HBV via blood contact with intact skin.

Other body fluids may contain infectious viral particles, and therefore, potentially at least, pose a risk for transmission of hepatitis B. These include amniotic, pericardial, peritoneal, pleural, synovial and cerebrospinal fluids, and semen and vaginal secretions. Exposures to non-blood-stained faeces, nasal secretions, sputum, sweat, tears, urine and vomitus are not thought to pose a risk of infection.[37,38] Hepatitis B transmission has been reported following human bites,[39–41] presumably related to inoculation of the biter's blood-stained saliva. This sort of exposure may be experienced by workers with mentally disturbed or subnormal patients or by police and prison officers.

Whilst percutaneous injuries are the most obvious route of exposure to HBV, in reality, many infections acquired by HCWs result from direct or indirect blood or body fluid exposures that inoculated HBV into cutaneous scratches, abrasions, burns, other lesions or on mucosal surfaces.[42] HBV has been demonstrated to survive in dried blood at room temperature on environmental surfaces for at least 1 week.[43]

### Magnitude of risk

The risk to HCWs of acquiring HBV infection at work will depend on the prevalence of HBV carriers among the patient population, the frequency of needle-stick/sharps injuries, the risk of transmission per exposure (considered above), and the immune status of the HCW population in regard to HBV (see below, under prevention).

The prevalence of HBV carriers in the patient population will vary widely according to the population under study. The UK is a low prevalence area with an estimated hepatitis B surface antigen (HBsAg) carriage rate of 0.1%. Elsewhere, carriage rates may exceed 20%. Rates may vary within identifiable population subgroups – e.g. in the UK, in hospitals for those with learning disabilities, prevalence rates between 1 and 12% have been reported.[44]

Blood exposure incidents are, unfortunately, all too common in a hospital setting, the most obvious occurrences being during operative procedures. Studies of sharps injuries in operations report rates of 1–7%.[45–48] Risk factors include long procedures, high blood loss, major operations and wound closure with staples. Other potential exposures include glove tears and perforations,[49–51] and eye splashes with blood and other bodily fluids.[52–54] Other HCWs, including nurses, laboratory, domestic and portering staff also suffer blood exposures.[55] Widely differing rates are reported in different studies, and there may be considerable inaccuracies due to under-reporting.[56–58]

In an Italian national surveillance programme of occupational exposures to blood-borne pathogens, only 13% of exposures to a known infected source were to HBV. There were 1155 exposures to known HBsAg-positive sources in the period 1986–1998.[59] No HBV seroconversions were observed, even in 158 known susceptible HCWs, 117 of whom received post-exposure prophylaxis (PEP). The Communicable Disease Surveillance Centre of the Health Protection Agency in the UK has been running an active surveillance scheme of needle-stick injuries in HCWs, in collaboration with over 200 occupational health departments in England, Wales and Northern Ireland. In this study, incidents of HCW exposure to HBV were a small minority compared with HCV and HIV.[60] No HBV seroconversions were reported after 219 HBV exposure incidents in the period 1997–2002.

### Prevention of occupational acquisition of HBV

The principal strategies for the prevention of occupationally transmitted HBV infection are (1) vaccination of at-risk personnel, (2) institution of appropriate infection control policies, and (3) institution of appropriate policies for management of exposure incidents.

## Hepatitis B vaccine

Recombinant surface antigen-based vaccines for prevention of HBV infection have been in routine use for some years. They are demonstrably safe and efficacious, although protection from vaccination is not 100% (see below). The key issues surrounding vaccination policies are (1) who should be vaccinated, (2) what is a protective response to vaccine, (3) what is the need for boosters, and (4) how should non- or hypo-responders to vaccination be managed?

## Who should be vaccinated?

It is the recommended policy of the World Health Organization (and also of the European Union) that HBV vaccine be adopted as a universal vaccine of childhood, and this is now the case in the majority of countries around the world.[61] However, the UK currently operates a selective vaccination policy – that is, vaccine is targeted only at those subgroups of the population deemed to be at significant risk of exposure to HBV. In occupational terms, the official UK guidance[14] explicitly identifies 'Health-care workers including students and trainees', 'staff and residents of residential accommodation for those with severe learning disabilities', and a catch-all 'other occupational risk groups' including morticians and embalmers, certain sections of the police, ambulance, fire and rescue services, and prison service staff in regular contact with prisoners. With a selective policy, there is always going to be a grey area as to precisely which individuals within an organization do or do not require vaccination. In the past, this has led to considerable discussion and division. Hopefully, the experience accumulated over a number of years has now led to the adoption of more inclusive policies, rather than yet more attempts to justify withholding vaccine from certain subgroups of individuals, and the controversies over who to vaccinate have now become a non-issue.

Mandatory vaccination policies are more effective than voluntary ones. A recent Canadian report has demonstrated that between 10 and 60% of all exposure-prone procedure (EPP)-performing HCWs were not vaccinated under a voluntary system.[62] However, mandatory policies are difficult to implement, and therefore most countries rely on highly recommending HBV vaccination to HCWs, whilst reserving the right to determine the infection status of individuals who refuse vaccination.

## What is a protective response to vaccination?

The initial trials of plasma-derived vaccine demonstrated that protection acquired from vaccination was directly proportional to the level of anti-HBs achieved. Importantly, vaccinees who did not generate any detectable anti-HBs response were as susceptible to HBV infection as placebo recipients, and there is no evidence to suggest that such vaccinees may somehow be protected by non-humoral mechanisms (e.g. T-cell immunity) generated by the vaccine. However, a matter of some debate is precisely how much anti-HBs is required in order to be confident of protection. There is a strong lobby in favour of 10 IU/L as being a cut-off level above which the individual is deemed to be protected, and this is the official policy in the USA.[42] Certainly, there are vanishingly few reports of acquisition of either symptomatic acute HBV infection, or of the development of chronic hepatitis B infection in vaccinees who have attained this level of anti-HBs, although asymptomatic anti-HBc seroconversion, indicative of infection, is more common.[63] In the UK, a more cautious approach has been taken, whereby an antibody level above 100 IU/L is considered to be protective, and responses in the range 10–100 IU/L are regarded as suboptimal. One concern that led to this approach was the specificity/reliability of the anti-HBs detection assays operating at close to their lower limits of detection. A second concern is that on its own, a low level of anti-HBs may not represent a protective response to vaccination – chronic carriers of HBV frequently have low levels (i.e. <100 IU/L) of anti-HBs in their sera.[64,65] The importance of this latter phenomenon is discussed in more detail in the section concerned with prevention of HCW-to-patient transmission of HBV.

## What is the need for booster doses of vaccine?

The anti-HBs level in vaccines shows an inexorable decline over time since the last dose of vaccine. Thus, even individuals who make respectable levels of anti-HBs (>100 IU/L), as assessed 2 months after the last dose of vaccine, will eventually become anti-HBs-negative in the absence of further antigenic stimulation. Given that the degree of protection against infection is proportional to the level of anti-HBs achieved, this observation raises the possibility that protection will be lost over time as anti-HBs levels decline. Thus, initial vaccination policies built in stipulations for booster doses at various time intervals following the initial vaccination course. Some regimens tried to take into account individual variations in the half-life of anti-HBs, and therefore tailored the timing of booster doses according to serial measurements of anti-HBs, with boosters only being given as an individual's anti-HBs level declined below the 10 IU/L mark. Such policies proved to be very difficult to implement in practice, and a more pragmatic approach of universal boosting at specified time intervals, e.g. 5 years, became more commonplace. This accepted the fact that some individuals would dip below the magic 10 IU/L before receiving their booster, whilst others would still have protective levels of anti-HBs at the time of boosting, but the overall gain in efficiency of implementation of fixed interval policies outweighed these considerations. However, there is now a solid body of evidence that the immunological memory induced by the initial course of vaccination will protect against infection even if the serum anti-HBs levels have declined below the limit of detection at the time of a subsequent exposure. The exposure itself will provide a potent antigenic stimulation, leading to the induction of protective anti-HBs within 3–5 days, well within the prolonged 4–12 week incubation period of HBV infection.[66] The European Consensus Group on Hepatitis B Immunity (ECG on HBI) has

therefore argued that, for individuals who have made a demonstrably protective level of anti-HBs in response to a primary course of vaccination, there is no need for any subsequent boosters.[67] This is now the official policy in the USA.[42] In the UK, the extant guidance remains that issued in 1996,[14] which recommends a single booster dose 5 years after completion of the primary course.

## Management of non- and hypo-responders to vaccination

For almost all prophylactic vaccines in current use, no attempt is made to confirm the induction of immune responses to vaccination, even though it is known that most vaccines do not induce protection in 100% of vaccinees. However, in the specific context of HBV vaccine used as prophylaxis against occupational exposure, there is a need to check the immune response to vaccination. About 10% of otherwise healthy adults do not see the currently licensed surface antigen vaccines as immunogenic, and mount no detectable anti-HBs response. A further 10% make somewhat suboptimal responses in the range 10–100 IU/L. As non-response equates with non-protection, it is important that such individuals are aware of their non-immune status. Provided that the exposure incident is reported appropriately, they can still be offered passive protection with hepatitis B immunoglobulin (see below). The issue as to whether hypo-responders to vaccination are really at risk of acquiring HBV infection was discussed above. One possible explanation for a non- or suboptimal response to vaccination is, of course, that the vaccinee is already a carrier of HBV. This has important implications for the individual's own state of health, and for their fitness to practise within the health-care service (i.e. whether they may perform EPPs or not, see below). Thus, the need for anti-HBc or HBsAg testing must be discussed with the individual concerned.

The management of these individuals, apart from ensuring that they are aware of their status and its implications, centres around how to improve their anti-HBs levels. One obvious strategy is to offer more doses of vaccine. UK and USA recommendations are for non-responders to receive a complete second course of vaccine, and hypo-responders to receive an immediate booster dose.[14] Around 30–50% of non-responders can be coaxed into making a detectable anti-HBs by a second course of vaccine, although it is unlikely that titres will be very high.[68,69] Beyond these blanket recommendations, whether or not to continue with multiple further doses in those who still fail to mount a response is a decision which needs to be made on an individual basis. For instance, if the non-responder is a surgeon, there may be a greater degree of motivation to try to generate some anti-HBs than, say, for a psychiatrist.

A variety of strategies have been tried, mostly on a small scale, to improve anti-HBs responses to current vaccines.[70] These include the use of the intradermal (i.d.) route of vaccine administration, the addition of various immunostimulants given at the same time as vaccine doses, and the development of more immunogenic vaccines.

Large-scale prospective studies have demonstrated satisfactory anti-HBs responses to vaccine administered i.d. in comparison to the standard intramuscular route.[71–73] A number of small-scale studies have reported impressive results with the use of the i.d. route of administration in HCWs known to be non-responders to conventional courses of vaccination (see Table 46.1).[74–78] However, despite these promising results, it should be emphasized that current vaccines are not licensed for use in this way.

The effect of a variety of immunostimulants administered at the same time as vaccine has been studied, but results are generally disappointing. Addition of recombinant interleukin-2 to a booster dose of hepatitis B vaccine in previously non-responsive uraemic patients failed to improve response rates.[79] The response to i.d. vaccine of patients with end-stage renal failure was slightly augmented by the addition of erythropoeitin.[80] Interferon (IFN)-α given at the same time as a booster dose of vaccine in healthy non-responders failed to produce a sig-

**Table 46.1** Use of intradermal vaccination in healthy non-responders to intramuscular vaccine

| Reference | Intradermal regimen | n | Results |
|---|---|---|---|
| 74 | 3 doses 2.5 μg 2–3 weeks apart | 17 | 16 seroconverted, mean titre anti-HBs >100 IU/L |
| 75 | 4 doses 5 μg every 3 weeks | 25 | 22 seroconverted |
| 76 | 2–5 doses 20 μg | 9 | 8 achieved anti-HBs >100 IU/L |
| 77 | 5 μg every 2 weeks up to 5 doses | 31 | 29 achieved anti-HBs >10 IU/L |
| 78 | 5 μg every 2 weeks up to 4 doses | 6 | 6 seroconverted |

nificant increase in response rates.[81] There is a report of enhancement of anti-HBs responses by use of granulocyte-macrophage colony stimulating factor, but this was a very small trial (15 patients) with no control group.[82]

A hope for the future is that more immunogenic vaccines will appear, generating such solid responses that all issues relating to non- and hypo-responders will disappear. Vaccines containing pre-S1 and pre-S2 regions of the HBsAg, in addition to the conventional S region, may circumvent genetic non-responsiveness to the S antigen. One trial of such a triple vaccine demonstrated 70% seroconversion in 100 HCWs who had failed to seroconvert after at least four doses of a licensed HBV vaccine containing the S component only.[83] However, production of that vaccine is technically difficult, and 7 years after the initial trial, there is no sign that it will become widely available. New adjuvant formulations may significantly improve anti-HBs responses to conventional S only vaccines. One such adjuvant, AS04, which contains 3′-deacylated monophosphoryl lipid A and alum, achieved response rates in non-responders (titre <10 IU/L after four doses of conventional vaccine) of over 90% after three further doses, compared with only 68% of those who received further doses of conventional vaccine. More impressively, the titre of anti-HBs reached after the third dose was over a log higher with the AS04 adjuvenated vaccine.[84] New approaches to vaccine development include DNA vaccines. A novel DNA vaccine against HBV has been shown to induce protective levels of anti-HBs in non-responders to conventional vaccine.[85]

## Infection control policies

Approaches to reduction of hazardous exposures to blood-borne viruses (BBV) can be categorized as: (1) the taking of special precautions for patients or specimens known or suspected to be infected, or (2) the adoption of universal precautions to cover all patients and specimens, the principle of which is that the level of precautions taken depends on a risk assessment of the procedures being undertaken, rather than on the patient's infection status. The key elements of universal precautions are hand disinfection after contact with patients, use of barrier precautions (e.g. gloves, gowns, goggles as appropriate), and minimal manipulation and safe disposal of sharp instruments. In recent years, there has been a major shift towards the latter approach, one of the main reasons being that for every patient known to be infected with a BBV, there will be many more in routine clinical practice where this status is not known or even suspected. There is also evidence to suggest that knowledge of a patient's high-risk status may not reduce the risk of blood exposures during surgical procedures,[46] whilst practising universal precautions does reduce the frequency of blood exposures among trained HCWs.[86,87]

Strategies designed to prevent the occurrence of inoculation injuries are essential to reduce the risk of occupationally acquired BBV infection. Possibilities include the development of safer surgical techniques including methods of passing instruments between personnel, avoidance of the use of sharp instruments where alternatives exist (e.g. when suturing), and better training of junior surgeons using surgical rigs.[51,88,89] Other infection control measures include double gloving,[90] spectacles or visors to avoid eye splashes,[52,53] impermeable gowns to prevent skin contamination,[91] effective systems for collecting and disposing of used sharps,[92] and safer needle and syringe combinations that reduce needle handling[93] and automatically protect the needle after use.[94]

## Post-exposure prophylaxis policies

Despite great attention to education and introduction of innovative infection control policies, it is unlikely that the risk of suffering a needle-stick injury will ever be zero. Thus, an important part of any strategy dealing with occupational exposure to HBV is the development of an effective policy for management of exposure incidents. This should include testing the source patient (if known) for HBsAg and HBeAg, establishing the worker's immune status for HBV, and administering HBIg and HBV vaccine as necessary. There should be clear lines of responsibility for the management of exposure incidents, including out-of-hours arrangements.

The risk arising from an exposure will be dependent on the type of exposure, the HBV status of the source, and the HBV status of the person exposed. Guidelines in the UK[95] define a significant exposure as one which is percutaneous (needle-stick or sharps injury, bite), mucocutaneous (non-intact skin, conjunctiva or mucous membrane) if involving blood, or sexual. The HBV status of the source may be known, determinable, or unknown and not determinable. The HBV status of the person exposed is categorizable as known responder to vaccine, known non-responder to vaccine, unknown, or known HBsAg carrier. On the basis of these variables, an algorithm was constructed to indicate the appropriate post-exposure prophylaxis (PEP) needed in different circumstances, a simplified version of which is shown in Table 46.2. The options for action to be taken vary from nothing through to administration of HBV vaccine and HBIg. The efficacy of HBIg given post-exposure in the occupational setting is estimated to be of the order of 75%.[34] The most recent guidelines in the USA are provided by the Public Health Service.[42]

**Table 46.2** HBV post-exposure prophylaxis for reported exposure incidents

| | Significant exposure | | | Not significant exposure | |
|---|---|---|---|---|---|
| HBV status of exposed person | HBsAg-positive | Unknown source | HBsAg-negative | Continued risk | No further risk |
| Not vaccinated or anti-HBs response unknown | Accelerated vaccine course | Accelerated vaccine course | Standard vaccine course | Standard vaccine course | No HBV prophylaxis |
| Known vaccine responder | Booster | Consider booster | Consider booster | Consider booster | No HBV prophylaxis |
| Known vaccine non-responder | HBIg × 1 consider booster | HBIg × 1 consider booster | No HBIg consider booster | No HBIg consider booster | No HBV prophylaxis |

## Transmission of HBV from infected workers

### HBV transmission from infected workers via exposure-prone procedures

There are over 45 reports of HCW-to-patient transmission of HBV in the literature, with over 400 infected patients, and transmission rates of 6–15%.[96] This was first recognized as a problem due to outbreaks of HBV infection occurring in renal dialysis units. In the UK, between 1975 and 1990, there were 12 outbreaks of HBV infection associated with infected HCWs, with 91 infections identified (reviewed in Heptonstall[97]). In 11 of 12 instances, the HCW was HBeAg-positive (the status of the 12th was unknown). In five outbreaks where tracing and testing of exposed patients was performed (i.e. lookback), acute icteric HBV infection occurred in 1–2% of patients, with an overall transmission rate (including asymptomatic cases) of 4–9%. In a report of 20 such outbreaks in the USA involving over 300 infected patients, all 17 surgeons who were tested were HBeAg-positive.[98]

The risk of an infected HCW transmitting HBV is dependent on the type of work performed, with obstetric/gynaecological and cardiothoracic surgeons being the two most frequently implicated in HCW-to-patient transmission of HBV. Within those specialties, there are clearly high- and low-risk procedures. For one obstetrician, a lookback covering 247 patients identified transmission in 19% of 108 high-risk, 1% of 107 medium-risk and 0% of 32 low-risk procedures.[99] The term 'exposure-prone procedures' (EPPs) was coined in 1991 by the Centres for Disease Control and Prevention[98] to describe procedures where there is a risk of percutaneous injury to the HCW, and where, if such an injury does occur, the worker's blood may contact the patient's tissues or mucous membranes. This is more likely when manipulation of needles or other sharp instruments in a body cavity without clear vision or in a restricted space is necessary.

### Magnitude of risk of transmission from infected workers

In the absence of any control measures, this will depend on the prevalence of HBV infection amongst HCWs performing EPPs, and the frequency of virus transmission from such HCWs per EPP. Using an estimate of the latter of 5% for HBeAg-positive surgeons, based on the reported range of 4–9% in UK outbreaks,[97] it is possible to work out the likely number of HBV transmissions by HCWs performing EPPs occurring per year in any country, by feeding into the equation the HBV prevalence band of the country concerned, and the total number of EPPs performed per year in that country. An example of this is given in Table 46.3.[101]

### Prevention of transmission of HBV from workers

The realization that HCWs who are chronic carriers of HBV are a potential infection risk for their patients has resulted in the introduction of a variety of measures to reduce this risk and thereby protect patients. In 1981 in

**Table 46.3** Risk of transmission of HBV by prevalence band of country of origin of health-care worker (HCW)

| Prevalence band | Typical prevalence | Risk per 500 000 EPPs | Approximate risk per EPP |
|---|---|---|---|
| Low (<2%) | 0.3 | 75 | 1 in 7000 |
| Intermediate (2–7%) | 5 | 1250 | 1 in 400 |
| High (≥8%) | 10 | 2500 | 1 in 200 |

EPP, exposure-prone procedure. Estimated transmission during EPP is 5%. Adapted from Department of Health 2002.[101]

the UK, the Advisory Group on Hepatitis (AGH) recommended restriction of the working practices of individuals who had been shown to be associated with the spread of infection, but stopped short of recommending routine screening of staff or patients. This was clearly not sufficient to prevent HCW-to-patient transmission of HBV. The UK guidelines have since been modified at various intervals, with the trend towards the introduction of ever more restrictive guidelines.

The first major revision occurred in 1993.[102] The explicit intention of these new guidelines was to remove HBeAg-positive HBV carriers from the pool of HCWs allowed to perform EPPs, a decision based on the extensive evidence available at that time that HBV-transmitting HCWs were all HBeAg-positive (where HBeAg/anti-HBe testing had been performed). The guidelines stipulated that HCWs performing EPPs should:

> undergo a course of HBV vaccination and have their response checked 2–4 months after completion of the primary course; non-responders to vaccine should, after appropriate counselling and consent, be tested for HBsAg; those found to be HBV carriers should then be tested to determine their HBeAg/anti-HBe status; those found to be HBeAg-positive were then banned from performing EPPs. No restrictions were placed upon infected health-care workers who were HBeAg-negative.

The implementation of this guidance undoubtedly had a profound effect on those surgeons found to be HBeAg-positive, who were faced with the end of their surgical careers. Indeed, in one infamous incident, a surgeon substituted another blood sample for his own to hide the fact that he was HBeAg-positive. He was subsequently struck off the medical register and given a prison sentence, having transmitted infection to 24 patients.[103] There was also a significant effect on the health-care service through the loss of surgical expertise.

In the years since 1993, despite these guidelines, there have been nine HCWs who have transmitted infection to at least 15 patients, three of whom died of fulminant HBV infection (see Table 46.4). This is considerably less than the 75 transmissions per year estimated to arise in the absence of any regulatory guidelines (see Table 46.3 above), but was nevertheless disappointing. In all instances, the guidelines had been correctly instituted. The first eight surgeons in Table 46.4 were known to be anti-HBe-positive HBV carriers (and therefore entitled to perform EPPs), whilst the ninth case was an HBeAg-positive junior house surgeon who did not perform EPPs (and where, presumably, transmission occurred through failure of infection control procedures).

Detailed investigation of all transmission incidents has demonstrated the reason for failure of the guidelines – all the anti-HBe-positive surgeons harboured precore mutants and had high levels of HBV DNA. The first four cases of surgeon-to-patient HBV infection from anti-HBe-positive surgeons have been reported in detail.[104] In the light of these unexpected incidents, the AGH recommended yet further restrictions on HBsAg-positive HCWs wishing to perform EPPs. Clearly, HBeAg/anti-HBe markers are only surrogate guides to the infectivity of an HBsAg-positive individual, the true marker being HBV DNA. Options considered by the AGH included the banning of all HBsAg-positive HCWs, regardless of HBeAg/anti-HBe status, or finding some compromise policy based on HBV DNA levels. The former course of action, whilst perhaps more likely to be acceptable to the general public, was felt to be unfair to those HCWs with very low levels of HBV DNA who were likely to pose very little risk to their patients, and would result in unnecessary loss of surgical expertise. The revised guidelines, published in 2002,[105] therefore contained the following recommendations:

> HCWs found to be HBsAg-positive should have their HBeAg/anti-HBe status determined; there was no change to the guidelines for those found to be HBeAg-positive; HCWs without HBeAg should be further tested for HBV DNA; a cut-off value of $10^3$ copies per mL was set – with HCWs with DNA levels below this level allowed to continue to perform EPPs, subject to annual testing of their HBV DNA levels, and HCWs with DNA levels above this cut-off banned from EPPs.

Given the critical role of the HBV DNA level, procedures were put in place to ensure accurate testing. The assays are performed in only two designated laboratories. Two samples from each HCW are required, taken a few days apart. Each sample is tested twice, and then referred to the other laboratory for testing. The assay used is the same in both laboratories (different commercially available assays may give readings differing by as much as 0.5 $\log_{10}$), and the assays are internally controlled.[106] To date, around 400 anti-HBe-positive HCWs have been

**Table 46.4** HCW-to-patient transmissions of HBV in the UK since the 1993 guidelines

| Surgical speciality | Number infected (number died) | Exposed patients tested |
|---|---|---|
| 1. General | 1 | – |
| 2. O&G | 3 | 92 |
| 3. O&G | 1 | 111 |
| 4. General/urology | 1 | 21 |
| 5. Orthopaedic | 1 (1) | 188 |
| 6. Orthopaedic | 1 (1) | – |
| 7. Cardiac | 2 | 125 |
| 8. General | 3 (1) | – |
| 9. Surgical house officer | 2 | – |

HCW, health-care worker; O&G, obstetrics and gynaecology.

tested in this way. Around 60% have DNA levels above the cut-off and have therefore had to stop performing EPPs.

The construction and implementation of this policy has not been without controversy. Two particularly difficult issues are (1) what should the cut-off HBV DNA level be, and (2) should HCWs with DNA levels above the cut-off be allowed to take suppressive drugs such as lamivudine or adefovir, in order to bring their HBV DNA levels into the range which would allow them to carry on their operative careers?

Whatever cut-off level is set, there will always be individuals whose level falls just above. The cut-off level was set taking into account: (1) the levels of HBV DNA in those anti-HBe-positive surgeons known to have transmitted infection – the lowest recorded level was $4 \times 10^4$ copies/mL;[107] (2) the degree of reproducibility of the assays used to measure viral load, where serial testing of the same sample may yield results within a range of half a log about the mean; (3) potential variability in viral load within individuals over time – as a guide, it was estimated that viral load may oscillate by half a log either side of a mean value in a given individual over time, although there are reports that HBV DNA loads may vary by more than this in certain individuals .[108] The agreed level of $10^3$ copies per mL is felt to err on the side of caution. Other countries have opted for different cut-offs, e.g. the Netherlands excludes HCWs with HBV DNA levels $>10^5$ copies/mL.[109] All policies are kept under review, and cut-off values may need to be adjusted to take into account any further transmission episodes should they occur.

The availability of effective and safe antiviral agents capable of suppressing HBV replication creates a new dilemma for policy-makers. If anti-HBe-positive HCWs can maintain their HBV DNA levels below $10^3$ copies per mL by taking lamivudine, adefovir, or even both, then should that individual be allowed to perform EPPs? One concern would be that maintenance of safe levels of HBV DNA would then be dependent on compliance with therapy, something which cannot easily be guaranteed. A second issue is whether long-term suppressive therapy may fail due to the emergence of drug-resistant mutations, resulting in the return of unacceptably high DNA levels. Lamivudine resistance arises in around 15% of patients per year of therapy in both HBeAg-positive[110] and anti-HBe-positive patients,[111] and although current data suggest that adefovir resistance is not as common, there is no doubt that it can occur.[112] The rapidity of emergence of lamivudine resistance is dependent on the initial HBV DNA level.[113,114] Thus, in HCWs with HBV DNA levels in the range, say $10^3$–$10^5$ copies per mL, emergence of resistance may be significantly less likely than the figure quoted above. Also, when resistant mutants do appear, the HBV DNA level rises only gradually, and even then may not return to the pretreatment load. Thus, a persuasive argument can be constructed to allow anti-HBe-positive HCWs to return to EPPs whilst on suppressive therapy, with the built-in safety measures that the pretreatment viral load was no greater than $10^5$ copies/mL, and that the HCW concerned agrees to have regular HBV DNA viral load assays every 3 months. The residual risk of patients acquiring HBV infection under those circumstances must be extremely low. Such a policy would condone the use of antivirals for the sole purpose of allowing a HCW to resume his/her operative career, an indication which is unlicensed. Thus far, no regulatory authorities have adopted a policy allowing HCWs on antiviral therapy to perform EPPs.

One additional 'loophole' arising from the 1993 guidelines has also been dealt with. It is now recommended that HCWs should be screened for HBsAg prior to the onset of vaccination. HBsAg-positive individuals may harbour low levels of anti-HBs.[64,65] Thus, if only non-responders to vaccine are tested for HBV carriage, as was stipulated by the 1993 guidelines, carriers with anti-HBs levels >10 IU/L after vaccination would be allowed to proceed to EPPs without any further testing. There have been several anecdotal reports to the AGH of HBV-infected HCWs with detectable anti-HBs, with some even >100 IU/L. The importance of this loophole is tragically demonstrated by surgeon 8 in Table 46.4. As a low-level apparent anti-HBs responder, this individual received a number of booster doses of vaccine, and at one stage achieved an anti-HBs titre of 252 IU/L. It was only when he was associated with transmission of HBV to a patient that his true carrier status was identified. He was anti-HBe-positive, but with a viral load of $>2 \times 10^5$ copies/mL at the time of transmission.

Policies such as those adopted in the UK and the Netherlands, which allow known HBV-infected HCWs to continue to perform EPPs, should emphasize that any needle-stick or sharps injury to the HCW received whilst performing an EPP must be reported appropriately, regardless of the HCW's HBV DNA level. The transmission risk to the patient is not simply dependent on the HBV DNA load of the HCW – other factors, such as the amount of blood to which the patient is exposed, are important. A risk assessment must therefore be performed to determine whether or not to offer PEP (HBIg and/or HBV vaccine) to the patient.

Infected workers may occasionally pose a risk in other settings. Outbreaks of hepatitis B infection among butchers have been attributed to transmission from an infected worker.[21,115] It is important for workers in any occupation where cuts and other injuries leading to bleeding are common to be trained in proper methods of treating wounds and cleaning up any blood spillages.

## Hepatitis C

### Occupational acquisition of HCV by workers

#### *Evidence of risk in different occupational groups*

As with HBV, HCV is blood-borne. Occupational exposure to HCV infection is therefore likely to occur through the same routes as for HBV, i.e. needle-stick and sharps injuries, and mucosal splashes. Indeed, there are well-documented examples of acquisition of HCV infection by workers through needle-stick injury,[116–119] some of which have been confirmed by molecular evolutionary analysis.[120] One would expect HCWs to be the occupational group most at risk, and most data concerning prevalence of HCV infection in different occupational groups are derived from such individuals. One case-control study[121] found that health-care employment in the UK was associated with a threefold greater risk of acquiring HCV infection. However, most serosurveys report a low prevalence of anti-HCV in this group, higher than the prevalence in blood donors in some[122–124] but not all[125–127] studies, although it should be acknowledged that blood donors are not an ideal control group. Where comparisons are made to seroprevalence rates in other population groups, most reports indicate that HCWs are no more likely to have markers of HCV infection,[54,123,128,129] although there are one or two exceptions to this.[130,131] Similarly, studies that concentrate on markers of HCV infection in dental-care workers report either a slight increase[6,132] or no difference[133–136] in prevalence compared to control populations. Extensive data from the USA derived from emergency responders (including fire-fighters, paramedics and emergency medical technicians) suggest a prevalence of anti-HCV no different to that among appropriate referent groups in the general US population.[137,138] Taken together, the data in Table 46.5,[54,83,122–135,137–153] showing an absence of consistently higher HCV seroprevalence rates in these occupationally exposed groups, make it difficult to conclude that HCWs are more likely than the general population to have markers of HCV infection. This is in marked contrast to the body of evidence discussed earlier in relation to HBV infection, at least before routine HBV vaccination was introduced. One outstanding exception to this reassuring conclusion is a paper demonstrating anti-HCV positivity in 38% of 50 barbers in Sicily who shaved themselves with the same instruments as those used for their customers, as compared with 0% of 50 local blood donors.[153] However, there are no other data relating to this particular occupational group to verify or rebut these alarming data.

#### *Modes of occupational transmission of HCV to workers*

As with HBV, the most obvious routes of occupational transmission of HCV are via needle-stick or sharps injuries. There is also at least a theoretical risk of transmission by exposure of mucous membranes to blood splashes – transmission by a blood splash into the conjunctiva has been reported.[154] There are no reports of transmission of HCV via blood contact with intact skin.

#### *Magnitude of risk*

The risk of transmission of hepatitis C to HCWs depends on the same variables as previously discussed for hepatitis B virus.

(1) The prevalence of HCV infection in the patient population. This will vary considerably. In a London teaching hospital, 8.5% of 200 needle-stick source patients were positive for hepatitis C antibodies between January 1989 and January 1992, compared with 13.9% of 173 source patients tested between January 1992 and June 1993.[155] The HCV infection in one-quarter of the source patients was only discovered as a result of follow-up of the needle-stick injury. None of 102 patients in a UK hospital for patients with learning disabilities was found to have antibodies for hepatitis C.[156] The prevalence of HCV infection in dialysis units is highly variable, e.g. from 0 to 39%.[157] The clientele attending accident and emergency departments may have a high prevalence of blood-borne virus infection – of 2523 patients attending an inner-city emergency department in the USA, 18% were positive for HCV, and 5% were positive for HBsAg.[158] (2) The frequency of blood exposures among HCWs capable of allowing transmission. This was discussed in detail earlier. Published estimates of this frequency vary from annual rates of 0.1 to 5 per surgeon.[59,159] (3) The risk of transmission following an exposure to an infected source. A number of prospective studies have addressed the risk of seroconversion after a needle-stick injury from a source patient with anti-HCV antibodies – these are summarized in Table 46.6.[59,83,139,157,160–180] Adding the data from all reports in the literature, there are 39 acquisitions of HCV infection in HCWs from 2506 (1.6%) needle-stick exposures, although these figures may not be entirely accurate, as it is difficult to rule out double reporting of incidents/transmission events in studies by the same sets of investigators. The Centres for Disease Control and Prevention (CDCP) guidelines in the USA quote an average incidence of anti-HCV seroconversion after accidental percutaneous exposure from an HCV positive source of 1.8%.[181] Many countries have set up long-term surveillance schemes to monitor trends in HCW exposure to and transmission of

**Table 46.5** HCV seropositivity rates in occupational groups

| Country | Year | No. positive/ no. tested | % | Comparison group (if any) and comments | Reference |
|---|---|---|---|---|---|
| (a) Health care workers | | | | | |
| Germany | 1992 | 6/1033 | 0.6 | 5/2113 (0.24%) blood donors | 122 |
| Italy | 1992 | 45/495 | 4.8 | 39/3575 (1.1%) blood donors<br>58/576 (10.1%) factory workers | 128 |
| | 1995 | 61/3073 | 2.0 | | 139 |
| | 2003 | 39/1800 | 2.2 | Higher than blood donors, lower than general population | 123 |
| | 1996 | 12/472 | 2.5 | 8/285 (2.8%) healthy individuals | 54 |
| USA | 1993 | 23/167 | 1.4 | ↑ if markers HBV infection, blood Tx needle-stick | 140 |
| | 1993 | 7/943 | 0.7 | 0.4% blood donors | 124 |
| | 1995 | 7/770 | 0.9 | ↑ if surgeon | 141 |
| UK | 1994 | 3/1053 | 0.28 | | 83 |
| | 1997 | 4/1949 | 0.2 | | 142 |
| | 2001 | 30/10654 | 0.28 | | 143 |
| France | 1996 | 9/557 | 1.6 | | 144 |
| | 1996 | 2/328 | 0.7 | 0/112 (0.0%) other workers | 130 |
| Belgium | 1994 | 5/120 | 4.1 | Haemodialysis nurses, ↑ with ↑ years on dialysis ward | 145 |
| Sweden | 1994 | 6/880 | 0.7 | Same order as first-time blood donors | 125 |
| Hungary | 2001 | 13/477 | 2.7 | ↑ with age | 146 |
| India | 1994 | 0/90 | 0.0 | | 147 |
| | 2000 | 0/200 | 0.0 | | 148 |
| Pakistan | 1996 | 4/95 | 4.2 | 13/91 (14%) controls | 129 |
| Syria | 2001 | 6/189 | 3.2 | 1% in general population | 131 |
| Lebanon | 2001 | 2/502 | 0.4 | Comparable to local blood donors | 126 |
| Turkey | 2003 | 2/702 | 0.3 | 23/5670 (0.4%) blood donors | 127 |
| S. Africa | 2002 | 7/402 | 1.8 | | 149 |
| Argentina | 1994 | 7/439 | 1.6 | | 150 |
| (b) Dental-care workers | | | | | |
| UK | 1997 | 2/167 | 1.2 | 0.5% estimate in general population | 132 |
| | 1992 | 0/94 | 0.0 | | 133 |
| USA | 199? | 7/343 | 2.0 | Oral surgeons ↑ with age, years in Thomas practice | 134 |
| | | 2/305 | 0.7 | Dentists practice ↑ with age, years in Thomas practice | |
| | 1991 | 8/456 | 1.75 | 1/723 (0.44%) blood donors | 151 |
| Swiss | 2001 | 1/1056 | 0.1 | 0.5–1.0 estimated blood donors | 135 |
| (c) Emergency responders | | | | | |
| USA | 2003 | | 1.3–3.6 | No difference from appropriate referent groups in general US population | 137 |
| | 2002 | 5/406 | 1.2 | Fire-fighters | 138 |
| | | 2/274 | 0.7 | Corrections personnel | |
| | | 0/29 | 0.0 | Police officers | |
| | 1999 | 64/2136 | 3.0 | Fire-fighters | 152 |
| | 1991 | 9/437 | 2.1 | Fire-fighters | |
| | 1992 | 5/382 | 1.3 | Fire-fighters and emergency medical technicians | |
| | 2000 | 35/1314 | 2.7 | Fire department personnel | |
| | 2000 | 5/154 | 3.2 | Paramedics | |
| (d) Others – barbers | | | | | |
| Italy | 1995 | 14/37 | 38.0 | 0/50 local blood donors | 153 |

Tx, transfusion.

**Table 46.6** Risk of HCV infection following parenteral occupational exposure to blood from infected patients

| Reference | Year | Location | No. of parental exposure | No. of HCV infections | % infected | Comments |
|---|---|---|---|---|---|---|
| 160 | 1991 | Japan | 110 | 3 | 2.7 | |
| 161 | 1992 | Italy | 30 | 0 | 0 | |
| 162 | 1992 | Spain | 81 | 0 | 0 | |
| 163 | 1992 | Italy | 117 | 3 | 2.6 | |
| 164 | 1992 | Japan | 68 | 7 | 10.0 | HCV RNA-positive source |
| 165 | 1993 | Italy | 30 | 0 | 0 | |
| 166 | 1993 | Japan | 88 | 3 | 3.4 | |
| 167 | 1994 | USA | 50 | 3 | 6.0 | |
| 168 | 1994 | Spain | 53 | 1 | 2.0 | |
| 157 | 1994 | Italy | 61 | 0 | 0 | |
| 83 | 1994 | UK | 24 | 0 | 0 | |
| 139* | 1995 | Italy | 97 | 1 | 1.0 | |
| 169* | 1995 | Italy | 436 | 4 | 0.9 | 4/331 hollow-bore needles, 0/105 sharps |
| | | | 85 | 0 | 0 | Mucocutaneous exposures |
| | | | 125 | 0 | 0 | Skin contaminations |
| 170* | 1995 | Italy | 61 | 0 | 0 | |
| 171 | 1996 | Japan | 56 | 3 | 5.4 | |
| 172 | 1997 | Japan | 56 | 2 | 5.4 | |
| 173 | 1998 | Spain | 443 | 3 | 0.7 | |
| 174 | 1998 | Japan | 251 | 4 | 1.6 | |
| 175 | 1999 | Pakistan | 53 | 2 | 3.8 | |
| 176 | 1999 | Kuwait | 24 | 0 | 0 | |
| 59* | 1999 | Italy | 3076 | 12 | 0.39 | Percutaneous |
| | | | 557 | 2 | 0.36 | Mucocutaneous |
| | | | 473 | 0 | 0 | Non-intact skin |
| 177 | 2001 | Italy | 257 | 0 | 0 | 80% of exposures were percutaneous |
| 178 | 2002 | Italy | 68 | 0 | 0 | |
| 179 | 2002 | Holland | 23 | 0 | 0 | |
| 180 | 2002 | Taiwan | 14 | 1 | 7.1 | |

Adapted and reproduced, with permission, from Henderson 2003.[189] Some patients may overlap (refs 160 and 166). *Some patients may be counted more than once in these studies by the same set of investigators.

blood-borne viruses. In the HPA surveillance study in the UK referred to earlier, in the period 1997–2002, there were 498 HCWs who were known to have been exposed to an HCV-positive source and from whom follow-up data were available. Percutaneous injuries accounted for 70% of exposures, nearly two-thirds from hollow-bore needles. There were 2 seroconversions, although in 62 HCWs, it was not clear whether a 6-month follow-up sample had been tested or not.[60]

In the most recent reports from the Italian Surveillance scheme (SIROH), 41 participating hospitals reported transmission of HCV arising from 12/3076 percutaneous (0.39%, 95% CI 0.20–0.68), 2/557 mucous (0.36%, 0.04–1.29) and 0/473 (0%, 0–0.78) non-intact skin contaminations.[59] In the particular setting of cardiothoracic surgery, a total of 987 percutaneous and 255 mucocutaneous exposures were reported, of which 257 source patients were anti-HCV-positive. No seroconversions were observed.[177] Using estimates of the probabilities that: (1) a HCW might sustain a percutaneous injury during a surgical procedure, (2) the source patient is HCV-infected, and (3) the virus is transmitted to the surgeon after a single exposure, Yazdanpanah *et al.*[159] constructed a mathematical model from which they estimated a risk between one in 1000 and one in 10 000 for a surgeon acquiring HCV infection from an infected patient. These figures equate to between two and twenty surgeons out of a total of 20 000 in France acquiring HCV infection each year.

### *Prevention of occupational acquisition of HCV*

The cornerstone of any strategy to reduce the risk of HCWs acquiring HCV infection from their patients must be the institution of appropriate infection control

measures, including the use of safe practices to reduce hazardous exposures. This topic was discussed earlier in relation to prevention of hepatitis B transmission from patient to HCW, and the comments therein apply equally well to prevention of HCV transmission. Prevention strategies include ensuring adequate education of HCWs about phlebotomy and intravenous cannula insertion, availability of suitable sharps-disposal containers, introducing safety cannulas, reducing unnecessary use of sharps through less invasive techniques, using blunt needles for suturing, wearing double gloves, and using injury-avoidance work practices when handling sharps.[182] However, even the most rigorous set of infection control guidelines will not prevent completely the occurrence of needle-stick accidents and other potential exposures. Thus, there is a need to consider the post-exposure management of a HCW who has suffered an exposure to blood, whether from a patient of known or unknown HCV status. In contrast to the situation with HBV, there is no vaccine for either pre- or post-exposure active immunization. Also, passive protection with pooled gamma-globulin is not recommended,[181] although there are data pre-dating the characterization of HCV that immune serum globulin prophylaxis reduced the risk of post-transfusion non-A, non-B hepatitis,[183] and there is a provocative report of prevention of sexual transmission of hepatitis C by use of intramuscular immune serum globulin.[184] There are now a number of reports of successful treatment of acute HCV infection with IFN therapy.[174,185–187] Rates of virus clearance when treatment is initiated early following infection are very much higher than in patients with chronic HCV infection. A multicentre German study reported viral clearance in 43 of 44 (98%) such patients, including 14 whose route of acquisition of infection was a needle-stick injury.[186] A French study reported that IFN induced clearance of virus in 12 of 13 patients with acute infection.[187] These encouraging data therefore allow construction of a rational approach to the management of HCV-related needle-stick exposures.

The UK recommendations[188] state that a baseline serum sample from the recipients of such incidents should be stored. Where possible, serum from the source patient should be tested for evidence of infectivity (i.e. HCV RNA). Further samples from the recipient should be taken at 6, 12 and 24 weeks, for HCV RNA and anti-HCV testing. If any of these samples are positive, then comparison with the baseline sample will indicate whether the infection arose from the needle-stick injury. As soon as there is evidence of patient-to-HCW transmission of HCV, the HCW should be referred to an appropriate specialist for consideration of early IFN therapy, and the associated high chance of viral clearance. The only management issue that remains difficult to categorize is precisely when therapy should be started. There is at least a 25% chance that an acutely infected HCW will spontaneously clear the infection, thus sparing him/herself 6 months' worth of IFN therapy, with its attendant side-effects and cost. Thus, there may be some benefit in delaying therapy for, say, 3 months after diagnosis, in order to assess whether spontaneous clearance might occur.

These principles of management are broadly in line with those recommended by American[189] and Australian authorities,[182] although details as to the precise timing and frequency of recipient sampling and antibody and HCV RNA testing may vary. In the period following the percutaneous exposure, the current CDC recommendations state that 'health care professionals exposed to HBV- or HCV-infected blood do not need to take any special precautions to prevent secondary transmission during the follow-up period. However, they should refrain from donating blood, plasma, organs, tissue or semen'.[181] Allowing HCWs to continue their normal professional practice is sensible for two reasons. Firstly, the chances of the HCW becoming infected are low, as is the risk of onward transmission from the HCW to patients (see below). Should the HCW become HCV-infected, the identification and appropriate post-exposure management of patients on whom the HCW performed EPPs during the follow-up period would not be difficult. Secondly, it would be totally impracticable to operate any other policy in this regard. The effect of occupational health departments standing down all surgeons post-needle-stick injury until such time as acquisition of HCV infection had been ruled out would be chaotic, given the unpredictable nature of needle-stick injuries and their unfortunate frequency in HCWs. This would also have the effect of discouraging surgeons from reporting exposure incidents.

Guidelines for the post-exposure management of HCWs who suffer blood exposures can only be implemented if the HCW concerned reports the incident to the appropriate authority. As mentioned under hepatitis B, there is evidence that reporting of such incidents is suboptimal, even after high-risk exposures.[56–58]

## Transmission of HCV from infected workers

### *Evidence of HCV transmission from infected workers*

Hepatitis C can be transmitted from infected HCWs to patients during EPPs, in a similar way to hepatitis B. A number of HCW-to-patient transmissions of HCV infection have been reported (see Table 46.7).[30,109,190–192] The UK has the largest number of incidents, with five surgeons having transmitted HCV infection to patients, although only one of these has been published in full thus far.[190] Using the latest data from the five lookbacks available from the Department of Health, there have been 14 or 15

**Table 46.7** Transmission of HCV infection from health-care worker to patient

| Reference | Location | Surgeon | No. transmissions/no. procedures | % |
|---|---|---|---|---|
| 30 | Spain | Cardiothoracic | 5/222 | 2.3 |
| 190 | UK | Cardiothoracic | 1/278 | 0.36 |
| 191 | Germany | O&G | 1/2286* | 0.04 |
| 192 | Germany | Orthopaedics | 1/207 | 0.48 |
| Unpublished | UK | O&G | 8/3900† | 0.21 |
| Unpublished | UK | General | 3/1400† | 0.21 |
| Unpublished | UK | General | 1–2/578‡ | 0.17–0.35 |
| Unpublished | UK | O&G | 1/243 | 0.4 |
| Unpublished | US | Reconstructive | 0/268§ | 0.0 |
| Unpublished | US | Cardiac | 3/unknown¶ | |

O&G, obstetrics and gynaecology.

*Transmission occurred in 1/489 high-risk and 0/1850 low-risk procedures.

†The denominators in these two reports are rounded figures, as exact number of patients studied in lookback is not available.

‡Two lookback patients tested positive for anti-HCV, one being the index case that led to the lookback. It is not clear yet whether the surgeon is thought to be the source for the second case.

§Reported as personal communication from MJ Alter in 109.

¶Listed in Table 7 of ref. 109.

identified transmissions from 6919 patients (0.20–0.21%, see Table 46.7).

The transmission rates for each surgeon are remarkably consistent (Table 46.7), with all except the Spanish cardiothoracic surgeon being <0.5%. Taking all reports together, the best-case transmission rate is 23/10180 (0.23%, assuming only one transmission from the third UK surgeon, and taking into account all procedures performed by the German gynaecologist), and the worst-case rate is 24/8383 (0.29%, counting both cases from the third UK surgeon, and only the high-risk procedures of the German gynaecologist).

In addition to these well-documented surgeon-to-patient incidents, there have been a number of transmissions associated with anaesthetists. The first of these occurred in Australia, where five patients who underwent surgery during the same session were identified as having HCV infection (two presented with acute hepatitis 5 and 7 weeks after surgery, the other three were discovered by lookback). It has been suggested, although not proven, that transmission occurred due to contamination of the reuseable part of the anaesthetic circuitry via coughing up of respiratory secretions from the first HCV-infected patient on the list, who was known to have other risk factors for HCV.[193] A Spanish anaesthetist infected over 200 patients as a result of contamination of the patients' narcotics used by the HCW for self-injection,[194] whilst an Israeli substance-abusing anaesthestist infected over 30 patients by the same mechanism.[195] A German anaesthesiology assistant acquired HCV infection occupationally, and then transmitted this to five other patients through lack of adherence to infection control protocols.[196] More recently, in the USA, an anaesthetist transmitted to a patient (A) 3 days before he himself became jaundiced. A lookback exercise identified 2/348 patients (A+B) treated by him as having the same virus. It appears likely that he acquired HCV infection from patient B and then transmitted it to patient A.[197] An outbreak of HCV infection in attendees of an endoscopy practice in New York City arose from the anaesthetist re-entering a multidose vial with the same needle and syringe. All 12 newly infected patients had procedures that followed one chronically infected patient, over the subsequent 3 days.[198]

### *Modes of occupational transmission of HCV from workers*

In retrospective investigations, it is often impossible to identify the precise mechanism of transmission of a blood-borne virus. The assumption, for the surgeons at least, is that the surgeon suffers a percutaneous injury, perhaps not apparent at the time, leading to recontact of the surgeon's blood with the patient's open wound. The incidents arising from infected anaesthetists, and indeed, well documented nosocomial outbreaks of HCV,[199] serve to illustrate that unspecified breakdowns in infection control procedures are also a major risk factor for HCV transmission.

### *Magnitude of risk of transmission from infected workers*

In the light of proven HCW-to-patient transmission of HCV, Ross and colleagues[200] have constructed a model to determine the risk of occurrence of this adverse event. This was based on the premise that the risk to an individual patient of acquiring HCV infection from an infected HCW during an exposure-prone procedure is the product of the probabilities that (1) the surgeon is infected with

HCV, (2) the surgeon might suffer a percutaneous injury during the procedure, (3) an HCV-contaminated instrument might recontact the wound, and (4) HCV may be transmitted as a result of the exposure. Using published data for each of the parameters, they conclude that for a surgeon of unknown HCV status, the risk of transmission in any one operation is of the order of one in 135 000 to one in 1.2 million. As with any model, the accuracy of the calculated risks is dependent on the accuracy of the underlying assumptions, and the authors acknowledge that their model has certain limitations. However, it does allow comparison of the risk of HCW-to-patient transmission of HCV with that of the risks for HBV and HIV transmissions. For known infected surgeons, the calculated risks per operation are one in 1750 to one in 16 000 for HCV, one in 400 for HBV, and one in 40 000 for HIV.[200] A simpler approach to estimating transmission risk was adopted by the UK Department of Health.[101] On the basis that one in 200 surgeons currently practising in the UK is infected with HCV (i.e. the same prevalence as in the general population), and that an infected surgeon transmits infection in one in 400 (0.25%) operations (a reasonable assumption, see Table 46.7), then the chances of an individual patient acquiring infection from a surgeon of unknown status is one in 80 000. Thus, if half a million exposure-prone procedures are performed annually, somewhere between five and ten patients per year will acquire HCV infection through this route.

### *Prevention of transmission of HCV from workers*

Constructing sensible guidelines for the prevention of HCW-to-patient transmission of hepatitis C is fraught with difficulties. In contrast to hepatitis B, there is no vaccine available for pre-exposure prevention, and no passive immunization for post-exposure protection. A balance must be struck between protecting the patient on the one hand, and safeguarding the rights of HCWs on the other. The two extreme options are as follows.

**1** To take no action. This policy acknowledges that some HCWs are infected with HCV, and therefore there will always be a background rate of HCW-to-patient transmission of HCV. However, the risk to any individual patient undergoing an EPP is low (e.g. one in 80 000 in the UK, see above), and in any case much lower than other recognized surgical complications such as a wound infection or acquisition of methicillin-resistant *Staphylococcus aureus* (MRSA). Given that at least one-quarter of infected patients would be expected to clear virus spontaneously, and that perhaps only 20% of chronically infected patients develop cirrhosis over a period of 20 years, the annual risk of a life-threatening event arising from such acquisition of HCV is extremely small (e.g. one in $10^7$). For the UK, such a policy would amount to an acceptance of 5–10 HCV infections per year arising through this route.

**2** To screen all HCWs who perform EPPs for HCV infection, and exclude all those who are chronically infected. This would result, in the UK, in the removal of one of 200 such HCWs, representing an enormous loss of expertise. The net effect on the nation's health would be far more detrimental than in scenario (1) above, as surgical waiting lists would increase owing to the absence of trained surgeons. Furthermore, in the absence of a prophylactic vaccine, HCWs would be at risk of acquiring HCV infection from their patients (e.g. a one in 1000 annual infection rate).[159] Thus, there would be no alternative but to continue screening all HCWs performing EPPs at intervals, most likely on an annual basis. This raises a sword of Damocles over the career of every such HCW, with the possibility that they may have to end their chosen career at any time, and would undoubtedly have a negative effect on recruitment into EPP-performing specialties. Finally, if all HCWs were being screened for evidence of HCV infection, it is likely that pressure would be brought to bear to introduce screening of all patients. Not only would this be costly, but it would inevitably lead to discrimination against patients found to be HCV-infected.

There are proponents of each of these extreme policies to be found in the literature. In the United States, mandatory testing of surgeons and other HCWs for HCV is not recommended as a measure to protect patients.[201] However, Mele and 43 co-authors from the Italian Association for the Study of the Liver suggest that 'Healthcare workers who directly perform invasive procedures must undergo serological testing and … those … positive for … anti-hepatitis C virus and hepatitis C virus RNA must abstain from directly performing invasive procedures' ,[96] although they then benignly suggest that 'no other limitations in their activities are necessary'.

Suggested alternatives to these two extremes include introducing mandatory tests for HCV in HCWs at enrolment, but not preventing any HCW found to be HCV-infected from performing their duties.[202] A recent European Consensus Group discussed the issues surrounding both HBV- and HCV-infected HCWs.[109] National guidelines for HCV-infected HCWs were identified for only five (Belgium, Germany, Italy, UK, USA) of the 13 countries represented at the meeting. The Group recommended that HCWs who perform EPPs should know their HCV status – ideally before they begin their EPP posts. The report is somewhat opaque as to whether this injuncture should apply to all HCWs currently performing EPPs, or only to those entering an EPP-based career. It is difficult to see how such a policy of 'test but don't ban' might alleviate the problem of HCW-to-patient transmission of HCV, and it creates the additional dilemma of whether a HCW who knows him/herself to be infected

with HCV is required to inform his/her patients of this in advance.

In the UK, the recommendations of the Advisory Group on Hepatitis, subsequently adopted by the Department of Health, represent a traditional compromise.[203] HCWs who are known to be infected with HCV are banned from performing EPPs. There is no recommendation for blanket screening of all HCWs performing EPPs, although any HCW with reason to suspect that he/she might be infected by way of having a risk factor for infection is encouraged to be tested. However, screening is introduced for all HCWs entering training in a specialty which requires them to perform EPPs. The aim of this is to block the recruitment of HCV-infected individuals into the pool of HCWs who perform EPPs. However, for these HCWs, it is clearly an advantage to know their HCV status at this stage, as any infected individuals can then make an informed career choice. Implicit in this policy is the realization that a small number of patients (5–10 per year) will acquire HCV infection from infected HCWs.

## Hepatitis D

Hepatitis D infection is rarely of occupational relevance, except in particular circumstances. Staff who work with animals experimentally infected with hepatitis D should be screened for hepatitis B infection before starting the work and immunized against hepatitis B. This is in order to protect them, indirectly, from hepatitis D infection, which they can contract only if they already have hepatitis B infection, contracted by any means. Staff working with patients who might have hepatitis D infection should in any case be immunized against hepatitis B.

## Hepatitis E

There is little information about occupational associations with hepatitis E infection. It may pose a risk for individuals whose work takes them to areas of endemicity and poor hygiene, as for hepatitis A. An outbreak of acute HEV infection has been described among military personnel in Ethiopia,[204] and in a serosurvey in Brazil, 6 of 97 gold miners from the Amazon region were found to have antibodies to hepatitis E, thought to be related to poor sanitation in the mining camps.[205]

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# Chapter 47
# Neonatal and paediatric infection

Deirdre Kelly, Elizabeth Boxall

Viral infections in neonates have a wide clinical spectrum ranging from an asymptomatic carrier to end-stage acute or chronic liver failure. Most of the hepatitis viruses that affect children have been discussed in detail elsewhere in this book. This chapter will highlight the clinical and therapeutic differences in children. Table 47.1 summarizes the important features of all of the viruses associated with hepatitis in children.

## Neonatal viral hepatitis

Neonates who are infected with hepatitis viruses may have been infected *in utero* or perinatally. The clinical presentation includes intrauterine infection, acute liver failure or an asymptomatic carrier.

### Intrauterine infection

The commonest intrauterine infections or congenital infections are the TORCH infections which are due to toxoplasmosis, rubella, cytomegalovirus, herpes or herpes simplex (Table 47.2). Less common infections include human herpes virus 6, herpes zoster or enteric viruses (echoviruses, Coxsackie A and B viruses, adenoviruses).[1]

#### *Clinical presentation and diagnosis*

The clinical presentation of intrauterine infection is similar for all forms of congenital infection which affect the liver. Most infants are small for gestational dates or are born prematurely. Jaundice and hepatosplenomegaly are constant features. Conjugated hyperbilirubinaemia occurs within 24 hours of life. Serum aminotransferases are frequently elevated (2–4 times normal), while alkaline phosphatase and gamma-glutamyl transpeptidase may be normal or only mildly elevated. Blood glucose and serum albumin may be low, particularly if the infant is premature.

Investigations should include an abdominal ultrasound scan, which will demonstrate hepatosplenomegaly. A radioisotope scan is only necessary if biliary obstruction is a feature and will demonstrate poor hepatic uptake with normal biliary excretion in congenital infection with a neonatal hepatitis. Liver histology demonstrates a neonatal hepatitis with multinucleated giant cell transformation and rosette formation. There may be extramedullary haematopoiesis and cholestasis.

#### *Toxoplasmosis*

Toxoplasmosis is not a viral infection but is an important differential diagnosis in this group of children. Infection is likely following maternal infection in the third trimester, and neonatal hepatitis is an important feature associated with central nervous system involvement with chorioretinitis, hydrocephaly or microcephaly. Intracranial lesions lead to convulsions, nystagmus and raised intracranial pressure. Spiramycin therapy may prevent progression of central nervous system and liver disease

#### *Rubella virus*

Rubella is a toga virus which causes a benign infection in older children. Infants of mothers infected in the first trimester of pregnancy are either stillborn or have a number of congenital malformations. Although congenital infection with rubella virus is rare because of widespread immunization, it is still a significant problem in the developing world.[2] The main clinical features include congenital cataracts, hepatitis, hepatosplenomegaly and congenital heart disease – which includes patent ductus arteriosus or pulmonary artery stenosis, mental retardation and sensorineural deafness. The diagnosis is based on the characteristic clinical features, the liver histology, which shows typical giant cell hepatitis, and the presence of IgM antibodies to rubella. The liver disease is usually self-limited, but occasional patients have persistent cholestasis and progress to cirrhosis.

A recent review which evaluated patients 60 years after the initial intrauterine rubella infection found that >80% were still alive. The most prominent persistent problem was mild aortic valve stenosis in 68%, but none of the survivors had persistent liver disease.[3]

**Table 47.1** Infections associated with hepatitis in childhood

| Aetiology | Antecedent history | Diagnostic investigations | Extrahepatic manifestations | Remarks |
|---|---|---|---|---|
| *Primary hepatotropic viruses* | | | | |
| Hepatitis A virus (HAV) | Neonatal intensive care unit epidemics | Anti-HAV IgM | None reported | Usually asymptomatic |
| Hepatitis B virus (HBV) | HBsAg-positive mothers: blood or blood products | HBsAg | Rash, arthritis? | Acquisition of persistent infection usually asymptomatic; rarely acute icteric or fulminant hepatitis |
| Hepatitis C virus (HCV) | Infants of HCV RNA-positive mothers, HIV-positive mothers, and/or intravenous drug abusers | HCV RNA by PCR; demonstration of HCV antigen in the liver; anti-HCV at 1 year | | More common if with high maternal viraemia (>$10^6$ copies/mL) or co-infection with HIV |
| Hepatitis D virus (HDV) | Anti-HDV-positive mothers | Anti-HDV IgM; HDV antigen in the hepatocytes | | Needs co-infection with HBV to manifest the disease |
| Cytomegalovirus (CMV) | Maternal primary or recurrent CMV infection | CMV-specific IgM; viral isolation in urine (PCR) and saliva; cytomegalic inclusion bodies in hepatocytes | Microcephaly; intracranial calcifications; chorioretinitis | |
| Epstein-Barr virus (EBV) | Maternal primary or recurrent EBV infection | Anti-EBV IgM; EBV DNA by PCR in blood | Low birth weight; congenital anomalies | |
| *Systemic viruses* | | | | |
| Rubella virus | Maternal infection, especially in the first trimester of pregnancy | Anti-rubella IgM; viral isolation from pharyngeal secretions, urine, CSF | Blueberry skin lesions; cataract; heart disease | Now uncommon in developed countries due to immunization |
| Herpes simplex virus (HSV) | Maternal HSV, especially in the genitalia | HSV-specific IgM; viral isolation from vesicular fluid, CSF, liver | Skin vesicles; keratoconjunctivitis; encephalitis | |
| Varicella zoster virus (VZV) | Maternal varicella less than 5 days before to 7 days after delivery | VZV-specific IgM, viral isolation from vesicular fluid and liver | Skin vesicles; pneumonitis | |
| Adenovirus | Vaginal delivery; premature rupture of membrane; maternal upper respiratory tract infection | Neutralizing or complement-fixing antibody to adenovirus; viral isolation in the liver | Thrombocytopenia; coagulopathy; pneumonitis | |
| Coxsackie virus | Epidemics of enteroviral disease in the community | Viral isolation in faeces, blood, CSF | Myocarditis; meningoencephalitis; pneumonitis; generalized myositis; thrombocytopenia | |
| Human immunodeficiency virus (HIV) | HIV-positive mothers | HIV proviral DNA in blood, HIV RNA by PCR; IgA anti-HIV; anti-HIV when age >1 year | Lymphadenopathy; intrauterine growth retardation | |

PCR, polymerase chain reaction; CSF, cerebrospinal fluid; HBsAg, hepatitis B surface antigen.

### *Cytomegalovirus*

Cytomegalovirus (CMV) is a DNA virus which is a common cause of congenital infection in humans, affecting 0.2–2.4% of all live-born infants, most of whom are asymptomatic. Severe infection is associated with intrauterine retardation or prematurity.[4] It accounts for 46% of neonatal hepatitis in Taiwan.[5] Seropositivity for CMV increases with age, ranging from 52% in infants aged <1 year to 60% in children between 4 and 7 years old in China. A higher rate of CMV antibodies has been observed among urban than rural children, in those who

**Table 47.2** Intrauterine infection: diagnosis and treatment

| Disease | Diagnostic test | Treatment |
|---|---|---|
| Rubella | IgM-specific antibodies | None |
| Cytomegalovirus | Urine for viral culture/PCR; IgM antibodies | Ganciclovir |
| Herpes simplex | EM/viral culture of vesical scraping | Acyclovir |
| Human herpes virus 6 | Seology, PCR | Acyclovir |
| Herpes zoster | Serology, PCR | Acyclovir |
| Human immunodeficiency virus (HIV) | Anti-HIV, PCR (EDTA blood); CD4 count | Retroviral therapy |
| Parvovirus B19 | IgM antibodies | |
| Syncytial giant cell hepatitis | Giant cell hepatitis on liver biopsy | Ribavarin |
| Toxoplasmosis | IgM-specific antibodies | Spiramycin |

EM, electron microscopy; EDTA, ethylenediaminetetraacetic acid; CD, cluster designation.

were breastfed, and among day-care centre attendees.[6] Acquisition is later in countries with better socio-economic conditions.[7]

### *Transmission*

Primary infection in the second or third trimester is likely to result in symptomatic CMV infection during the neonatal period,[8] while perinatal infection may occur through infected maternal genital secretions, urine, saliva or breast milk.[9] Infected infants excrete CMV for many years in urine and saliva and are a risk to other children and pregnant women. Infected blood products or donor organs may also be a source for CMV transmission of importance to transplant recipients.

### *Clinical features*

All infected infants have intrauterine growth retardation. Clinical features include fetal ascites, jaundice and hepatosplenomegaly and a petechial rash in 60–80%. CMV infection may affect the central nervous system, with microcephaly, intracranial calcification and chorioretinitis. Progressive sensorineural deafness, developmental delay or cerebral palsy may develop later in childhood. It is important to differentiate the neonatal hepatitis secondary to CMV from other causes of neonatal cholestasis, including biliary atresia, particularly as co-infection may exist.[10,11]

Although usually self-limiting, CMV infection may progress to hepatic fibrosis or cirrhosis[12,13] or non-cirrhotic portal hypertension.[14] Persisting neurodevelopmental abnormalities tend to be the most significant problem.[15]

### *Diagnosis*

Diagnosis is made by isolation of virus from any site (urine, saliva, bronchoalveolar lavage, blood, or liver) or by the presence of specific IgM antibody to CMV in the neonate.[16] Samples must be collected soon after birth to eliminate the possibility of post-natally acquired infection. Immunofluorescence staining using monoclonal antibody to detect the early CMV antigen following inoculation into cell cultures is a sensitive method, whereas CMV DNA detection by genome amplification techniques (e.g. polymerase chain reaction; PCR) is the most specific method in the diagnosis of CMV hepatitis, particularly post-transplant.[17] PCR can be performed on EDTA blood, urine and liver biopsies. Liver biochemistry is compatible with neonatal hepatitis syndrome and includes nuclear inclusion bodies and giant cell formation (see above).

Liver biopsy demonstrates giant cell hepatitis with multinucleated giant cell transformation. Hepatocyte necrosis, cholestasis, extramedullary haematopoiesis and fatty infiltration with minimal inflammatory cells are common, but the classic inclusion bodies of CMV are rarely seen in neonatal infection (Fig. 47.1). Occasionally, portal fibrosis and bile duct proliferation may be seen, similar to findings in extrahepatic biliary atresia.[18]

### *Prevention and treatment*

It is not possible to prevent perinatal infection, as vaccination is not available despite attempts to develop an

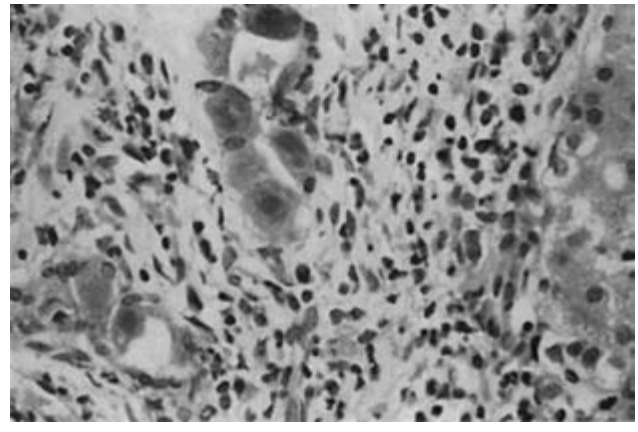

**Figure 47.1** Intranuclear inclusion bodies in CMV infection.

attenuated, live CMV vaccine, and also because infection takes place in the second or third trimester.[19,20] It is not usually necessary to treat neonatal infection as it is a self-limiting illness, but severe cases may respond to ganciclovir 7.5 mg/kg twice a day for 2 weeks followed by 10 mg/kg three times a week for 3 months.[21] CMV persists in a latent form that can be reactivated later.

#### *Human immunodeficiency virus (HIV) infection*

Infants with congenital HIV infection may present with hepatosplenomegaly, but conjugated hyperbilirubinaemia in the neonatal period is rare. Neonatal hepatitis in HIV-positive infants is more likely to be associated with concomitant congenital CMV infection.[22] Congenital HIV infection may present clinically as hepatitis with jaundice and hepatosplenomegaly typically at ~6 months of age.[23]

## Acute liver failure in the neonate

Acute liver failure in neonates is rare, but potentially fatal without liver transplantation. The aetiology includes inborn errors of metabolism, mitochondrial disorders, disorders of fatty acid oxidation, generalized sepsis and viral infection.

### Clinical presentation and diagnosis

Infants may present with intrauterine retardation, hypoglycaemia, jaundice, coagulopathy and encephalopathy within hours or weeks of birth. It is important to exclude other causes of liver failure and to treat with appropriate therapy (Table 47.3).

**Table 47.3** Causes of fulminant viral hepatitis in children

| Aetiology | Disease | Incidence |
|---|---|---|
| Neonates | | |
| | Herpes virus 6 | Rare |
| | Echovirus | Rare |
| | Adenovirus | Rare |
| | HBV | Frequent |
| | Parvovirus | Rare |
| | Coxsackie | Rare |
| Older children | | |
| | HAV | Frequent |
| | HBV | Rare |
| | HDV | Rare |
| | NA–G | Common |
| | EBV | Rare |
| | Herpes viruses | Rare |

HAV, hepatitis A virus; HBV, hepatitis B virus; HDV, hepatitis delta; NA–G, non A–G virus.

### Herpes simplex virus

Herpes simplex virus (HSV) (*Herpesvirus hominis*) is a DNA virus that causes a generalized disease with associated hepatitis. The incubation period is 2–12 days and it is transmitted by close bodily contact, or perinatally by contact with maternal body fluids.

#### *Clinical features*

Neonatal HSV infection presents with a severe multisystem disorder with encephalitis, severe hepatitis, or acute liver failure[24,25] due to either type 1 or type 2 virus, although type 2 virus shed from the infected cervix at birth is more common. Infants have progressive jaundice, hepatosplenomegaly, raised hepatic transaminase levels and coagulopathy, most commonly during the second week of life. Skin vesicles are present in 60% of adult patients, but the typical herpetic skin, mouth or eye lesions may not be present in neonates.[26] Fatal herpetic hepatitis has been described.[25,27]

#### *Diagnosis*

Diagnosis is made by the isolation of virus from vesicle swabs or other tissue/aspirates or demonstration of HSV antigens by direct immunofluorescence on vesicle swabs, nasopharyngeal aspirates, skin scrapings or biopsy tissue. Intranuclear inclusion bodies and multinucleate giant cells in scrapings or biopsy tissue are a feature of herpes viruses. The demonstration of specific neutralizing antibodies in the sera is confounded by the presence of passively transferred maternal antibody, although the demonstration of a specific IgM response would confirm infection in an infant. As with all perinatal infections, active disease in the mother and premature rupture of membranes are risk factors. Active genital lesions at the time of delivery present the greatest risk, although infection can occur in the absence of maternal lesions. Liver biopsy shows areas of necrosis with viral inclusions in intact hepatocytes.[28] Scrapings from vesicular skin lesions reveal HSV.

#### *Treatment*

Acyclovir (10–20 mg/kg per dose every 8 hours) is the drug of choice for disseminated HSV infection, with best results being obtained when treatment is started on the first day of illness. Liver transplantation may be indicated for acute liver failure if multi-organ failure is not present.[29]

### Enteric viruses

#### *Echovirus*

The enteroviruses are a rare cause of systemic viral infection in the newborn period, but are associated with severe hepatitis or acute liver failure. The incidence is highest in late summer and early autumn. Vertical infection at the time of birth is associated with more severe disease in the infant. Most infants present between 1 and 5 weeks old with lethargy, jaundice, very high levels of hepatic aminotransferases and severe coagulopathy. Meningitis may also be present. Echovirus serotypes 3, 6, 7, 9, 11, 14, 19 and 21 have all been reported in severe infections with hepatitis,[30] although echovirus serotype 11 appears to be most virulent for neonates. There is no specific treatment, but liver transplantation is effective for acute liver failure.

#### *Coxsackie virus*

Coxsackie virus is a human enterovirus which normally causes a self-limiting gastrointestinal disease. In neonates Coxsackie A and B viruses present in a similar way to echovirus with fulminant hepatitis, but with myocarditis or heart failure.[31,32]

#### *Adenovirus*

Adenoviruses are respiratory tract pathogens which also cause acute gastroenteritis, haemorrhagic cystitis, meningoencephalitis, hepatitis and myocarditis in the newborn and immunosuppressed patients.[33,34]

Neonatal adenovirus infection produces a severe illness with hepatomegaly, thrombocytopenia and coagulopathy in the first 10 days of life. Mortality with acute liver failure is of the order of 85–90%.[35] There is widespread necrosis of the liver with Feulgen-positive intranuclear target-like inclusions in the hepatocytes. On electron microscopy, paracrystalline arrays of adenovirus virions may be seen. There is no known treatment for adenovirus hepatitis, but supportive management of acute liver failure is essential. Hepatic function in survivors is normal.

### Syncytial giant cell hepatitis

Syncytial giant cell hepatitis is secondary to paramyxovirus infection. The liver disease varies with the age of the patient. In neonates, syncytial giant cell hepatitis presents with a severe hepatitis, with moderately elevated serum aminotransferases and may progress to chronic cholestasis and decompensated cirrhosis over 6–12 months. Some infants may have a chronic active hepatitis with autoimmune haemolytic anaemia. In older children, fulminant hepatic failure is common, while rapidly progressive chronic hepatitis occurs in adults.

The diagnosis depends on demonstrating the characteristic syncytial-type giant cells and viral inclusions consistent with the morphology of paramyxoviruses[36] on liver histology and electron microscopy. Spontaneous recovery is uncommon. Treatment with the antiviral agent ribavirin may be effective.[37] Most infants require liver transplantation.

## Acute hepatitis in older children

### Hepatitis A virus (HAV)

#### *Prevalence*

HAV has a worldwide distribution. It is endemic in early childhood in areas with poor living conditions and low socio-economic status. Seropositivity to HAV was noted in 70% of children 3–4 years of age in Liberia,[38] in 76% of children between 9 and 11 years in Venezuela[39] and in 98% of children between 6 and 10 years in India.[40] The mean age of infection increases with improvement in sanitation. In Thailand, with its economic growth and improved levels of public sanitation, the prevalence of antibody to hepatitis A among children 10–15 years old has fallen from 50–70% in 1977 to 20–25% in 1992.[41,42] In Hong Kong, the prevalence of anti-HAV antibody has declined over a 10-year period from 45% to 17% for subjects between 11 and 20 years of age.[43] In the UK, only 10% of individuals <15 years of age have serological evidence of past HAV infection, and the current annual incidence of hepatitis A in 5–14-year-olds is between 0.5% and 1.9%.[44]

Transmission of the disease is by faecal contamination of drinking water or food and by person-to-person contact. Epidemics and sporadic cases of HAV in developed countries are usually associated with infected food handlers and contaminated food or water.[45] Outbreaks of hepatitis A have been described in day-care centres[46] and institutions for children with learning disabilities.[47] Rarely, parenteral transmission of hepatitis A has been implicated. An outbreak of symptomatic HAV infection in both children and adult haemophiliacs caused by inadequately treated factor VIII concentrate has been reported.[48] One case of vertical transmission has been described leading to an epidemic in a neonatal intensive care unit,[49] although during an epidemic of hepatitis A affecting 431 pregnancies in Shanghai, China, no neonate had detectable anti-HAV IgM.[50]

#### *Clinical features*

Asymptomatic infection has been described in 84% of those infected between 1 and 2 years old..[51] Clinical cases

of HAV are usually seen in older children, who are more likely to be symptomatic in both developing and developed countries.[52,53] In an epidemic in the UK, only one of 43 children aged <5 years became icteric compared with one of five between eight and 10 years of age.[53]

After a prodromal period of 4–10 days, jaundice is the first sign of liver disease. The following clinical features were recorded in 281 cases (aged 3 months to 12 years): jaundice (99%), dark urine (85%), anorexia (83%), lethargy (81%), vomiting (72%), abdominal pain (64%), fever (57%), diarrhoea (18%), pale faeces (17%), rash (7%) and arthritis (5%).[54] Liver function tests returned to normal between 1 and 18 months after onset. Atypically, children with HAV infection may have clinical and ultrasonographic features suggestive of acute cholecystitis.[55] Enlargement of lymph nodes located in the hepatic hilum, pancreatic area and lesser omentum may be present in acute HAV infection.[56] The majority (99.9%) of children with HAV infection recover completely.

### *Diagnosis*

Diagnosis of acute hepatitis A is by detection of hepatitis A specific IgM class antibody in serum. Virus particles can be demonstrated by immune electron microscopy in faeces in the incubation period and early acute phase of infection. Virus RNA can be amplified by genome amplification techniques from faeces and acute phase sera for epidemiological studies.

### *Complications*

As in adults, the most serious complication of the disease is fulminant hepatic failure, with encephalopathy and coagulopathy and progressive jaundice. In one hospital-based study, fatal fulminant hepatitis A was reported in 3 of 2174 (0.14%) patients. Two were children, aged 5 and 15 years.[57] The survival of patients with grade III–IV hepatic encephalopathy with specialized intensive care was 67%.[58]

In 3–20% of patients with icteric hepatitis, a relapse may occur within 2–12 weeks after initial improvement of their liver function tests.[59] These episodes are associated with a rise in alanine aminotransferase (ALT) levels and a recurrence of jaundice. Patients usually recover completely.[60] HAV in the stools has been demonstrated during episodes of relapse, implicating HAV as the causative agent of relapsing hepatitis.[61]

### *Prevention*

Active immunization against HAV infection is now possible with the use of formalin-inactivated hepatitis A vaccine derived from the HM175 or CR 325F′ virus strains.[62] HAV vaccine was protective in 97% of 38 000 Thai schoolchildren aged 2–16 years.[41] Vaccine protection against both disease and infection occurs within 1 month after administration of two doses and persists for at least 1 year.[63] The vaccine is safe and well tolerated. The most common reaction is soreness at the site of injection.

HAV vaccine is also effective in the control of HAV outbreaks in low endemicity areas. Only one of 404 (0.25%) schoolchildren from Slovakia who were given at least one dose of 360 EIU HAV vaccine developed icteric hepatitis as compared with 8 of 157 (5%) unvaccinated children.[64] The vaccine was also more effective than post-exposure IgG prophylaxis in reducing the epidemic.

Protective antibody titres (>20 mIU/mL) in 106 vaccinees persisted for at least 30 months after the third dose of vaccine, with 94% having titres >200 mIU/mL.[62] A recent consensus statement concluded that immunity lasted at least 10 years and that there was no evidence to support the need for booster doses in healthy groups of patients, although more information was needed in groups of patients at special risk.[63]

## Hepatitis B virus (HBV)

### *Prevalence*

Hepatitis B infection is a major public health problem, with an estimated 350 million hepatitis B surface antigen (HBsAg) carriers worldwide. It is primarily a disease of childhood in endemic areas of the world such as Africa and China, where 15–20% of the population are HBsAg carriers.[65] Infection in the perinatal period or early infancy occurs in 40% of infants in Taiwan, but in only 1–5% in sub-Saharan Africa. The difference is in part explained by the higher prevalence of hepatitis B e antigen (HBeAg) in Chinese (40%) than African (15%) mothers. In Africa, the majority of infections are acquired between 6 months and 6 years of age, with other family members implicated as a source of infection.[65,65a]

In areas of intermediate endemicity (Italy, Japan, Spain, Greece, Portugal), where 2–10% of the population are HBsAg carriers, infection occurs in both adults and children. In Italy, the HBsAg carrier rate among pregnant women was 5% and the prevalence of hepatitis B markers in children <11 years was 1.7%.[66] In areas of low endemicity (north-west Europe, North America, UK, Australia), infection in infancy and childhood is uncommon and <1% of the population has chronic HBV infection.[67]

### *Transmission*

Infection is either blood-borne or through contaminated body fluids. The mode of transmission is by parenteral exposure (e.g. blood transfusion) or percutaneous ex-

posure (e.g. use of unsterilized instruments, tattooing, acupuncture, ear piercing). Inapparent spread of infection may also occur through intimate contact with body secretions and skin lesions and sharing of household utensils among family members.

Maternal–infant transmission is an important means by which the virus is maintained in the population. HBsAg can be detected in up to 5% of samples of cord blood taken at delivery from HBsAg-positive mothers, which is an indication of infection *in utero*.[68–70] One important mode of infection is inoculation or ingestion by the newborn of infected maternal blood and vaginal secretions around the time of delivery. The risk of infection is 15–40% if the mother is HBsAg-positive, but increases to 70–90% if the mother is HBeAg-positive.[71] In the presence of maternal anti-HBe, the risk of viral transmission to the infant is only 10–20%.[67] However, if their offspring are infected, they are at risk of developing fulminant liver failure.[72–75] Some 70% of mothers with acute hepatitis B during pregnancy may transmit the virus if infection occurs during the third trimester of pregnancy, and 90% will do so if it occurs within 8 days of delivery. There is no increased risk of abortion or malformation.

### *Clinical features and prognosis*

More than 80% of infants born to HBeAg- and HBsAg-positive mothers become chronic carriers.[76] Rarely, infection during the perinatal period may cause an acute icteric or fulminant hepatitis.

The clinical features of acute hepatitis B infection, by either perinatal or horizontal transmission include jaundice (97%), dark urine (79%), lethargy (69%), anorexia (55%), fever (52%) and vomiting (45%). The clinical course is more insidious than hepatitis A infection.[54] Extrahepatic manifestations include arthritis (14%) and skin eruptions (10%) (e.g. maculopapular or urticarial rash). The arthritis is symmetrical and polyarticular, involving the hands and the knees, and may last for about 3 weeks. The Gianotti-Crosti syndrome has been described with hepatitis B infection and consists of fever, lymphadenopathy, hepatomegaly and a non-pruritic, erythematous papular exanthem of the face and extremities.[77]

The majority (>90%) of children with acute hepatitis B infection recover completely within 6–12 months. Out of 28 children, 96% cleared the HBsAg, and only one patient became a chronic carrier. Anti-HBs seroconversion occurred after 6 months in 77% and in 96% after 1 year.[54] A study from Italy showed that hepatitis B-infected children recovered more slowly than children infected with hepatitis A, with 30% becoming chronically infected for up to 2 years, but some of these showing later resolution of infection.[78]

Fulminant hepatitis, may occur in <1% but is more frequently observed when there is co-infection or superinfection with the delta agent[79] (see section on hepatitis D virus) or in neonates born to mothers who are anti-HBe-positive.[72] Mutations in the precore region of the HBV DNA have been implicated.[80,81]

### *Diagnosis*

Acute hepatitis B is diagnosed serologically by the presence of HBsAg in serum along with IgM antibody to HBcAg. Other markers which can be assayed to indicate progression of the acute infection are the transition from HBeAg to AntiHBe and changes in the titre of HBsAg and HBV DNA. Most acute HBV will resolve with clearance of HBsAg and development of antiHBs and natural immunity.

## Hepatitis E virus (HEV)

### *Prevalence*

The disease occurs in the form of waterborne epidemics on the Indian subcontinent, south-east and central Asia, Africa and North America. In developed countries, sporadic cases are related to HEV infection among people returning from visits to endemic areas. The incubation period is 6 weeks (range 2–9 weeks) after primary exposure. It has a low secondary attack rate among exposed household members.

Population-based studies have shown that HEV infection is infrequent among children <10 years of age. In India, where HEV is endemic, only 5 of 103 (5%) children were noted to be anti-HEV IgG-positive.[40] Similarly, in areas where HEV epidemics have not been reported, the presence of anti-HEV IgG was noted in 2 of 73 (3%) children aged 1–10 years from Hong Kong[82] and in 1 of 300 (0.3%) Taiwanase children aged 6–10 years.[83]

Co-infection with hepatitis A and hepatitis E has been reported.[82,84] Clinically overt disease is observed mostly among young adults between 15 and 40 years of age (2.95%), and the prevalence is only 1.4% in subjects under 14 years.[85]

### *Clinical features*

The clinical features of the disease are similar to acute hepatitis A or B. Of 23 Sudanese children with icteric hepatitis, concomitant symptoms included vomiting (61%), fever (48%) and arthralgia (30%).[84] A striking and unexplained feature is the severity of HEV infection in pregnant women, with a mortality rate as high as 20%. Vertical transmission has been described with significant perinatal morbidity and mortality.[86]

Complete recovery usually follows acute infection in children. Progression to chronic liver disease has not been observed. The frequency of secondary cases in household members is low. Superinfection with HEV inpatients with chronic liver disease may cause severe decompensation.[87]

### *Diagnosis*

Acute HEV is diagnosed by the presence of IgM and IgG antibody to recombinant HEV antigen.[87a] HEV RNA can be demonstrated in acute phase serum and stool by reverse transcription-polymerase chain reaction (RT-PCR). HEV RNA in serum precedes peak ALT. IgG antibody is detectable for up to 5 years after an acute infection, but the duration of persistence is not known.

### *Treatment and prevention*

There is no specific treatment for HEV infection. Recombinant hepatitis E vaccines given either orally or intramuscularly have been shown to protect experimental animals against challenge[88,89] and are now undergoing clinical trial.[90]

## Hepatitis F

In a proportion of fulminant non A–E hepatitis, small particles resembling bunya viruses or toga viruses have been observed in liver by electron microscopy. A feature of these infections is the reinfection of transplanted livers with the production of a haemorrhagic fever-like illness.[91,92] The role of these viruses is still to be established.

## Hepatitis G

Molecular methods have assisted in the recognition of further 'hepatitis' viruses. Hepatitis G virus (HGV) or GBV-C is a transfusion-transmissible agent related to hepatitis C which has been implicated in both acute and chronic infections, although most people with HGV viraemia have no biochemical evidence of any liver disease.[91,92] Vertical transmission has been described with molecular studies showing relationship between maternal and infant virus.[95] Transmission of HGV occurred at a lower rate than HCV in co-infected women, although transmission was more frequent from mothers who were also infected with HIV.[96–99] Such transmission events were not associated with abnormal liver function in babies followed up for periods up to 1 year.[100]

The prevalence of HGV infection in children has not been established. However, in a study of children who had undergone liver transplantation, the HGV prevalence was 30% and infection was persistent, but not associated with chronic hepatitis.[101]

## Other transfusion-transmissible viruses

TT virus is a transfusion-transmissible virus named after the first patient with this infection.[102] It may be spread parenterally and by the faecal-oral route. It is highly prevalent in the general population, and its role in causing otherwise unexplained acute hepatitis is not established. Another virus, which has been shown to be parentally transmitted, is SEN virus.[103] Both of these viruses have been shown to be transmitted perinatally. However, the long-term outcomes in children have yet to be described.[99,104,.105]

## Non A–E hepatitis

It is clear that acute or fulminant hepatitis may be caused by other, unidentified viruses, labelled non A–E hepatitis or non A–G hepatitis. Children and young adults are affected with a severe hepatitis illness with little spontaneous recovery. Non A–E hepatitis is the commonest viral cause of fulminant hepatic failure requiring liver transplantation in children the UK and the USA. It may be further complicated by transient bone marrow suppression or aplastic anaemia: in one series of 32 children and young adults who underwent liver transplantation for non-A, non-B hepatitis, 28% developed aplastic anaemia.[106] Furthermore, in a series of eight children with both acute liver failure and bone marrow failure, two had parvovirus B16 infection, and six had non A–G hepatitis.[107] Management is supportive, with early consideration of liver transplantation in view of the poor chance of spontaneous recovery.

## Measles virus

Measles is a 120–250-nm RNA virus of the paramyxovirus family that causes febrile exanthema in children and young adults and is transmitted by droplet infection from nasopharyngeal secretions. The presence of abnormal hepatic transaminases without jaundice is common in young children,[108,109] while hepatobiliary disease is more common in adolescents and adults.[110] Spontaneous resolution is usual. Measles has been proposed as a trigger for a type 1 autoimmune hepatitis occurring within 3 months of infection.[111]

### *Diagnosis*

Measles virus can be isolated from throat swabs and saliva samples and infection can be confirmed by specific IgM antibody.

## Parvovirus B19

Human parvovirus (HPV) B19 infection in childhood

presents with erythema infectiosum or haemolytic anaemia which may lead to an aplastic crisis. Infection during pregnancy may lead to fetal ascites in the infant. HPV B19 infection is now associated with hepatic dysfunction with raised hepatic aminotransferase enzymes. Fulminant liver failure (with and without aplastic anaemia) in association with HPV B19 is described.[107]

## Herpes viruses

The herpes viruses are a family of icosahedral double-stranded DNA viruses which may cause acute hepatitis. They often become latent, persisting in the host after primary infection despite high levels of neutralizing antibody. Reactivation occurs in immunosuppressed patients. Replication by herpes virus DNA polymerase is inhibited by antiviral agents.

## Herpes simplex viruses 1 and 2

Herpes simplex virus (HSV) hepatitis is rare outside the neonatal period in an immunocompetent host.

## Cytomegalovirus

Cytomegalovirus (CMV) usually occurs in neonates (see above) but may cause a mild illness in childhood similar to infectious mononucleosis. Hepatic involvement with raised transaminases, alkaline phosphatase and bilirubin resolves without treatment and a chronic course is unusual.

## Epstein-Barr virus

Epstein-Barr virus (EBV) is a herpes virus which causes infectious mononucleosis. The pharynx is the initial site of replication, but the virus spreads throughout the lymphatic system, where it persists for life and may be periodically reactivated. In developing countries, 90% of children are infected before 4 years of age. In affluent societies it is a disease of adolescents and adults.[112] Transmission is by oropharyngeal secretions with an incubation period of 30–40 days.

### *Clinical features*

In childhood, EBV infection is asymptomatic or associated with mild non-specific symptoms. Primary infection in adolescence or early adulthood is associated with acute infectious mononucleosis (IM) or 'glandular fever' which is characterized by pharyngitis and malaise. Splenomegaly and lymphadenopathy are present in 50%, with hepatomegaly in 20% and jaundice in 5%. Although elevated aminotransferase enzymes (up to five times normal) are detected in up to 80%, hepatitis is usually mild with a complete recovery.

Approximately 30% of cases of childhood haemophagocytic lymphohistiocytosis have been described in association with primary EBV infection.[113,113a] Fatal X-linked lymphoproliferative disease (Duncan syndrome) occurs in boys unable to mount a cytolytic T-cell response.[114]

### *Diagnosis*

The diagnosis is made by finding atypical lymphocytes in the peripheral blood, viral isolation from throat washings or by serological assays. Antibody to viral capsid antigen (VCA): IgM is positive in early acute infection, IgG is usually present at clinical presentation, then declines, but persists for life. Antibody to Epstein-Barr nuclear antigen (EBNA) increases during convalescence, persists for life and may increase during reactivation. Antibody to early antigen (EA) transiently increases during infection and reactivation.

The Paul-Bunnell test demonstrates acute non-specific antibody production in response to EBV infection. Unlike in adults, where the Paul-Bunnell test is usually diagnostic of IM, in up to 50% of children and immunosuppressed patients the heterophile antibodies are not detectable.

Detection of EBV-encoded products including latent membrane protein (LMP), nuclear antigen (EBNA) and EBV encoded RNA (EBER) by immunohistochemistry, and detection of EBV genome by *in situ* hybridization permits specific detection of EBV in infected tissues.[115] In transplant recipients, PCR detection and quantitation of EBV RNA in blood monitors viral load, so that immunosuppressive therapy can be adjusted appropriately.

Liver histology shows inflammation with mononucleocyte infiltration in the portal tracts, Kupffer cell hyperplasia, cholestasis and foci of liver cell necrosis. Progression to hepatic cell necrosis and chronic hepatitis has been reported.[116]

### *Treatment*

Treatment is supportive in most cases, but the combination of intravenous acyclovir and prednisolone may improve pharyngeal symptoms and fever in 12 of 15 (90%) patients with fulminant mononucleosis.[117]

EBV-related lymphoproliferative disease post-transplantation is a serious disease which is treated by decreasing immunosuppression, high dose acyclovir, surgical removal of localized disease, autologous T-cell therapy[118] or chemotherapy in selected cases.[119]

## Human herpes virus 6

Children are infected with human herpes virus 6 (HHV6)

in the first year of life[120] and may develop roseola infantum (exanthem subitum), characterized by a fever for 3–5 days, which subsides as a rose-pink macular rash becomes apparent.

Liver dysfunction in association with HHV6 infection includes an infectious mononucleosis-like syndrome, hepatitis and fulminant hepatic failure.[121] Serological diagnosis is based on the appearance of specific HHV6 IgM antibody or a fourfold rise in IgG titre.

Antiviral therapy is usually not required, except in immunosuppressed patients. Acyclovir, ganciclovir, cidofovir and famciclovir are all effective against herpes viruses.

## Varicella zoster

Varicella zoster occasionally effects the liver. The infection is spread by direct contact with a patient, who may transmit the disease from 1 day before onset of rash until all the vesicles have become dry. The incubation period is 10–21 days.

In older children, liver disease is unusual except in immunosuppressed children with HIV infection or post-transplant recipients who require intravenous acyclovir and famciclovir.[122,123]

### *Prevention*

Varicella zoster immune globulin (125 units/10 kg) is recommended for newborns whose mother had varicella zoster infection 5 days before to 2 days after delivery and for premature infants with exposure to chickenpox. For the immunosupressed, varicella zoster immune globulin should be given within 96 hours of exposure to a patient with varicella infection.

Attenuated live varicella vaccines are available and, if possible, varicella vaccination should be administered before transplantation or before patients undergo chemotherapy for haematological malignancies.

## HIV

HIV is a 105–120-nm RNA-containing retroviral agent that replicates by reverse transcription. It causes acquired immunodeficiency syndrome (AIDS) and impairs the immune system by attacking the helper/inducer (T4) lymphocytes and binding to the CD4 receptor. Transmission may occur by the transplacental or perinatal route or by exposure to infected blood or blood products.

The initial manifestation may be a cholestatic hepatitis,[23] as reported in seven infants who presented between 5 and 10 (median 7) months of age. All seven infants died, six secondary to HIV-related complications and one from liver failure. In a longitudinal study of 31 children with perinatally acquired HIV infection followed up from 2 to 82 months (mean 30), 18 (58%) had biochemical or histological evidence of hepatic involvement. Clinical outcome in this study showed that liver disease in HIV-infected infants is indicative of a poor prognosis. Nine (50%) infants with liver disease died from persistent multiple viral infections as opposed to only one of 13 without hepatic involvement.[124]

Histological findings include giant cell transformation (23%), CMV inclusion (17%), Kaposi sarcoma (13%), lymphoplasmacytic infiltrate (7%) and granulomatous hepatitis secondary to fungal infection (3%). Non-specific liver abnormalities included steatosis (47%), portal inflammation (24%) and periportal necrosis (10%).[125] Chronic active hepatitis with predominantly T8 lymphocytes, as determined by tissue immunochemistry, was demonstrated in five of twelve (42%) children with HIV infection.[113] The development of primary malignant fibrosarcoma of the liver has been reported in an 8-year-old African girl with HIV infection.[126]

## Flavivirus

### *Dengue haemorrhagic fever*

Dengue haemorrhagic fever (dengue shock syndrome) is a mosquito-borne infection (*Aedes aegypti*) common in the western Pacific and south-east Asia. The disease is characterized by a sudden onset of fever, myalgia and headache followed 2–3 days later by bleeding diathesis, encephalopathy and cardiovascular collapse. Definitive diagnosis is made by rising titres to dengue antigen.

Hepatitis, with hepatomegaly and transaminase elevation, is present in 90% of patients as part of multi-organ involvement. Aspartate aminotransferase (AST) levels increase on the third day of illness, reach a peak level (nine times normal) on days 7–8, and normalize 3 weeks after onset of illness. Pathological lesions in the liver are varying degrees of fatty metamorphosis, focal mid-zonal necrosis, Kupffer cell hyperplasia and the presence of non-nucleate cells with vacuolated acidophilic cytoplasm, resembling Councilman bodies in the sinusoids.[127] The dengue viral antigen may be demonstrated in the Kupffer cells. Fulminant hepatitis is rare but fatal.[128]

Survival is directly related to correction of fluid and electrolyte loss. For prevention, eradication of the mosquito vector is essential.

### *Yellow fever virus*

Yellow fever virus is a member of the flavivirus (group B arbovirus), a spherical 35–45-nm particle containing a single-stranded RNA genome.[129] It causes a disease endemic in West Africa and some parts of South America

and is transmitted to humans by the mosquito vector, *Aedes aegypti* or *Aedes africanicus*. There is an incubation period of 3–6 days, with viraemia for 5–10 days.

Diagnosis is made by viral isolation or by demonstration of viral antigen in the acute-phase blood samples. Available serological tests for antibody determination include complement fixation, haemagglutination inhibition and enzyme-linked immunoabsorbent assay (ELISA).[130]

*Clinical features*

The disease varies in severity from subclinical to fatal illness. In the severe form, jaundice, renal and cardiovascular symptoms and bleeding diathesis are present on days 4–5 of illness.

The liver is tender, but not enlarged, and splenomegaly is unusual. The mortality rate in hospitalized patients approaches 40–50%. Liver pathology reveals widespread mid-zonal coagulative necrosis with eosinophilic degeneration of hepatocytes (Councilman bodies), microvesicular steatosis and minimal inflammation. For patients who survive, hepatocellular regeneration is rapid and post-necrotic fibrosis does not occur.

*Treatment*

Treatment of yellow fever is supportive. The disease may be prevented with the administration of a live attenuated vaccine to infants 6 months or older residing in an endemic area. Travellers to endemic areas should be vaccinated at least 10 days before arrival.

### Viral haemorrhagic fevers

This group of viruses includes, yellow fever, Lassa fever, Marburg and Ebola fever and they are endemic in Africa, South America and Asia. They are characterized by massive hepatocellular necrosis and disseminated intravascular coagulation (DIC), and a very high mortality.

#### *Arenavirus (Lassa fever)*

The Lassa fever virus is a single-stranded RNA virus that infects the African house rat, *Mastomys natalensis*. The rodent excretes the virus in the urine and saliva, and humans become infected by consumption of contaminated food or water. Nosocomial infection may likewise occur by close contact with infected secretions and excretions from patients. Diagnosis is confirmed by viral isolation from the throat, urine or blood, or by detection of specific Lassa antibodies present in the second week of illness by immunofluorescence or by rising antibody titres.

Epidemics of Lassa fever have been reported in Nigeria, Sierra Leone and Liberia. Lassa fever infection during pregnancy has been reported to cause an 87% fetal and neonatal loss. The risk of maternal death is higher if the infection occurs in the third rather than in the first or second trimester of pregnancy.[131]

*Clinical features*

The clinical features are variable and range from an asymptomatic infection to a fatal disease. In children, the disease is heralded by a slowly rising temperature, headache, conjunctivitis, pharyngitis, abdominal pain and diarrhoea. Patients either recover spontaneously or deteriorate, with anasarca and a bleeding diathesis.[131a]

The liver is the main target organ, although jaundice has not been reported. Lassa virus hepatitis occurs in three phases: active hepatocellular injury (<20% necrosis), necrosis, or early recovery with regeneration. All three phases may be present at the time of death.[132]

*Treatment*

Ribavirin, a broad-spectrum antiviral agent, may be effective for Lassa virus infection.[133]

## Acute liver failure (ALF)

Viral hepatitis is responsible for most cases of fulminant hepatic failure in children of all ages.[134,135] In a recent study from the National Institutes of Health (NIH) ALF study, 63% of cases in children under 2 years of age and 35% of cases in children over 2 years old had viral or presumed viral hepatitis (personal communication, R. Squires, 2003).

### Hepatitis A

Acute HAV infection is a common cause of fulminant hepatic failure. The prevalence among patients of all ages with fulminant hepatic failure in published series has varied from 1.5% to 31% from both developing countries[136]and developed countries.[137] In the USA, HAV causes <5% of fulminant hepatic failure and only one of the 86 cases in the NIH ALF study was due to HAV (personal communication, R. Squires, 2003).

### Hepatitis B

In contrast, the overall rate of fulminant hepatic failure in acute HBV infection is estimated to be about 1%. It is uncommon to document HBV infection in children with acute liver failure from Western Europe and the USA, as demonstrated in the NIH ALF study, except in infants born to HBV-positive, HbeAg-negative mothers,[72] in endemic areas,[138] or after bone marrow transplant.[139] The prognosis of HBV-related fulminant hepatic fail-

ure is generally worse than with other aetiologies, with spontaneous recovery occurring in fewer than 20% of cases.[140,140a]

### Hepatitis C

HCV is an unusual cause of fulminant hepatic failure in children.[140] HDV and HEV have rarely been associated with fulminant hepatic failure in children, HGV and TTV do not cause fulminant hepatic failure.

Sporadic non-A–G hepatitis is diagnosed when there is evidence of acute hepatitis in the absence of markers for hepatitis virus infection, the absence of clinical and/or serological evidence of systemic infection with other infectious agents, no exposure to drugs or toxins, and negative markers of autoimmune disease.[135] The prognosis in non-A–G fulminant hepatic failure is poor, with the rate of spontaneous recovery from 5% to 30% indicating the need for early referral for transplantation.

### Other viruses

EBV may cause fulminant hepatic failure, almost always in immunocompromised hosts, with the EBV most frequently implicated. Paramyxovirus, parvovirus B19 and toga virus have been identified in some cases.

## Acute viral hepatitis in transplant recipients

### CMV

CMV hepatitis in liver transplant recipients is common and must always be considered in the differential diagnosis of spiking fever elevated transaminase levels, especially if there is no evidence of rejection on liver biopsy.[141]

#### *Prevention and treatment*

Transplant recipients should be given CMV-negative blood or blood products. Prophylaxis includes intravenous immune globulin (IgG, 0.5 mg/kg) at weekly intervals for 6 weeks and oral acyclovir for 3 months after transplantation;[142] or high-dose acyclovir for 3 months or ganciclovir (5 mg/kg twice a day for 1 week) followed by acyclovir (800 mg four times a day for 3 months).[143]

CMV is successfully treated with a combination of specific immune globulin and either acyclovir (800 mg four times a day) or ganciclovir (5 mg/kg twice a day) for 14 days.[143]

### EBV

EBV-associated lymphoproliferative disease (LPD) (Fig. 47.2) after liver transplantation has been reported in 4–11% of recipients.[144] A higher incidence has been observed in paediatric liver transplant recipients younger than 5 years of age who were seronegative pretransplant, and treated with FK506. Post-transplant lymphoproliferative disease is particularly common following small bowel transplantation, because of the intensive immunosuppression required.[145]

### Adenovirus

Adenovirus hepatitis is a severe illness in paediatric liver transplant recipients, mainly as a result of serotype 5, which has a high mortality.[146]

## Chronic hepatitis

### Hepatitis B

Chronic hepatitis B is defined as failure to clear HBsAg after 6 months and is more likely if infection occurs early in life, if it is asymptomatic, or if there is a general defect in the immune system.[147] More than 80% of infants infected during the first year of life become chronic carriers as compared with a rate of 6–10% if infection occurs after the sixth year of life.[148] HBeAg loss occurs at an annual rate of 10–16%.

In a prospective study of 420 HBsAg carriers in Taiwan in whom infection was detected from <1 month to 17 years of age and observed for 1–12 years, spontaneous loss of HBsAg occurred in only 10 patients (0.6% per year).[147] Annual HBsAg clearance rate was significantly higher in those who were anti-HBe-positive than those with HBeAg (1.7% versus 0.4%). Carrier children whose mothers were HBsAg-negative had a higher incidence of HBsAg clearance (5.6% versus 0.8%).

In perinatally infected children, HBeAg clearance was 30% by the age of 10 years, but only 4% had HBsAg clearance.[149] Liver histology in this group showed that 60% of the children had mild hepatitis and fibrosis, while 18% showed evidence of moderate to severe fibrosis despite almost normal transaminases.

Of infants who acquire HBV infection perinatally, boys have a higher risk of developing histologically aggressive disease with progression to cirrhosis,[150,150a] seven being less than 4 years of age. A male preponderance (76%) has also been found among children with HBV-related hepatocellular carcinoma (HCC).[151]

HBV infection may have an important role in the development of HCC in children. In Taiwan, 51 consecutive children with HCC had detectable HBsAg, either in the blood or in the liver tissue.[146] In Germany, seven of eleven (64%) cases of HCC in childhood had positive HBsAg serology.[152] Integrated HBV DNA sequences have been found in neoplastic liver tissues of children with previous HBV infection.[153]

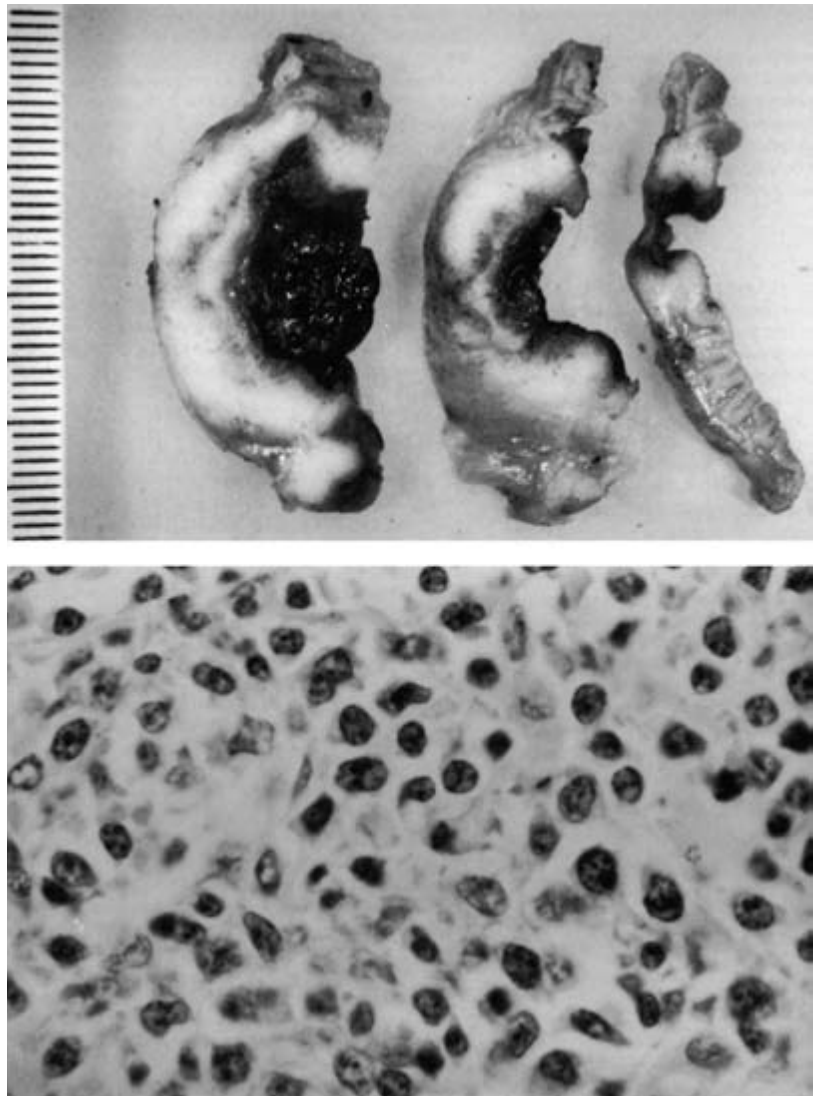

**Figure 47.2** EBV-induced lymphoproliferative disease of the gut in a child after liver transplantation. (a) Macroscopic and (b) microscopic appearance.

### *Perinatal transmission*

The risk of perinatal transmission of hepatitis B is directly associated with the infectivity of the mother, which was first demonstrated by the presence of circulating HBeAg[154] and later by direct measurement of HBV DNA.[155] Intervention such as amniocentesis[156] and delivery by caesarean section do not affect the transmission rate,[157] although lower 'microtransfusions of HBsAg' during delivery occur when elective caesarean section is chosen.[158] Studies on placental tissue show that there is evidence of cell-to-cell transfer of hepatitis B antigen by immunohistochemistry staining[159] and HBV DNA by *in situ* hybridization,[160] showing a gradient of infection from the maternal to the fetal side. These studies also showed a direct association between transplacental HBV infection and maternal HBeAg positivity (OR = 17.07, 95% CI 3.39–86.01): threatened pre-term labour (OR = 5.44, 95% CI 1.15 –25.67), higher levels of maternal HBsAg and HBV DNA, and active infection of the placenta particularly in layers close to the fetal side.[159,160] Breastfeeding does not affect the outcome in babies of HBV carrier mothers,[161] especially in the post-vaccination era.[162]

Persistent infection in neonates of HBV HBeAg-positive mothers may be associated with the transfer of maternal HBeAg, a soluble antigen, across the placenta. The HBeAg antigen shares epitopes with the virus core antigen and induces a specific unresponsiveness of helper T cells to HBcAg in the neonates.[147] It has been demonstrated that cord blood mononuclear cells from neonates born to HBeAg-positive mothers do not prolif-

erate in response to HBcAg, suggesting that transplacental exposure to HBeAg induces non-responsiveness in the developing fetal immune system.[163] HBeAg contains several sequence similarities with HBcAg, the principal target of cytotoxic T cells involved in hepatocellular damage leading to HBV clearance.[164] Such immune tolerance has been demonstrated in a transgenic mouse model and it is postulated that the function of HBeAg is to induce tolerance *in utero* and that it may be strategically important in the natural history of hepatitis B.[165,166]

### *Prevention of perinatal infection*

The options for prevention of perinatal transmission of hepatitis B are described below.

First, passive immunization using hepatitis B immunoglobulin (HBIG) was shown to protect babies of HBeAg-positive mothers, but repeated doses of globulin were required and although some babies developed active immunity, it was observed that post-natal infection led to a persistent infection as the passive protection waned.[154,167,167a]

Secondly, active immunization with hepatitis B vaccine is effective in the prevention of perinatal transmission of hepatitis B in babies of HBeAg-positive mothers. It is also as an effective means of reducing the carriage of hepatitis B in populations in areas of high and intermediate endemicity.[168,169]

In Taiwan, the prevalence of HBsAg in children <5 years old has decreased from 9.3% (31/332) in 1984 to 2% (9/457) in 1989 as a result of a mass hepatitis B immunization programme.[170] Other studies have shown that hepatitis B vaccine alone given for three or four doses during the first year of life is effective in preventing perinatal transmission.[171] This scheme of prevention has important economic implications for countries that are unable to afford hepatitis B immunoglobulin. In Gambia, where the majority of infections occur after 5 months of life, the prevalence of HBV infection in 1–14-year-old children after mass immunization with vaccine alone has decreased from 60% (474 of 791) in 1984 to 9% (57 of 629) in 1993.[172] Integrating HBV vaccination into the routine childhood immunization programme (i.e. DPT) may be the most cost-effective means of preventing infection. The introduction of mass immunization has already had an impact on the incidence of HCC in children in Taiwan.[173]

The third option is combination of active and passive immunization. Many studies have shown that the administration of hepatitis B immunoglobulin and hepatitis B vaccine within 48 hours of birth and followed by further doses of vaccine in a variety of possible schedules (e.g. 0, 1, 6; 0, 1, 2, and 6 or 12 months) has been shown to prevent perinatal transmission of HBV in up to 95% of infants of HBsAg carrier mothers.[174] Delayed first dose of the vaccine beyond 7 days increases the risk of infection.

In areas of low endemicity, the current recommended policy is the screening of all pregnant women for HBsAg. Newborns of HBsAg-positive mothers, whether they are HBeAg-positive or not, should be given specific immunoglobulin and HBV vaccine. In the UK, only babies born to mothers who are not anti-HBe-positive are given HBIG as well as vaccine based on risk assessment and in order to limit the exposure of newborns to blood products.

Universal vaccination, however, is the only effective measure to eradicate infection within populations. The choice is either to integrate hepatitis B vaccination with childhood immunizations or to vaccinate adolescents before they begin risk behaviour. Different strategies will be appropriate for different countries, according to their local epidemiology.

For infants who have received the full series of immunizations, protective anti-HBs levels (>10 mIU/mL) may persist up to 10 years of age.[175–178] An adequate anamnestic response, most frequently around 4 years, has been shown in vaccinated children, whose anti-HBs titres fall below protective levels, thus questioning the recommendation for HBV vaccine boosters at 5 years. The vaccine is ineffective in infants who acquire the infection *in utero* and are HBsAg-positive at birth.

HBV 'escape mutant' strains with alteration in the 'a' determinant of the surface protein have been described in vaccinated infants.[178a] Studies in Taiwan since the introduction of universal vaccination show an increasing accumulation of these variants in the population in children who fail to be protected by immunization.[179] Mathematical modelling studies are optimistic that the rate of accumulation of these variants and the decreasing likelihood of exposure of susceptible individuals to these variants suggest that it would take 20 years till the variants became dominant.[180]

The fourth option comprises antiviral treatment during pregnancy. Antiviral treatment during pregnancy effectively prevents mother to baby transmission of HIV[181] and has prompted the investigation of the use of lamivudine in pregnancy in women with persistent hepatitis B.[182] A single case report in which complete viral suppression on lamivudine therapy failed to protect the baby from infection despite active and passive immunization is not encouraging.[183] In contrast, lamivudine treatment during the last month of pregnancy in eight mothers with very high viraemia (>$10^9$genome equivalents/mL) reported that only one baby was not protected (12.5%), compared with a failure rate of 28% (7 of 25) in historical controls.[182] Further evaluation of lamivudine in pregnancy requires larger controlled tri-

als. Antiviral treatment in pregnancy would reduce or eliminate the use of HBIG in newborns, thus limiting exposure of children to blood products, and concentrate immune prophylaxis on vaccine alone.

### *Management of chronic HBV infection*

Chronic HBV carriers should have persistent medical supervision, in order to identify natural seroconversion, progress of liver disease, for consideration of antiviral therapy and for detection of HCC. It is important to screen and immunize family members and to provide support and education for the whole family with regard to minimizing transmission while avoiding social isolation or stigmatization.

### *Treatment*

The aim of treatment is to eradicate the virus, reduce transmission and prevent or ameliorate the underlying liver disease. The decision to treat depends on disease activity, histology, likelihood of response and treatment-associated side-effects. To date, treatment is only partially successful because of side-effects, poor efficacy and viral resistance. In children as in adults, success of treatment is based on the rates of clearance of HBeAg and of seroconversion to anti-HBe, rarely to anti-HBs. Agents for which efficacy has been claimed include interferon (IFN)-α with or without prednisolone priming and lamivudine.

#### *IFN*

IFN-α has been assessed in controlled studies, but these are difficult to compare because of small numbers and different forms of IFN-α given in different doses and for different durations. In children, the response rates to IFN-α therapy are very variable, but not dissimilar to those reported in adult studies. Differences in response rates depend on route of infection, ethnic origin, disease activity and treatment regime. Sustained clearance varies between 0 and 50% of those treated, and is usually around twice that of untreated controls.[184]

Predictors of IFN responsiveness include: active hepatitis on histology; low HBV DNA levels (<1000 pg/mL); high serum aminotransferase enzymes (more than twice upper limit of normal), short duration of disease, Mediterranean origin, horizontal transmission.[185] IFN therapy does not benefit children with perinatally acquired infection who have normal or minimally elevated aminotransferase enzymes.

IFN is limited by its side-effects, although children tolerate treatment better than adults. Fever and flu-like symptoms are invariable during initiation of treatment, and bone marrow suppression is common. Autoimmune thyroid disease and alopecia are frequent, and mental disturbance including severe depression may occur in older children. IFN treatment is contraindicated in patients with cirrhosis or prior to seroconversion, as a flare of immune activity with increased immune damage may lead to decompensation of liver function.

The transient rise in transaminases that occurs during treatment is associated with increased likelihood of HBeAg clearance. Pretreatment with corticosteroids ('prednisolone priming') and their withdrawal *prior* to commencing IFN may exacerbate the host immune response, facilitating seroconversion. In a randomized controlled trial of IFN-α with or without prednisolone pretreatment in 90 Chinese children (aged 2–17 years), slightly better results were achieved with prednisolone for 6 weeks, followed by IFN-α for 16 weeks.[186] Prednisolone priming and IFN may reduce the time to seroconversion long-term in both adults and children.[187,188] The higher rate of anti-HBe seroconversion in European children may be related to horizontal infection with higher pretreatment transaminases.

Based on European experience, consensus recommendations for IFN therapy include: children over the age of 2 years, who are HBeAg- and HBV DNA-positive, with low–intermediate HBV DNA levels and abnormal aminotransferase enzymes.[189] IFN (5 mU/m$^2$) is given three times per week for 6 months. Retreatment in nonresponders is not indicated. IFN is contraindicated in children with decompensated liver disease, bone marrow depression, severe renal or cardiac disorder and autoimmune disease.

#### *Lamivudine*

Lamivudine is a pyrimidine nucleoside analogue which is incorporated into viral DNA and competitively inhibits viral reverse transcriptase. It reduces plasma HBV DNA by 97% within 2 weeks of treatment,[190] and to undetectable levels within 4 weeks.[191] Loss of HBeAg after 1 year of treatment occurs in 17–33% of those receiving lamivudine compared with 11–13% in controls and is higher in those with elevated transaminases before treatment in both adults and children.[192,193] Prolongation of treatment for up to 3 years improves seroconversion, but increases resistance. Relapse following completion of therapy is common, but the main limitation to treatment is the development of resistance with a specific HBV mutation (tyrosine-methionine-aspartate-aspartate or YMDD mutation) in the polymerase gene.[194] The mutant virus may revert to wild-type after lamivudine therapy is withdrawn.

A dose ranging study of lamivudine in children has confirmed the dose to be 3 mg/kg/day (maximum 100 mg).[195] A subsequent international, randomized, double-blind, placebo-controlled trial of 286 children

with chronic HBV demonstrated a complete response (HBeAg clearance and undetectable HBV DNA after 52 weeks of treatment) in 23% compared with 13% with placebo.[74] The response rate was higher in those with ALT >2 (34% compared with 16%). The YMDD variant emerged in 18%. A number of small studies have compared the combination of IFN and lamivudine, but have not shown improved efficacy or tolerance.[196,197]

*Adefovir*

Adefovir is a purine analogue, which inhibits viral replication and increases natural killer cell activity and endogenous IFN activity.[198] HBV strains resistant to lamivudine and famciclovir are susceptible to adefovir.[199] A recent multicentre study of 185 adults has demonstrated histological, biochemical and virological improvement, but seroconversion rates were low.[200] A pharmacokinetic and dose-finding study in children is underway, and the agent appears to be well tolerated. A clinical trial is planned.

*Recommendations for treatment of hepatitis B infection*

The effect of treatment must be compared to the natural seroconversion rate and the potential side-effects. In a recent study, with a mean follow-up period of 4.9 ± 2.9 years, clearance of HBeAg occurred in 58% of 90 children treated with IFN, lamivudine or IFN then lamivudine compared with 37% of 68 untreated controls. Loss of HBsAg occurred in 10 treated and one untreated patient. The cumulative probability of HBeAg clearance and HBsAg clearance 10 years after the initial visit were both significantly higher in the treated patients.[201] At present, clear guidelines are available for IFN treatment, but the availability of pegylated IFN (PEG-IFN) and other antiviral agents may improve outcome.

*Hepatitis B in the immunosuppressed patient*

Patients co-infected with HBV and HIV, organ transplant recipients and chronic carriers requiring chemotherapy may respond to lamivudine,[184] either as pre-emptive treatment[202] or in the treatment of acute or fulminant hepatitis.[203]

### *Liver transplantation*

End-stage liver failure due to chronic HBV is an uncommon indication for transplantation in children and will recur unless prevented with immunoglobulin or lamivudine.[204] In fulminant HBV infection, HBV reinfection is uncommon.[205]

## Hepatitis C virus (HCV)

### *Prevalence*

HCV accounted for >90% of transfusion-transmitted hepatitis. Screening tests for anti-HCV for blood and organ donors was introduced in the early 1990s. HCV is efficiently transmitted by infected blood or organs.[206,207] Many haemophiliac children became infected with hepatitis C as the result of the preparation of clotting factor from large pools of blood products.[208] A further cohort of infected children have been identified through 'look-back' exercises carried out by blood services to identify patients infected by anti-HCV-positive donors before donor screening was introduced.[209,211] In children with haemophilia, anti-HCV seroprevalence ranged from 50 to 82%.[208] Studies on HCV-infected children identify two major groups: those infected prior to blood donor screening and those infected perinatally from HCV-infected mothers.[199,212] Potential acquisition of HCV infection was high in children exposed to multiple donors, either by receiving multiple blood transfusions or by repeated infusion of pooled products.

The reported prevalence of anti-HCV antibody in healthy children varies from 0.9% to 14.5%.[213,214] The prevalence was higher in lower than upper (16% vs 8%) socio-economic groups and in rural than urban (21% vs 12%) children.

In high-risk group children, the reported prevalence of anti-HCV ranges from 55% to 83% in thalassaemics[215] and from 80% to 95% in haemophiliacs.[216] In 5 of 27 (18.5%) anti-HCV-positive dialysis patients (age 25.9 ± 1.6 years), the length of time on haemodialysis, and hence presumably the number of units of blood transfused, was the most predictive factor for HCV infection (mean of 105 months for those who were anti-HCV-positive vs 41 months for the 22 who were anti-HCV-negative.[217] Of 50 leukaemic children with known chronic liver disease followed for 1–12 years after chemotherapy, 11 (22%) remained persistently positive for anti-HCV, 10 of whom were viraemic as determined by PCR.[218] Of 203 paediatric cancer survivors, 41 (20%) were anti-HCV-positive as detected by a combination of first-, second- and third-generation assays.[219] A retrospective analysis on the prevalence of HCV infection in 149 paediatric liver transplant recipients from France investigated 9 months to 5 years post-surgery demonstrated 14 (9%) children to be HCV RNA-positive,[220] with six having evidence of HCV infection before and eight after surgery.

Among children with chronic cryptogenic liver disease, HCV antibodies were found in 16 of 33 (48%) children in Italy,[221] and 14 of 144 (9.7%) Taiwanese children,[222] but only in one of 163 (0.6%) children in the UK.[199]

### *Perinatal transmission*

Mother to baby transmission of HCV occurs in <10% of pregnancies. The seroprevalence of HCV infection in pregnant women in the UK is estimated to be 0.2%, with an estimated 1150 pregnancies occurring each year in women infected with HCV and 70 infants acquiring infection.[223] In a study of infants born in the UK and Ireland, the vertical transmission rate was 6.7% overall, and 3.8 times higher in HIV co-infected than HIV-negative women.[224] In a systematic review of studies of transmission risks for infants born to HCV mothers, Thomas and colleagues[225] established that transmission from mothers with an HCV viraemia of $<10^6$ copies/mL was very rare. Only two out of thirty (6.6%) transmitting mothers had a viraemia of $<10^6$ copies/mL and only 8 transmissions out of 976 infants followed up were identified from non-viraemic mothers. Studies reviewed were those with adequate follow-up of the children, as defined by PCR at 6 months or serology at 18 months. The results of this review demonstrated that maternal viraemia is a more significant risk than co-infection with HIV. Mothers without HIV co-infection generally had lower HCV viraemia.[224,226–228] The systematic review also examined method of delivery and infant feeding. The conclusions were that there is no evidence of a positive effect in caesarean section, and that in HIV-negative women there is no difference in the transmission rate in breastfed and non-breastfed infants, although HCV RNA has been demonstrated in colostrum.[220] These conclusions have been confirmed by later studies published from the European Paediatric Network.[229] Recommendation for caesarian section and avoidance of breastfeeding should be considered for those women who are also HIV-positive. Transmission may be higher in mothers with transfusion-acquired infection or in intravenous drug abusers.[228]

### *Diagnosis*

HCV infection is suggested by the serological detection of anti-HCV antibody by enzyme-linked immunoassay (EIA) and confirmed by HCV RNA in children. The detection of anti-HCV is variable and may initially be absent. It may persist for up to 10 years after clearance of viraemia. It may also be undetectable in patients with HCV infection who are immunocompromised.

### *Screening the newborn infant*

Determination of HCV status in the newborn infant is difficult, as maternal anti-HCV may persist in the newborn for up to 18 months. Loss of maternal anti-HCV varies from 50% by 8 months to 95% by 13 months.[224] The estimated proportion of uninfected children who continue to have detectable maternal antibody at 18 months is <0.1%.[230]

HCV RNA detection is the only way to confirm infection in infancy. The specificity of HCV RNA detection by PCR in one study was 97% irrespective of age,[63] but the sensitivity was only 22% for infants under 1 month, but 97% after 1 month. A recent study suggests that in some babies with chronic infection, HCV RNA may remain undetectable until 8 months of age.[231]

### *Clinical features*

Children with chronic HCV infection are usually asymptomatic. Of 606 children with chronic HCV infection, only 8% had symptoms or signs, which included anorexia, weight loss, abdominal pain, hepatomegaly and splenomegaly.[212] Most children have minimal disturbance of liver enzymes, even in the presence of hepatic inflammation. In those with persistently elevated liver enzymes, the existence of other diseases, particularly hepatitis B and autoimmune liver disease, must be considered. In a multicentre study of 77 (mean age 4 years, 35 boys) Spanish and Italian children with chronic HCV infection and no underlying systemic disease, only 17 (22%) were symptomatic (asthenia, anorexia, abdominal pain) at presentation. The rest were diagnosed incidentally on routine screening.[232] At presentation, only 9 (32%) of 28 patients showed histological features of chronic active hepatitis, whereas 11 had chronic persistent hepatitis and 8 had chronic lobular or non-specific reactive hepatitis. The clinical features and outcome were similar in both the post-transfusion and community-acquired cases. During a mean observation period of 6 (1–14) years, the majority of patients remained asymptomatic (81%) with inactive histological disease (29/40, 72%), but only 10% achieved sustained aminotransferase normalization.

A study on the natural history of hepatitis C in 76 children born in the UK, who acquired their infection by blood or blood products (68%), organ transplants (3%), or through perinatal transmission (28%), showed that those who were HCV RNA-positive had a very low rate of clearance of HCV RNA without treatment. Those children who had liver biopsies all showed mild to moderate inflammation and fibrosis.[233] The long-term outcome of chronic hepatitis C is unknown, although uncommon progression to cirrhosis and end-stage liver disease has been described in childhood.[234,234a]

### *Prevention*

There is currently no effective vaccine and prevention is focused on reducing the risks of parenteral acquisition by screening blood donors for anti-HCV, and the

use of vapour-heated and wet-heated clotting factor concentrates which decrease the risk of HCV transmission. Most developed countries have also introduced either HCV RNA or HCV core antigen testing in addition to HCV antibody testing of blood donations in order to identify blood donors in early stages of infection and increase the safety of the blood supply.

Programmes of enhanced surveillance and detection of HCV infection in adults may benefit women of childbearing age who could be treated before becoming pregnant, and reduce the risk of vertical transmission.

### *Treatment*

Children with chronic HCV infection require annual review to monitor viraemia, detect seroconversion, assess liver dysfunction and its progression, consider optimal timing of antiviral therapy and detect HCC.

The aims of antiviral treatment are to: eradicate HCV infection (loss of HCV RNA in peripheral blood); prevent progression of liver inflammation and fibrosis; normalize liver function; and improve quality of life.

Current treatment options include: IFN monotherapy, combination therapy with IFN (or PEG-IFN) and ribavirin

#### *IFN monotherapy in children*

IFN monotherapy produced a favourable response in 50% of treated adult patients, as indicated by normalization of serum transaminase levels and histological improvement, but only 20–30% had a sustained response after treatment withdrawal. An analysis of 19 trials of IFN-α for children with HCV infection compared the effect of IFN in 366 children compared with 105 untreated controls:[235] 54% (range 0–91%) initially responded to treatment, with a sustained response in 36% (0–73%); 27% of children with genotype 1 had a sustained response compared with 70% in those with other genotypes. The spontaneous clearance rate in untreated children was 5%. Treatment regimes varied, with duration of therapy ranging from 6 to 18 months. Most studies used 3 MU/$m^2$ by subcutaneous injection three times weekly.

#### *Combination therapy: IFN/ribavirin*

Ribavirin is a guanosine nucleoside analogue, which although ineffective as monotherapy,[236] is more effective with IFN-α. In adults, combination therapy has superceded monotherapy as sustained response rates are improved. Response is higher in female adults with baseline viral load <3.5 million copies/mL, minimal histological disease, age <40 and genotypes 2, 3 or 4.

Small studies of combination therapy in children indicate that the combination of IFN and ribavirin is as effective as in adults and achieves more sustained HCV RNA clearance than IFN-α alone.[237,238] As in adults, children with genotype 2 or 3 had the highest response (70%) compared with genotype 1 (40%).[237] Similar efficacy has been demonstrated in children with previous malignancy and co-infected with HIV.[239] The most common side-effects are related to IFN (flu-like symptoms, neutropenia and depression) and weight loss, which resolves when treatment is discontinued. Ribavirin causes a haemolytic anaemia in the first 12 weeks, which resolves with time or discontinuation of the drug. Six months of treatment with IFN (3 MU/$m^2$ three times per week) and ribavirin (15 mg/kg/day) is recommended, with a further 24 weeks in patients with genotype 1 if clearance of HCV RNA has occurred in the initial 24 weeks.

#### *Future treatment strategies*

PEG-IFN may be more effective than IFN monotherapy[240] and is more likely to reduce the rate of progression of fibrosis and improve necroinflammatory damage.[241] A pharmacokinetic study has established the dose in children and clinical trials of combination therapy are planned.[242]

## Hepatitis delta

### *Prevalence*

Considerable regional variation in HDV infection has been noted.[243,244] The role of environmental and genetic factors or HBV strain is unknown. In the Amazon Basin of Brazil, infection rates increase with age from 5% in children <10 years, to 26% in those between 10 and 14 years and to >40% in subjects >15 years of age. Among the Yucpa Indians of Venezuela, HDV prevalence varies significantly with age, being highest in those between 15 and 19 years of age (44%) and then progressively lower in those between 5 and 14 years (38%), >24 years (18%) and <5 years (14%) of age.[245] Serological evidence of HDV infection was demonstrated in 34 of 270 (12.5%) Italian children who were HBsAg carriers,[79] but in only 4 of 247 (1.6%) Taiwanese children who had acute or chronic HBV infection.[246] In the Guangzhou area, 6 of 44 (13.6%) Chinese children had superinfection with the delta agent, a prevalence rate identical to that in adults.[247]

### *Clinical features*

In 34 Italian children with serological evidence of HBV-associated delta agent infection,[79] histological features at presentation included chronic active hepatitis (71%), chronic persistent hepatitis (18%) and cirrhosis (12%); 38% showed histological deterioration of liver disease

after 2–7 years of follow-up. This is in contrast to 236 delta-negative HBsAg carriers, with improvement in 55% and deterioration in only 7%. A study of 30 anti-HDV-positive and 30 anti-HDV-negative long-term leukaemia survivors demonstrated that anti-HDV-positive children had significantly higher transaminase levels 3 years after cessation of chemotherapy and more frequent histological evidence of chronic hepatitis or cirrhosis, suggesting that HDV infection is a major cause of morbidity and an adverse prognostic factor in terms of leukaemia-free survival.[248]

In the Amazon Basin, delta virus infection was demonstrated in 74% of patients with fulminant hepatitis (Labrea hepatitis) and in 100% of patients with chronic hepatitis B.[243] Thirty-two of the 44 (73%) cases of fulminant hepatitis were in children <15 years of age, 31 of whom were boys. In the same study, 44 delta virus-negative HBsAg carriers were followed up for 1–5 years; among these, four boys <10 years of age became superinfected with HDV and three developed fulminant hepatitis, which was fatal in two. Post-mortem studies of patients with fulminant hepatitis showed eosinophilic necrosis and microvesicular steatosis of the liver. A similar type of fulminant hepatitis with the same epidemiological and histological features has been described in Colombia (Santa Marta hepatitis) in 81 patients, with the majority (63%) being males and children <15 years old (58%). In 69%, the delta antigen was demonstrated in the liver in combination with microvesicular steatosis, granular eosinophilic necrosis, portal lymphocytic inflammation and characteristic morula cells.[249]

### *Prevention and treatment*

There is no specific treatment for HDV infection. As the greatest risk of HDV infection occurs in HBV carriers, infection may be prevented by giving only anti-HDV-negative blood or blood products to HBsAg-positive patients. HBV vaccination should prevent and limit the spread of HDV. Successful IFN-α treatment of a child with chronic HBV and HDV infection has been reported.[250] The incidence of HDV infections has reduced with increasing use of hepatitis B vaccines.

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# Chapter 48
# Management of hepatocellular cancer

Helen L Reeves, Jordi Bruix

## Background

HCC is the commonest primary liver tumour, the incidence of which is rising worldwide.[1] The major risk factors for HCC vary to some extent with its geographical distribution, but include cirrhosis of the liver regardless of its aetiology, viral infection and chemicals or toxins. Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection contribute to HCC development in as many as 80% of cases. Chemicals include aflatoxin B1 (AFB1) uptake, cigarette smoking and heavy alcohol consumption. Each is an independent risk factor, but they do have synergistic effects.[2,3] The prevalence of hepatitis C has major clinical implications as it is the major contributor in Western developed countries where HCC is increasing.[4] The rising incidence of non-alcoholic fatty liver disease (NAFLD)[5] associated with diabetes or obesity is also a major cause for concern, as not only do these conditions predispose to the development of HCC,[5–7] but emerging data support accelerated HCV disease in fat diabetics.[8] The synergistic effect of alcohol and increased risk of HCC development in the presence of hepatitis B and C or diabetes is well recognized.[6] The reality is that these diseases are all increasing, and whatever the aetiology or synergistic mechanisms leading to chronic liver disease, it has been shown that currently HCC is the leading cause of death in cirrhotics.[2,9–11] Thus, both prevention and treatment of this neoplasm are major health concerns.

## Risk factors and prevention of disease

It is now widely accepted that cancers develop on a background of accumulated genetic change conferring survival advantages to abnormal cells. As most patients develop HCC after a long-standing hepatitis, it is reasonable to relate the persistent inflammatory changes to cancer formation. The chronic proliferative state of the injury and repair process, associated with cell necrosis, death and regeneration, all increase the risk for DNA mutation and subsequent accumulation.[12] However, continuous severe inflammation is also a feature of autoimmune hepatitis, where the incidence of HCC in the absence of cirrhosis is extremely rare.[13] Even in the presence of autoimmune-mediated cirrhotic disease, HCC is less common than in the viral or alcoholic liver diseases.[14] Inflammation or cirrhosis alone, therefore, do not appear to be the sole predetermining features of malignant disease. With this in mind, effective prevention of HCC should, wherever possible, prevent the occurrence of the causative liver disease, i.e. 'primary prevention'. Often this is not possible and 'secondary preventive' measures – preventing the progression of active inflammatory disease to cirrhosis – are the focus of health-care workers.

In HBV infection, the virus itself may have direct genetic or epigenetic effects, explaining why tumours often arise in pre-cirrhotic HBV-related disease. In most tumours, HBV gets integrated into host genome and this is accompanied by rearrangement and increased mutagenesis.[15–17] The active inflammation and proliferation may act synergistically by both increasing the opportunity for such events to occur and promoting their selection. HBV is the main cause of HCC in the world and it could be prevented by vaccination. Vaccination of children in Taiwan has led to a drastic decrease in the incidence of HCC,[18] indicating a clear cost-effective benefit of widespread immunization programmes in developing countries with high rates of infection.

The viral replication of HCV, an RNA virus, does not involve a DNA synthesis step, and thus does not integrate into host genome. Unsurprisingly, therefore, the strongest association with HCC development is in the context of cirrhotic disease. As there is no effective vaccine against HCV, primary preventive measures are limited to health education highlighting the methods of viral transmission. Secondary prevention, namely preventing the progression of inflammatory disease to cirrhosis, is of critical importance for those infected with the virus. This includes antiviral therapy in pre-cirrhotic disease and advice about limitation of other factors, such as alcohol, which adversely affect disease progression. Chemoprevention of HCC, treating individuals with established cirrhosis with interferon (IFN), has also

been proposed, but current data regarding this strategy are conflicting in nature.[19–21]

Most of the non-viral causes of HCC including chronic alcohol intake, obesity-related liver diseases, metal storage diseases and aflatoxin exposure are characterized by the generation of reactive oxygen species (ROS) leading to oxidative stress. In fact, oxidative stress as a mechanism of injury is also evident in viral disease. Either the ROS themselves or the products of lipid peroxidation may be involved in hepatocarcinogenesis by reacting directly with DNA and causing mutations in cancer-related genes, or by reacting with proteins involved in the regulation of DNA repair, the cell cycle and apoptosis. Primary prevention in these diseases is currently limited to health education, which may well be stepped up in coming years, given the increasing health burden attributable to alcohol and obesity in Western countries. Whether or not increased oral intake of anti-oxidants reduces the risk of cancer development is unknown. The vitamin A derivative, polyprenoic acid, may have chemo-preventive properties.[22] Its anti-tumour effects, however, are attributed to it being a nuclear hormone receptor ligand, rather than as a result of any anti-oxidant effect. Other ligands for the nuclear hormone receptor family, including peroxisome proliferator-activator receptor PPARγ ligands, acting either on their own receptor or possibly as heterodimers on retinoic acid receptors, have also shown promise inhibiting tumour cell growth *in vitro*.[23]

A proviso which should be remembered is that, apart from specific measures directed at high-risk individuals, the success of preventive strategies is likely to also depend on an increased awareness of the frequency of chronic liver disease in the general population. Whether population screening for the presence of chronic liver disease will ever be a cost-effective measure remains an area of active debate.[24]

## Surveillance and recall of high-risk individuals

The mean annual incidence of HCC in cirrhotic patients in Western countries is 3–4%. The risk increases in parallel with liver function deterioration and specific characteristics imply a higher risk. Thereby, male sex and increased alpha-fetoprotein (AFP) concentration constitute the most powerful risk predictors,[2] while others such as irregular regeneration, high proliferative stage and presence of dysplastic foci are less widely accepted. Surveillance for HCC in these patients has been advocated for years, even though a benefit in survival has not been clearly demonstrated. The purpose of surveillance is to recognize HCC at an early stage, when the tumour could be cured.

The failure of previous studies to show a survival benefit is probably a result of a number of factors, including poor ultrasound (US) performance, the absence of clear-cut diagnostic criteria and the absence of curative treatments. It is now generally believed that, even accounting for the contribution of lead-time bias (i.e. patients apparently surviving longer, simply because their tumours were diagnosed earlier) and length-time bias (the interval between screening misses the more aggressive tumours that will be detected upon symptoms appearance), improved methods of surveillance, diagnosis and treatment will lead to improved cost-effectiveness of surveillance programmes. Consequently, the European Association for the Study of the Liver panel of experts suggests 6-monthly abdominal US scanning and AFP measurements for high-risk individuals.[2] Nevertheless, AFP is known to have reduced sensitivity, and probably it merely serves to identify patients at higher risk and its repeated measurement does not reach proper cost-efficiency,[25] as surely happens with most biochemical assessments in cirrhotic patients. The recall procedures for individuals in whom an abnormality is detected are shown in Fig. 48.1, while the criteria required to confirm HCC diagnosis are shown in Table 48.1. In brief, nodules <1 cm that are detected in a cirrhotic liver are malignant in <50% of cases, and therefore close follow-up every 3 months to detect any increase in size is recommended. Whether or not fine-needle aspiration or biopsy of these small lesions would help clinical management is currently unknown. Fine-needle biopsy is recommended for nodules 1–2 cm in diameter (Fig. 48.2), but even in these larger lesions does not necessarily rule out malignancy if negative, as cytohistology is falsely negative in 30–40% of these cases. For nodules >2 cm within a cirrhotic liver, the diagnosis of HCC can be made based on the concomitant findings of two imaging techniques – CT (Fig. 48.3) or MRI scan (Fig. 48.4) – with contrast-enhanced US confirming characteristic arterial hypervascularization. Alternatively, it can be made by a single imaging study confirming arterial hypervascularization in asso-

**Table 48.1** Diagnostic criteria for hepatocellular carcinoma (HCC)

| Cytohistological criteria |
|---|
| Non-invasive criteria (cirrhotic patients) |
| 1. Radiological criteria: two coincidental imaging techniques* |
| a. Focal lesion >2 cm with arterial hypervascularization |
| Combined criteria: one imaging technique associated with AFP |
| 1. Focal lesion >2 cm with arterial hypervascularization |
| a. AFP levels >400 ng/mL |

*The four techniques considered are: ultrasound (US), spiral CT, MRI and angiography.

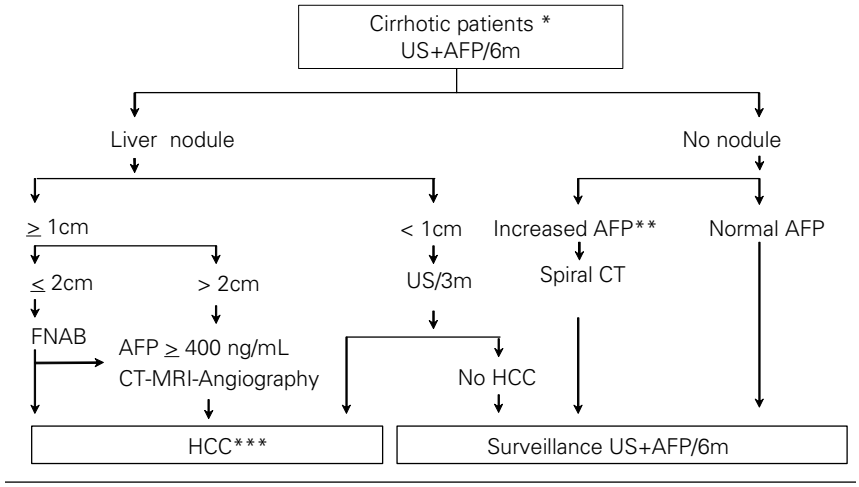



**Figure 48.1** Screening strategy as proposed by the panel of experts of the EASL Conference on Clinical Management of Hepatocellular Carcinoma. US, ultrasound; AFP, alpha-fetoprotein; FNAB, fine-needle aspiration biopsy; CT, computed tomography; MRI, magnetic resonance imaging; HCC, hepatocellular carcinoma. *Available for curative treatments if diagnosed with HCC. **AFP levels to be defined. ***Pathological confirmation or non-invasive criteria.

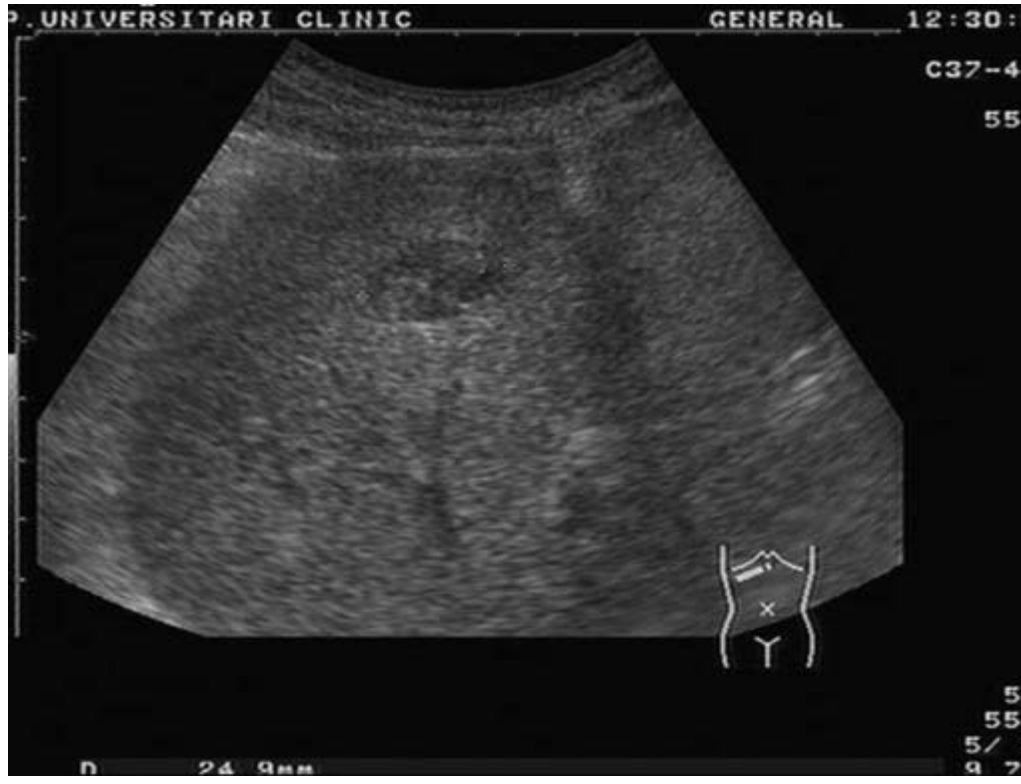



**Figure 48.2** Hepatic ultrasound (US) depicting a nodule measuring 25 mm in diameter. Diagnosis can be ensured by biopsy or, if detected within a cirrhotic liver, the coincidental findings of two imaging techniques.

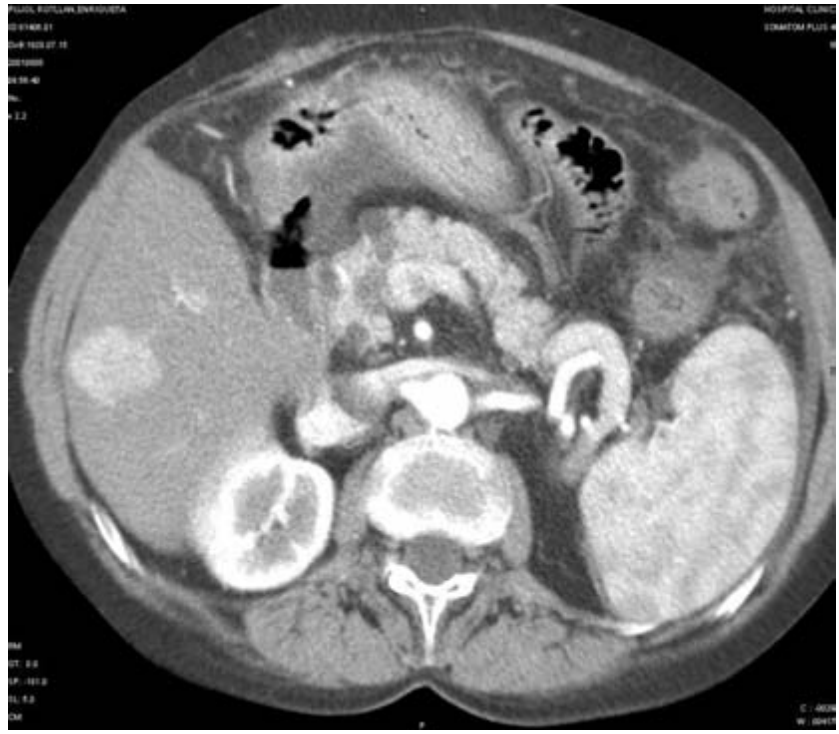


**Figure 48.3** Computed tomography (CT): the right hepatic lobe contains a solitary tumour with evident arterial enhancement after contrast administration. The washout of contrast during the venous phase is characteristic of hepatocellular carcinoma (HCC).

ciation with an AFP >400 ng/mL.[2] US, spiral computed tomography (CT) and MRI are the imaging modalities conventionally used to assess disease extension. With the improvements in these techniques, angiography for diagnostic and staging purposes is rarely necessary.

Further data are needed to confirm the benefit of other proposed markers such as protein induced by vitamin K absence (PIVKA),[26,27] glypican-3[28,29] and AFP fractions[30] in clinical practice.

## Staging systems in HCC

Generally speaking, the prognosis of solid tumours is largely related to tumour stage, and it is typically this that guides management decisions. In HCC, however, the degree to which the underlying liver function is impaired is an additional important determinant of outcome. This makes staging and prognostication of patients with HCC difficult. Unsurprisingly, therefore, a worldwide consensus is lacking and HCC staging remains a very controversial area. A well validated and internationally accepted staging system should stratify patients into subgroups with significantly different outcomes and should enable health-care providers to give accurate information to their patients about long-term life expectancy, as well indicate the most adequate treatment and predict outcome after treatment. Historically, HCC has been classified by the TNM[31] or Okuda staging systems.[32]

The TNM system has been modified repeatedly on account of its poor accuracy. Its general use is limited because it relies predominantly on pathological findings, which are often unavailable, and liver function is not considered. In contrast, the Okuda classification excludes pathology and is based on tumour size (on imaging/surgery) and liver function. As with the TNM scheme, it can be unhelpful in individuals with intermediate forms of HCC.

Several scoring systems have been recently proposed to stratify patients according to expected survival. Schemes have been proposed in Uganda,[33] the United States,[34] Barcelona,[35] France,[36] Italy,[37] Austria,[38] Hong-Kong[39] and Japan.[40] Although all of them divide patients into strata with different prognoses, their major usefulness is the identification of end-stage patients with grim prognosis, but none provides any link to treatment in-

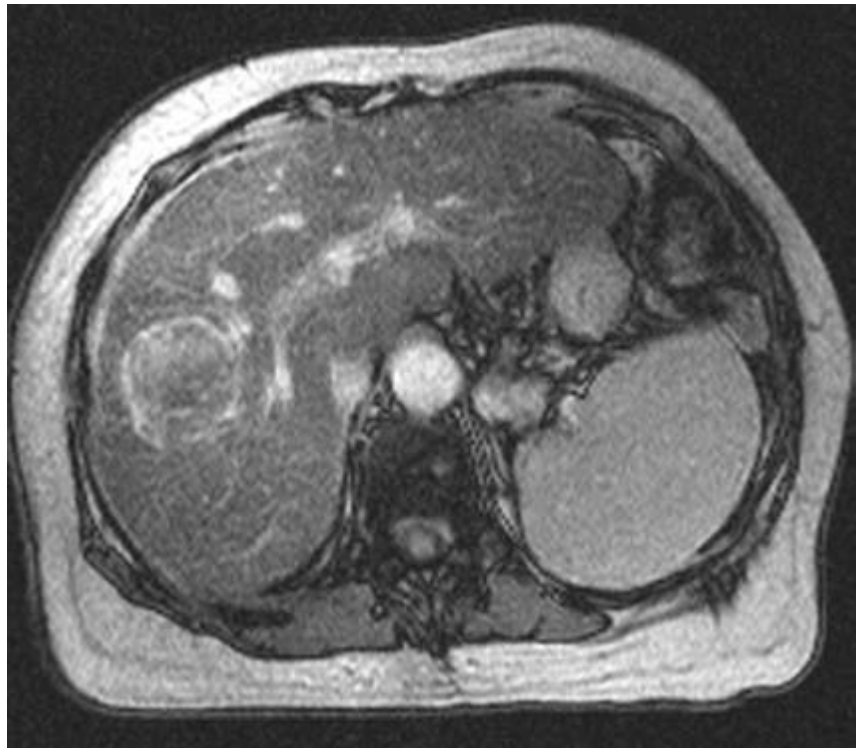


**Figure 48.4** Magnetic resonance imaging (MRI) showing a hepatocellular carcinoma (HCC) located in the right lobe.

dication. Furthermore, survival data of the strata have neither been reproduced from different geographical origins, nor do they retain their prognostic power in comparison with other staging systems. The lack of reproducibility suggests heterogeneity between the different patient groups that may preclude the introduction of a universal scoring/staging system.

The Barcelona-Clínic-Liver-Cancer (BCLC) staging system[41,42] was constructed on the basis of results obtained in the setting of several cohort studies and randomized clinical trials (RCTs) by the Barcelona group. The proposal, shown in Fig. 48.5, is not a scoring system, but rather a regularly updated staging classification resulting from the combination of the data of several independent prognostic studies in different disease stages and treatments. As a whole, it has become a widely used clinical tool for treatment decision-making. It includes variables related to tumour stage, liver functional status, physical status and cancer-related symptoms. It identifies those with early disease who may benefit from curative therapies, those who may benefit from palliative treatments, as well as those with a particularly poor outlook who should receive symptomatic care only. Patients diagnosed at an early stage may achieve a 5-year survival between 50% and 70%. Those that are diagnosed at an intermediate or advanced stage belong to the so-called non-surgical HCC category and their survival depends on the existence of cancer-related symptoms and the presence of vascular invasion or extrahepatic spread. Thereby, those who are asymptomatic without invasion or dissemination may achieve a 50% survival at 3 years, while the median survival for those with an adverse profile is <1 year (Fig. 48.6).

The hope for the future perhaps lies in the genomic and proteomic characterization of HCC. This should make it possible to identify a group of genes and/or proteins that define a molecular classification of HCC with predictive power for survival and response to treatment modalities.

## Treatment

Treatment options for HCC patients include curative interventions, such as resection, liver transplantation and percutaneous ablation, as well as palliative chemoembolization. Before any decision regarding treatment is made, however, the initial patient assessment should address and answer a number of key issues. These are discussed below, but are also summarized in the treatment algorithm shown in Fig. 48.5

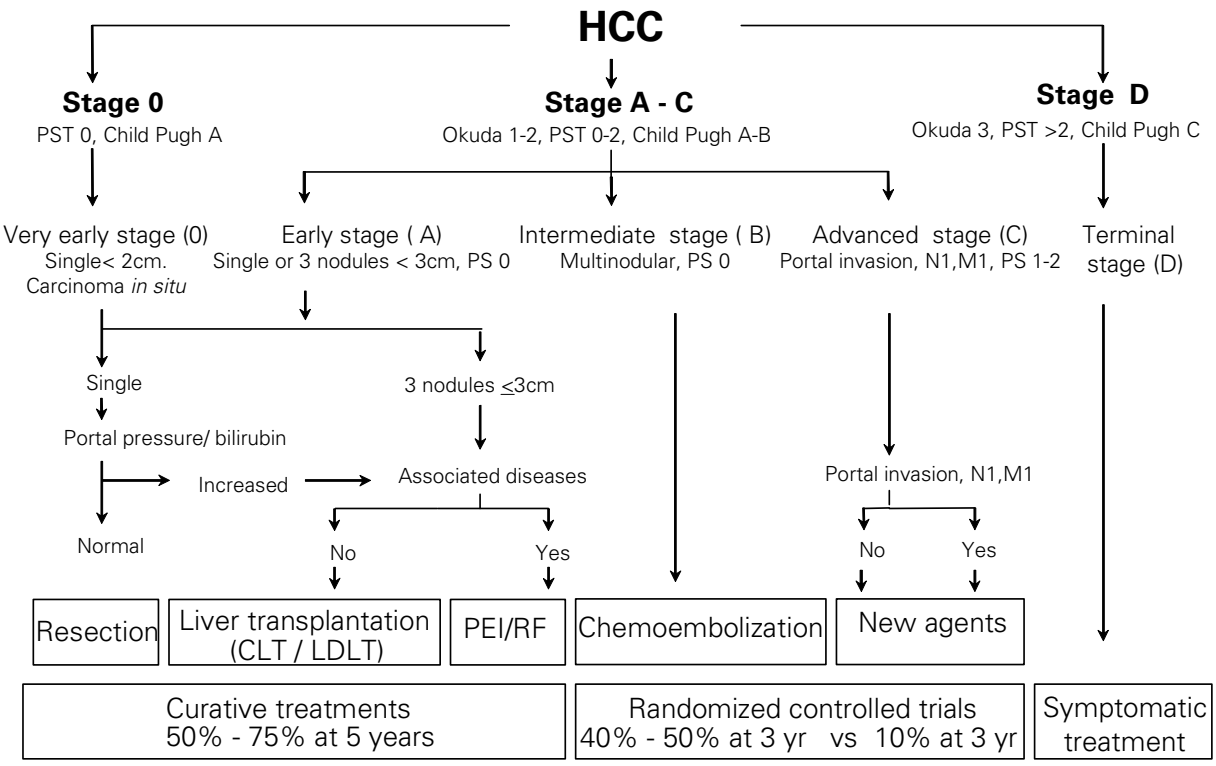



**Figure 48.5** Staging system and treatment strategy according to the BCLC criteria. Patients are stratified according to their clinical, tumour and biochemical characteristics into major strata: early, intermediate, advanced and terminal. Treatment selection is decided according to stage allocation. HCC, hepatocellular carcinoma; CLT, cadaveric liver transplantation; LDLT, living donor liver transplantation; PEI, percutaneous ethanol injection RF; radiofrequency

## The status of the non-tumorous liver

Knowledge of the status of the non-tumorous liver is essential. Patients with non-cirrhotic disease are good candidates for liver resection or other potentially liver damaging therapies such as chemoembolization. The vast majority of HCC patients, however, have either established cirrhosis (85–90%) or non-cirrhotic chronic liver disease. The management of these patients presents a challenge. Clearly, individuals with decompensated cirrhosis cannot undergo any invasive therapy that may precipitate or accelerate liver failure. These include both surgical and some locoregional approaches such as chemoembolization. The first-line option for these decompensated individuals, if they meet additional criteria, is liver transplantation.[2,42] In fact, only individuals with Child Pugh A chronic liver disease should ever be considered for resection and only after a more sophisticated assessment to identify the subgroup that will perform well after surgery. This includes individuals with a normal serum bilirubin and/or those without even mild portal hypertension (PHT; portal venous pressure gradient <10 mmHg).[43,44]

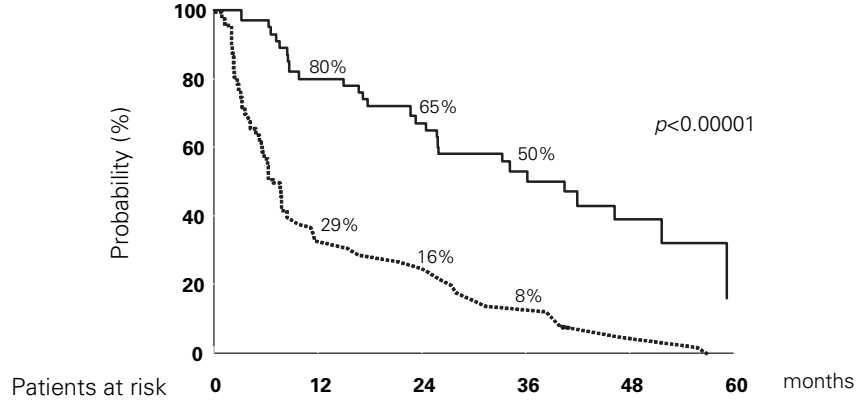



**Figure 48.6** Survival of patients with non-surgical hepatocellular carcinoma (HCC) who were untreated because of their allocation to the untreated control arm within prospective randomized trials. Asymptomatic patients without vascular invasion or extrahepatic spread may achieve a 50% survival at 3 years, while only 10% of those with symptoms and/or vascular invasion/extrahepatic spread reach this time-point.

## The size and extension of the tumour

Evaluation of tumour extension, with a search for the presence of daughter nodules, extrahepatic disease and/or portal vein thrombosis, is necessary in all cases. This can be performed using a combination of US and CT scan or MRI. Angiography has largely been superseded by these less invasive techniques. The search for extrahepatic disease may include a chest CT scan or a bone scan. The presence of tumoral portal invasion or metastatic disease leads to a bleak prognosis and precludes curative intervention.

## The general condition of the patient

The general condition of the patient should be assessed before taking any therapeutic decision. This includes consideration of co-morbid conditions that may increase perioperative or intervention-related morbidity and mortality, but also the assessment of cancer-related symptoms as reflected by their performance status (PS) score. This is used to quantify functional status in cancer patients[45] and if heavily affected (PS = 3–4) serves to identify patients with advanced or terminal disease that will not benefit from any form of active intervention.[46]

## Resection

This is the treatment of choice for HCC in non-cirrhotic patients, i.e. 5% of cases in Western countries and up to 40% of cases in Asia owing to the increased frequency of HBV-related disease. In cirrhotic patients, however, the 3-year survival post-resection 20 years ago was <50%.[47,48] Several major advances have steadily improved these figures in recent years, including earlier diagnosis in asymptomatic phases of disease, accurate staging of the disease identifying favourable tumour characteristics, and refined functional evaluation of the underlying liver function enabling the prediction of outcome after surgical intervention.

Most groups restrict the indication for resection to patients with a single tumour, as shown by state of the art imaging techniques. The size of the tumour is not a clear-cut limiting factor – the risks of vascular invasion and dissemination increase with size, but occasionally tumours may grow slowly to a huge size, without evidence of invasion or satellite disease. In these cases, the risk of recurrence post-resection is not significantly increased compared to smaller tumours. Some groups perform chemoembolization of larger tumours, hoping to convert larger non-surgical lesions into resectable ones.[49] Others use portal vein embolization of the hepatic lobe hosting the tumour to induce compensatory liver growth in the non-affected lobe in order to increase its functional capacity if a major resection is planned.[50] The benefits of these interventions in large series are not well established. Large RCTs defining the risks and benefits of these practices are required.

For many years, the selection of candidates with potentially resectable tumours has been based upon the Child Pugh score. However, even Child Pugh A patients may perform poorly. The Japanese groups rely on the indocyanine retention test of hepatic function, using the rate of hepatic clearance of the dye to guide their decisions regarding the appropriateness and extent of liver resection.[51,52] Portal pressure and bilirubin have been established as the best parameters to select optimal surgical candidates in Europe.[42,44] Clinically relevant PHT can be defined as the presence of either a hepatic vein pressure gradient of ≥10 mmHg,[43] oesophageal varices, or splenomegaly with a platelet count of <100 000/mm$^3$. Subjects without relevant PHT and normal bilirubin achieve 5-year survival rates of 70%, whereas this decreases to 50% in patients with PHT, and to 25% in those with PHT and a raised bilirubin.[44]

Even restricting resection to the best candidates, tumour recurrence rates exceed 50% at 3 years. When present, it influences long-term survival. The most powerful predictors of postoperative recurrence are the presence of microvascular invasion, poor differentiation and satellite lesions.[44,53–55] This reflects the fact that the majority of recurrences result from dissemination from the primary tumour, rather than being 'new' or *de novo* HCC.[56] Currently, there is no effective method proven to diminish recurrence. Preoperative chemoembolization or adjuvant chemotherapy have no efficacy and may complicate the intervention.[57] Preliminary studies indicating benefit from internal radiation[58] and adoptive immunotherapy[59] (using activated lymphocytes) require validation, as do the apparent successes of retinoids[22] and IFN in preventing *de novo* tumours,[60] as has been also suggested after percutaneous ablation.[61]

## Liver transplantation

Individuals with HCC played a major role in the pioneering days of liver transplantation. At a time when the success of this radical intervention was unknown, the risks were considered acceptable for HCC patients, owing to the lack of alternative treatments and a dismal life expectancy. Although recurrence was high in the early years (32–54%) and survival poor (<40% at 5 years), this practice changed the treatment strategy for HCC and was instrumental in defining the criteria we now use for selection of favourable candidates.[62] By selecting the 'optimal patients', i.e. those with a single HCC ≤5 cm or up to three nodules ≤3 cm, the 5-year survival is 70% with a recurrence rate below 15%.[44,63,64]

Although many believe that transplantation should be the first-line option for treatment of early HCC, as it

cures both the tumour and the underlying disease, the shortage of donors and long waiting list times impose a grim reality even for those eligible at the time of first assessment. The United Network of Organ Sharing (UNOS) data indicate that almost as many patients are excluded while waiting, as a result of progressive disease, as are effectively transplanted.[65] In the United States, UNOS adopted the Model for End-Stage Liver Disease (MELD) system to rank or prioritize patients awaiting orthotopic liver transplantation. A composite score based on the bilirubin, prothrombin time, creatinine and aetiology is used for non-cancer patients and a variable score was initially granted to HCC patients.[66] Patients in stage I (single <2 cm) received 24 points and patients in stage II (single 2–5 cm or three ≤3 cm) 29 points. However, this resulted in an unfair priority for HCC patients as compared with non-cancer patients. Thus, points were reduced to 20 and 24, respectively, and depending on the results obtained the system may be again changed.[67] In fact, one of the main issues when priorities are established is to ensure an equitable distribution of organs. As the MELD score favours the transplantation of HCC patients, of which there are many in some centres, there are concerns that it may increase the drop-out rate and subsequent death of non-cancer patients. At the same time, it is mandatory to avoid excessive priority for too ill patients with dismal outcome after transplant. These aspects are not yet solved and no firm recommendation can be given.

According to estimates from available data, a waiting time of 6 months would reduce the drop-out of HCC patients from transplant waiting lists to <10%. For waiting times above this, several strategies to impede tumour progression while waiting for a donor have been proposed. Systemic chemotherapy has no efficacy despite encouraging preliminary results.[68] Robust RCTs comparing active intervention versus no therapy for these individuals are lacking. Most groups perform transarterial chemoembolization upon listing because it reduces tumour burden and delays tumour progression.[49,69] As this treatment may induce liver failure and death in patients with decompensated disease, it cannot be applied to all candidates. Patients with small tumours can be ablated by percutaneous ethanol injection or radiofrequency ablation.[70] One potential drawback of this practice is seeding due to tumour puncture. The risk is mostly restricted to peripheral tumours that cannot be accessed through a rim of non-tumoral liver,[71] and is more likely using larger needles. As ethanol injection uses thinner needles, this may be the most appropriate course of action, but direct puncture of nodules without a rim of non-tumour liver should always be avoided. A laparoscopic approach and radiofrequency ablation for peripheral tumours may circumvent this issue.

An alternative solution to the long waiting list times is to expand the numbers of available livers. While strategies here include the use of marginal livers as well as domino or split-liver transplantation, it is the introduction of living donor liver transplantation (LDLT)[72] that possibly has the greatest potential. Over 2000 adult LDLTs using the right hepatic lobe have been performed throughout the world. The drawbacks are the 0.5% donor mortality and 20% recipient morbidity. At present, recipient survival post LDLT appears similar to cadaveric transplantation, but the long-term outcome is unknown.[73] There is a suggestion, for example, that reinfection of the graft in HCV patients is more severe in recipients of live than of cadaveric transplantations.[74] While accepting that long-term data are still awaited and live donation programmes are likely to face the problems of patient and/or donor refusal, blood group incompatibility, medical contraindications and funding restrictions, there may be potential within LDLT practice to extend the limits currently applied to HCC transplantation. As the transplant can be done without delay, with highly accurate staging, a set of expanded criteria have been proposed by several groups.[75,76] In the Barcelona Liver Unit we proposed the following limits for live donation: single tumour ≤7 cm, three nodules ≤5 cm, five nodules ≤3 cm, or a down-staging to conventional criteria after local or regional therapy lasting more than 6 months.[46] It is expected that survival will still exceed 50% at 5 years, but the applicability of this programme is clearly reduced. In general, live donation may not only benefit some individuals with more extensive disease, but may also reduce waiting list times for cadaveric recipients.

Initial concerns that immunosuppression post-transplant may accelerate the growth of recurrent or metastatic HCC[77] are now thought to be unfounded,[78] adherence to selection criteria being the single key factor predicting postoperative recurrence. There are currently no data indicating that systemic chemotherapy pre- or post-transplant for these individuals has any impact on disease recurrence. Some centres administer systemic chemotherapy to all individuals with satellites or vascular invasion at pathological examination post-transplant, but there are no data to support this policy.

### *Percutaneous treatments*

Destruction or ablation of tumour cells can be achieved percutaneously by the injection of chemical substances (ethanol, acetic acid and boiling saline) or by inserting a probe that modifies local tumour temperature (radiofrequency, microwave, laser and cryotherapy). These are the best options for patients with early HCC who are ineligible for resection or transplantation.[79–84] Whether

or not they may be the best option for all patients is unknown due to the lack of RCTs comparing them with resection. Some Japanese centres, however, do offer ablation as first-line therapy.[85]

Percutaneous ethanol injection (PEI), usually performed under US guidance, is the best known and studied of the percutaneous therapies. It is highly effective for small HCCs and has a low rate of adverse effects. It achieves complete tumour necrosis in 90–100% of HCCs <2 cm in diameter. This is reduced to 70% in 2–3-cm tumours and 50% in 3–5-cm tumours.[80,86] The injection is repeated on successive days and the efficacy of ablation is assessed at 1 month by dynamic CT.[2] The absence of contrast uptake in the tumour reflects tumour necrosis, while the recognition of contrast uptake constitutes treatment failure. The 5-year survival of Child Pugh A candidates with a complete response is 50%.[81] Complete necrosis in tumours >3 cm, however, is rarely achieved, possibly because of the presence of septae preventing the ethanol accessing the whole tumour volume. Overall, the rate of recurrence is similar to that seen for surgical resection and occurs within the vicinity of the treated nodule.

Radiofrequency ablation (RFA) is the most extensively used alternative to PEI. It can be applied through single or multiple cooled-tip electrodes, either percutaneously, laparoscopically or intra-operatively. It is claimed to achieve the same objective response as PEI in significantly fewer sessions.[87,88] Theoretically, it may be superior in tumours >3 cm by disrupting intra-tumoral septae. The main drawbacks of RFA are its higher cost and associated mortality of 0.5% and morbidity of 10%. Superficial tumours should not be treated percutaneously by direct puncture because of the risk of tumour seeding.[71] In addition, tumours in close proximity to the hilum or gallbladder should not be treated by RFA as there is a high risk of damaging the biliary tree. Treatment of tumours in close proximity to the heart or major vessels should also be avoided.

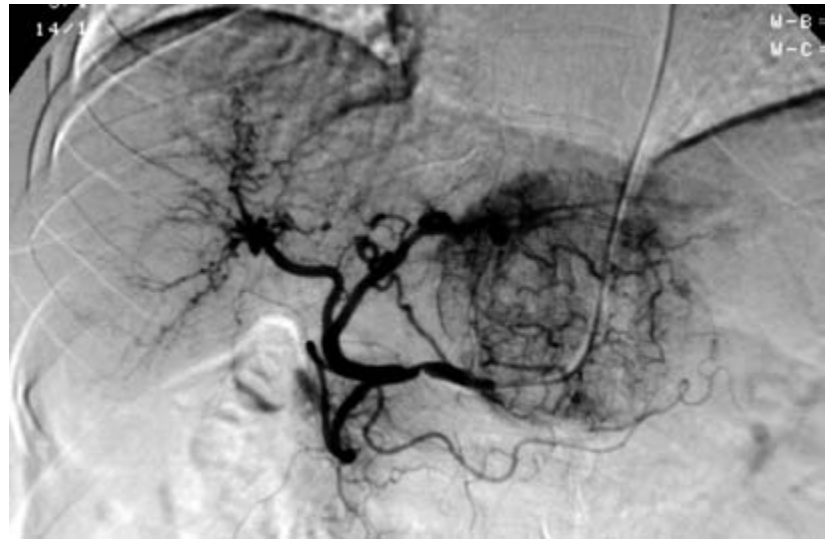
## Palliative therapy

As the majority of HCC patients over the last 25 years have been diagnosed at advanced stages of disease, where no standard therapy has been universally established, a whole host of alternative therapies have been advocated. In the main, relatively small studies have analyzed the effectiveness of treatments such as embolization, chemoembolization, arterial or systemic chemotherapy, internal radiation with $^{131}$I, proton beam radiation, hormonal compounds and immunotherapy.[89] Systematic review and meta-analyses of all available studies indicate that only chemoembolization has a beneficial impact on survival.[89] In contrast, oestrogen blockade with tamoxifen unequivocally lacks anti-tumoral effect and has no impact on survival.[89] All other treatment approaches, including internal irradiation with $^{131}$I,[90,91] octreotide[92,93] and IFN[94,95] have been assessed in the setting of too few small studies that do not have the statistical power as yet to provide solid conclusions. Initially positive results with IFN[94] and octreotide[92] have since been contradicted.[93,95] As the majority of intra-arterial chemotherapy trials have not included a no-treatment arm, no definitive conclusions can be drawn from these. Similarly, systemic chemotherapy (most often doxorubicin) has marginal activity (<10% response rate) with no proven impact on survival.[96,97]

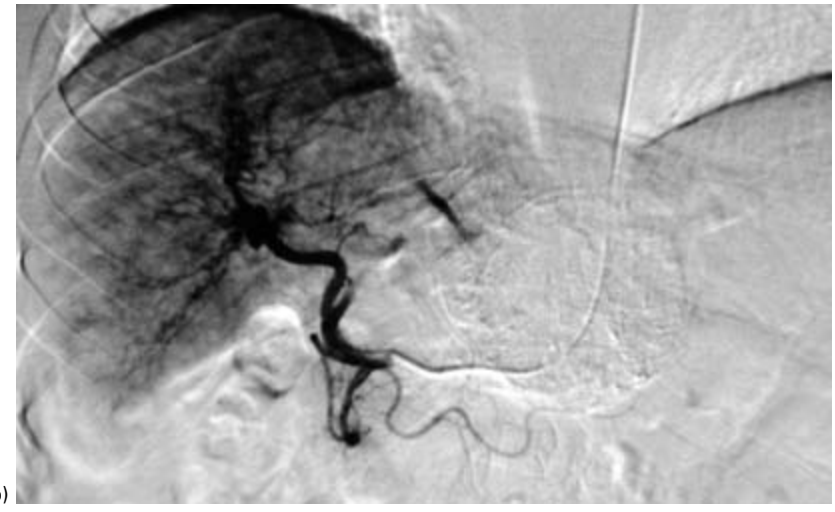
## Arterial embolization

The majority of hepatic blood flow in a normal liver arises from the portal vein (70%), while the remainder arises from the hepatic artery. In contrast, HCC are predominantly supplied by the hepatic artery. Arterial neo-angiogenic activity in malignant tumours typically results in lesions that are hypervascular. This characteristic forms the pathologic basis of both the typical diagnostic radiological features of HCC and the therapeutic rationale supporting arterial obstruction. Embolization of the arterial blood supply to the tumour is a practice typically considered for individuals with non-surgical HCC who are also excluded from percutaneous ablation (often because of multifocal disease or tumour size), but in whom there is no extrahepatic disease. The main contraindication is the lack of portal blood flow (secondary to portal vein obstruction with thrombosis or tumour, portosystemic anastomosis or hepatofugal flow), as in these individuals the arterial supply may be the predominant liver blood supply and any intervention obstructing this may precipitate liver failure. Patients with advanced disease (Child Pugh C) should also be excluded from this treatment.

Obstruction of the hepatic artery induces extensive tumour necrosis. Gelfoam prepared as 1-mm cubes is the most commonly used agent, but polyvinyl alcohol, alcohol, starch microspheres, blood clots and metallic coils have all been used. Hepatic artery obstruction is performed during an angiographic procedure and is known as transarterial, or transcatheter arterial embolization (TAE). The procedure requires the advancement of the catheter into the hepatic artery and then lobar and segmental branches (Fig. 48.7a). The aim is to interrupt blood flow to the tumour in as selective a way as possible (Fig. 48.7b), avoiding injury to the surrounding non-tumour liver as much as possible. This may not be possible if treating multifocal HCC involving both right and left hepatic lobes. The injection is done slowly to avoid the backward flow of gelfoam particles that could embolize arterial vessels outside the liver. Care to avoid obstruction of the cystic artery and ensuing necrosis of the gallbladder is standard practice.




(a)



(b)

**Figure 48.7** (a) Hepatic angiography depicting a large hypervascular mass located in the left lobe corresponding to a hepatocellular carcinoma (HCC). Impaired liver function and associated conditions precluded surgical resection and liver transplantation. Large tumour size excluded the patient from percutaneous ablation and the recommended treatment was transarterial chemoembolization. (b) The catheter was advanced selectively into the left hepatic artery feeding the tumour, and at that time-point chemotherapy mixed in lipiodol and gelatin fragments was injected until blood flow interruption was achieved. The figure shows that all blood flow to the tumour is completely abolished.

When TAE is combined with the prior injection into the hepatic artery of chemotherapeutic agent, most commonly doxorubicin, mitomycin or cisplatin, the procedure is known as transarterial chemoembolization (TACE). It is usual to suspend the chemotherapeutic agent in lipiodol, an oily contrast agent that is selectively retained within the tumour, enhancing the exposure of the tumour cells to the chemotherapy. The dose of chemotherapy administered should be distributed between the affected lobes. Common practice is to inject 25% of the total volume into the tumour-free lobe, aiming to act on any potentially undetected tumour cells.

The side-effects of intra-arterial injection of chemotherapy are the same as for systemic administration, namely nausea, vomiting, bone marrow suppression, alopecia and renal impairment. In addition, hepatic artery obstruction with induced necrosis of the tumour may be associated with what is known as 'post-embolization syndrome'. This is seen in >50% of individuals and consists of fever, abdominal pain and a moderate degree of ileus. It is usually self-limiting and most patients can be discharged within 48 hours. Fasting is required for 24 hours prior to the procedure and intravenous hydration is mandatory. Prophylactic antibiotics are not routinely used. Post-procedure fever is usually a reflection of tumour necrosis, but if it persists severe infectious complications such as a hepatic abscess or cholecystitis should be considered.

A partial response, accompanied by delayed tumour progression and vascular invasion, is induced by both TAE and TACE in 15–55% of patients[69,98] and can be assessed by the decrease in concentration of tumoral markers and/or the identification of intra-tumoral necrosis and reduced tumour burden on dynamic CT scan or MRI. However, the residual tumour typically recovers its blood supply and repeated treatment is necessary. In the cumulative meta-analysis of all RCTs[69,98–103] a significant survival benefit (41% at 2 years versus 27% in the control group) has been shown for chemoembolization, but never confirmed for embolization alone.[89]

## The future

Despite significant advances in the management of this disease over the last 20 years, the majority of patients are still diagnosed at advanced stages of disease and have little hope of cure. Future improvements will more than likely include advances at the level of prevention in the form of immunization and antiviral therapies, at the level of detection and staging with ever better imaging modalities, as well as at the treatment level, with the increasing opportunities to expand the donor pool and improve surgical and possibly ablative techniques. Hopefully, however, the application of advances in genomics and proteomics will also play a major role. Several human tumours, including breast,[104–106] prostate,[107,108] and lung cancer,[109] non-Hodgkin's B lymphoma[110] and melanoma,[111] have recently been classified, with respect to their stage, prognosis and/or response to treatment, according to gene or protein profiling using microarray[112,113] or proteomic technologies.[114] These molecular classifications are still being validated, but there is hope that they may be far more accurate in predicting prognosis or response to therapies than traditional staging systems.

The basis of molecular classification lies in the accumulation of genetic defects and associated changes in gene and protein expression that take place as tumour pathways are initiated and subsequently progress. Chronic liver disease not only creates an environment where DNA damage is likely to occur, but the persistent repair and regenerative response to injury enables the clonal selection and perpetuation of any cells with damaged genes afforded a proliferative or survival advantage. Whatever the aetiology of the chronic liver disease, genetic change contributes to the creation of clones of abnormal cells we term low-grade dysplastic nodules (LGDN). In association with additional genetic changes these LGDN progress to high-grade dysplastic nodules (HGDN), early HCC, and subsequently intermediate and later stages of disease. An extensive literature suggests that the different stages of HCC development can be defined by changes in DNA, mRNA or proteins.[115,116] Some of these changes may have direct clinical relevance. For example, both HSP70[117] and Glipycan-3[29] have been proposed as markers of early HCC and may possibly distinguish HGDN and early HCC. Similarly, a recent gene array in HCV-related HCC cases has proposed a '50 gene set', including TP53, the RAS oncogene and TNF families as genes which could be used in combination to confirm diagnosis of an HCC.[118,119] Some genes, including p16, SOCS-1 and PEG10, are reportedly associated with tumour progression, while nm23-H1, ostepontin and Rho C form part of a proposed 'genetic signature' predicting disseminated HBV-related HCC.[120]

It will probably be a number of years before studies such as these are validated and their practical value is assessed. Ideally, we would like to identify DNA changes, study their origin and impact, and devise effective means of preventing their occurrence, detecting and monitoring their presence, as well as developing means of inhibiting their deleterious effects. In practical terms for HCC, this might mean chemo-preventive strategies inhibiting the development of chromosomal instability in chronic liver disease, sensitive serum biomarkers capable of detecting early disease, serum biomarkers for staging, monitoring disease and predicting response to therapeutic strategies, and the development of novel targeted therapies. With these approaches, in combination with improvements in the strategies that have already had a dramatic impact on HCC management, we may see the prognosis of this once universally dismal tumour change yet further in the coming years.

## Conclusion

Hepatocellular cancer (HCC) is a common malignancy with a particularly poor prognosis. It is associated with chronic liver diseases, particularly those secondary to viral infection or toxic agents such as alcohol. Over the last 10–20 years, significant advances have been made in both the ways we detect these tumours and the ways in which we treat them. Unfortunately, although tumours diagnosed early may be cured by resection, liver transplantation or ablation, the majority of patients with this disease are still diagnosed at an intermediate or advanced stage. Advances in care for these individuals have not been as successful, but a significant palliative survival benefit has recently been confirmed for some of those receiving transarterial chemoembolization of their tumour. The principal focus of this chapter is to review not just the therapies available, but also the current clinical practices for identifying tumours and staging the disease. Early detection, established recall procedures and accurate staging have a major influence on the success of treatment application.

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# Chapter 49
# Application of molecular biology to the diagnosis of viral hepatitis

Jean-Michel Pawlotsky

In the past decade, improvements in molecular biology-based techniques have yielded highly valuable tools for use in the management of liver diseases related to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. Molecular tools have been used for years to diagnose these infections. Nowadays, molecular assays have also become mandatory in the management of antiviral therapy. They are particularly useful to establish treatment indications, tailor therapy to the individual patient and assess virological responses and resistance to antiviral drugs. This chapter reviews current molecular biology-based techniques and assays, and their practical use in the management of viral hepatitis.

## Molecular biology-based techniques

### Detection and quantification of viral genomes

Viral genomes are generally present in relatively small amounts in body fluids of infected patients, hindering their detection by simple molecular hybridization-based techniques. Thus, their detection and quantification requires a preliminary 'amplification' step. This can be achieved by using two categories of molecular biology-based techniques, namely target amplification and signal amplification.

#### *Target amplification techniques*

The principle of target amplification techniques is to synthesize a large number of copies of the viral genome (amplicons) in a cyclic enzymatic reaction. In the 'classical' target amplification methods, the amplicons are detected at the end of the reaction by various methods and the amount of viral genomes in the clinical sample can be quantified through competitive amplification. In the 'real-time' target amplification techniques, detection and quantification are performed during the reaction in the closed tube, by a non-competitive method.

*Polymerase chain reaction*

The polymerase chain reaction (PCR) method[1] uses several temperatures and one enzyme, a thermostable DNA polymerase. The amplicons are double-stranded DNAs. PCR can be applied to DNA viruses (such as HBV) directly, after extraction of nucleic acids or lysis of the viral envelope and capsid. In contrast, a reverse transcription step is required for RNA viruses (such as HCV), in order to synthesize a complementary DNA (cDNA) for use as a template in the PCR reaction. Each complete PCR cycle doubles the number of DNA copies. After n cycles, $2^n$ copies of each DNA molecule present at the beginning of the reaction are theoretically synthesized. In fact, the reaction is saturable and reaches a plateau, generally after 35–45 cycles. Detection of PCR amplicons is classically based on specific hybridization to immobilized oligonucleotide probes. Amplicon-probe hybrids are revealed in an enzymatic reaction, followed by detection of a coloured or luminescent signal. Quantification is based on competitive amplification of the viral template with a known amount of synthetic standard added to each reaction tube. The relative amounts of viral template and standard amplicons are measured at the end of the procedure and the results are read from a standard curve established in parallel.

*Real-time PCR*

The principle of real-time PCR is to detect amplicon synthesis and to deduce the amount of viral genomes in the starting clinical sample during rather than at the end of the PCR reaction.[2] This method is more sensitive than classical PCR and is not prone to carryover contamination. The dynamic range of quantification of real-time PCR techniques is consistently wider than that of classical target amplification techniques, making them particularly useful for quantifying the full range of viral loads observed in untreated and treated patients with HCV or

HBV infection.[3–13] Several real-time PCR methods can be used in various devices, such as the TaqMan™ technology, the fluorescence resonance energy transfer (FRET) method, the SYBR Green I dye method or methods using molecular beacons.[14] Real-time PCR can be partly or fully automated and adapted to high throughput settings. Whatever the technique used, software is used to calculate the threshold cycle in each reaction from which there is a linear relationship with the initial amount of DNA. In each run, parallel processing of a panel of quantified standards is used to establish a standard curve for quantification.

*Transcription-mediated amplification*

In transcription-mediated amplification (TMA),[15] the reaction is isothermal and uses two enzymes, a reverse transcriptase and a T7 RNA polymerase. The amplicons are single-stranded RNAs. After lysis of the viral envelope and capsid, the viral genome (DNA or RNA) is captured by oligonucleotide probes and bound to magnetic microparticles. Amplification involves autocatalytic isothermal production of RNA transcripts with the two enzymes. Each newly synthesized RNA re-enters the TMA process and serves as a template for the next round of replication, resulting in exponential amplification of the target RNA. TMA reactions, like PCR reactions, reach a plateau after a certain number of cycles. Detection of TMA amplicons is based on specific hybridization to immobilized oligonucleotide probes. Amplicon-probe hybrids are revealed in an enzymatic reaction, followed by detection of a coloured or luminescent signal. Quantification can be performed by competitive amplification of the viral template with a known amount of synthetic standard added to each reaction tube.

### *Signal amplification techniques*

In signal amplification techniques the viral genomes are first hybridized to a holder, by means of specific 'capture' oligonucleotide probes. Then the signal emitted by the hybrids is amplified for detection and measurement.

*Hybrid capture*

In the hybrid capture system,[16] HBV DNA is hybridized to specific RNA probes to create RNA-DNA hybrids. The hybrids are captured on a solid phase coated with universal capture antibodies specific for RNA-DNA hybrids, and are detected with multiple antibodies (resulting in signal amplification) conjugated to a revelation system based on chemiluminescence. Luminescence is proportional to the initial amount of the template, and quantification is based on a standard curve generated simultaneously with known standards.

*Branched DNA technology*

In the 'branched DNA' (bDNA) assay, the third generation of which is currently available,[17–23] viral genomes are specifically captured on microwells by hybridization with oligonucleotide probes. A preamplifier molecule is added to the bDNA complex and synthetic bDNA amplifier molecules are hybridized to immobilized target hybrids in the microwells. Signal amplification is achieved through the multiple repeat sequences within each bDNA amplifier molecule that serve as sites for hybridization with alkaline phosphatase-conjugated oligonucleotide probes. Detection is based on alkaline phosphatase-catalyzed chemiluminescence emission from a substrate. As for hybrid capture, luminescence is proportional to the initial amount of the template nucleic acid, and quantification is based on a standard curve generated simultaneously with known standards.

## Analysis of the viral genome sequence

Viral genome sequence analysis is aimed at identifying signature sequences and/or amino acid substitutions at specific positions. In practice, signature sequences are used to classify viral strains into phylogenetic groups of clinical interest, called genotypes (including HBV and HCV types, and HCV subtypes).[24,25] It is also possible to identify amino acid substitutions known to be associated with viral resistance to antiviral drugs (for instance, lamivudine or adefovir resistance of HBV), or with particular forms of liver disease (for instance, precore and core promoter mutations in HBV).[26–30] The entire genome of HBV or HCV cannot be routinely analyzed, and available techniques therefore study particular genomic regions. Genome sequence analysis is based on direct sequencing, which provides the full sequence of the analyzed fragment, or on alternative techniques that identify specific sequences at given positions.

### *Direct sequencing of PCR amplicons*

Direct sequencing of PCR amplicons is performed after the sequencing reaction in an automated DNA sequencer. It can be used to determine the exact nucleotide and deduced amino acid sequence of the analyzed fragment. This can be applied to the detection of known clinically relevant motifs or mutations, and can also be used for 'blind' analysis aimed at detecting new motifs or mutations. The sequences must be analyzed and interpreted carefully, because the co-existence of variant viral populations in the same blood sample (quasi-spe-

cies distribution of viral genomes) may lead to sequence ambiguities.[31] Identification of amino acid substitutions at specific positions relies on direct examination of the generated sequences, whereas genotype determination is based on phylogenetic analysis of the generated sequences relative to reference sequences. Direct sequencing is the gold standard for genomic sequence analysis.

### *Reverse hybridization of PCR amplicons*

As direct sequencing is labour-intensive and only available in specialized laboratories, alternative techniques have been developed for routine clinical use. These techniques necessitate prior knowledge of the target motifs or mutations. By far the most widely used assays are based on reverse hybridization of PCR amplicons.[32–38] In these assays, PCR amplification of the region of interest is followed by stringent hybridization with oligonucleotide probes fixed to a solid phase. The probes are designed to be complementary to the various possible sequences. Revelation is based on a colorimetric reaction. Hybridization to a particular probe means that the analyzed fragment has the complementary sequence. This technique has the potential to detect mixed populations representing 10% or more of a quasi-species mixture.

## Application of molecular biology to HBV infection

### Available assays

#### *HBV DNA detection and quantification*

Table 49.1 shows the commercial assays that can currently be used to detect and quantify HBV DNA. HBV DNA quantitative units used in the various assays (pg/mL or copies/mL) do not represent the same actual amount of HBV DNA in a given clinical sample. The World Health Organization (WHO) has established an international standard for universal standardization of HBV DNA quantification units, and an HBV DNA international unit (IU) has been defined.[39] This IU must be preferred to any other quantitative unit and should now be implemented in all commercial HBV DNA quantitative assays. This will be particularly useful for establishing clinically relevant thresholds and universal recommendations for clinical decisions based on HBV DNA load. Table 49.1 shows the conversion factors from relative units used in the current assays to HBV DNA IUs.

Figure 49.1 shows the dynamic ranges of quantification of the current commercial assays stated by the manufacturers, in log IU/mL. The dynamic range of

**Table 49.1** Available HBV DNA detection and quantification assays and conversion from non-standardized, assay-specific units into IUs

| Manufacturer | Assay | Method | Dynamic range of quantification* | Conversion to IUs |
|---|---|---|---|---|
| Digene Corp., Gaithersburg, MD, USA | HBV Digene Hybrid-Capture™ II | Hybrid capture signal amplification in microplates | 142 000–1 700 000 000 copies/mL | To be determined |
| | Ultrasensitive HBV Digene Hybrid-Capture™ II | Hybrid capture signal amplification in microplates after centrifugation | 4700–57 000 000 copies/mL | To be determined |
| Roche Molecular Systems, Pleasanton, CA, USA | Amplicor HBV Monitor™ v2.0 | Manual quantitative RT-PCR | 1000–4 000 000 copies/mL | To be determined |
| | Cobas Amplicor HBV Monitor™ v2.0 | Semi-automated quantitative RT-PCR | 200–200 000 copies/mL | 1 IU = 5.6 copies/mL |
| | Cobas TaqMan 48 HBV | Real-time quantitative RT-PCR | 30–110 000 000 IU/mL (lower limit of detection if the assay is used as a qualitative, non-quantitative assay: 6 IU/mL) | 1 IU = 5.8 copies/mL |
| Bayer Corporation, Tarrytown, NY, USA | Versant TM HBV DNA 3.0 Assay | Semi-automated branched DNA signal amplification | 357–17 857 100 IU/mL | 1 IU = 5.6 copies/mL |

*The HBV DNA units are those currently given by the assays; they should be interpreted according to the conversion factors to and from IUs.

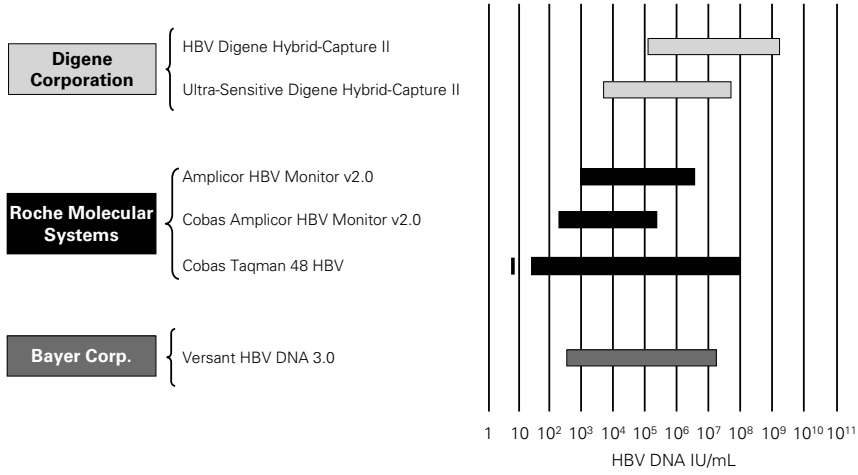


**Figure 49.1** Dynamic ranges of quantification of the current HBV DNA assays relative to the range of potential HBV DNA levels observed during infection, in log IU/mL. The dynamic range of quantification is indicated by a horizontal bar. When the lower limit of detection differs when the assay is used as a qualitative assay, non-quantitative from the lower limit of quantification, it is indicated as a vertical bar.

quantification can be extended to higher values by diluting and retesting high viral load samples falling above the upper limit of quantification of any of these assays, but the dilution step is time-consuming, increases the cost and may affect accuracy. By far the broadest dynamic range of quantification is achieved by real-time PCR techniques,[3–5,7–9,11,12,40] and this will be further improved in the future. This is particularly useful in the case of HBV infection, due to the great variability of baseline viral loads, sometimes reaching very high levels, and potentially very low HBV DNA loads observed upon antiviral therapy.

All of these assays have been shown to be specific and accurate within their respective dynamic ranges of quantification.[16,18–20,41–46] The possible influence of the HBV genotype on quantification has not been extensively studied. It is generally recommended not to take into account HBV DNA load variations of less than threefold (i.e. ± 0.5 $\log_{10}$), whereas variations of more than threefold (i.e. 0.5 $\log_{10}$) can reliably be considered to reflect significant changes. However, in the case of HBV infection where HBV DNA load may spontaneously fluctuate, it is recommended that baseline viral loads be established on the basis of several determinations before therapy.

### *HBV genome sequence analysis*

HBV genome sequence analysis has three potential applications, including HBV genotype determination, identification of precore and core promoter mutations, and identification of antiviral drug resistance mutations.[47,48] The clinical utility of these determinations is currently under study. All of them can be achieved by means of 'home-made' sequencing. Genotype determination is based on sequence comparison with banks of reference sequences, eventually through phylogenetic analysis, that allows classification of the typed HBV strain based on sequence similarities. Previously known precore and core promoter mutations can be identified by sequencing the corresponding region after PCR amplification.[47] Finally, mutations known to confer HBV resistance to lamivudine are identified by analyzing the sequence of the HBV polymerase at known amino acid positions.[27–30] Additional mutations, recently suggested to confer HBV resistance to adefovir dipivoxil, can also be identified through direct sequencing of the HBV polymerase.[26]

Commercial assays are being developed to be used in these indications. Line probe assays are now available to determine the HBV genotype, identify precore and core promoter mutations and identify mutations of the HBV polymerase known to confer resistance to lamivudine.[35,49–54] Compared with direct sequencing, these assays have the advantage of potentially detecting mixtures of quasi-species variants in patients' blood. On average, minor variants representing 10% or more of the viral populations can be identified together with the major population. This may be particularly interesting to diagnose HBV resistance to specific inhibitors before the resistant population has completely replaced the 'wild-type' sensitive one. If confirmed to be clinically relevant, resistance mutations to adefovir and future antiviral drugs will be added in the future generations of these assays.

## Practical use

### *Screening of blood donations for HBV*

Routine screening of blood donations for HBsAg and total anti-HBc antibodies has drastically reduced the risk of post-transfusion hepatitis B, which was estimated to be approximately 1/137 000 donations in the 1997–1999 period in the United States and 1/475 000 donations in France during the same period. No extra donors would

be rejected on the basis of pooled HBV DNA testing than by using the most sensitive HBsAg tests on an individual basis. However, HBV DNA can be detected on average 21 days prior to the appearance of HBsAg, even when HBsAg is assayed with the most sensitive tests. In addition, HBV DNA testing may identify 'occult' HBV infection in HBsAg-negative donors.[55] Thus, individual HBV DNA screening of blood donations by sensitive molecular biology techniques could improve viral safety. The same applies to organ, tissue and cell donors.

### *Diagnosis of HBV infection*

No molecular biology-based assays are necessary for the diagnosis of acute hepatitis B, which is based on serological testing. Chronic hepatitis B is defined by HBsAg persistence in serum for >6 months. In this setting, HBV DNA detection-quantification is necessary to determine whether or not HBV is replicating and the level of HBV replication.[56] When HBV DNA is detected, the quantitative result should be interpreted according to HBeAg status, alanine aminotransferase (ALT) activity and histological status, including the activity and degree of fibrosis.

In the presence of HBeAg, the diagnosis of replicating chronic hepatitis B is made whatever the viral load.[56] In contrast, the interpretation of HBV DNA quantification is difficult in HBeAg-negative/anti-HBe antibody-positive patients (precore mutant HBV), who generally have lower replication levels than HBeAg-positive patients. The current highly sensitive assays, which can detect HBV DNA in the vast majority of HBsAg carriers (Fig. 49.1), make it necessary to define a clinically relevant replication threshold. The best discriminatory threshold (or interval, as overlaps may exist) may vary from patient to patient, or between different patient subgroups, and remains to be established in appropriate clinical studies using highly sensitive and accurate HBV DNA assays. These thresholds may indeed be particularly important in making appropriate therapeutic decisions.

### *Assessment of disease severity and prognosis*

Histological examination of liver biopsy material is still the best way of assessing the severity of chronic hepatitis B and establishing the prognosis. HBV DNA detection also provides valuable prognostic information. Indeed, active HBV replication is associated with a significant risk of progression to chronic hepatitis B complications (including cirrhosis and hepatocellular carcinoma – HCC).[56] This risk is low in the absence of detectable HBV DNA, except in patients with cirrhosis, who may subsequently develop HCC despite the absence of HBV replication. However, most of the HBsAg carriers are now HBV DNA-positive with highly sensitive assays. It has been suggested that HBV DNA loads lower than approximately $10^2$–$10^3$ IU/mL were associated with inactive liver disease. Nevertheless, the possible prognostic significance of HBV DNA load, and informative thresholds, need to be determined in appropriate clinical studies with the highly sensitive assays.

### *Treatment of HBV infection*

HBV DNA detection and quantification is a key tool for treatment monitoring in chronic hepatitis B.

#### *Decision to treat*

The decision to treat patients with chronic hepatitis B must be taken individually, on the basis of precisely weighed parameters. Elevated serum ALT activity, a liver biopsy showing chronic hepatitis with or without cirrhosis, and the presence of significant levels of HBV DNA are strong arguments for initiating antiviral therapy.[56] As stated above, no precise clinically relevant HBV DNA thresholds are known, and prospective trials are needed to determine HBV DNA loads (in IU/mL) above which patients with chronic hepatitis B should be treated (and below which they should not be treated).

#### *Selection of optimal therapy*

Patients with chronic hepatitis B can now be offered two treatment options: (1) short-term treatment with pegylated interferon-α (PEG-IFN-α) administered weekly[57,58] or HBV inhibitors administered daily[59–67] with the aim of achieving a sustained response (such as HBeAg or HBsAg seroconversion); (2) long-term 'suppressive' treatment with specific HBV inhibitors,[59–67] that would ideally combine a nucleoside analogue and a nucleotide analogue in order to delay the occurrence of HBV resistance in patients who will be treated for several years. The individual indication for these two treatment options is still debated.[56,78] Algorithms combining several clinical, biological, virological and histological parameters need to be established in order to define which is the best first-line option for each individual patient. In this context, it is likely that HBV DNA quantification will help in selecting optimal therapy. For instance, the HBeAg-positive patients with a low HBV DNA level, who generally have high ALT values, are more likely than those with a high HBV DNA level to have a sustained response to short-term therapy.[56,68] However, the precise HBV DNA cut-off point that discriminates between 'low' and 'high' pretreatment replication levels needs to be determined. Further studies are needed to define the best individual tailoring options and the utility of HBV DNA quantification in this process.

*Treatment monitoring*

HBV DNA quantification is critical in treatment monitoring. A significant antiviral effect of therapy can be defined as a reduction of HBV DNA levels of 1 log or more relative to baseline (i.e. a baseline viral load divided by 10 or more). The magnitude of this antiviral effect can be measured by the log HBV DNA reduction compared to baseline. However, the interpretation of antiviral effects of therapy may be complicated by the fact that baseline HBV DNA loads are often fluctuating, especially in HBeAg-negative patients. Thus, baseline viral load should be established by several determinations prior to treatment initiation. The criteria for 'successful' inhibition of HBV replication (antiviral efficacy) are not defined. While treatment efficacy is easy to establish in the case of an HBe seroconversion, the log reduction or target HBV DNA thresholds to be reached in non-HBe seroconverters or HBeAg-negative patients have not been precisely determined, especially in the light of the subsequent outcome of liver disease. This is urgently needed for patients receiving long-term 'suppressive' therapy based on nucleotide/nucleoside inhibitors. Overall, HBV DNA load quantification is a crucial tool to monitor HBV treatment, but the end-points of treatment efficacy need to be precisely determined.

*Assessment of HBV resistance to nucleoside/nucleotide analogue HBV inhibitors*

HBV resistance to lamivudine is frequent. It is related to the selection of HBV mutants bearing amino acid substitutions located within or close to the catalytic site of HBV DNA polymerase.[69–79] Cross-resistance with newly developed nucleoside analogues, such as FTC, entecavir or LdT, has been described both *in vitro* and *in vivo*. HBV resistance to adefovir has recently been shown to occur, although it is delayed and less frequent than lamivudine resistance.[26] Its molecular mechanisms are still under study, but HBV polymerase mutations conferring adefovir resistance have been identified. Cross-resistance with tenofovir remains to be demonstrated.

The assessment of HBV resistance to specific inhibitor molecules is based on HBV DNA load monitoring. There is currently no consensus on the rhythm at which such determinations should be performed. Initial treatment efficacy should be assessed on average 3 months after treatment initiation. Subsequently, HBV DNA determinations can be performed every 3 months (like during HIV treatment with highly active anti-retroviral therapy) or every 6 months. If HBV resistance is suspected, it is necessary to eliminate non-viral causes of antiviral treatment failure, such as poor or non-adherence to therapy, or metabolic causes of lack or reversion of the drug's antiviral effect. Primary HBV resistance to an antiviral inhibitor molecule is defined by the lack of a significant antiviral effect of the drug, i.e. the inability of the drug to decrease baseline HBV DNA load by more than 1 log. Secondary resistance occurs in patients who experienced a significant HBV DNA reduction (1 log or more) upon therapy and relapsed while on treatment. It is defined by a re-increase of viral replication of 1 log or more compared with the HBV DNA nadir. This relapse must be confirmed by a second determination 1 month later. Highly sensitive assays with a broad dynamic range of quantification must be preferred to monitor HBV treatment responses.

Resistance mutations can be detected by direct sequencing or reverse hybridization.[27,29,35,49,53] These techniques currently have no routine indications, given the small number of available molecules. However, identification of antiviral resistance mutations might be useful in future when several drugs and combination therapy options become available.

## Application of molecular biology to HCV infection

### Available assays

#### *HCV RNA detection and quantification*

Table 49.2 lists the commercial assays that can currently be used to detect or quantify HCV RNA. Qualitative, non-quantitative assays are based on target amplification, i.e. PCR or TMA. They can detect the presence of HCV RNA, but they cannot measure HCV viral load. Qualitative assays have been clinically useful because they are more sensitive for HCV RNA detection than most of the currently available quantitative assays. They will probably lose their clinical utility with the advent of highly sensitive quantitative assays.

Quantitative HCV RNA assays are based on bDNA technology, competitive PCR or real-time PCR. Until recently, the quantitative units used in the various assays did not represent the same amount of HCV RNA in a clinical sample. WHO has established an international standard for universal standardization of HCV RNA quantification units and an HCV RNA international unit (IU) has been defined.[80,81] It is currently used in all commercial HCV RNA quantitative assays.[82–86] The use of standardized IUs for HCV RNA quantification has been recommended by the 2002 National Institutes of Health Consensus Conference on Hepatitis C in order to allow recommendations and guidelines to be derived from clinical trials and applied in clinical practice with any quantitative HCV RNA assay.[87]

The manufacturers' stated dynamic ranges of quantification of the currently available tests are shown in Fig. 49.2. The dynamic range of quantification can be extend-

**Table 49.2** Available diagnostic HCV RNA detection and quantification assays

| Manufacturer | Assay | Method | Lower limit of detection | Dynamic range of quantification* |
|---|---|---|---|---|
| Roche Molecular Systems, Pleasanton, CA, USA | Amplicor™ HCV v2.0 | Manual qualitative RT-PCR | 50 IU/mL | None |
| | Cobas Amplicor™ HCV v2.0 | Semi-automated qualitative RT-PCR | 50 IU/mL | None |
| | Amplicor™ HCV Monitor™ v2.0 | Manual quantitative RT-PCR | 600 IU/mL | 600 to <500 000 IU/mL |
| | Cobas Amplicor HCV Monitor™ v2.0 | Semi-automated quantitative RT-PCR | 600 IU/mL | 600 to <500 000 IU/mL |
| | Cobas TaqMan 48™ HCV | Real-time quantitative RT-PCR | 10 IU | 30–200 000 000 IU/mL |
| Bayer Corporation, Diagnostics Division, Tarrytown, NY, USA | Versant™ HCV RNA Qualitative assay | Manual qualitative TMA | 6 IU/mL | None |
| | Versant™ HCV RNA 3.0 assay | Semi-automated quantitative branched DNA signal amplification | 615 IU/mL | 615–7 700 000 IU/mL |
| Abbott Diagnostic, Chicago, IL, USA | LCx™ HCV RNA Quantitative assay | Semi-automated quantitative RT-PCR | 23 IU/mL | 23–2 300 000 IU/mL |
| | HCV Quant ASR | Real-time quantitative RT-PCR | 25 IU/mL | 25–50 000 000 IU/mL |
| National Genetics Institute, Los Angeles, CA, USA | SuperQuant | Semi-automated quantitative RT-PCR | 30 IU/mL | 30–1 470 000 IU/mL |

RT-PCR, reverse transcriptase-polymerase chain reaction; TMA, transcription-mediated amplification.
*Quantitative assays only.

ed by diluting and retesting high viral load samples, but the dilution step is time-consuming, increases the cost and may affect accuracy.[14,88] Real-time PCR assays yield the broadest dynamic range of quantification, which covers well the range of HCV viral loads generally observed in untreated and treated patients. All currently available assays have satisfactory specificity. Their stated specificity is higher than 95% in all instances, and reaches 98–99% in most cases. They accurately quantify HCV RNA within their respective dynamic ranges of quantification, with no difference among the various HCV genotypes.[21,82–93] It is generally recommended not to take into account HCV RNA load variations of less than threefold (i.e. ± 0.5 log), which may be related to the intrinsic vari-

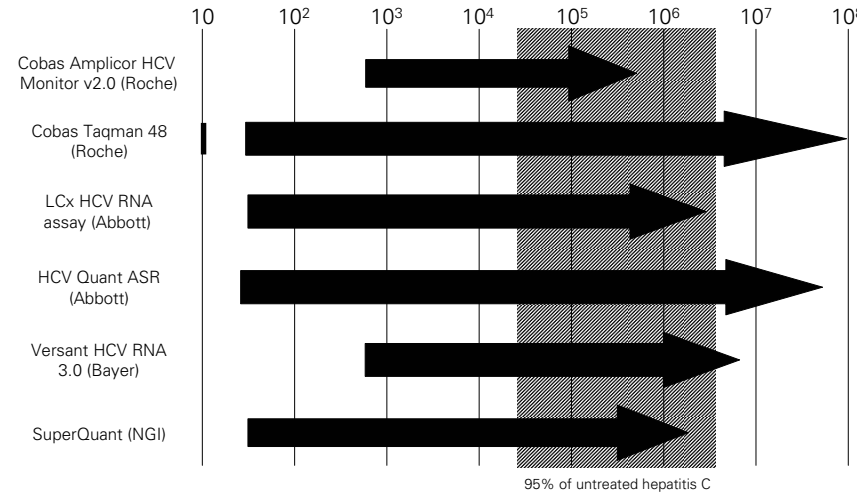


**Figure 49.2** Dynamic ranges of quantification of the current HCV RNA assays relative to the range of potential HCV RNA levels observed during infection, in log IU/mL. The grey area indicates the range of HCV RNA levels found in approximately 95% of untreated patients with chronic hepatitis C. The dynamic range of quantification of each assay is indicated by an arrow. When the lower limit of detection differs when the assay is used as a qualitative assay, non-quantitative from the lower limit of quantification, it is indicated as a vertical bar.

ability of the assays. In contrast, variations of more than threefold (i.e. 0.5 log) can reliably be considered to reflect significant differences in HCV RNA load. However, when samples from the same patient taken at different time-points are tested in the same laboratory with the same technique, the precision of HCV RNA quantification is of the order of ± 0.2 log. This is important when assessing the log drop of HCV RNA during treatment of patients with HCV genotype 1 infection.

### *HCV genotype determination*

Table 49.3 shows the commercial assays based on molecular methods that can be used to determine the HCV genotype. Genotyping based on sequence analysis in the NS5B region is considered the gold standard for genotype determination.[25] However, most of the assays target the 5′ non-coding region of HCV genome, which can be amplified by PCR in nearly all patients. In the 5′ non-coding region, mis-typing is exceptional, whereas mis-subtyping is frequent (approaching 10% of cases). This has no clinical consequences, as clinical decisions are not currently based on the HCV subtype.

## Practical use

### *Screening of blood donations for HCV RNA*

Routine screening of blood donations for anti-HCV antibodies has drastically reduced the risk of post-transfusion hepatitis C.[94] HCV RNA detection in pooled or individual donations by molecular biology techniques (e.g. qualitative PCR or TMA assays) has been implemented in blood banks in the European Union and the United States in order to reduce the 'serological window' between HCV infection and the detection of specific antibodies, and to improve the viral safety of blood products.[95,96] This measure has made it possible to further reduce the residual risk of HCV transmission, which is currently less than 1/1 000 000 donations in France (S. Laperche, personal communication). We recently reported cases of HCV RNA detection in the absence of anti-HCV antibodies in organ and tissue donors,[97] suggesting that HCV RNA screening in the setting of organ, tissue and cell transplantation might be useful to further reduce the risk of HCV transmission. This could be of particular interest in organ donors, who were shown to have a higher prevalence of HCV infection than the general population. However, molecular biology techniques will have to be modified to allow single determinations performed in emergency for use in this setting.[97]

### *Diagnosis of HCV infection*

A sensitive HCV RNA assay, able to detect 50 IU/mL or less, should be used for the diagnosis of HCV infection. This lower limit of detection is achieved by the current qualitative assays and by some quantitative assays (Table 49.2).[87]

#### *Acute hepatitis C*

In acute HCV infection, anti-HCV antibodies are detected in only 50–70% of patients at the onset of symptoms. In the remaining patients, anti-HCV antibodies usually emerge after 3–6 weeks.[98–100] Thus, HCV RNA must be sought in patients with acute hepatitis who have no serological markers of viral hepatitis (including anti-HCV antibody negativity).[87] HCV RNA detection strongly suggests acute hepatitis C, which is confirmed by subsequent antibody seroconversion. In contrast, negative HCV RNA detection makes this diagnosis very unlikely. When both anti-HCV antibodies and HCV RNA are present during acute hepatitis, it may be difficult to discriminate among acute hepatitis C, an acute exacerbation of chronic hepatitis C, and acute hepatitis of another cause in a patient with chronic hepatitis C. Finally, when anti-HCV antibodies are present in the absence of

**Table 49.3** Available HCV genotyping assays

| Manufacturer | Assay | Method | Ability to identify types | Ability to identify subtypes |
|---|---|---|---|---|
| Innogenetics, Ghent, Belgium | Inno-LiPA HCV II or Versant® Genotyping Assay | Reverse hybridization of PCR amplicons | 1, 2, 3, 4, 5, 6 | 1a, 1b, 1a/1b, 1, 2a/2c, 2b, 2, 3a, 3b, 3c, 3, 4a, 4b, 4c/4d, 4e, 4f, 4h, 4, 5a, 6a, 10a |
| Bayer Corporation, Diagnostics Division, Tarrytown, NY, USA | Trugene™ HCV 5′NC Genotyping kit | Direct sequencing of the 5′ non-coding region of HCV genome followed by sequence comparison with an HCV genotype sequence database | 1, 2, 3, 4, 5, 6 | 1a, 1b, 1c, 2a, 2b, 2c, 2d, 3a, 3b, 3c, 3d, 3e, 3f, 3g, 4a, 4b, 4c, 4d, 4e, 4f, 4g, 5a, 6a, 6b |
| Abbott Diagnostic, Chicago, IL, USA | HCV Geno ASR | Multiple real-time PCR amplifications of the 5′ non-coding and NS5B regions with genotype-specific primers | 1, 2, 3, 4, 5, 6 | 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a |

HCV RNA, acute hepatitis C is unlikely. However, HCV RNA should be retested a few weeks later, as transient negative HCV RNA detection can occur at the late stage of acute HCV infection before the infection becomes chronic.[88]

*Chronic hepatitis C*

In patients with symptoms of chronic hepatitis, HCV RNA testing must be performed when anti-HCV antibodies are present, to assess viral replication and to confirm the diagnosis of HCV infection.[87,88,101,102] HCV RNA testing should also be performed when no antibodies are found and hepatitis C is suspected in haemodialyzed or immunosuppressed patients who may (occasionally) have seronegative chronic hepatitis C, especially if the immunodepression is profound.[87]

*Mother-to-infant transmission*

Babies born to HCV-infected mothers usually carry HCV antibodies for between a few months and 1 year, due to passive transfer from the mother.[103–106] The diagnosis of HCV infection in the baby is thus based on HCV RNA detection.[87] The dates at which the test should ideally be performed are not precisely known. In case of HCV RNA negativity, the lack of transmission is confirmed by the gradual disappearance of HCV antibodies. If the baby is infected, HCV RNA detection may be positive at birth or only later during the first year of life, while anti-HCV antibodies persist. Due to a higher rate of spontaneous resolution in children than in adults,[107] HCV RNA persistence must be confirmed after the first year of life in the babies identified to be HCV RNA-positive at birth or soon afterwards.

*Accidental exposure*

HCV RNA can generally be detected in serum within a few days to a few weeks after accidental exposure to HCV-infected blood. Elevated ALT activity and seroconversion are observed a few weeks later in the absence of therapy.[87] Antiviral therapy, which prevents progression to chronicity in a large proportion of cases, is not an emergency and may be started once the diagnosis of acute hepatitis C is made.[108]

### *Assessment of disease severity and prognosis*

No virological parameters can be used to assess the severity of HCV-related liver disease (which is currently evaluated by means of liver biopsy or serological markers of activity and fibrosis), or to establish the prognosis. In particular, neither the HCV genotype nor HCV viral load at the time of liver biopsy appear to correlate with necroinflammatory activity or the extent of fibrosis, or to predict subsequent outcome. Therefore, repeated HCV RNA determinations are not needed during follow-up of untreated patients.

### *Treatment of HCV infection*

The reference therapy for chronic hepatitis C is now a combination of weekly PEG-IFN-α and daily ribavirin administration.[87] Molecular biology-based techniques are particularly useful for managing the successive phases of treatment. Figure 49.3 presents an algorithm for treatment decisions based on molecular biology techniques.

*Decision to treat*

Chronic hepatitis C is curable. Therefore, in the absence of contraindications to antiviral therapy, treatment is theoretically indicated for all patients with HCV RNA positivity, whatever their ALT levels, severity of liver disease or extrahepatic manifestations.[87] The strategy, however, varies according to the HCV genotype, because of consistently different outcomes of therapy.[109–111] Thus, HCV genotype determination is mandatory when treatment is envisaged.[87] Patients infected with HCV genotypes 2 or 3 should be treated in the absence of contraindications, because >80% of them will achieve a sustained virological response (defined by an HCV RNA clearance maintained for 24 weeks after treatment withdrawal), the end-point of therapy that corresponds to a cure of infection in the vast majority of cases.[109–111] In patients infected with other genotypes, who have lower rates of sustained virological response (40–50% in genotype 1),[109–111] or in patients with genotypes 2 or 3 and relative contraindications to therapy, the decision to treat must be weighed up according to the natural prognosis of HCV-related liver disease.[87] ALT levels, liver biopsy or serological markers of fibrosis, extrahepatic manifestations and the patient's motivation for therapy are useful in deciding which of these patients should be treated.[87]

*Selection of optimal therapy*

Both the duration of treatment and the dose of ribavirin must be tailored to the HCV genotype.[87,110] Patients with HCV genotypes 2 and 3 must be treated with PEG-IFN-α and 800 mg/day of ribavirin for 24 weeks.[87] Patients infected with HCV genotype 1, as well as those infected with genotypes 4, 5 or 6 pending further studies, must receive a higher dose of ribavirin (1000–1200 mg/day according to body weight) and must be treated for 48 weeks (according to the virological response to therapy in genotype 1).[87]

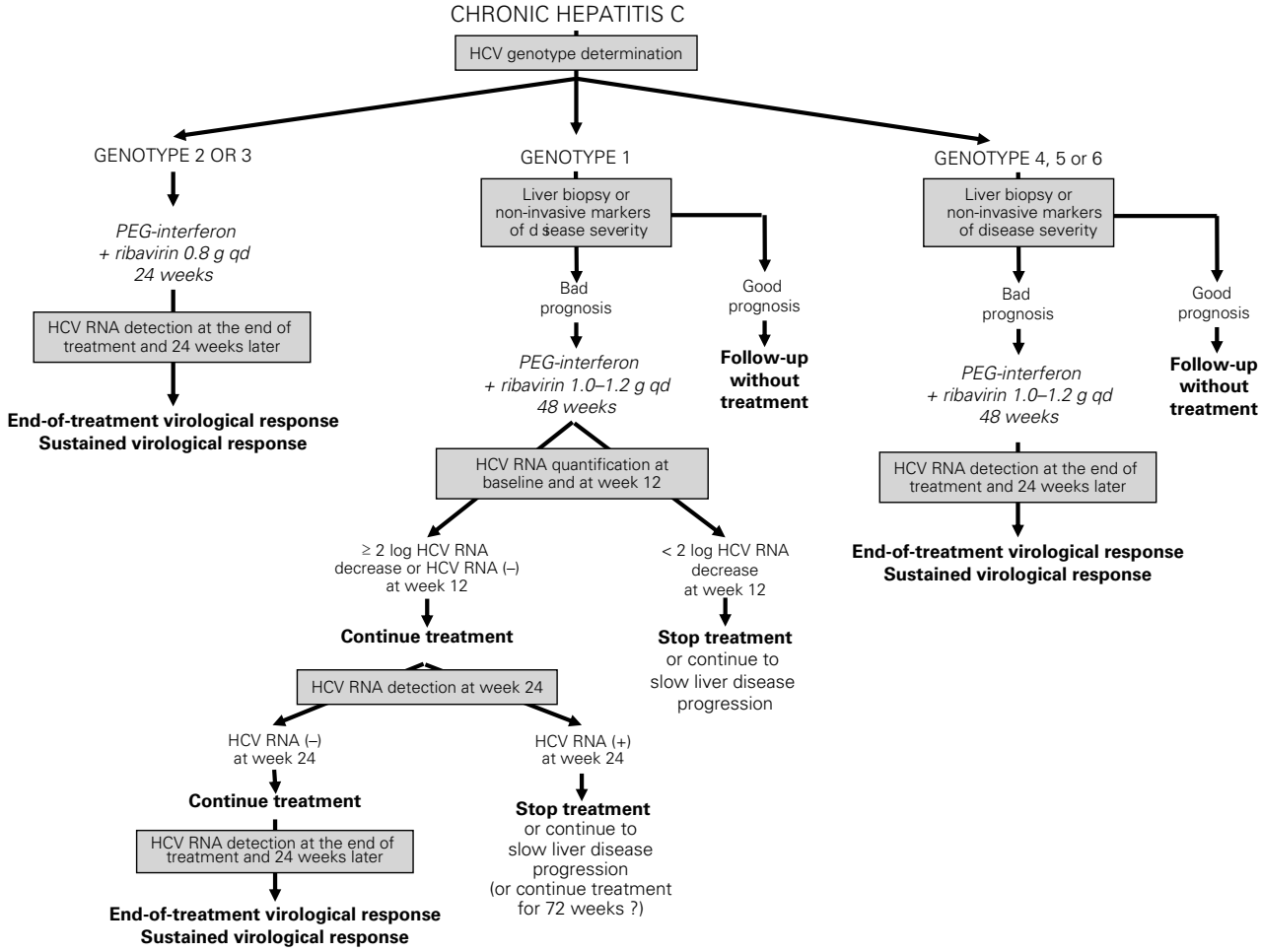


**Figure 49.3** Algorithm for treatment decisions in chronic hepatitis C based on molecular biology techniques.

*Treatment monitoring*

HCV RNA detection at the end of treatment is highly predictive of post-treatment relapse. It is better achieved with a highly sensitive technique (lower limit of detection ≤50 IU/mL).[87] HCV RNA non-detection at the end of treatment demonstrates a virological response. These patients should be retested for HCV RNA 24 weeks later with a sensitive method to assess the sustained virological response, which corresponds with a cure of infection in the vast majority of cases.[87]

HCV RNA assay before and after 12 weeks of treatment is used to monitor genotype 1 chronic hepatitis C treated with PEG-IFN-α and ribavirin.[87,109,112] Treatment can be continued when a 2 log (100-fold) drop in HCV RNA level occurs, or when HCV RNA is undetectable at week 12. Otherwise the likelihood of achieving a sustained virological response is virtually nil, and treatment can be stopped, or continued in an attempt to slow liver disease progression (without clearing the virus).[87,109,112] In patients who continue on therapy, HCV RNA can be again assessed at week 24 with a sensitive assay. If HCV RNA is undetectable (<50 IU/mL), treatment should be continued until week 48. If HCV RNA is detectable (≥50 IU/mL), the likelihood of a sustained virological response is virtually nil.[87,112] These patients could, however, benefit from longer (72 weeks) therapy,[113] pending further studies. The end of treatment, and most importantly the sustained virological response, must be assessed at the end of treatment and 24 weeks later, respectively, with a sensitive HCV RNA assay.[87] The potential utility of assessing the virological response during therapy in patients infected with a genotype other than 1 remains to be determined.

## Application of molecular biology to other hepatitis viruses

No molecular biology tool is useful for the diagnosis of hepatitis A or E, which is based on serological assays.

Hepatitis delta virus RNA detection is the most sensitive and specific marker of infection, but is not widely used in clinical practice.

## Conclusion

In the past decade, the introduction and constant improvement of molecular biology-based techniques have provided invaluable tools for the management of chronic viral hepatitis. They can now be used to test blood donations, diagnose active infection, help to establish the prognosis, guide treatment decisions and assess the virological response to therapy. Further work is required to fully standardize assays and quantification units, improve automation, and better define clinically relevant thresholds that can be used to establish universal recommendations for patient care. The development of increasingly sensitive and accurate assays for viral genome detection and quantification will improve the assessment of the response to antiviral therapy and permit earlier detection of viral resistance. Together with the development of new antiviral drugs and novel therapeutic approaches, these improvements will allow us to optimize the treatment of chronic viral hepatitis and improve the global results.

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# Chapter 50
# Hepatitis in HIV-infected persons

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There are many important epidemiological and clinical interactions between human immunodeficiency virus (HIV) and the hepatotropic viruses. The pattern of hepatotropic virus infection varies according to the risk group. In homosexual men, for example, hepatitis A virus (HAV) and hepatitis B virus (HBV) infections are common, whereas the HIV-infected intravenous drug user or recipient of blood products is more likely to be exposed to hepatitis C virus (HCV). Recent reports, however, describe acute HCV infection among HIV-positive homosexual men, and there are concerns that underlying HCV may increase the risk of anti-retroviral drug toxicity. Co-infection with GBV-C virus may, however, be in some way beneficial for patients with HIV infection.

It is increasingly recognized that HIV infection can modify the clinical course of several other viral infections, whether these occur before the onset of HIV infection, simultaneously or subsequently. The clinical importance of this is increasing with the improved survival of the HIV-infected individual.

## Hepatitis A

Several outbreaks of HAV infection have occurred in homosexual men[1] and intravenous drug users.[2] However, similar seroprevalence rates of previous hepatitis A infection were reported in homosexual (32%) and heterosexual (30%) men attending a large London clinic for sexually transmitted diseases.[3] HAV vaccination has been recommended for people in high-risk groups, although the antibody response is reduced in the presence of HIV infection.[4]

## Hepatitis B

### Epidemiology

HIV and HBV have the same routes of transmission and co-infection is therefore common. In one study, >90% of patients with HIV infection had HBV markers of current or past infection.[5] One study of a predominantly male homosexual clinic reported a co-infection rate of 6%.[6] High seroprevalence rates are also seen in sub-Saharan patients and in patients with a history of intravenous drug use.

### Immunopathology

Direct interaction of viral infections at the cellular level would require that both viruses infect and replicate within the same cell lines. In an analysis of infected lymphoid cells from patients with chronic HBV infection, one study reported the presence of HBV DNA in peripheral blood mononuclear cells (PBMCs) in all patients with HIV and HBV infection, but in only 50% of those who were HIV-negative.[7]

Experimental models suggest that HIV can infect and replicate within CD4-expressing sinusoidal endothelial cells[8] and Kupffer cells.[9] Resultant functional changes have also been described leading to reduced synthesis of von Willebrand factor and reduced secretion of endothelin-1.[10] Human hepatoma cell lines, which are non-CD4-expressing, can also be infected with HIV.[11] Although hepatomegaly, steatosis and abnormal transaminase values are more commonly seen in patients taking anti-retroviral drugs, such findings are seen from time in untreated patients and it is unclear whether this can be attributed to a direct effect of HIV on the liver.

Direct interactions at the molecular level are thought to occur. The long terminal repeat (LTR) of the HIV genome, for example, has several sequences in which cellular transcription factors can bind and upregulate expression of viral genes (Fig. 50.1). NF-κB, a transcription factor, is generally bound to an inhibitor I-κB within the cytoplasm of the cell and on activation is separated from I-κB, passes to the nucleus, and binds to the LTR. Cytokines such as tumour necrosis factor (TNF)-α, can induce NF-κB,[12] and it is thought that the X gene product of HBV can act in a similar way.[13] Studies with hepatocellular carcinoma and lymphoblastic T-cell lines have demonstrated that the expression of the X gene is capable of upregulating the expression of reporter genes under the control of the HIV LTR[14] (Fig. 50.2).

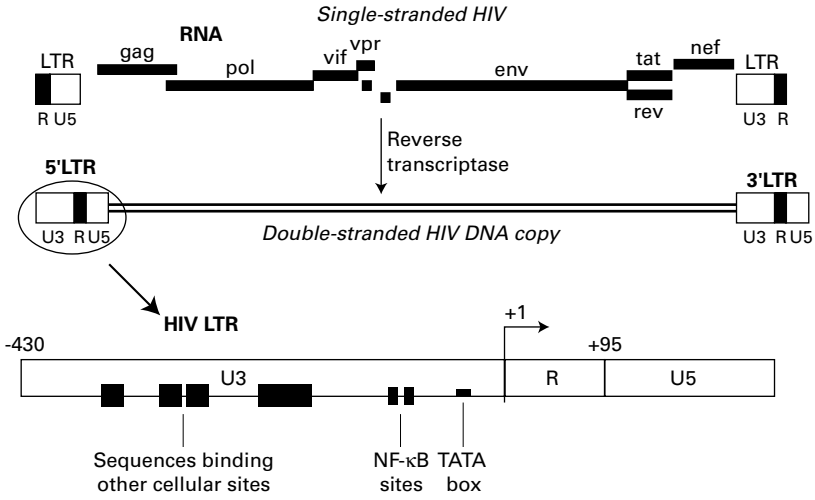


**Figure 50.1** HIV genome and promoter sites on HIV long terminal repeat (LTR).

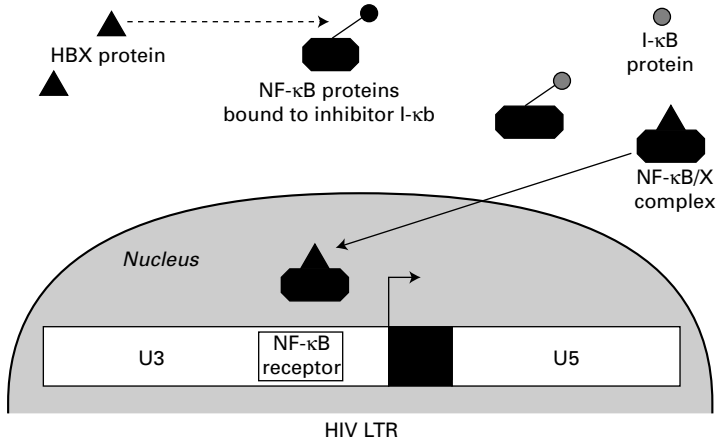


**Figure 50.2** Possible relationship between NF-kB and HBx proteins.

HIV infection leads to a progressive decline in immune function, affecting principally the cell-mediated response, but also with effects on the humoral response. Much of the liver damage seen with hepatitis B is thought to be related to recognition and clearance of infected hepatocytes by cytotoxic T cells.[15] The HIV-mediated reduction of T-cell function and number is inevitably associated with increased HBV replication and less immune recognition of infected hepatocytes. A similar pattern had already been observed in HIV-negative HBV-infected patients receiving immunosuppressive therapy.[16,17] Studies have shown that when HIV-associated immunosuppression develops, there is an increase in HBV DNA levels but a reduction in transaminase levels and a decrease in the hepatic necroinflammatory score.[18–21] Conversely, the immune restoration associated with effective anti-retroviral therapy can lead to immune recognition and destruction of infected hepatocytes, leading to an increase in the transaminase levels.[22]

## Clinical outcome

### *Acute infection (Table 50.1)*[18–21,23–29]

Retrospective analysis of the 1980 Centers for Disease Control multicentre HBV vaccine study has yielded valuable data on the interaction of HIV and HBV.[23] In unvaccinated individuals, the risk of chronic carriage of HBV was 21% in those with HIV infection compared with 7% of those who were HIV seronegative. The average duration of HIV infection before HBV infection was only 8.5 months. Peak transaminase values were similar in both groups, but the duration of elevated transaminase values (>200 IU/L) was significantly prolonged in the HIV-positive group. In an Australian study[18] the risk of chronic HBV infection was 23% (7/31) in HIV-positive individuals, compared with 4% (2/46) in those who were HIV-negative. HIV-infected patients with higher CD4 counts were more likely to clear infection.

**Table 50.1** Effect of HIV on HBV infection

| Effects | References |
|---|---|
| Acute infection | |
| Increased carriage following acute infection | Hadler *et al.*,[23] Bodsworth *et al.*[18] |
| Milder acute disease | Bodsworth *et al.*[24] |
| More severe acute disease with prolonged transaminitis | Hadler *et al.*[23] |
| Chronic infection | |
| Higher HBV DNA load | Krogsgaard *et al.*,[20] Perrillo *et al.*,[21] Bodsworth *et al.*,[24] Goldin *et al.* [19] |
| Reduced hepatocyte necrosis | Krogsgaard *et al.*,[20] Perrillo *et al.*,[21] Bodsworth *et al.*,[24] Goldin *et al.*[19] |
| Reduced response to treatment | McDonald *et al.*,[25] Brook *et al.*[26] |
| Increased risk of HBV reactivation/reinfection | Levy *et al.*[27] |
| Vaccine | |
| Reduced response to vaccination | Collier *et al.*,[28] Loke *et al.*[29] |

### *Chronic infection (Table 50.1)*

In a study by Goldin *et al.*,[19] the necroinflammatory scores of liver biopsies from HBV-infected patients with and without HIV infection were compared. A greater expression of hepatitis B e antigen (HBeAg) and HBV polymerase, but reduced necroinflammation and fibrosis, were seen in the HIV-positive group. Clinical studies of the interactions of HIV and HBV are often hampered by lack of data on the relative timing of the two infections. In a Norwegian study,[30] 80 individuals with HIV infection were monitored, and the outcome of those with anti-HBc antibodies was compared with those who were anti-HBc-negative. The relative risk for progression to AIDS was 3.6 for those with markers of HBV infection.

In the early Multicenter AIDS cohort study[31] HBV infection showed no significant effect on progression to AIDS over 2.5 years, but more recent studies in the setting of decreasing HIV-related mortality rates show an increased risk of liver-related mortality in this group.[32]

There have been several reports of clinically important reactivation of HBV in HIV-infected individuals. This has also been described in patients on cessation of immunosuppressive therapy for other conditions.[33] The mechanism of HBV reactivation in HIV infection is not understood and does not necessarily correlate with changes in the CD4 count or other quantifiable changes in immune function. This diagnosis should be considered in a patient with HIV and HBV infection who develops a flare in the transaminase levels. HBeAg may be negative, however, and the diagnosis may depend on the exclusion of other causes of liver disease, by the detection of HBV DNA levels[27] or by appropriate immunohistochemical stains of liver biopsy material (Table 50.2). Very high levels of HBV may be associated with the development of fibrosing cholestatic hepatitis. This was mainly reported in liver transplant patients prior to the availability of lamivudine and has also been recorded in patients with HIV and HBV infection.[34] It is thought that at very high levels of HBV replication the virus may have a direct cytopathic effect.

## Response to treatment

### *Interferon-α*

It was readily apparent from the early interferon (IFN) trials for chronic HBV infection that underlying immunosuppression was associated with a reduced chance of sustained response. Several studies have now confirmed that the chance of a successful response to IFN is markedly reduced in the setting of HIV infection.[25,26] It is not known whether the poor response to IFN is because of reduced T-cell function or the abnormal cytokine milieu in HIV infection, with high levels of acid-labile IFN,[35] downregulation of IFN-α receptors,[36] or the presence of IFN antibodies.[37] In one study comparing the effects of IFN monotherapy with the combination of corticosteroid

**Table 50.2** Diagnostic and staging tests for chronic hepatitis in anti-HIV-positive patients

| HBV | HDV | HCV |
|---|---|---|
| HBeAg/HBsAg | Anti-HDV | Anti-HCV (ELISA) |
| Anti-HBs/Anti-HBc | IgM/IgG | HCV RNA |
| HBV DNA | Serum HDV Ag | HCV genotype |
| Liver biopsy with immunohistochemical staining | HDV RNA<br>Liver biopsy with HDV Ag staining | Liver biopsy and histology |

ELISA, enzyme-linked immunoabsorbent assay.

therapy and IFN,[38] two of three HIV-positive patients in the combination group cleared HBV infection. Both had CD4 counts >500/mm$^3$.

The availability of pegylated IFNs (PEG-IFNs) has led to renewed interest in IFN as therapy for chronic HBV infection. This option is only suitable for patients with high CD4 counts as: (1) IFN is less likely to work in patients with CD4 lymphopenia and (2) IFN therapy can be complicated by a decline in the CD4 count.

### *Nucleoside analogues*

HBV and HIV both have reverse transcriptase (RT) activity and many HIV RT inhibitors also have activity against HIV. Zidovudine has only modest activity against HBV but lamivudine has good antiviral activity against both viruses.[39] A lamivudine-containing regimen is therefore a good option for HIV-infected patients where there are clinical concerns regarding HBV disease. Clinicians must be aware, however, that if they decide to change the anti-retroviral regimen, there may be a flare of hepatitis B if lamivudine is discontinued and the new regimen has no anti-HBV activity. The recommended approach is to either continue the lamivudine in addition to the new anti-HIV regimen or to switch to a regimen containing another drug with both anti-HIV and anti-HBV activity such as tenofovir.

With effective treatment for active HBV the transaminase values decline. An increase near the initiation of anti-retroviral therapy raises the possibility of anti-retroviral drug toxicity or immune lysis of infected hepatocytes as part of immune restoration. Subsequent increases in the transaminase values raise the possibility of the emergence of HBV resistance,[40] and this can be confirmed by the detection of HBV DNA levels and the presence of mutation in the YMDD motif.[41] The main approaches are to switch to or to add tenofovir to the regimen.[42,43] Adefovir at high dose has activity against both HIV and HBV but toxicities included renal tubular damage.[44] At low dose (10 mg/day), it appears to be safe[45] and still has anti-HBV activity with activity against lamivudine-resistant HBV.[46] There are concerns, however, that the use of adefovir in this setting may limit the future use of tenofovir as an anti-HIV agent. Many clinicians use both tenofovir and lamivudine in patients where there are concerns about HBV. However, despite the theoretical advantages to such a combination, this has not been proven in clinical practice and leaves limited options when HBV resistance develops. Entecavir, emtricitabine, clevudine and other newer nucleoside analogues may also prove useful.

## Prevention

Protective levels of antibody develop in 90–95% of immunocompetent adults after vaccination with hepatitis B vaccination. The response is less in those with chronic disease such as renal failure,[47] and studies have shown that the response is also reduced in those with HIV infection.[28,29] It has also been noted that the levels of both naturally acquired[48] and post-vaccination anti-HBs antibody levels decrease more rapidly in the patient with HIV.

In one study described by Carne *et al*.[49] HBV vaccination was administered to 35 homosexual men, and only one of the eighteen HIV-negative men failed to develop an antibody response, whereas eight of the seventeen HIV-positive group failed to respond. One study[23] described a high rate of chronic infection in HIV-infected individuals who had received vaccine at about the time of HBV infection.

# Hepatitis C

## Epidemiology

Hepatitis C virus (HCV) infection is particularly common in intravenous drug users or in those exposed to blood products administered before the introduction of HCV screening of blood donors. These risk groups are also exposed to HIV infection, and the presence of both infections is common. Although the risk of sexual transmission of HCV appears low in the setting of a monogamous heterosexual relationship, there are reports of increased prevalence of chronic HCV among homosexual men and more recent reports of acute HCV among HIV-positive homosexual men.[50] There are concerns that the higher levels of HCV RNA seen in the setting of HIV infection may be a factor in this, and may also explain the higher rates of vertical transmission seen with HIV-positive mothers.[51]

The increased survival of HIV-infected individuals with effective anti-retroviral therapy has resulted in patients living long enough to develop the life-threatening complications of HCV infection.[52] Hospitals in Europe and North America have reported that, although there are less patients requiring inpatient care for HIV-related opportunistic infections (OIs), there is increasing use of inpatient facilities for patients with decompensated liver disease and other complications of HCV infection.

## Immunopathology and clinical outcome (Table 50.3)[51,53–60]

### *Effects of HIV infection on HCV disease*

Some studies have suggested a higher rate of HCV chronicity in HIV-infected individuals. In one retrospective study of haemophiliac patients,[61] persisting HCV viraemia was seen in 23 of 25 (92%) HIV-positive patients compared with 20 of 32 (62%) HIV-negative patients.

**Table 50.3** Effects of HIV on HCV infection

| Effects | References |
|---|---|
| Higher HCV viral load | Eyster *et al.*,[53] Telfer *et al.*[54] |
| Increased vertical transmission | Giovannini *et al.*[51] |
| Increase in loss of HCV antibodies | Chamot *et al.*[55] |
| No adverse effect on survival | Wright *et al.*[56] |
| More severe liver disease | Martin *et al.*,[57] Sanchez-Quijano *et al.*,[58] Soto *et al.*,[59] Benhamou *et al.*[60] |

There was no correlation with the CD4 count, but more frequent HCV genotype 1 infection (61% vs 30%) was seen in the HIV-positive group. In many of the studies, the durations of both HIV and HCV infections are unknown.

Our understanding of the immunopathology of chronic HCV infection is limited. There is evidence, however, that HCV has a cytopathic effect on hepatocytes, and more severe disease is seen in immunosuppressed patients receiving corticosteroids and other immunosuppressive agents.[62] An adverse effect on the natural progression of HCV would therefore be expected in patients with HIV infection, and an early report described rapidly progressive liver disease in patients with HCV and HIV infection.[57] The progression of HCV-related liver disease in 32 HIV-positive patients was compared with 44 patients who were HIV-negative.[58] Within 15 years after HCV infection, cirrhosis developed in 8 of 32 (25%) HIV-positive patients and in only 2 of 31 (6.5%) in the HIV-negative group. In one large study of 547 patients, mainly drug users, with chronic hepatitis C more rapidly progressive disease was reported in those with HIV infection.[59] In the histological component of the study, 13 of 87 (14.9%) patients with HIV infection developed cirrhosis compared with only 7 of 272 (2.6%) who were HIV-negative. In patients with haemophilia, there have been several reports of more rapidly progressive liver disease when the patient had HIV and HCV infection.[63,64] HCV RNA levels were higher in those with HIV infection, and this observation has been reported by others.[53,54]

In a detailed histological study, the liver disease progression rate in HIV/HCV-infected individuals was compared with the rate in matched HIV-negative patients.[60] The patients were matched according to other hosts factors known to affect progression (sex, age at time of infection, chronicity of infection, alcohol consumption) and a more rapid rate of progression was seen in those with HIV infection.

Not all studies have confirmed these findings. In HIV-positive homosexual men with HCV infection, one study[56] showed no effect of HIV on HCV progression and vice versa. A total of 512 patients was studied. The group comprised mainly non-drug-using male homosexual patients, 224 of whom had AIDS-defining diagnoses. Seventy-four patients had positive anti-HCV antibody tests and available follow-up (mean 28 months) data were analyzed from 66 patients and compared with matched HCV-negative controls. The presence of HCV viraemia did not shorten survival in those with or without AIDS.

### *Effects of HCV on HIV progression and response to anti-retroviral therapy*

Prior to the availability of more effective anti-retroviral therapy, a large Italian study[65] of 416 patients with HIV infection compared the clinical outcome in HCV-positive (214 patients) and HCV-negative patients. HCV infection had no effect on progression to AIDS or rate of decline of CD4 counts.

The Swiss cohort study[66] evaluated the response to anti-retroviral therapy in HIV-positive patients with and without HCV infection. Although both patient groups had a similar response in terms of reduction of HIV viral load, there appeared to be a reduced CD4 response in those with HCV infection, suggesting that HCV itself had some immunomodulatory effect. A Spanish study reported that HCV-infected patients had a delayed CD4 increase with anti-retroviral therapy.[67] This effect, however, has not been confirmed by a US study[68] which also reported an increase in HCV RNA levels following institution of anti-retroviral therapy.

A syndrome of red fingers in patients with HIV and HCV infection has been reported[69] and, as with HIV-negative patients, there have been reports of cryoglobulinaemia and glomerulonephritis in patients with HIV and HCV infection.

There may be diagnostic difficulties when testing HIV-positive patients for HCV infection. Just as with HBV infection, a reduction in anti-HCV antibodies has been described in HIV-infected patients,[55] and a high number of indeterminate recombinant immunoblot assays has also been noted.[70,71] Although the more recent tests have less false-negative rates, testing for HCV RNA should be considered in these patients and also in those with suspected acute infection where it may take several months to develop an antibody response (Table 50.2).

## Treatment

### *Acute hepatitis C*

Acute hepatitis C is generally asymptomatic and therefore under-recognized. Acute hepatitis C infection has been increasingly diagnosed in HIV-positive patients[50] many of whom, whilst apparently stable on anti-retroviral therapy, have developed a sudden increase in the transaminase level. A positive HCV RNA test and subsequent positive anti-HCV antibody test suggests acute HCV infection. In view of the high levels of response with early treatment of acute HCV infection in HIV-negative individuals (see Chapter 33), similar approaches have been tried in the HIV-positive group. It is also suggested that baseline checks for hepatitis C and annual reviews are performed in patients with HIV infection.[72]

### *Chronic hepatitis C*

#### *IFN monotherapy*

In view of the poor response rates with IFN as therapy for HBV in HIV-positive patients, most of the early HCV trials excluded HIV-positive patients. A large Spanish study[73] found that the response rate was higher in those with a CD4 count >500/mm$^3$, and that following a sustained virological response (SVR) a subsequent relapse was unlikely.[74]

#### *Combination therapy*

Although there have been many studies with standard IFN-α and ribavirin as therapy for HIV/HCV-infected patients, most have evaluated therapy in a small number of patients with different HCV genotypes and different stages of HIV and HCV disease.[75–78] The results demonstrated a lower SVR than is seen in HIV-negative patients and that, just as with the IFN monotherapy studies, the success rate was higher in those with higher baseline CD4 counts.

There have now been several trials investigating the efficacy of PEG-IFNs in combination with ribavirin in HIV/HCV co-infected patients. A Spanish trial reported SVR rates of 28%,[79] the French RIBAVIC[80] trial 27% and the US ACTG 5071 trial with PEG-IFN and ribavirin 27%.[81] The biggest study has been APRICOT (AIDS Pegasys Ribavirin Co-infection Trial) with an overall SVR of 40%.[82] In this study, 868 patients were randomized to receive a year of therapy with standard IFN and ribavirin, PEG-IFN and placebo or PEG-IFN and ribavirin. The results are summarized in Table 50.4. The mean CD4 counts were 520–542/mm$^3$ in the three groups.

Assessing the viral load response at 12 weeks may help discontinue ineffective therapy at an early stage,[79] but further studies are required to determine whether 6 months of therapy are adequate for patients with genotypes 2 and 3 infection. Viral kinetics suggest that co-infection may be associated with slower clearance of HCV.[83]

It has also been suggested that, particularly in those with advanced liver disease who have failed to respond to standard treatment, there may be a role for longer treatment regimens.

However, there have been concerns regarding the potential toxicities of IFN and ribavirin in patients with HIV and the interactions of these agents with anti-retroviral drugs.

IFN can cause CD4 lymphopenia. One Italian group described a sudden decrease in the CD4 count after the administration of IFN.[84] Another group confirmed this and described patients who then went on to develop AIDS-defining diagnoses after the sudden decrease in the CD4 count.[85] Patients on anti-retroviral therapy appear more likely to have a significant decline in haemoglobin as a result of ribavirin.[86]

In terms of interactions IFN and zidovudine are both myelotoxic, so careful monitoring of the blood count is required whilst on therapy. There have been laboratory concerns regarding interactions of ribavirin and other nucleoside analogues.[87–89] Although clinical studies assessing the efficacy of anti-retroviral therapy in patients on IFN and ribavirin have questioned the relevance of these *in vitro* findings,[90] there have been concerns raised regarding lactic acidosis and pancreatitis in patients on regimens that included didanosine.[91–93]

**Table 50.4** APRICOT study[82]

| Parameter | Interferon + ribavirin | Pegylated interferon + placebo | Pegylated interferon + ribavirin |
|---|---|---|---|
| Number treated | 285 | 286 | 289 |
| Overall SVR (%) | 12 | 20 | 40 |
| SVR genotype 1 (%) | 7 | 14 | 29 |
| SVR genotype 2 (%) | 20 | 36 | 62 |
| Discontinued (%) | 39 | 31 | 25 |

SVR, sustained virological response.

*Other approaches*

Successful anti-retroviral therapy is an important strategy for the patient with HIV/HCV infection. It is hoped that with immune restoration the rate of progression of HCV liver damage may be slowed.[94–96]

Heavy alcohol consumption is not uncommon in this group of patients, and it is important to educate patients regarding the dangers of heavy alcohol use in the setting of HCV-associated liver damage.

*Hepatotoxicity of anti-retroviral therapy*

Several anti-retroviral agents are hepatotoxic and there have been concerns regarding the risks of severe hepatotoxicity in patients with existing liver disease such as HCV infection. Several groups have looked at this aspect. The Swiss cohort study found no increase in treatment changes in the HCV-infected patients,[66] whereas a large prospective study reported an increased incidence of hepatotoxicity in patients with HCV co-infection.[97] Many clinicians avoid didanosine and stavudine in patients about to embark on IFN and ribavirin, but otherwise use similar anti-retroviral regimens in patients with HCV co-infection and monitor the liver function tests more rigorously in this group.[98]

*Liver transplantation*

The early experience of liver transplantation for individuals with HIV infection was disappointing, with high mortality rates. Survival rates are improving with more effective anti-retroviral therapy and careful selection of patients, but liver transplantation for HIV-positive patients is only available in a limited number of centres.[99]

## Hepatitis D

### Epidemiology

As with HCV infection, hepatitis D virus (HDV) is not a major infection of homosexual men, so the combination of HIV and HDV infection is mainly seen in those infected by intravenous drug use or exposed to contaminated blood products.

### Immunopathology

HDV is thought to have a direct cytopathic effect on hepatocytes, and more rapidly progressive liver disease would be expected in immunosuppressed states.

Normally, HDV has an inhibitory effect on HBV replication, but some reports suggest that this effect is lost with HIV infection.[100] It has also been suggested that HDV can suppress HCV replication in patients with HIV infection.[101] In one cross-sectional study, 15 patients with HIV and HDV infection were compared with 29 patients with only HDV infection.[102] This study confirmed that HIV reduces the suppressive effect of HDV on HBV replication, but found no effect of HIV infection on HDV replication and no increase in the severity of the liver disease in those with HIV and HDV.

The clinical outcomes over 6.5 years in patients with chronic delta hepatitis were compared with the outcomes in those who had concurrent HIV infection, HCV infection, or both.[103] Although 52 patients remained asymptomatic, 34 developed hepatic dysfunction and 16 patients died because of liver failure. Ten patients died from AIDS and two from unrelated causes. Deaths were recorded in 4 of 20 patients from the HDV alone group, 3 of 11 patients with co-existent HCV, 3 of 12 patients with HIV and 18 of 43 patients with HCV and HIV infection. It is likely that with improved survival in patients with HIV infection, liver failure will be seen more commonly in those co-infected with HCV and HDV. With progression of HIV infection, a failure to detect anti-HDV antibodies has been reported[104] and this may complicate the diagnosis of HDV infection in this group of patients (Table 50.2).

### Treatment

The rate of successful response following IFN administration of HIV-negative individuals is disappointingly low, and there is very little published information regarding the treatment of those with HIV and HDV infection.

## Hepatitis E

Although there are reports of an increased seroprevalence of anti-hepatitis E virus (HEV) antibodies in Italian injecting drug users[105] and homosexual men,[106] it is likely that most HIV-positive patients exposed to HEV infection live in tropical areas where there are large outbreaks of HEV infection, and as yet few data are available regarding the relative seroprevalence of HIV and HEV infection or the severity of disease in HIV-infected patients. It has been suggested that false-positive tests can occur[107] in HIV-infected patients and that in homosexual men, foreign travel rather than sexual practice can predispose to HEV infection.[108] If the severity of HEV infection in pregnancy is related to immune changes, it may be that more severe disease will be seen in patients with underlying HIV-associated immunosuppression.

## Hepatitis GBV-C

There have been some interesting observations in patients with HIV and GBV-C co-infection. It is thought

that GBV-C can be transmitted sexually[109] and patients co-infected with GBV-C tend to have higher CD4 counts and some, but not all, studies have reported a survival benefit.[110,111] One recent study described a survival benefit at 5–6 years post-HIV seroconversion but not at 12–18 months.[112] GBV-C infects PBMCs, and it is not clear if the apparently beneficial effect of co-infection relates to the interaction with HIV at the cellular level or to an indirect action related to alterations in the cytokine balance promoting a T-helper-1 cytokine profile.[113]

## Conclusion

There are many epidemiological, molecular, and clinical interactions between HIV and the hepatotropic viruses. With advances in the therapy of HIV infection and improved survival of patients, these are likely to become of increasing clinical and economic importance.

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# Chapter 51
# Treatment of extrahepatic diseases caused by hepatitis B and hepatitis C viruses

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## Introduction

Hepatitis B (HBV) and hepatitis C (HCV) viruses are well-recognized causes of chronic hepatitis, cirrhosis and even hepatocellular carcinoma. Apart from liver disease, these viral infections are known to be associated with a spectrum of extrahepatic manifestations. Hepatotropic HBV and HCV are capable of triggering immune complex formation and autoimmune reactions. The prevalence of clinically significant extrahepatic manifestations is relatively low, but it can be associated with significant morbidity and even mortality. An awareness and recognition of these manifestations is of paramount importance in facilitating early diagnosis and in offering treatment. Such treatments may be highly successful, as for HBV polyarteritis nodosa (PAN), but they may also be less effective, and patients may continue with disabling extrahepatic manifestations.

Patients with chronic viral hepatitis commonly have immunological manifestations, including autoantibodies and concurrent immune diseases. These immunological findings may resemble those of autoimmune hepatitis and they are not disease-specific. High titre autoantibodies are uncommon in chronic viral hepatitis as are multiple concurrent autoantibodies. These findings reflect an autoimmune-predominant disorder in which the viral infection may be coincidental or facilitative. Concurrent immunological disorders may be viral antigen-driven and associated with immune complex deposition (cryoglobulinaemia, glomerulonephritis, cutaneous vasculitis and polyarteritis) or autoantigen-driven (autoimmune thyroiditis and Sjögren's syndrome) and associated with host-specific rather than virus-specific factors. Genetic predisposition influences immunological expression. Seropositivity for antinuclear antibodies is associated with HLA A1-B8-DR3, and concurrent immunological diseases are associated with the DR4 allele. Patients with chronic hepatitis B and C can have similar immune features, but patients with chronic hepatitis C more commonly have autoantigen-driven processes.

## Extrahepatic diseases caused by HBV infection

### Background

Although most patients infected with HBV remain symptom-free, a wide clinical and histopathological heterogeneity bears witness to complex interactions between the virus, the immune response and other factors: for example, hepatitis C or delta virus or human immunodeficiency virus (HIV) co-infections; consumption of cytotoxic, immunosuppressive or glucocorticoid medicines; or alcohol abuse. In acute primary HBV infection, in adults almost 75% of patients remain symptom-free. Fortunately, about 90–95% of patients recover spontaneously. However, the prognosis can be worsened by fulminant hepatitis (about 1% of acutely infected patients), or evolution to chronic infection (about 5–10% of acutely infected patients in adulthood). Chronic HBV infection affects 350 million people in the world, and 25–40% will eventually die of either cirrhosis-related complications or primary liver cancer.[1] Both acute and chronic HBV infections have been well recognized as responsible for a variety of extrahepatic manifestations including polyarteritis nodosa, glomerulonephritis, polyradiculoneuritis, essential mixed cryoglobulinaemia, porphyria cutanea tarda, polyneuritis and thyroid dysfunction.[2]

Classically, acute viral hepatitis B may be preceded by a prodromal pre-icteric phase characterized by arthralgias or arthritis and urticarian skin rashes. Rarely, other types of symptoms, including renal, central or peripheral nervous system manifestations, may also occur. Although extrahepatic manifestations do exist with all hepatitis viruses, they are more commonly associated with acute HBV infection. The most common pattern is that of diffuse polyarthralgia occurring several days

before the onset of icterus, and may be associated with fever and some urticarian maculopapular lesions. All these manifestations are usually transient and clear up as the hepatitis progresses. Rarely, extrahepatic manifestations of HBV infection may persist for months, usually reflecting the transition to chronic hepatitis. High viral replication levels and persistent infection may favour production of soluble immune complexes in antigen excess, which deposit on specific sites including skin, kidney and small/medium-calibre arteries. Different pathogenetic mechanisms have been identified in the pathogenesis of vasculitis. Immune complexes are mainly thought to be responsible for vasculitis including polyarteritis nodosa, glomerulonephritis and mixed cryoglobulinaemia. Nevertheless, other immune system factors can also participate in the development of the disease, e.g. cytokines and lymphocytes. Furthermore, glomerular deposits of HBs/HBc/HBe antigens together with immunoglobulins and C3 have been documented together with low complement levels in the serum. Immunostaining and ultrastructural findings indicate that hepatitis B 'e' antigen (HBeAg) is probably a responsible candidate antigen.[2] However, a polyarteritis nodosa case was recently reported in association with an HBeAg-negative precore mutant infection.[3]

## Manifestations

### *Polyarteritis nodosa*

Three decades ago, HBV was recognized as being associated with a significant number of patients with polyarteritis nodosa (PAN).[4,5] PAN is due to inflammation and necrosis of medium-sized arterial walls that lead to microaneurysm formation and organ infarction. Exposure to HBV before the onset of PAN is well documented in 60% of cases.[6] The immunological process responsible for PAN usually occurs within 6 months of the primary HBV infection. Hepatitis is rarely diagnosed as it remains mostly silent before the PAN becomes clinically patent. Clinical manifestations are characteristic of those commonly described with historical classical PAN, with gastrointestinal involvement (especially perforation and bleeding), malignant hypertension, renal infarction and orchiepididymitis.[6,7] The outcome of HBV-PAN is more serious unless the appropriate antiviral treatment is prescribed.[7] Sequels are the consequence of pathological damage including vascular nephropathy, as well as central and/or peripheral nervous disorders. Hepatic manifestations associated with the course of PAN are clinically moderate, and transaminase elevations are mild in most cases. Whenever performed, liver biopsies have shown a chronic hepatitis pattern in agreement with the overwhelming progression to chronicity.[2] Although most cases of PAN are associated with the wild-type HBV strains that strengthen the involvement of HBeAg in pathogenesis of PAN, a few cases challenge this view, as they have been reported to be associated with precore mutation which abrogates the formation of HBeAg. However, no autoimmune mechanism could be incriminated as antiviral therapy was successful in eradicating the disease in close connection with replication inhibition.[3]

For many years, HBV-related PAN was treated in the same way as non-virus-related PAN, and patients received corticosteroids combined with plasma exchanges. This treatment was often effective in the short term. In a prospective randomized study enrolling 71 patients, addition of cyclophosphamide was even shown to be beneficial in preventing relapses and improving the quality of the clinical response to therapy during the long-term follow-up.[8] However, exclusive immunosuppressive therapy showed the occurrence of relapses and complications related to virus persistence like chronic hepatitis or liver cirrhosis, showing that when corticosteroids and immunosuppressive agents are prescribed to HBV-related PAN, the outcome is poorer than in non-viral PAN.[9] Later, a first-generation nucleoside analogue (i.e. vidarabin) was used to treat HBV-related PAN based on its efficacy in chronic hepatitis B together with plasma exchanges commonly used in PAN, both therapies being combined in a physiopathological approach to treatment.[10] The rationale of the therapeutic sequence was: (1) to rapidly control the most severe life-threatening manifestations of PAN by an initial corticosteroid therapy; (2) to control the course of PAN and restore immune reactivity by removing immune complexes by plasma exchanges; (3) to inhibit HBV replication by vidarabin administration; and (4) to enhance immunological clearance of HBV-infected hepatocytes and favour HBe antigen to anti-HBe seroconversion by abrupt cessation of corticosteroid therapy. Thirty-three patients were treated following this schedule (1 week of 1 mg/kg/day corticosteroids, followed by a 3-week course of vidarabin combined with plasma exchanges), inducing a full clinical recovery in 75% of patients, and HBeAg to anti-HBe seroconversion in about 50% of patients, while hepatitis B surface antigen (HBsAg) to anti-HBs seroconversion was obtained in about 20% of patients. Later studies enrolling small numbers of patients replaced vidarabin by interferon (IFN)-α, allowing efficient recovery from PAN and HBeAg to anti-HBe seroconversion in about two-third of patients, and HBsAg to anti-HBs seroconversion in about half of patients, and rare relapses (about 6%) over the long-term follow-up.[6,11] The dose of IFN-α was comparable to that prescribed for hepatitis B, the duration of administration not exceeding 6 months for the majority of patients. The triple therapy combining plasma exchanges, corticosteroids and IFN-α was again shown to be successful for treating a PAN case associ-

ated with a precore promoter mutant HBV infection.[3] Other similar antiviral approaches were also successful including IFN-α alone,[12–14] or combined with famciclovir[15] or lamivudine.[16,17] Lamivudine has also been tested as a successful single first-line antiviral therapy for HBV-related PAN.[18]

In summary, although therapeutic options for HBV-related PAN must primarily inhibit HBV replication, they should also control lesions immediately due to the severe life-threatening manifestations of PAN. To this end, plasma exchanges are able to rapidly clear the immune complexes responsible for the disease. This rapid intensive intervention is the most appropriate way to minimize life-threatening disease complications and/or sequels. We can probably expect that new antiviral therapies and novel drug combinations will bring further improvement in terms of definitive suppression of the virus.

### *Glomerulonephritis*

Although most cases of glomerulopathies are idiopathic, they may be associated with a variety of chronic infections and immune complex diseases. HBV is a well known cause of membranous glomerulonephritis (MGN), membranoproliferative glomerulonephritis (MPGN), mesangioproliferative glomerulonephritis (MSPGN) and IgA nephropathy. The association between HBsAg and nephrotic syndrome is usually due to membranous GN, probably immune complex-mediated.[19] Patients commonly present with a nephrotic syndrome and microscopic haematuria. Almost half of them have hypertension and 20% have renal impairment. Remission of nephrotic syndrome parallels elimination of HBV antigens, especially HBeAg, with resolution of proteinuria occurring within 6 months of clearing the HBeAg in many cases.

There are few available data on the treatment of HBV-related GN. Treatment such as corticosteroid or immunosuppressive therapy is not effective, the renal prognosis being similar to idiopathic MPGN, and may actually increase the morbidity and mortality.[20,21] Only limited series of patients have been reported. Most cases treated with IFN-α published so far have had either membranous or mesangiocapillary GN, and response to IFN-α seems to be better in membranous as compared with mesangiocapillary GN. Chung *et al*.[22] treated four patients with HBV-associated MPGN with IFN-α for 6 months. Although two of these patients showed a transient decrease in proteinuria to less than the nephrotic range with anti-HBe seroconversion, the other two did not. Serum creatinine levels did not change significantly during the treatment. Lisker-Melman *et al*.[23] gave IFN-α to five patients with HBV-related GN for at least 6 months. The four patients with membranous GN were good responders with a 24-hour urinary protein falling to <2 g/day at 1 year. The patient with MPGN failed to obtain a sustained response. In a case report,[24] one patient with an HBV-related MPGN was shown to be in complete remission of the nephrotic syndrome after IFN-α therapy, and this persisted despite the reappearance of viral markers in the serum when IFN-α was withdrawn. In another case report,[25] one patient with HBV-related MSPGN was shown to benefit from IFN-α therapy too, corroborating data obtained from two other HBV-related MSPGN patients treated with IFN-α.[22] Proteinuria subsided with or without seroconversion to anti-HBe and loss of HBeAg. The response to IFN-α therapy found in HBV-infected patients with GN seems to be higher than seen in patients without GN. Conjeevaram *et al*.[26] have reported a series of 15 patients with chronic hepatitis B and GN (membranous 10, membranoproliferative five) treated with IFN-α, and have shown a long-term serological response with sustained loss of HBeAg and HBV DNA in eight (53%) patients. Most of these eight virological responders also showed a gradual and marked improvement in proteinuria. Lin[27] reported the effect of IFN-α therapy in 40 children with HBV-related membranous GN who failed to respond to corticosteroid treatment. All 20 patients who received IFN-α were free of proteinuria after 1 year of follow-up, and 50% of these cases showed anti-HBe seroconversion. By contrast, in the untreated group only 35% were free of proteinuria, and none showed anti-HBe seroconversion.

Disadvantages of IFN-α include expense, the requirement for subcutaneous injections, and frequent side-effects that may be severe. In contrast, the incidence of adverse events with lamivudine is similar to placebo. Lamivudine has recently been shown to be efficient for inducing remission of HBV-related membranous GN and nephrotic syndrome.[28,29]

## Extrahepatic diseases caused by HCV infection

### Background

HCV is an RNA virus that is a major cause of acute and chronic hepatitis. Both acute and chronic hepatitis C are asymptomatic in most patients. However, chronic hepatitis C is a slowly progressive disease and results in severe morbidity in 20–30% of infected persons. Chronic hepatitis C is associated with a host of extrahepatic manifestations. Patients who definitely need treatment are those with liver fibrosis progression, and/or with extrahepatic manifestations and those who can transmit the virus. Furthermore, treatment of HCV should be systematically discussed in patients co-infected with HIV and HCV. In discussing the extrahepatic manifestations of HCV, it is necessary to distinguish between syndromes

in which the role of HCV is well established (e.g. essential mixed cryoglobulinaemia and glomerulonephritis) and those in which it is still poorly understood although clinically relevant (e.g. thyroid dysfunction). Other, less common manifestations, such as other autoimmune-related disorders, are also of interest.

Between 1996 and 1997, 321 patients chronically infected with HCV were prospectively enrolled in a study designed to determine the prevalence of extrahepatic manifestations associated with HCV infection, to identify associations between clinical and biological manifestations.[30] In all, 38% of patients presented at least one clinical extrahepatic manifestation including arthralgia (19%), skin manifestations (17%), xerostomia (12%), xerophthalmia (10%) and sensory neuropathy (9%). The main biological abnormalities were mixed cryoglobulinaemia (MC; 56%), thrombocytopenia (17%) and the presence of the following autoantibodies: antinuclear (41%), rheumatoid factor (38%), anticardiolipin (27%), antithyroglobulin (13%) and anti-smooth muscle cell (9%). At least one autoantibody was present in 70% of sera. A definite connective tissue disease was noted in 14% of patients, mainly symptomatic MC and systemic vasculitis.

Few treatment-related data are available on the management of the numerous extrahepatic manifestations and autoimmune disorders associated with chronic HCV infection. Recent studies have demonstrated that antiviral therapy, as well as corticosteroids and immunosuppressive therapies, may be effective in managing extrahepatic HCV manifestations, although discontinuation often produces relapses.[31,32] Very recently, anti-CD20 (rituximab) therapy has given promising results and opened new hopes in the therapeutic management of HCV-induced vasculitis.[33] However, the potential improvement of symptoms and reduction of viraemia must be balanced against the adverse effects of medication, which include IFN-α-induced autoimmune phenomena, ribavirin toxicity and possible exacerbation of HCV infection caused by immunosuppressive agents as well as anti-CD20 therapy.[34]

## Manifestations

### *Glomerulonephritis*

HCV infection has been associated with glomerular disease, the foremost being MPGN with or without MC.[35,35] Cases of MGN and focal segmental glomerulosclerosis (FSGS) have also been reported.[36,37] Treatment of these patients with IFN-α therapy has been disappointing, with in some cases a favourable outcome, but often with relapse of viraemia and recurrence of renal disease after cessation of therapy.[38,39] The addition of the nucleoside analogue ribavirin have meant that treatment successes for HCV have improved significantly, but only a few case reports deal with treatment outcomes for HCV-associated renal diseases.[40,41] It has been shown that ribavirin could be used in combination with IFN-α in dialysis patients, the cornerstones of therapy being reduced ribavirin doses and high-dose erythropoietin treatment, as well as ensuring adequate iron stores for erythropoiesis.[42] One study involved 20 patients with HCV-associated glomerulopathy (MPGN in 85% of these cases, MGN in 10% and mesangioproliferative glomerulonephritis in 5%) who received IFN-α therapy for 12 months. In IFN-α-resistant subjects, IFN-α was combined with ribavirin. MC was not constant, and was encountered in only 60% of the cases. Twelve months of antiviral therapy combining IFN-α and ribavirin resulted in viral clearance in 25% of cases associated with significant proteinuria decrease (4 g to 1.10 g/24 h, $p = 0.001$), serum albumin increase (25 to 35.5 g/L, $p = 0.012$), and C3 and C4 concentration returning to normal.[43] The use of pegylated interferon (PEG-IFN) has recently been tested for two of seven patients treated with IFN-α and ribavirin for HCV-related renal insufficiency: two with cryoglobulinaemia, vasculitic manifestations and glomerulonephritis, four with MPGN and one with FSGS.[44] The aim of this study was to retrospectively analyze the outcome of these patients, regarding viral and renal response, as well as their tolerability to treatment, showing that IFN-α and ribavirin could with reasonable safety be used in HCV-related vasculitis and GN irrespective of renal function. Six of seven patients became HCV RNA-negative by PCR, and four of seven maintained both virological and renal remission with 12–32 months of follow-up. One of seven maintained virological and partial renal remission. One patient did not tolerate IFN-α, but was in renal remission with low-dose ribavirin. One vasculitis patient responded with complete remission, but relapsed virologically and had a minor vasculitic flare after 9 months. Only one patient with vasculitis required low-dose immunosuppression in addition to antiviral therapy. The renal outcome was favourable in all cases. In all patients, serum albumin normalized and albuminuria decreased substantially to levels <0.5 g/24 h, except for one patient. The renal damage was most probably irreversible, considering the chronic changes seen in the renal biopsy. However, the rapid loss of renal function had been halted. Haematuria was no longer detectable at follow-up in five of seven cases, and glomerular filtration rate normalized in two of seven patients, or stabilized in the remainder of the cases.

### *Sicca syndrome*

Although sicca syndrome seems to be associated with HCV, it may differ from the typical Sjögren's syndrome. It affects women preferentially and is usually not accompa-

nied by anti-SSA or anti-SSB antibodies. This syndrome is histologically detected in 15–50% of patients. The main studies that include large series of patients show that xerostomia and xerophthalmia are observed in about 15% of HCV patients.[45] Improvement of HCV-related sicca syndrome under antiviral therapy has not been clearly reported. Adequate management should include the replacement of oral and ocular fluids, and a multidisciplinary approach involving different specialities (odontology, ophthalmology, gynaecology and dermatology).

### *Pulmonary disease*

There are few data on the treatment of pulmonary alveolitis in patients with chronic HCV infection. The severity of this situation suggests the need for aggressive treatment combining antiviral and immunosuppressive therapies and plasmapheresis. Some studies have evaluated the presence of an inflammatory process of the lower respiratory tract in patients with MC associated with HCV. Bronchoalveolar lavage and pulmonary function tests including diffusion capacity for carbon monoxide (DLCO) were carried out, and indicated a subclinical T-lymphocytic alveolitis in HCV-related MC patients that was not associated with a risk of deterioration in lung function.[46] A few case reports have been published so far on symptomatic pulmonary alveolitis due to HCV-related MC, and patients may respond dramatically to plasmapheresis and the addition of high-dose corticosteroids,[47] or may be refractory to conventional immunosuppressive treatment.[48]

### *Articular manifestations*

Arthralgias are common in patients with chronic HCV infection, and were reported in 20% of patients in a large series.[49] Paracetamol medication should be considered as a first therapeutic approach, and the use of non-steroidal anti-inflammatory drugs (NSAIDs) requires individual evaluation as they are contraindicated in patients with cirrhosis.[50] Furthermore, anti-cyclooxygenase 2 drugs may be considered with a good clinical response and no worsening of liver function.[51]

Arthritis occurs less frequently than arthralgias, with a prevalence below 5%, and is usually related to a cryoglobulinaemic syndrome. They are mono- or oligoarticular and non-erosive arthritis, affecting large and medium-sized joints. In small published series, patients with HCV-related arthritis have been shown to be able to benefit from IFN-α therapy (n = 4).[52] Other studies conducted on small series of patients reported the usefulness of NSAIDs associated with low doses of prednisone (n = 3),[50] or hydroxychloroquine plus low doses of prednisone (n = 19).[53] The use of immunosuppressive agents, such as methotrexate and azathioprine, should be considered for severe and refractory arthritis in individual cases, with close monitoring of liver function, blood cell counts and HCV RNA levels.

### *Systemic lupus erythematosus*

The most frequent extrahepatic manifestations of HCV including arthralgia, myalgia, sicca syndrome and nuclear antibodies, may mimic a connective tissue disease, particularly systemic lupus erythematosus (SLE). Reports on the association between SLE and HCV infection are scarce, and to our knowledge, so far no study has reported the benefit of antiviral therapy and clearance of HCV RNA on the outcome of the presumed HCV-related SLE syndrome. A retrospective case-control study of 19 patients with SLE and anti-HCV antibodies versus 42 randomized SLE patients without anti-HCV antibodies showed that prevalence of cryoglobulin was higher in SLE patients with anti-HCV antibodies, but without MC syndrome. HCV infection had moderate signs of biochemical and liver pathological severity. One patient treated with IFN-α had a sustained virological response without SLE flare. Corticosteroid therapy did not seem to alter the HCV course.[54] Furthermore, IFN-α therapy itself, with or without combination with ribavirin, is able to induce SLE or an SLE-like syndrome in 0.15–0.7% of patients, with a generally good outcome after discontinuance of the drug.[55]

### *Thyroiditis*

Although chronic HCV infection is associated with a high prevalence of thyroid autoantibodies, only a few patients spontaneously develop autoimmune thyroid dysfunction.[56] Most HCV patients with thyroid dysfunction are middle-aged women with asymptomatic hypothyroidism and do not require specific treatment. However, no study has so far really clarified whether HCV does play a pathological role in the development of thyroid dysfunction and autoimmune thyroiditis.[57]

By contrast, thyroid dysfunctions developing in HCV-infected patients under IFN-α therapy are common (50% of cases with anti-thyroperoxidase (anti-TPO) antibodies prior to treatment) and well documented. They are different from those occurring without IFN-α.

A prospective study has recently demonstrated that the appearance of thyroid autoantibodies during treatment of HCV with IFN-α is accompanied in most cases by the occurrence of a destructive process in the thyroid gland with hyper- or hypothyroidism, and that the thyroid clinical outcome of these patients is strictly correlated to the continuation of IFN-α treatment.[58] The presence of thyroid autoantibodies before initialization of antiviral treatment should not necessarily be regarded as a contraindication for IFN-α therapy, depending

on the severity of the HCV-related hepatopathy and likelihood of response to IFN-α therapy. Screening for serum antibodies and thyroid-stimulating hormone (TSH) is highly recommended before, during and until 6 months after discontinuation of IFN-α therapy. Because most patients with increased antibody titres or thyroid dysfunction (especially hypothyroidism) under IFN-α therapy recover after completing therapy, interruption of IFN-α therapy may not always be required, although treatment must be interrupted in patients with severe symptoms or frank hyperthyroidism.[58a] After specific treatment of hyperthyroidism and recovery, IFN-α therapy may be reconsidered.

### *Skin manifestations*

Among extrahepatic disorders associated with HCV infection, dermatological diseases occupy a central part. The most frequent dermatological manifestations associated with chronic hepatitis C include MC with leukocytoclastic vasculitis, porphyria cutanea tarda and lichen planus.

Patients with HCV-associated vasculitis may present palpable purpura, urticaria or livedo reticularis, most of which present as leukocytoclastic vasculitis associated with cryoglobulinaemia.[59] The most frequent clinical situation is palpable purpura in the legs, requiring non-aggressive management consisting of changes in posture, rest and, in some cases, low doses of corticosteroids. Severe cutaneous complications, such as ulcers, necrosis and gangrene, require intensive immunosuppressive therapy with higher doses of corticosteroids, pulses of cyclophosphamide, intravenous gamma globulins or prostacyclins.

Porphyria cutanea tarda is due to uroporphyrinogen decarboxylase deficiency in the liver that may be driven, at least in part, by HCV infection. Most patients with porphyria cutanea tarda have iron overload with increased plasma iron and ferritin levels and hepatic siderosis similar to that observed in individuals with haemochromatosis. Treatment of porphyria cutanea tarda remains mainly based on low-dose choloroquine and phlebotomy. It is probable that previous phlebotomy increases the percentage of response to IFN-α when patients present with HCV infection. Although the indication for phlebotomy appears clear in patients with iron overload, the rationale for chloroquine prescription, even though proven to be useful, has not been clearly established as its therapeutic mechanism is poorly understood.[60]

Lichen planus with cutaneous eruption and sometimes mucosal lesions may be associated with HCV infection. Cutaneous biopsy specimens can show lymphoid infiltrates without CD20+ B cells. Although patients have HCV RNA in the serum, the viral genome is usually not detected by reverse transcription and polymerase chain reaction (RT-PCR) in skin biopsies, and lichen planus show an inconsistent response to IFN-α and ribavirin therapy. Thus, lichen planus appears to be related to the pattern of immune dysregulation induced by HCV, probably in a host with an underlying susceptibility to autoimmune disease, but therapeutic viral response does not correlate with clearing of lichen planus.[61]

However, other skin disorders, such as Adamantiadis-Behçet syndrome, erythema multiforme and nodosum, malacoplakia, urticaria and pruritus, may also be linked to HCV. Further studies are necessary to establish or refute any aetiopathogenetic role of HCV in these conditions. Skin manifestations are also part of the clinical picture of other extrahepatic disorders associated with HCV infection, such as thyroid dysfunction and HCV-related thrombocytopenia.

The response to IFN-α therapy in skin diseases is unpredictable with some patients ameliorating, others remaining stationary and others deteriorating. Lichen planus, psoriasis and vitiligo may be induced or exacerbated by IFN-α therapy.[62–64]

### *Idiopathic autoimmune cytopenia*

Thrombocytopenia is frequently seen in HCV-infected patients. Furthermore, to a much lesser extent, autoimmune haemolytic anaemia and neutropenia are sometimes described.[65] Although thrombocytopenia in patients with advanced liver disease can result from splenic sequestration due to portal hypertension, the degree of thrombocytopenia observed in patients with HCV appears to be significantly greater than reported in other forms of severe liver disease. This would suggest a possible immunopathic mechanism for the development of thrombocytopenia with HCV infection.[66] Thrombocytaemic patients infected with HCV share many clinical features of idiopathic immune thrombocytopenic purpura. They are characterized by the presence of autoantibodies against platelet membrane proteins, and they have large platelets on peripheral smear, and normal or increased megakaryocytes on bone marrow examination.[67] An immunopathic mechanism for the development of thrombocytopenia is also supported by numerous reports of other immunological abnormalities in patients with HCV. Treatment with steroids is effective in these patients, but poses a risk of aggravating the HCV infection. Patients with HCV-related thrombocytopenia may have a good response to corticosteroids alone (around 50% of patients), although with a higher rate of mortality observed in case of liver cirrhosis.[65] Patients who fail to respond to corticosteroids may respond to intravenous immunoglobulins or cyclophosphamide, or may require splenectomy.[68–70]

As treatment with steroids may be effective but can exacerbate the viral infection, an antiviral approach has

been tested as a therapeutic strategy for HCV-related thrombocytopenia. IFN-α is the primary therapeutic modality for HCV infection. IFN-α can be associated with the development of thrombocytopenia.[71] However, if viral replication and host immune response to the virus are responsible for the accelerated platelet clearance, then successful IFN-α treatment of the HCV could result in improvement in the platelet counts. Although IFN-α has documented efficacy in the treatment of HCV, its use in the treatment of HCV thrombocytopenia is controversial. In a small study,[72] eight patients with severe HCV-related thrombocytopenia (platelet count $<50 \times 10^9$/L) were treated with IFN-α (3 MU three times a week for 24 weeks). Only three patients tolerated the full course of treatment, and all three had improvements in their platelet counts to $>50 \times 10^9$/L. Two other patients had improvement in platelet counts to $>50 \times 10^9$/L with a shorter duration of treatment (6 and 16 weeks, respectively). Duration of response ranged from 4 to at least 18 months, with the shortest responses observed in the two patients treated with a shorter course of IFN-α. Responding patients showed improvement in hepatic transaminases, reduction in cryoglobulin and anticardiolipin antibodies, and HCV plasma RNA. Relapse was associated with an increase in these laboratory markers of HCV infection. It was concluded that IFN-α could be an effective treatment in patients with HCV-related thrombocytopenia. A larger study involving 20 patients strengthened evidence for the benefit of IFN-α for HCV-related thrombocytopenia.[73] In this study, 20 naïve HCV-infected patients with thrombocytopenia (platelet count $<140 \times 10^9$/L for at least 6 months, without portal hypertension and/or hypersplenism) received leucocyte IFN-α (3 MU three times per week for 12 months) and were followed up for 12 months. During treatment, platelet counts decreased to <10–20% of pretreatment values in most patients. Three of the four patients with a complete virological response showed a platelet increase during treatment and throughout the follow-up period.

In summary, although steroids may have a role in the short-term treatment of patients with HCV-related thrombocytopenia, their deleterious effect on HCV infection should limit their use in these patients. Antiviral therapy with IFN-α, combined with ribavirin if possible, should be attempted. A remission can be expected in virological responders. In non-responders, treatment with ribavirin alone may be considered.

### *Myositis*

HCV-related polymyositis should be considered as an extrahepatic condition of chronic HCV infection for which therapeutic management is difficult, needing careful individual evaluation when considering the use of corticosteroids and/or IFN-α. Indeed, different therapeutic options have been used, with controversial results. Some studies reported the benefit of steroids or intravenous gamma globulins in achieving a reduction in symptoms and muscular enzyme levels,[50,74] while others reported that steroids could increase hepatic transaminases leading to severe liver damage.[74] In some patients, cytotoxic agents were successfully added to steroid therapy.[75] On the other hand, some authors have used IFN-α monotherapy to treat HCV-related polymyositis with the same controversial outcome: some patients responded well, others presented severe aggravation of polymyositis after IFN-α discontinuation, and several worsened or precipitated into myositis under IFN-α therapy.[74,76]

### *Neurological manifestations*

Patients infected by HCV frequently complain of fatigue, lassitude, impaired memory and a perceived inability to function effectively, even in the absence of clinically significant liver disease. However, it is not known whether social, psychological or biological factors may cause these complaints. HCV infection itself very likely affects cerebral functions, as demonstrated by proton magnetic resonance spectroscopy, showing elevations in basal ganglia and white matter choline/creatine ratios in patients with histologically mild hepatitis C, compared with healthy volunteers and patients with hepatitis B.[77] Corroborating these findings, several studies have shown that patients with HCV infection score worse than matched controls on health-related quality of life indices, and their scores improve with successful antiviral treatment.[78] The mechanisms involved are poorly understood, but HCV can infect cells of monocytic lineage, raising the possibility that HCV may infect brain cells as well. An alternative explanation is a centrally mediated effect of peripherally derived cytokines.[79] In a much more severe manifestation, although exceptional, patients with HCV infection may present central nervous system (CNS) vasculitis related to cryoglobulinaemia, for which intravenous pulse and oral corticosteroid therapy combined with cyclophosphamide, followed by warfarin and IFN-α, was shown to be effective in some case reports.[80,81]

More frequently, HCV-infected patients may develop peripheral neuropathy (PN), generally due to MC. However, PN may be associated with HCV infection without MC, for which mechanisms are even less understood and might involve vasculitis and even PAN.[82] In contrast to other HCV-related extrahepatic manifestations, PN does not respond as favourably to the different therapeutic regimens. In addition, it has been reported that IFN-α may cause a worsening of PN in patients with HCV-related MC, despite the improvement of hepatic function.[83] Among patients with HCV-related PN in which a virological response to IFN-α therapy was reported,

only a few of them presented a favourable response for the PN.[30,40,82,84,85] Addition of ribavirin to IFN-α in patients resistant to other therapies (IFN-α monotherapy, corticosteroids, immunosuppressive agents), showed a stabilization or a slight improvement in two of the four treated patients.[40] The best result was obtained when plasmapheresis was added to IFN-α and/or immunosuppressive agents. Finally, the management of HCV-related PN should be based on its severity and response to treatment. Combined antiviral therapy (ribavirin and IFN-α) should be considered as the initial option in patients with slight to moderate neuropathy, with addition of corticosteroids in the case of non-response or worsening of the PN. Patients who fail this triple therapy should be considered for intravenous immunoglobulins, keeping in mind that plasmapheresis seems to be the best option in severe or refractory cases.

### *Cryoglobulinaemia and systemic vasculitis*

MC is a chronic immune complex-mediated disease.[86] MC is the most frequent biological peculiarity in chronically HCV-infected patients, found in >50% of patients, and there seems to be a south–north gradient in the prevalence of HCV-associated cryoglobulinaemia. It is only symptomatic in less than one-third of cases – i.e. vascular, renal and neurological lesions.[87] Oligoclonal or monoclonal B-cell expansions are significant molecular features of bone marrow[88] and liver.[89] It is estimated that almost 10% of patients with MC progress to frank B-cell non-Hodgkin's lymphoma.[90]

Cryoglobulins are cold precipitable immunoglobulins that are accounted for by two or more immunoglobulin isotypes.[91] In type II MC, cryoglobulins are composed of a monoclonal rheumatoid factor (usually, IgM kappa) against polyclonal IgG. In type III MC, all components are polyclonal. The different autoantibodies and circulating immune complexes produced, including the cryoglobulins, deposit in small vessels and are responsible for systemic vasculitis and damage of various organ. The primary role of HCV in the mechanism of cryoprecipitation is mainly suggested by its selective concentration in cryoglobulins.[92,93] The association of haplotype human leucocyte antigen (HLA) B-8 and DR-3, MC and HCV infection has been demonstrated.[94]

Although HCV is increasingly recognized as a cause of systemic vasculitis, limited data are available regarding the optimal treatment of this potentially serious condition. Based on the close correlation between HCV infection and MC, treatment with IFN-α has been strongly advocated.[95] IFN-α produces significant clinical improvement in 40–70% of MC patients.[38,96–98] Its efficacy is closely associated with inhibition of HCV replication. Reduction of HCV RNA in responsive patients usually precedes a decline of cryopricipitates. However, the improvement is generally short-lived and >80% of responders have a relapse within 6 months.[98] In addition, in patients with cryoglobulinaemic neuritic or nephropathic complications or active skin ulcers, IFN-α frequently precipitates neuropathies or aggravates renal failure and delays ulcer healing.[99]

As a small fraction of patients with HCV-positive MC achieve long-term recovery after IFN-α therapy, the effectiveness and safety of combination therapy with IFN-α and ribavirin was tested in non-responders or those who relapsed after one or more courses of IFN-α. A retrospective study[100] analyzed 27 patients with chronic hepatitis C complicated by systemic vasculitis, and treated by IFN-α plus ribavirin therapy for at least 6 months. At the end of the combined therapy, 16/27 (59%) patients were negative for HCV RNA, and 15/27 (55%) were complete clinical responders. Among the remaining 11 virological non-responders at the end of therapy, 8/11 (73%) had at least a partial clinical response. Only 14 patients had a 6-month follow-up after discontinuation of IFN-α plus ribavirin therapy. Among them, 9/14 (64%) showed a virological, clinical and immunological sustained response 6 months after the end of therapy. All (nine of nine) virological sustained responders and a few virological non-responders (one of five) were also clinical and immunological complete responders. However, three of five non-responders could benefit from the therapy and had partial clinical and immunological responses. This study demonstrated that IFN-α plus ribavirin can achieve a complete clinical response in a substantial proportion of patients with HCV-related systemic vasculitis, and that complete clinical response correlates with the eradication of HCV.[100] A more recent and prospective study involving 27 patients with HCV infection and MC did not show such a high efficiency of IFN-α plus ribavirin combined therapy administered for 1 year, as only 18.5% of patients completely recovered (viral clearance and all symptoms of the disease). However, most patients (85%) improved clinically during the combined therapy.[101]

This prompted a search for innovative therapeutic strategies with the aim of reducing or depleting the B-cell clonal expansion that sustains production of IgM rheumatoid factor molecules. One such approach involves the use of monoclonal antibodies directed to CD20 antigen, a transmembrane protein expressed on pre-B lymphocytes and mature lymphocytes.[102] Rituximab, a humanized murine monoclonal antibody of this kind, is highly effective for *in vivo* B-cell depletion.[103] Peripheral blood B lymphocytes become undetectable after a single infusion and recover 6–9 months after discontinuation of treatment.[104] Rituximab was originally approved for the treatment of low-grade B-cell non-Hodgkin's lymphoma,[105] and has since become a promising therapeutic approach for diffuse large B-cell lymphomas, mantle cell

lymphoma,[106] hairy cell leukaemia[103] and chronic lymphocytic leukaemia.[107] It has also been used in several other haematological disorders including pure red cell aplasia and haemolytic anaemia,[108] primary cold agglutinin disease,[109] post-transplantation B-lymphoproliferative disorder,[110] Waldenström's macroglobulinaemia,[111] and idiopathic thrombocytopenic purpura.[112] A recent controlled Italian study involving 20 patients with MC and HCV-positive chronic active liver disease, resistant to IFN-α therapy and non-improved by low or moderate doses of corticosteroids, has revealed that an intravenous infusion of 375 mg/m$^2$ rituximab once a week for 4 consecutive weeks could safely induce a rapid improvement of clinical signs and complete response in 80% of patients (disappearance of purpura, weakness arthralgia, improvement of peripheral neuropathy, and decline of cryoprecipitates).[33] This complete response was maintained in 75% of patients during the whole of follow-up (12 months). Complete response was associated with a significant reduction of rheumatoid factor activity and anti-HCV antibody titres. Decline of IgG anti-HCV titres in the cryoprecipitates was usually associated with a favourable response. Molecular monitoring of the B-cell response revealed disappearance/deletion of peripheral clones in the responders and stability in the non-responders. However, complete response was associated with an increase of HCV RNA titres, suggesting that combination of anti-CD20 therapy together with antiviral combination could be of optimal benefit.

### *Non-Hodgkin's lymphoma*

In connection with MC, which is a low-grade benign lymphoproliferative disorder (LPD), HCV infection may be associated with other lymphoproliferative disorders (LPDs), especially malignant B-cell non-Hodgkin's lymphoma.[113] The association between HCV and B-cell non-Hodgkin's lymphoma has been controversial. There is increasing evidence of the pathogenesis of HCV-associated lymphoma.[114] Another clue pointing towards the relationship between B-cell non-Hodgkin's lymphoma and HCV has been brought by the establishment, from a HCV-associated human lymphoma, of a B-lymphoma cell line which continuously produces HCV.[115] MC is characterized by the clonal expansion of B cells that may evolve into low-grade or high-grade non-Hodgkin's lymphoma.[116] HCV infection is most frequently encountered in patients with lymphoplasmocytoid lymphoma or immunocytoma-lymphoma, marginal zone lymphomas of the lymph node, diffuse primary hepatosplenic large B-cell lymphomas and splenic lymphomas with villous lymphocytes.[117] However, the pathogenetic mechanisms involved are still poorly understood. A direct role of HCV infection in the genesis of these B-cell lymphoproliferative disorders was suggested initially by epidemiological studies and is supported by recent studies, which analyzed the monoclonal B cells that proliferate in these disorders. How HCV induces B-cell lymphoproliferative disorders is still unclear. It is probably not due to a direct change of phenotype in B cells after viral infection, but it may be due to an HCV antigen-driven process. Support for this hypothesis comes from the analysis of monoclonal B cells found in these disorders, which use a restricted repertoire of immunoglobulin variable region genes that are similar to those used by B cells that secrete anti-HCV antibodies. The fact that monoclonal IgM is resolved in HCV-infected patients who responded to antiviral treatment supports the linkage between antigen persistence and B-cell proliferation. Finally, the linkage between benign B-cell proliferation and overt lymphoma is supported by the identification of a pre-malignant B-cell clone that subsequently converted to an overt B-cell lymphoma. The molecular basis for viral-induced B-cell proliferation is still unknown. One possibility is that HCV stimulates the proliferation of monoclonal B cells via their HCV-specific B-cell receptor on the cell surface. Binding of the HCV envelope proteins to a cellular ligand, CD81, may also enhance this antigen-driven process. A recent report on regression of splenic marginal zone lymphoma after antiviral treatment with IFN and ribavirin has significantly strengthened the cause–effect relationship between HCV infection and lymphoma.[118]

A significantly higher prevalence of bcl-2 recombination [t(14;18) (q32;q21)] – a B-lymphocyte-specific chromosomal rearrangement – was shown in peripheral blood mononuclear cell (PBMC) samples from HCV-infected patients, especially in the case of HCV-related MC, as well as clonal expansion of translocated B lymphocytes, suggesting that inhibition of B-lymphocyte apoptosis by bcl-2 overexpression plays a key role in LPD pathogenesis.[119] The pathogenetic importance of t(14;18) in LPDs has been supported by animal studies. Mice transgenic for t(14;18) develop an indolent polyclonal follicular lymphoproliferation that progresses to a monoclonal high-grade malignancy.[120] However, in humans the t(14;18) translocation is not sufficient to induce clinical neoplasm and may be found in apparently healthy people, with its prevalence increasing with age. In some individuals, however, t(14;18) may represent an early stage in a complex, multi-step process that leads to malignant lymphoma, and inhibition of abnormal B-cell expansions at earlier stages of the pathogenic process may prevent the development of some neoplasias.[121,122]

Limited data are available with respect to the effects of antiviral treatment on clonal HCV-related B-cell expansion. These data generally refer to patients with frank LPDs, especially MC with or without non-Hodgkin's lymphoma.[119,123,124] One study has investigated the effect of antiviral treatment on immunoglobulin heavy-chain gene (IgH) rearrangement and t(14;18) translocation in

the PBMCs of 29 HCV-infected patients. Fifteen of 29 patients were treated with either IFN-α or combination therapy with IFN-α and ribavirin for 6–12 months. IgH rearrangement became negative in seven of nine treated patients compared with only one of eight of non-treated patients ($p$ <0.02). The t(14;18) translocation became negative in six of seven treated patients compared with one of six non-treated patients ($p$ = 0.03). Disappearance of IgH rearrangement or t(14;18) translocation was strongly associated with virological response to treatment. Two t(14;18)+ patients developed B-cell lymphoma during follow-up. Antiviral treatment appeared to be effective in eliminating the clonal proliferation of B cells in patients with chronic HCV infection and might have prevented the subsequent development of lymphoma.[123]

Furthermore, antiviral therapy has shown anti-oncogenic activity on malignant B lymphocytes. Twenty patients with HCV infection and MC had a bone marrow biopsy, showing a massive monomorphous infiltration by plasmacytoid lymphocytes, indicating the presence of low grade non-Hodgkin's lymphoma in six of twenty patients. After IFN-α therapy for 1 year and a subsequent few months' follow-up after therapy withdrawal, bone marrow examination showed that B-lymphocytic monoclonal infiltrate had disappeared in three patients.[124] In another study, IFN-α with or without ribavirin was shown to induce complete remission of eight of nine HCV-related splenic lymphomas with villous lymphocytes after the loss of detectable HCV RNA, while none of the six HCV-negative patients had a response to IFN-α therapy.[117]

## Conclusion

Hepatitis B and C infections are a major public health problem worldwide, as they may lead to liver failure and/or primary liver cancer. Furthermore, although to a much lesser extent, these infections may induce extrahepatic lesions, with a significant impact on morbidity and mortality of HBV- or HCV-infected patients. As limited data are available, more information is needed before definitive therapeutic recommendations for the extrahepatic HBV or HCV features can be established.

PAN is the most dramatic manifestation of primary HBV infection, while glomerulonephritis and nephrotic syndrome may develop during chronic HBV course. The pathogenesis of both vasculitis and glomerulonephritis is still believed to be mediated by circulating immune complexes, the formation of which is favoured by high HBV replication. No evidence for an autoimmune pathogenic mechanism can be substantiated in PAN, contrary to other types of systemic vasculitis unrelated to HBV. The spectacular immediate and long-term benefits of combining plasma exchange with antiviral therapy add further support to this in the treatment of PAN. Antiviral therapy alone without plasma exchange may control HBV-related glomerulonephritis. Traditional immunosuppressive and corticosteroid therapy should no longer be used.

The optimal treatment strategy for HCV-related extrahepatic manifestations remains to be defined. Both antiviral and immunosuppressive therapies, either alone or in combination, seem likely to have an important role, although these treatments should be administered with caution, and the exacerbation or precipitation of autoimmune symptoms should be monitored closely. The first goal of the treatment should be the definitive eradication of HCV by combined therapy with IFN-α and ribavirin without adding immunosuppressive drugs if possible. HCV eradication may be enough to suppress extrahepatic manifestations. However, persistence or relapse, and sometimes worsening, of extrahepatic lesions may occur, and conventional corticosteroid therapy, immunosuppressive agents, intravenous immunoglobulins and/or plasmapheresis should be added in specific severe cases. Anti-CD20 therapy is a new potentially promising therapeutic approach for MC-mediated lesions, but data have to be confirmed in large cohorts of patients, alone if antiviral therapy is contraindicated or combined with antiviral therapy to prevent high viral replication rates and subsequent possible worsening of the liver disease. When long-term immunosuppressive therapy becomes warranted, this will require very close monitoring of liver function and HCV levels, and combined antiviral therapy should be added if at all possible. The search for new antiviral drugs, possibly combined with immunosuppressive agents, is urgently needed because of the significant morbidity and mortality associated with some HCV-related extrahepatic manifestations.

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# Chapter 52
# The histologist's role in the diagnosis and management of chronic hepatitis B and C

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The role of the histopathologist in the diagnosis and management of viral hepatitis has changed significantly in recent years and will no doubt continue to evolve in the future, as more effective antiviral drugs and new diagnostic techniques become available. The objectives of this chapter are to outline the current role of histopathology and to suggest how this is likely to change.

## Diagnosis

The actual diagnosis of hepatitis B (HBV) and C (HCV) is almost always made before the liver biopsy is taken and is usually done by a combination of serology and polymerase chain reaction (PCR). Very occasionally, unsuspected HBV is diagnosed by the histopathologist by the presence of ground-glass cells (and confirmed by immunohistochemistry) or HCV by the presence of the combination of the characteristic features described below. In contrast hepatitis D (HDV) is frequently first diagnosed (or at least confirmed) on liver biopsy (see below). More common than diagnosing chronic viral hepatitis on liver biopsy is making the diagnosis of another form of liver disease in a patient known to have chronic viral hepatitis. This has important implications for managing these patients and will be discussed in the next section.

### Histopathological features of chronic viral hepatitis

#### *HBV*

(See Plates 52.1–52.3, found between p.786–7.)The specific histopathological features of HBV are: the presence of immunopathic liver damage, ground-glass cells and the immunohistochemical demonstration of HBV antigens in the liver.[1,2] As the damage in HBV is immune-mediated, a common finding in HBV is the presence of lymphoid cells in close apposition to hepatocytes showing degenerative changes. The ground-glass cells seen in HBV need to be distinguished from those seen, much less frequently, in other conditions, such as Lafora bodies in mycoclonic epilepsy. More frequently, they need to be distinguished from the effects of drugs, especially anticonvulsants such as diphenylhydantoin, which induce the smooth endoplasmic reticulum ('turned on hepatocytes'). This is easily done by staining for hepatitis B surface antigen (HBsAg). There is a limited but significant role for immunohistochemical staining in cases of HBV in addition to confirming the diagnosis. It is only necessary in a very small number of cases. Staining with hepatitis B core antigen (HBcAg) (or hepatitis B 'e' antigen; HBeAg) can be used to assess disease activity.[3] Active disease is characterized by more widespread staining with more cytoplasmic (rather than purely nuclear) staining. In cases with the precore, mutant staining for HBeAg will be negative; HBeAg staining correlates to the presence of wild-type virus. In immunosuppressed patients, there may be decreased necroinflammatory activity in the presence of large amounts of viral antigen.[4] Immunohistochemistry is especially useful, as some of these patients may in the past have been considered to have cleared HBV and even be HBsAb-positive.

#### *HDV*

(See Plates 52.4 and 52.5, found between p.786–7.) HDV is associated with increased histological activity with prominent cytopathic changes.[1,2,5,6] The histopathologist may see unusually active lobular inflammation with prominent apoptosis in a patient with HBV and confirm the diagnosis of HDV using immunohistochemistry. Even if the clinician is the first to raise the possibility of HDV, the definite diagnosis may first be made by immunohistochemical staining of a liver biopsy, as the demonstration of HDV antigen in the liver biopsy remains the gold standard for making the diagnosis.[7] Furthermore, this can usually be done more rapidly than the corresponding serological tests. Reliable anti-HDV antibodies are, however, not commercially available.

### *HCV*

(See Plates 52.6 and 52.7, found between p.786–7.) There are no diagnostic features of HCV. However, as compared with other causes of chronic hepatitis the presence of two or more of the characteristic changes (lymphoid aggregates/follicles, hepatitic bile duct damage and large droplet fatty change) strongly suggests the diagnosis.[1,2,8,9] Lymphoid aggregates may, however, also be seen in HBV and autoimmune hepatitis. Although hepatitic bile duct damage is very often seen, bile duct loss is not a feature of HCV, and if present, the usual causes of ductopaenia need to be excluded.[10] The presence of canalicular cholestasis should always suggest a superimposed pathological process. Excess alcohol intake is not uncommon in patients with HCV. Fatty change is particularly associated with infection with HCV genotype 3.[11] Ballooning degeneration, neutrophils, etc. are only seen when there is significant alcohol-induced liver disease in addition to the HCV. Biopsies from patients with HCV often show relatively mild portal and periportal inflammation with more prominent lobular changes as compared with cases of HBV. Granulomas for which no other cause can be found are more common with HCV than HBV.[12] Plasma cell-rich inflammatory infiltrates may be seen in cases of HCV, and this is often associated with positive serum antinuclear antibodies, although their presence does not seem to be associated with an impaired responsiveness to antiviral treatment.[13] Unfortunately, there is still no routinely available immunohistochemical technique for demonstrating HCV in formalin-fixed, paraffin-embedded tissue.[14] However, it is possible to assay by PCR and even genotype HCV from formalin-fixed, paraffin-embedded liver biopsies.[15] There has been considerable debate about the effect of co-infection with HGV in patients with HCV. A recent Japanese study has suggested that HGV RNA-positive patients show significantly more severe bile duct damage, perivenular fibrosis, pericellular fibrosis and 'irregular regeneration' of hepatocytes than the livers of the HGV RNA-negative patients.[16] Overall, however, most studies have suggested that there is no significant impact of HGV co-infection on disease severity.[17]

## Histopathology of chronic viral hepatitis in liver transplant patients

### *HBV and HDV*

Biopsies taken from patients with recurrent HBV in their liver grafts show a spectrum of histopathological appearances.[18] Over three-quarters of them show the changes of chronic hepatitis or cirrhosis, often with ground-glass cells. This is usually indistinguishable from HBV in non-transplant patients. The second commonest finding is of a mild, non-specific hepatitis. A fibrosing cholestatic hepatitis is seen in a minority of patients.[19] This is characterized by fibrosis (both portal and perisinusoidal), cholestasis (intracellular and canalicular), severe ballooning of hepatocytes and mild portal inflammation. Overall, the severity of the pathological changes is more severe than in patients with recurrent HCV. This is associated with high levels of HBsAg and HBcAg expression. It should be noted that recurrent HDV may recur without demonstrable markers of HBV infection. In these circumstances HBV is not necessarily cytopathic. Coagulative necrosis of zone 1 associated with fibrinoid necrosis of hepatic arteries has also been described.

### *HCV*

(See Plate 52.8, found between p.786–7) The nature of the pathological changes may be the same as in non-transplant patients and a chronic hepatitis is seen in 80%.The pathological changes tend to be milder than is the case with HBV; only 5–10% progress to cirrhosis. The severity, in terms of both inflammation and fibrosis, tends to be more severe than in non-transplanted HCV patients. A match at one or two HLA DQ loci has been strongly associated with the severity of the damage seen. HCV has a direct cytopathic effect on bile duct epithelium. The level of liver injury does not correlate with the levels of HCV. One-third of treated patients develop ductopaenic rejection. Atypical features include severe bile duct injury and bile duct proliferation. This may be so marked as to mimic large duct obstruction. Features resembling the fibrosing cholestatic hepatitis seen with HBV may also be observed.[20] Nodular regenerative hyperplasia has also been described. When recurrence occurs within 6 weeks the differential diagnosis includes acute cellular rejection. Multiple biopsies help with this differential diagnosis as the portal inflammation, with lymphoid aggregate formation, and the lobular inflammation characteristic of HCV, develop with time. In cases which are difficult to categorize as either recurrent HCV or acute rejection, it is likely that each of these processes may be occurring together. In atypical cases, other causes of chronic hepatitis need to be considered. Unless it is certain that all the changes seen are all ascribable to HCV, they should not be scored (see below).

## Assessment of disease severity

Assessment of disease severity is, perhaps, the most important contribution the histopathologist makes to patient management. This involves the separate assessment of necroinflammatory activity (the *grade* of the biopsy) and the fibrosis (the *stage* of the biopsy). The terms grade and stage were coined by Scheuer, by analogy to tumour pathology, and represented a significant advance over the older nomenclature in which biopsies were subdivided according to the presence or absence of piecemeal necrosis alone (chronic persistent hepatitis or chronic active

hepatitis) with cirrhosis being a separate category.[21] In terms of nomenclature, many people prefer the term 'interface hepatitis' to 'piecemeal necrosis' as this is a more accurate description of the pathological process, which is apoptosis rather than necrosis. Still others prefer the less committed expression 'periportal inflammation'. Stage is usually considered the more important, as the severity of the inflammation may vary while, at least until recently, fibrosis was considered an irreversible process. [22,23]

Grade and stage are usually assessed using a semi-quantitative scoring system, although this can also be done by simply describing the severity of the necroinflammatory and fibrotic changes in words. In the hospital setting, the decision as to whether to score biopsies or not depends on agreement between clinicians and pathologists, which in turn depends on whether the actual scores are used to determine whether the patient is treated or not. Furthermore, when comparing sequential biopsies on the basis of their scores, it is highly desirable that they are scored by the same pathologist and at the same time. In clinical trials, some form of scoring system is almost always used. Either way, the biopsy must be of adequate size (see below) and be stained using both a conventional haematoxylin and eosin stain, which is used to assess the necroinflammation, and a histochemical stain that highlights the connective tissue present. This is usually done using the silver stain Gordon and Sweets reticulin (especially in the UK) or trichrome stain, such as Masson's trichrome (especially in the USA).

It has been shown that the sampling error depends partly on the size of the liver biopsy taken.[24,25] This will depend on the size and gauge of the needle. In one study, successive 5-mm portions of liver biopsies were uncovered. In 20 biopsies in which cirrhosis was diagnosed when 25 mm of the biopsy was exposed, it was only diagnosed in 14 when 20 mm was exposed and in 11 when 10 mm was exposed. In another study in which needle biopsies were taken just before post-mortem, even 25-mm biopsies may not be big enough. 100% diagnostic precision was only obtained when three 25-mm biopsies were taken. Although the length of the biopsy is important, it is really the number of portal tracts that is critical. It is generally regarded that the biopsy must contain at least three portal tracts to be assessed at all. It should be pointed out that 16 gauge needles are needed if whole cirrhotic nodules are to be included in the biopsy. The way in which the liver tissue is actually obtained is also important.[26] Suction needles (such as Menghini needles) tend to produce fragmented samples, especially from livers with advanced fibrosis, in which nodules of hepatocytes are obtained, leaving behind much of the fibrous tissue. This can make the confident diagnosis of cirrhosis very difficult. For the same reason, biopsies taken with suction needles are particularly unsuitable for image analysis. On the other hand, cutting needles (such as a Tru-cut needle) remove both fibrous tissue and parenchyma and make the assessment of fibrosis much easier. It should be noted that as the changes in HCV tend to be more focal than those in HBV, sampling error is more significant. The subjective nature of the process can also be minimized if the scoring is done by a small number of experienced histopathologists, especially if they are given the opportunity to get together to discuss the scoring system before using it.

There are several semi-quantitative scoring systems that have been used to assess fibrosis and inflammation in patients with any of the causes of chronic hepatitis. The most established is the histological activity index – HAI (Knodell) system.[27] In its original form the scores for portal, inflammation, piecemeal necrosis (including confluent necrosis) and lobular inflammation were added together to give the HAI. Later, when it was considered incorrect to combine scores for necroinflammation and fibrosis, the system was modified to keep them separate. An idiosyncrasy of this scoring system is that, in all the categories, the scores are discontinuous. For example, the scores for fibrosis are: 0 = normal, 1 = portal tract expansion, 3 = bridging fibrosis and 4 = cirrhosis. In other words, there is no score of 2 in this system. Although this feature has come in for much criticism, it has the practical effect of dividing fibrosis into two categories: normal/portal tract expansion and bridging fibrosis/cirrhosis, which many recent publications base their analysis of drug trials on even if they use other scoring systems. Because the HAI has been used for many years, it is useful for comparing the results of different studies and it continues to be used in many current trials.

The modified HAI (Ishak) system was developed to take into account many of the criticisms made of the HAI. Necroinflammatory scores and fibrosis scores are separated and the scoring system is continuous.[28] In addition, it offers a wider range of scores, especially for fibrosis. In essence, however, it is similar. The Scheuer scoring system, important conceptually as being the first to separate grade and stage, is used mainly in Australia. This scoring system is now widely used.[21] The French METAVIR scoring system is the best validated of all the scoring systems but has not been widely used outside France.[28a] Again, necroinflammation and fibrosis are assessed separately. In terms of grade, only piecemeal necrosis and lobular inflammation are considered important and then, using a cross table, combined to give an overall necroinflammatory grade. Many centres use their own modified versions of one of these scoring systems. Even if different pathologists use the same scoring system, their interpretations of the definitions used for particular aspects are often quite different (partly because of the ambiguities that some of the scoring systems contain) and this adds another source of variation. Regardless of the scoring system used, when the

results come to be analyzed it is common for fibrosis to be divided into only two categories: normal/portal tract expansion and bridging fibrosis/cirrhosis. This is based on the idea that it is only a change from one of these broader categories to another that is clinically significant. It should be noted that this is essentially what the often criticized HAI system does!

There are a number of generic problems with all these scoring systems. Firstly, the data they produce is ordinal (categorical) but not numerical, i.e. the increasing scores for any feature represent progressively more severe changes but the differences between consecutive scores are not equal. For example, a score of 4 does not mean that the changes seen are twice as severe as those seen if the score was 2. This problem is especially acute with the HAI scoring system because it is discontinuous. In any case, this means that adding scores together, as is done by adding the different inflammatory scores together to give the overall grade, is statistically invalid, although it is regularly done! Adding the scores of the three (or four) categories of necroinflammation to give a single number loses information and may be misleading. For example, if there is a biopsy with marked portal inflammation but only mild piecemeal necrosis and lobular inflammation, it may have the same overall score for grade as a biopsy with mild portal tract inflammation and more marked piecemeal necrosis and lobular inflammation. Most people would agree that the second biopsy actually represents more active disease. Also, when analyzing the data generated by a study, non-parametric tests need to be employed. Furthermore, the scores obtained from different studies are not suitable for meta-analysis. Secondly, there are problems with inter- and intra-observer variation. This has been examined and, overall, experienced liver pathologists have a reasonable level of agreement with most studies, indicating that this is better for stage than grade.[29] The METAVIR group have also shown that pairs of pathologists give more reproducible results than single pathologists. This is, however, rarely a practical possibility either in clinical practice or in trials, as it is so time-consuming (and expensive). Those scoring systems which allow a more detailed assessment of the pathological changes are inherently more likely to produce increased inter-observer variation. For this reason, it is preferable to use the simplest scoring system which will provide the information required. It is salutary at the end of a study to undertake an audit to assess the degree of intra-observer variation to get a feel for the reliability of the results. Thirdly, it is important to consider what difference in scores will be considered significant. Empirically, it is usually considered that a difference of 2 or more is clinically important. This makes some allowance for sampling and observer error. It is clearly essential that the scoring be done without knowledge of the sequence of the biopsies and all other clinical information. A way of comparing biopsies which obviates this problem (and which does not depend on using a particular scoring system) is to make a direct comparison between biopsies which have been blinded as to sequence with grade and stage being assessed separately. The small number of possible results makes this a statistically powerful approach. Another way of assessing fibrosis and inflammation, which is especially useful in small studies, is to rank all the biopsies after they have been blinded as to which patients they are from and to sequence. Finally, it needs to be questioned what the scores mean for the patient. Although it is accepted that higher grade or stage disease is more likely to progress, there are no data to suggest that any of these scoring systems predict this more accurately than any of the others.[30] Scoring adds time and cost to the liver biopsy evaluation, but may provide a useful means of training in biopsy interpretation, and can assist clinical decisions, as well as providing a means of validating surrogate markers of fibrosis.

If three or more consecutive biopsies are available from a particular patient, and it is known when they were taken, it is possible not only to look at the differences in fibrosis scores between the biopsies but also the changes in the rate of fibrosis, which also takes into account how far apart they were taken. Studies using this approach have provided valuable information on the rates of disease progression (e.g. Wright *et al*.[31]). Some studies have suggested a linear increase in fibrosis, while others have supported a non-linear increase. It is not clear if the differences are artefacts of the different scoring systems used in these studies.

Immunohistochemical techniques can also be used to assess fibrosis or hepatocyte damage. For example, the presence of activated stellate cells can be quantified using immunohistochemical markers, for example, to α-smooth muscle actin or synaptophysin.[32] Alternatively, the deposition of a particular type of collagen, for example, the C-terminal procollagen α 1 peptide or other extracellular matrix protein, can be directly demonstrated. The degree of liver damage can also be assessed directly by using markers for cell damage (such as TUNEL staining or Fas antigen [CD95] expression)[33,34] or indirectly by assessing liver cell proliferation (using cell cycle markers such as PCNA). In the case of hepatitis B, immunohistochemical demonstration of the presence, distribution (nuclear ± cytoplasmic) and extent of HBcAg or HBeAg expression can add useful information about disease activity (see above).

Many people feel that image analysis can overcome many of the disadvantages of the semi-quantitative scoring systems when it comes to assessing fibrosis.[36] It has been claimed that it is more objective, sensitive and reproducible, although this is not accepted by everyone. For image analysis, sections are stained with a collagen

stain, most frequently Sirius red, and the amount of 'redness' in the section is measured using a computer attached to a video camera. Sirius red binds to collagen stochiometrically, and it has been shown that this binding correlates with the amount of collagen in the tissue as measured chemically by its hydroxyproline content.[58] Other stains which have been used for image analysis are Mallory's trichrome and aniline blue. Fast green binds to non-collagen proteins and can be used to complement Sirius red staining. Image analysis has been applied to biopsies from patients with chronic viral hepatitis. For example, a decrease in the collagen content, especially in the space of Disse, has been demonstrated in patients with HCV after they have been treated.[37]

Image analysis can also be applied to the immunohistochemical stains, including those described in the previous paragraph. For example, in one study the relationship between hepatocyte proliferation and DNA ploidy patterns was examined using a TV image analysis method. The authors concluded that the higher G1 and lower S cell cycle phase fractions observed in patients with HCV reflected decreased hepatocyte proliferation. In addition, they suggested that the near-aneuploid DNA content of the HCV-infected liver samples may be a sign of increased genetic instability which may, in turn, contribute to the increased risk of liver cancer.[38]

There are, however, three major drawbacks to image analysis. Firstly it requires equipment that is not universally available and it is very time-consuming. Secondly, it is only the total amount of collagen present that is being measured, and no account is taken of its distribution. For example, a high score may be obtained in a biopsy with normal architecture because of the presence of septal portal areas and a low score from a cirrhotic liver because only fragments of parenchyma surrounded by wisps of connective tissue have been obtained. Furthermore, critical changes in liver architecture, such as bridging fibrosis which is the morphological basis of porto-systemic shunting, are best assessed by eye. Thirdly, as part of the process it is necessary to set a threshold above which fibrosis will be considered to be present. This is often done 'by eye' and therefore remains very subjective. Even when a computer programme is used to minimize this problem, it has been shown that the semi-quantitative scoring systems are actually a more robust measure of fibrosis. Image analysis should be used only in the research setting, and even here should be seen as complementing routine histological examination and not replacing it.[39,40]

## Role of liver biopsy[41]

### HBV

At the time of the initial assessment of patients with HBV a liver biopsy is recommended.[42] This is done to assess the degree of liver damage and to exclude other causes of liver disease. It has also been suggested that the histological findings may predict prognosis. Following treatment, or spontaneous HBeAg seroconversion, the histology improves. Conversely, the histological changes may transiently deteriorate at the time of this seroconversion with an increase in lobular inflammation when the HBeAg-expressing cells are being cleared. The pathology may also deteriorate following reactivation of the disease. In following up patients not initially considered for treatment, in those who have a serum HBV DNA level of $>10^5$ and continuingly abnormal liver function tests, a further liver biopsy may be indicated. Liver biopsy remains an important part of the investigation of patients with chronic hepatitis B. The dynamic fluctuations in biochemical and seriological markers may not provide reliable information regarding the cumulative liver damage.

### HCV

The value of the liver biopsy in managing patients with HCV has been reviewed recently .[43–45] The possible roles of the liver biopsy include the following.

**1** To assess how urgently treatment is needed.
**2** To provide a baseline for assessing disease progression.
**3** To exclude co-existing liver diseases.
**4** To predict the likely response to antiviral treatment.

It should be noted that conventional liver function tests do not reliably predict the severity of the underlying disease. For example, in a group of 19 patients with normal aspartate aminotransferase (AST)levels two showed non-specific reactive hepatitis and 14 showed the features of chronic hepatitis.[46]

#### *1. To assess how urgently treatment is needed*

Treatment of HCV is unpleasant, expensive and associated with significant side-effects. More than this it is only effective, long term, in approximately 50% of patients. Currently, therefore, many management protocols require a liver biopsy to select those patients most urgently in need of treatment. As most patients only come to clinical attention many years after contracting the disease, the biopsy allows the assessment of the long-term impact of the disease on the liver. A small number of studies have been carried out using histological assessments of stage and grade to predict disease progression. It has been shown, for example, that mild disease progresses very slowly, if at all, when followed up for over 10 years, while patients with moderate or severe disease progress to cirrhosis over 20 years and 10 years, respectively. Some studies on the cost-effec-

tiveness of treatment have, however, suggested that it is worthwhile to treat even patients with mild disease. This conclusion has not been universally accepted. In any case, these studies need to be repeated with the new generation of antiviral agents.

### *2. To provide a baseline for assessing disease progression*

Another reason put forward for performing a liver biopsy is to act as a baseline against which disease progression can be assessed. Related to this is the question of how long to wait before rebiopsying patients who have not been treated to see if treatment is now indicated. Although a wait of 3 years has been widely suggested, if a rebiopsy is considered necessary at all, then, given the information that has been obtained from disease progression studies, an interval of 5 years may be more sensible. The differences between the baseline and subsequent biopsies can be assessed by comparing the scores, obtained using one of the semi-quantitative scoring systems, of the respective biopsies. It is generally considered that a difference in scores of 2 or more points is significant. This allows for some degree of sampling error. It can also be done by making a direct comparison of the stage and grade of the biopsies. When this is done as part of a clinical trial, it is clearly essential that the biopsies are blinded as to their sequence. When making any of these comparisons, it is highly desirable that this be done by the same experienced liver pathologist. The management of patients with normal alanine aminotransferase (ALT) remains controversial. The majority of these patients tend to have mild disease (F0 to F4) and the rate of progression of patients with normal ALT levels (which tend in fact to be at the upper limit of normal) is slower, or even stable. However, progression can be observed with longer follow-up, particularly in those with F2 rather than F0 or F1 disease at baseline. The risk of progression is to some extent dependent upon the definition of persistently normal ALT. There is a relationship between age, steatosis and the presence or absence of ALT flares. Liver biopsy, or perhaps serum markers of fibrosis, may be important in deciding upon treatment.[47]

### *3. To exclude co-existing liver diseases*

(See Plate 52.9, found between p.786–7.) The commonest liver diseases co-existing in patients with chronic viral hepatitis are due to iron overload or alcohol. These diseases are important in two different ways. Firstly, they may need to be treated in their own right. Secondly, they are associated with accelerated progression of the HCV and/or impaired response to treatment. One study found that 2% of patients with HCV had a clinically suspected other diagnosis and an equal number had a clinically unsuspected second disease, and another study demonstrated that the iron in Kupffer cells correlates with disease activity and that it decreases with successful treatment. Parenchymal iron disposition is more significant and underlying haemochromatosis should be excluded. In patients who have thalassaemia in addition to their HCV, parenchymal iron deposition is likely to be associated with the effects of the underlying ineffective erythropoiesis.[48] There are no prospective studies analyzing the clinical importance of excluding co-existing liver diseases.

### *4. To predict the likely response to antiviral treatment*

The more advanced the fibrosis in a liver biopsy, the less likely the patient is to respond to antiviral therapy. This difference is less marked in patients treated with the newer pegylated interferons (PEG-IFNs), especially when used in combination with ribavarin. A significant difference in response rates between patients without significant fibrosis (either no fibrosis or only portal tract expansion) and those with significant fibrosis (bridging fibrosis or cirrhosis) who have been treated with these agents has been demonstrated ($p<0.01$ for a sustained viral response in a multiple regression analysis). The effect of histology on the responsiveness to antiviral treatment has to be interpreted in the light of other factors that also influence it. These factors include: viral genotype, drug dosage and duration of treatment. Overall, the histology is not a sufficiently significant factor in predicting responsiveness to antivirals to influence treatment decisions on its own.

The determination of hepatitis C in biopsy tissue using sensitive PCR suggests that cryptic hepatitis C infection may be more common than previously thought to be the case.[49]

## Assessment of the risk of malignant change

(See Plate 52.10, found between p.786–7) The development of liver cell cancer is one of the most important complications of chronic viral hepatitis, and a number of protocols have been recommended to assess the risk. Liver biopsy may provide information about which patients are at increased risk for developing malignant change. The presence of large cell dysplasia and small cell dysplasia are both associated with an increased risk of developing liver cell cancer but are fundamentally different processes.[50–52] The terms large and small cell dysplasia (or change) should be used for lesions up to 1 mm in diameter. For larger lesions the expression dysplastic (macroregenerative) nodule is preferred.[53,54] Large cell dysplasia is characterized by groups of cells with large nuclei and prominent nucleoli set

in cells with more than the usual amount of cytoplasm.[55] Consequently, they have normal nuclear:cytoplasmic ratios. Large cell dysplasia is associated with an increased risk of malignant change, but these cells have a lower proliferation index than the other cells in the liver and do not themselves become malignant and appear to be terminally differentiated hepatocytes. Large cell dysplasia should therefore be considered as a *marker* for malignant change but not as a precursor lesion.[56] For this reason, the term large cell *change* is preferred by some. Its presence should act to encourage closer follow-up of patients.

Small cell dysplasia, conversely, is characterized by cells with normal, or even smaller than normal, nuclei within cells with decreased amounts of cytoplasm which is often basophilic. These cells, therefore, have increased nuclear: cytoplasmic ratios and the cells are more crowded together than normal. Small cell dysplasia is a *precursor* lesion for the liver cell cancer and, practically, it may be very difficult to distinguish it from a well differentiated liver cell cancer.[51] Staining for reticulin and CD34 may help with this differential diagnosis. Because of this link, the term small cell dysplasia is more informative than the more neutral small cell change. Small cell dysplasia appears to be commoner in Japan than in Western countries.

Although hepatocellular carcinoma is the commonest neoplasm seen in patients with HCV, there are a number of recent reports of cholangiocarcinoma developing in these patients.[57]

## Extrahepatic manifestations of hepatitis infection

Histopathologists may also make important contributions to patient management with reference to the extrahepatic manifestations of HCV, especially those involving the kidney. Extrahepatic disease is seen in approximately 40% of cases. They include renal disease, neuropathy, lymphoma and Sjögren's syndrome with or without mixed cryoglobulinaemia, which are all strongly associated with HCV infection. Porphyria cutanea tarda and diabetes have also been linked to HC.[58,59]

## The future of liver biopsies in the management of chronic hepatitis

### Non-invasive tests for hepatic fibrosis

Serum glycomics test based on serum N-glycans profiles distinguishes cirrhotic patients from non-cirrhotic patients. Compensated cirrhosis was detected with 86% specificity and 79% sensitivity. Although scientifically interesting, the current methodology does not detect early fibrosis easily, which is easily seen on liver biopsy.[60]

There is considerable interest in biochemical tests that predict stages of fibrosis. The FibroTest, developed by Poynard and co-workers, utilizes the fibrosis index, which combines the blood measurement of five indirect markers of fibrosis (alpha 2 macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin and gamma-glutamyl transpeptidase; PGGT), adjusted for age and sex. The test results are obtained by automated measurement. The diagnostic value of this matrix index has been assessed. High area under the curve statistics (AUROC) of the order of 0.8 for METAVIR F3 and F4 fibrosis have been reported in patients with chronic hepatitis C. A high negative predictive value for excluding METAVIR F1 or Ishak F2 fibrosis has been reported. The role and cost-effectiveness of FibroTest as an alternative to liver biopsy in selected patients with chronic hepatitis C requires assessment. The limitations of the test require continuing independent assessment. The test has not been evaluated in patients with post-transplant hepatitis or renal disease, or in patients younger than 18 years.[61–65] False-positive tests may occur with haemolysis or Gilberts syndrome.

Other predictive models utilizing, for example, age, GGT, cholesterol, platelet count and prothrombin time,[66] platelets, aspartate aminotransferase (AST) and alkaline phosphatase (using a novel index, AST to platelet ratio index, APRI), have been developed.[67] These may exclude F2–F4 fibrosis with high accuracy. However, all these indices have been validated mainly in patients with hepatitis C and give different results in different populations of patients being studied. There has been a single study in patients with HBV.[68] At best, biochemical markers of fibrosis are surrogate tests and do not reflect the processes of fibrogenesis and fibrinolyis occurring in the liver. They remain less useful, for example, in liver transplant patients, and those with genotype 3 and hypercholesterolaemia, and HIV co-infection.

Ultrasound examination of the surface of the liver, its echotexture and the resistance within the hepatic artery may provide a useful assessment of the presence of cirrhosis, but it remains a crude assessment of advanced disease. Staging of fibrosis is not possible with ultrasound imaging.[69–71] Newer techniques (such as microbubble transit times) can provide, by non-invasive means, a diagnosis of cirrhosis with some sensitivity.[71a] Beaugrand *et al*.[71b] have recently evaluated a new non-invasive device to quantify liver fibrosis: the shear elasticity probe or fibroscan. The device is based on one-dimensional transient elastography, a technique that uses both ultrasound and low-frequency elastic waves, whose propagation velocity is directly related to elasticity. Fibrosis of the liver is related to liver elasticity. Liver elasticity measurements, obtained in minutes, are reproducible, with an AUROC of 0.88 and 0.99 for the diagnosis of F4 cirrhosis. The technique can fail in obese subjects.[74]

### Liver biopsy

Recent advances in the management of viral hepatitis,

especially HCV, are causing the role of the liver biopsy to be reassessed. The development of more effective antiviral therapies (such as the introduction of the PEG-IFNs), together with the use of combination therapy coupled with improved patient selection (including the observation that patients with HCV genotypes 2 and 3 have higher response rates), has led some to suggest that liver biopsies should no longer be considered essential. Liver biopsies add to the cost of investigation and treatment for hepatitis C. Although hitherto, biopsies have been considered an integral part of the investigation of chronic hepatitis C, the need for routine biopsies may change in the future. Liver biopsies are associated with slight risk, and can cause discomfort, pain and anxiety for patients. Direct and indirect costs of the procedure add to the cost of a relatively costly treatment. The cost-effectiveness of liver biopsy needs evaluation. Prior studies have examined the cost-effectiveness of a baseline liver biopsy and viral genotype to ascertain patients least likely to respond. Strategies involving a liver biopsy were more expensive, as fewer patients needed treatment to achieve a higher sustained virological response rate. However, this study was based on the results achieved with a 6-month course of IFN-α monotherapy, and is dated.[75] A more recent study compared the cost-effectiveness of immediate antiviral therapy with biopsy every 3 years plus combination therapy with IFN-α and ribavirin. Immediate antiviral therapy was thought to increase life expectancy by 1.0 quality life year compared with biopsy management and was considered cost-effective compared with biopsy management.[76]

Thus, a liver biopsy before treatment is likely to increase the lifetime costs of treatment, and may be unduly restrictive and a barrier to treatment. However, physicians and patients may continue to value the diagnostic and prognostic information provided by a liver biopsy. It is believed that not everyone who is a candidate for treatment needs to undergo the procedure, and there is a move towards selective rather than routine use of the biopsy. For patients with mild disease (defined histologically as the absence of hepatic fibrosis or minimal fibrosis), empirical treatment for those with a high likelihood of response, i.e. genotype 2 and 3, is being applied in several countries, following the recommendations of recent French and American Consensus Conferences. The incremental cost-effectiveness of this approach has not yet been proven, however. Studies that have examined periodic biopsy to monitor the progression of mild disease need evaluation against treatment results for a combination of PEG-IFN and ribavirin, and against the use of serum non-invasive tests or perhaps ultrasonagraphic assessment compared with periodic biopsy. Narrowing the focus of treatment to those most likely to progress to sequelae of chronic liver disease remains a critical challenge to maximize the available resources for treatment.

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# Chapter 53
# Disinfection and sterilization

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The purpose of this chapter is to discuss disinfection and sterilization strategies used in health-care and laboratory settings to prevent the transmission of infectious agents from contaminated medical devices and environmental surfaces to patients and health-care workers. Although these strategies are those used generally in health-care facilities to accomplish disinfection and sterilization for all groups of pathogenic micro-organisms, emphasis will be placed on the efficacy of these procedures against the human hepatitis viruses.

The effective use of antiseptic, disinfectant and sterilization procedures in health-care settings is important in the prevention of hospital-acquired infections. Historically, the use of physical agents, such as moist heat in the form of steam autoclaves or dry heat applied using convection or forced air ovens, has played the predominant role in sterilizing instruments, equipment and supplies in hospitals. In the 1960s, a number of heat-sensitive medical instruments requiring sterilization by low temperature methods were developed. Ethylene oxide was used for this purpose for many years, but increasing concerns about acute and residual toxicity to humans and the environment and very long sterilization cycles stimulated the development of a number of alternative low temperature sterilization technologies. These include systems employing hydrogen peroxide gas plasma or ozone, and in some parts of Europe, steam/formaldehyde. Liquid chemical germicides formulated and marketed as 'total immersion' sterilants have also been available for many years, but they are used almost exclusively to disinfect rather than sterilize heat-sensitive medical devices.[1–3]

The choice of which sterilization or disinfection procedure or which specific chemical germicide should be used for sterilization, disinfection or antisepsis or for environmental sanitization depends on several factors. No single chemical germicide or sterilization procedure is adequate for all purposes. Factors that should be considered in the selection of a specific sterilization or disinfection procedure include (1) the degree of microbiological inactivation required for the particular device, (2) materials compatibility of the device with the disinfection or sterilization procedure based on the device's nature and physical composition, (3) the cost and ease of using a particular procedure, and (4) acute and residual toxicity concerns for the patient, the health-care worker and the environment. As will be pointed out below, the efficacy of various chemical and physical agents against the human hepatitis viruses are difficult to determine because only hepatitis A virus (HAV) can be grown in tissue culture. However, it is clear that all the human hepatitis viruses are relatively susceptible to common sterilants and disinfectants. Consequently, extraordinary procedures or preparations are not needed to process medical devices or for housekeeping purposes or laboratory decontamination.[4]

## Regulation of chemical germicides

Chemical germicides used as disinfectants or antiseptics in most countries are regulated by the federal or central government and usually by the Public Health Service or Ministry of Health.

In the United States, chemical germicides are regulated by two federal government agencies: the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA). Chemical germicides formulated as sterilants or disinfectants had historically been regulated by the EPA, but recently the EPA and FDA agreed that sterilants or disinfectants used on medical devices (e.g. haemodialysis machines, endoscopes, high-speed dental hand-pieces, etc.) will be regulated by the FDA. The EPA requires manufacturers of chemical germicides formulated as sanitizers, disinfectants, hospital disinfectants or sterilant/disinfectants (sporicides) to test these products using specific, standardized assay methods for microbicidal potency, stability and toxicity to humans. For chemical germicides intended for use on medical instruments (as opposed to environmental or housekeeping surfaces), the FDA requires that manufac-

turers submit a pre-market application that may include additional specific microbicidal activity data, device/chemical compatibility data and detailed instructions to the user regarding the 'safe and effective use' of the product. The FDA also regulates all sterilization devices such as ethylene oxide, ozone and hydrogen peroxide gas plasma sterilizers, steam autoclaves and dry heat ovens.

In addition to pharmaceutical drugs, the FDA regulates chemical germicides formulated as antiseptics, used to inhibit or kill micro-organisms on the skin or in tissue. These types of chemical germicides are categorized basically by use pattern (e.g. antimicrobial handwashes, patient preoperative skin preparations, skin wound cleansers, skin wound protectants and surgical hand scrubs) and are not regulated or registered in the same fashion that the EPA regulates and registers a disinfectant. Currently, data are not available to accurately assess the efficacy of many of the antimicrobial antiseptic formulations on the market. Consequently, health-care workers must make product selection decisions based on information derived from the manufacturer, published studies in the literature and guidelines from expert groups.[5]

The US Centers for Disease Control and Prevention (CDC) does not approve, regulate or test chemical germicides formulated as disinfectants or antiseptics. Rather, the CDC recommends broad strategies for the use of sterilants, disinfectants and antiseptics to prevent transmission of infections in the health-care environment.[1,6,7]

## Definitions

The definitions of sterilization, disinfection, antisepsis and other related terms such as decontamination and sanitization are generally accepted in the scientific community, but some of these terms are misused. It is important not only to understand the definition and implied capabilities of each procedure, but also to understand how to achieve and in some cases monitor each state.

### Sterilization and disinfection

The term *sterilization* is one that students and professionals have memorized and recited seemingly for ever. It can be the simplest and the most complex concept depending on how it is viewed and how it is applied. The definition of sterilization can change depending on the user's vantage point. We choose to view this term somewhat like a hologram and will define it in the context of:

**1** the *state* of sterilization,
**2** the *procedure* of sterilization,
**3** the *application* of sterilization.

Any item, device or solution is considered to be sterile when it is completely free of all living micro-organisms. This state of sterility is the objective of the sterilization procedure and, when viewed in this context, the definition is categorical and absolute, i.e. an item is either sterile or it is not.

A sterilization *procedure* is one that kills all micro-organisms, including high numbers of bacterial spores, representatives of the most resistant microbial forms. Sterilization can be accomplished by heat, ethylene oxide gas, hydrogen peroxide gas plasma, ozone or radiation (in industry). From an operational standpoint, a sterilization procedure cannot be categorically defined. Rather, the procedure is defined as a process, after which the probability of a micro-organism surviving on an item subjected to the sterilization procedures is less than one in one million ($10^{-6}$). This is referred to as the 'sterility assurance level,' and it is this approach that is used by the medical device industry to sterilize large quantities of medical devices. Some criteria used in the production and labelling of a sterile device are listed in Table 53.1.

The *application* of sterilization principles in industry is much more sophisticated and controlled than sterilization procedures used in hospitals. However, steam autoclaves, ethylene oxide gas sterilizers, hydrogen peroxide gas plasma sterilizers and dry heat sterilization ovens used in health-care facilities have operational protocols that are validated by the manufacturer to accomplish sterilization, and all the variables that control for the inactivation of micro-organisms are either automated or built into simple controls in the devices. In addition, the sterilization cycles can be monitored with mechanical, chemical and/or biological indicators.

The *application* of the sterilization process takes into account additional considerations. This approach involves the use strategy associated with a particular medical device (or medical fluid) and the context of its degree of contact with patients. Spaulding in 1972[1] proposed that instruments and medical devices be divided into three general categories based on the theoretical risk of infection if the surfaces are contaminated at time

**Table 53.1** Criteria used in producing sterile devices

| |
|---|
| Good manufacturing practices |
| Use of biological indicators |
| Validated sterilization process |
| Sterility testing of a subsample of the batch subjected to the sterilization process |
| Process controls |
| Quality control of materials |
| Post-sterilization testing of devices for function |

of use. Briefly, medical instruments or devices that are exposed to normally sterile areas of the body require sterilization; instruments or devices that touch mucous membranes may be either sterilized or disinfected; and instruments, medical equipment or environmental surfaces that touch only intact skin or come into contact with the patient only indirectly can be either cleaned and then disinfected with an intermediate-level disinfectant, sanitized with a low-level disinfectant or simply cleaned with soap and water. These instruments or other medical surfaces are termed (with respect to their need to be sterile at time of use) 'critical', 'semi-critical' or 'non-critical', respectively. Selection of the appropriate disinfecting procedure in the last category (non-critical) will include consideration of the nature of the surface, as well as the type and degree of contamination, as shown in Table 53.2.

In the context of these categorizations, Spaulding (1972) also classified chemical germicides by activity level. The activity levels are listed in Table 53.3 and are as follows.

**1** *High-level disinfection*. This is a procedure that kills all viruses, fungi and vegetative micro-organisms but not necessarily high numbers of bacterial spores. These chemical germicides, by Spaulding's definition, are those that are capable of accomplishing sterilization (e.g. killing of all microbial forms including high numbers of bacterial spores) when the contact time is relatively long (6–10 hours). When used as high-level disinfectants, the contact times are comparatively short (5–45 minutes). These chemical germicides are very potent sporicides and, in the United States, are those registered with the FDA as sterilant/disinfectants or simply, high-level disinfectants.

**2** *Intermediate-level disinfection*. This is a procedure that kills vegetative micro-organisms including comparatively resistant vegetative microbial forms such as *Mycobacterium tuberculosis*. All fungi and most viruses are also killed. These chemical germicides often correspond

**Table 53.2** Relationship of germicide type, type of device or surface and process

| Type of germicide | Type of device or surface | Process |
|---|---|---|
| Sterilant/disinfectant | Critical (heat-sensitive rigid endoscopes) | Sterilization (sporicidal chemical, prolonged contact time) |
| | Semi-critical (medical instruments, re-usable heat-sensitive anaesthesia circuits, endotracheal tubes, laryngoscope) | High-level disinfection (sporicidal, chemical, short contact time) |
| Hospital disinfectant (with label claim for tuberculocidal activity) | Non-critical (medical equipment, blood-contaminated control knobs of medical equipment) | Intermediate-level disinfection |
| Hospital disinfectant/sanitizer | Non-critical (environmental surfaces: exteriors of machines, floors, walls, other housekeeping surfaces) | Low-level disinfection to soap and water washing |

**Table 53.3** Levels of disinfectant action according to type of micro-organism

| | Bacteria | | | Virus | | |
|---|---|---|---|---|---|---|
| Level of action | Spores | *Mycobacterium* spp.* | Vegetative cells | Fungi† | Non-lipid, small | Lipid, medium-sized |
| High | +‡ | + | + | + | + | + |
| Intermediate | –§ | + | + | + | ±¶ | + |
| Low | – | – | + | ± | ± | + |

Plus sign indicates that a killing effect can be expected; a minus sign indicates little or no killing effect. Adapted from Favero and Bond.[1]

*Laboratory potency tests usually employ *M. tuberculosis* var. *bovis*.

† Includes asexual spores but not necessarily chlamydospores or sexual spores.

‡High-level disinfectants are chemical sterilants (sporicides); inactivation of high numbers of bacterial spores can be expected only when extended exposure times are used, e.g. 6–10 hours for sterilization vs 5–45 minutes for high-level disinfection.

§Some intermediate-level disinfectants (e.g. hypochlorites) may show some sporicidal activity, while others (e.g. alcohols, phenolics) have none.

¶Some intermediate-level disinfectants (e.g. certain phenolics, isopropyl alcohol) may have limited virucidal activity, even though they readily inactivate *Mycobacterium* spp.

to EPA-approved 'hospital disinfectants' that are also 'tuberculocidal'.

**3** *Low-level disinfection*. This is a procedure that kills most vegetative bacteria, some fungi, and some viruses. Comparatively resistant vegetative forms such as *M. tuberculosis* are *not* killed. These chemical germicides are often ones that are approved in the USA by EPA as hospital disinfectants or sanitizers. General use strategies of sterilization and disinfection using a variety of physical or chemical agents (with specific reference to hepatitis viruses) are shown in Table 53.4.[1]

Spaulding's system for classifying devices and strategies for disinfection and sterilization is quite conservative. There is a direct relationship between the degree of conservatism as expressed by the probability of a micro-organism surviving a particular procedure and the microbicidal potency of the physical or chemical germicidal agent. For example, a sterilization procedure accomplished by steam autoclaving, ethylene oxide gas, or hydrogen peroxide gas plasma sterilization, by design and definition, will result in a one-in-one million probability of a surviving micro-organism if the procedure

**Table 53.4** Some physical and chemical methods for inactivating hepatitis viruses*

| Class | Class concentration or level | Activity |
|---|---|---|
| *Sterilization* | | |
| Heat | | |
| Moist heat (steam under pressure) | 250 °F (121 °C), 15 min | |
| | Prevacuum cycle 270 °F (132 °C), 5 min | |
| Dry heat | 170 °C, 1 h | |
| | 160 °C, 2 h | |
| | 121 °C, 16 h or longer | |
| Ethylene oxide | 450–500 mg/L, 55–60 °C | |
| Hydrogen peroxide gas plasma | Manufacturer's instructions | |
| *Disinfection* | | |
| Heat | | |
| Moist heat | 75–100 °C | High |
| Liquid† | | |
| Glutaraldehyde, aqueous‡ | Variable | High |
| *Ortho*-phthalaldehyde | 0.55% | High |
| Hydrogen peroxide, stabilized | 6–10% | High |
| Formaldehyde, aqueous§ | 3–8% | High to intermediate |
| Iodophors¶ | 40–50 mg/L free iodine at use-dilution | Intermediate |
| Chlorine compounds** | 500–5000 mg/L free available chlorine | Intermediate |
| Phenolic compounds†† | 0.5–3% | Intermediate |
| Quaternary ammonium compounds‡‡ | 0.1–2% | Low |

*Adequate precleaning of surfaces is vital for any disinfecting or sterilizing procedure. Short exposure times may not be adequate to disinfect many objects, especially those that are difficult to clean because of narrow channels or other areas that can harbour organic material. Although alcohols (e.g. isopropanol, ethanol) have been shown to be effective in killing HBV, we do not recommend that they be used generally for this purpose due to rapid evaporation and consequent difficulty in maintaining proper contact times. Immersion of small items in alcohols could be considered.

†This list of liquid chemical germicides contains generic formulations. Other commercially available formulations based on the listed active ingredients can also be considered for use. Information in the scientific literature or presented at symposia or scientific meetings can also be considered in determining the suitability of certain formulations. The following US FDA site lists cleared formulations: http://www.fda.gov/cdrh/ode/germlab.html.

‡Manufacturer's instructions regarding use should be closely followed.

§Because of the controversy regarding the role of formaldehyde as a potential occupational carcinogen, the use of formaldehyde is recommended only in limited circumstances under carefully controlled conditions of ventilation or vapour containment, e.g. disinfection of certain haemodialysis equipment.

¶Only those iodophors designed as hard surface disinfectants should be used, and manufacturer's instructions regarding proper use-dilution and product stability should be closely followed. Check product label claims for demonstrated activity against *Mycobacterium* spp. (tuberculocidal activity) as well as a spectrum of lipid and non-lipid viruses.

**See text.

††Check product label claims for demonstrated activity against *Mycobacterium* spp. (tuberculocidal activity) as well as a spectrum of lipid and non-lipid viruses.

‡‡Quaternary ammonium compounds are not tuberculocidal and may not have significant effect against a variety of non-lipid viruses. This class of germicide is used primarily for routine housekeeping throughout health-care facilities.

had initially been challenged with $10^6$ highly resistant bacterial spores. The risk of infection resulting from the use of an item that was subjected to this type of procedure, assuming that the procedure had been carried out properly, would appear to be zero. Correspondingly, the probability of contamination and the theoretical probability of infection associated with sterilization or high-, intermediate- or low-level disinfection with liquid chemical agents would increase as the overall germicidal potency of the selected germicidal agent or procedure decreased.

A process of liquid chemical sterilization would, at best, be three orders of magnitude less reliable than a conventional sterilization procedure. From a practical standpoint, this means that there is a lower level of confidence with such procedures, and if and when mistakes are made there is a higher chance of failure than with a sterilization procedure. When operational errors are made, the consequences are magnified when a procedure of lower overall potency is used. When less reliable sterilization procedures such as this are used, they should invariably be accompanied by very precise protocols, policies and quality assurance monitoring.

### Decontamination

Another term quite often used in health-care facilities is *decontamination.* A process of decontamination is one that renders a device or items safe to handle, i.e. safe in the context of being reasonably free from disease transmission risk. In many instances, this process is a sterilization procedure such as steam autoclaving, and this is often the most cost-effective way of decontaminating a device or item. Conversely, the decontamination process may be ordinary soap and water cleaning of an instrument, a device or an area. When chemical germicides are used for decontamination, they can range in activity from concentrated oxidative agents such as sodium hypochlorite, hydrogen peroxide or chlorine dioxide, which may be used to decontaminate spills of cultured or concentrated infectious agents in research or clinical laboratories, to low-level disinfectants or sanitizers when general housekeeping of environmental surfaces is the objective.

### Antiseptic

The term *antiseptic* is used to describe a substance that has antimicrobial activity and is formulated for use on or in living tissue to remove, inhibit growth of or inactivate micro-organisms. Quite often, the distinction between an antiseptic and a disinfectant is not made. However, the differences between a disinfectant and an antiseptic are very great and applications are significantly different. A disinfectant is a chemical germicide formulated for use solely on inanimate surfaces such as medical instruments or environmental surfaces. An antiseptic is formulated for use solely on or in living tissues. Some chemical agents such as iodophors can be used as active ingredients in chemical germicides that are formulated either as disinfectants or antiseptics. However, the precise formulations are significantly different, use patterns are different and the germicidal efficacy of each formulation differs substantially. Consequently, disinfectants should never be used as antiseptics and antiseptics should never be used to disinfect instruments or environmental surfaces.

## Factors that influence germicidal activity

Micro-organisms vary widely in their resistance to sterilants and disinfectants. The most resistant microbial forms are bacterial spores (e.g. typically from the aerobic spore-forming genera, *Bacillus* and *Geobacillus*) and few, if any, other micro-organisms approach the broad resistance of these organisms to either heat, chemicals or radiation. A number of factors, some of which are associated with micro-organisms themselves and others with the surrounding physical and chemical environment, can significantly influence the antimicrobial efficacy of chemical germicides.

Some factors are more important than others, but all should be considered when planning sterilization and disinfection strategies for medical and surgical devices and materials. Briefly, these factors are as follows.

### Type of micro-organism

Bacterial spores are more resistant than mycobacteria, fungi, vegetative bacteria and viruses. Some types of viruses are more resistant to germicides than others. As a general guide, one should define the state or degree of inactivation needed (i.e. sterilization or various levels of disinfection) and then choose the most appropriate germicidal agent and method of application.

### Number of micro-organisms

All other factors being equal, the greater the number of micro-organisms on a device, the longer it takes to kill this microbial population. It is for this reason that devices, especially those that are disinfected, should be thoroughly cleaned prior to being sterilized or disinfected.

### Intrinsic resistance of micro-organisms

Bacterial spores have already been mentioned, but very few species in the genera *Bacillus*, *Geobacillus* or *Clostridium* are actually responsible for hospital-acquired infections. However, organisms such as *M. tuberculosis* var. *bovis* and non-tuberculous mycobacteria, as well as naturally occurring gram-negative water bacteria such as *Pseudomonas aeruginosa* and other pseudomonads can, under some circumstances, be relatively resistant to chemical disinfectants. After bacterial spores, *Mycobacterium* spp. are considered one of the more resistant classes of micro-organisms. It is for this reason that chemical germicides approved as 'tuberculocides' are sometimes recommended for purposes of decontamination or disinfection when a higher activity germicide is sought. It is usually not a concern for transmission of *M. tuberculosis* (*M. tuberculosis* is transmitted via contaminated aerosols but not by surfaces), but rather a definition or specification that can be used to describe a germicide with a relatively broad range of germicidal activity. Resistance of certain non-lipid viruses is similar to that of mycobacteria.[8]

### Amount of organic soil present on the item to be disinfected or sterilized

Blood, faeces or other organic soil may contribute to failure of a disinfecting or sterilizing procedure in three ways. Organic soil may contain large and diverse microbial populations, may prevent penetration of germicidal agents or may directly inactivate certain germicidal chemicals. This factor, perhaps even more than others, underscores the necessity of precleaning items thoroughly prior to disinfection or sterilization.

### Type and concentration of germicide

Generally, with all other factors being constant, the higher the concentration of a germicide, the greater is its effectiveness and the exposure time necessary for disinfection or sterilization can be shorter. If a chemical agent is reused over a period of time, the product effectiveness may be reduced due to a variety of factors such as dilution or organic contamination.

### Time and temperature of exposure

With few exceptions, the longer the exposure times to a given chemical agent, the greater is its effectiveness. An increase in temperature will significantly increase germicidal effectiveness, but deterioration or evaporation of the agent along with an increase in corrosiveness may also occur.

### Other product- or process-related factors

The presence of organic or inorganic loads, pH and the degree of hydration of biological material may significantly affect the potency of certain chemical germicides. For these as well as other factors given above, care should be taken to examine closely and follow label instructions of proprietary germicides.

### Device-related factors

The device or item being disinfected or sterilized must be physically and chemically compatible with the chosen procedure to ensure effectiveness and continued function of the device or item. Also, factors such as ease of access and cleaning as well as the size of the device or item are important considerations. The manufacturer of the item being reprocessed is the best source of pertinent information in this regard.

## Inactivation of hepatitis viruses

Germicidal activity of physical and chemical agents against the human hepatitis viruses has been difficult to establish because most (HBV, HCV, HDV and HEV) have not yet been grown in tissue culture. With the exception of HAV, comparative virucidal testing, for the most part, has not been performed as it has for other types of viruses that can be conveniently cultured and tested in the laboratory.

### HAV

HAV appears to have the same degree of resistance to chemical germicides and reagents as other picornaviruses.[8,9] Table 53.5 presents activity against HAV by various physical and chemical agents.[9,10] These data underscore the importance, in practice, of thorough cleaning of surfaces to remove gross organic soil and, at the same time, reduce the level of viral contamination.

### HBV and HCV

As pointed out in other parts of this book, hepatitis B and C are diseases of major public health significance. Both viruses can be transmitted in health-care settings from patient to patient and from patient to staff member, and HBV has been shown to have an environmentally mediated mode of transmission. This is due, at least in part, to the comparatively high numbers of HBV in the blood of certain infected pa-

**Table 53.5** Effects of chemical agents and heat on HAV viability

| Inoculum/exposure conditions | Agent (exposure time) | Result (log reduction) |
|---|---|---|
| Tissue culture-derived HAV with 10% faeces added, dry inoculum, 1 min exposure | – 2% glutaraldehyde | >4 |
| | – 5000 mg/L $cl_2$ | >4 |
| | – 0.4% quaternary ammonium compound plus 23% HCl | >4 |
| | – 3000 mg/L available chlorine | <1 |
| | – iodophor, 75 mg/L $I_2$ | <1 |
| | – phenolics, with and without alcohol | <1 |
| | – quaternary ammonium compounds, with and without alcohol | <1 |
| | – 70% ethanol | <1 |
| | – 3.5% peracetic acid | <1 |
| | – 6% hydrogen peroxide | <1 |
| Tissue culture-derived HAV, room temperature, liquid inoculum | – 10 mg/L available chlorine (15 min) | 3 |
| | – 3 mg/L $I_2$ (15 min) | 3 |
| | – 300 mg/L peracetic acid (15 min) | <3 |
| | – alcohol (3 min) | 2.25 |
| | – alcohol (12 h) | 4.75 |
| HAV + chimpanzee faeces, 18% suspension, $10^6$ MID/mL, marmoset IV recovery* | – 500 mg/L available chlorine (10 min) | <4 |
| | – 5000 mg/L available chlorine (10 min) | 4† |
| | – 75 °C wet (10 min) | <5 |
| | – 75 °C wet (30 min) | ≥5 |
| | – 25 °C dry, 42% RH (1 month) | <5‡ |
| Tissue culture-derived HAV | – room temperature (1 week) | 2 |
| | – 60 °C wet (6–12 h) | >5.25 |
| | – 85 °C wet (1 min) | >5.25 |

MID, marmoset infective doses. Modified, in part, from Thraenhart 1991 (Tables 26–13, 26–14, 26–16).[9]
*McCaustland KA, Bond WW, Spelbring JA, unpublished data.
†104 MID per test, both animals inoculated with treated material were not infected.
‡From McCaustland *et al.*[10]

tients (sometimes as high as $10^8$–$10^9$/mL) and also, the ability to survive for a period of time after drying.[11] However, as these viruses cannot be grown in tissue culture, data used to verify disinfection and sterilization procedures have been deduced from experiments using human volunteers, blood products that received some degree of treatment and where disease or infection was followed in human recipients, or experiments in which chimpanzees were used to determine HBV inactivation using infectivity as a criterion. In addition, there have been other experimental approaches to demonstrate that certain physical and chemical agents can alter the immunological reactivity of hepatitis B surface antigen (HBsAg) as well as the morphological alteration of various components of the intact virus.

We have used HBsAg as a marker for HBV in order to determine the potential for environmentally mediated modes of transmission as well as inactivation capabilities of various chemical and physical agents.[12,13] Detection of HBsAg on environmental surfaces does not indicate positively the simultaneous presence of viable HBV, but it does serve as an indicator of contamination with potentially infective material. We and others have shown that HBsAg can be quantified and traced in environmental surfaces as an adjunct to longitudinal or epidemic investigations.[13–18]

Because there is no evidence to suggest that the resistance level of HBV is equivalent to or even approaches the demonstrated stability of the immunological reactivity of HBsAg, we proposed in 1977 that the immunological reactivity of HBsAg is much more resistant to physical and chemical stresses than is the infectious virion. Consequently, those chemical and physical stresses that were shown to destroy the immunological reactivity of HBsAg can be assumed to be effective against HBV. We further proposed that the resistance level of HBV be considered equivalent to that of *M. tuberculosis*, i.e. less resistant than bacterial spores but more resistant than most micro-organisms. Subsequently, even this assumption was shown to be overly conservative when a number of intermediate- to high-level disinfectants were shown to be effective against HBV.[4]

HBV has been shown to be inactivated by several moderately potent disinfectants, including 0.2% and 0.1% glutaraldehyde, 500 p.p.m. free chlorine from sodium hypochlorite, an iodophor disinfectant and isopropyl or ethyl alcohol.[11,19] Table 53.6 gives a summary of some of the inactivation potentials of various physical agents and germicides in tests using titred inocula and chimpanzee infectivity assays.[11,19,20]

As infectivity experiments using chimpanzees are not suitable for the quantitative determination of HBV inactivation, more reliance has been placed on other avenues of experimentation. Thraenhart *et al*.[21,22] developed a test referred to as the 'morphologic alteration and disintegration test' (MADT). This has been standardized and used in Germany for determining the effect of chemical germicides and physical agents on HBV. The hypothesis of MADT is that the physical destruction of intact HBV virus particles is correlated with the inactivation of infectivity using chimpanzee infectivity tests.[22] The MADT uses the human HBV but the test procedure is too complicated and cumbersome for routine use. It requires expensive equipment and personnel with considerable experience and skill in electron microscopy. The HBV suspensions required need to be highly concentrated and pure enough to allow the viral particles to be readily visualized and counted under the electron microscope. And some chemical germicides such as glutaraldehyde and alcohol act by fixing proteins and preserving the structural integrity of the material being treated. Virus particles exposed to them may appear morphologically unaltered, although being non-infectious. This could lead to false-negative results.

Testing based on HBV polymerase inactivation has also been used to test germicides.[23] This testing method has not been widely accepted because it is based on an indirect measure of virus infectivity and requires highly purified virus preparations. The sensitivity of the test is also questionable.

There have been recent studies that propose the use of animal hepatitis viruses as surrogates in germicide testing protocols. The Peking duck virus resembles HBV closely in viraemia, carcinoma production,[24] and inactivation profiles.[25,26] The duck hepatitis B virus (DHBV) infects primary duck liver cells *in vitro*, thus making the test more cost-effective compared with the use of chimpanzees or even ducklings as experimental animals.[25] The US EPA issued guidelines in August 2000[27] on protocols for testing the efficacy of disinfectants that use DHBV as a surrogate for HBV. Pugh and co-investigators[28] inoculated carriers with DHBV, dried them and then exposed them to specific germicides. After a specified contact time, the disinfectant–virus mixture is eluted off the test carriers, neutralized and then serially diluted for culture in duck hepatocytes. Detection of DHBV replication is performed either by immunofluorescence or by nucleic acid detection methods.

Chan-Myers and Roberts[20] tested *ortho*-phthalaldehyde (OPA), for efficacy against DHBV and bovine viral diarrhoea virus (BVDV), a surrogate for HCV. The virus cultures containing 5% horse serum as organic soil load were dried onto the bottom of petri dishes and a

**Table 53.6** Complete inactivation of HBV inoculum by chemicals and heat*

| Inoculum | Treatment | Reference no. |
|---|---|---|
| Human plasma (dry) | 10 min, 20 °C (all tests) | 11 |
| $10^6$ CID | 500 mg/L available chlorine sodium hypochlorite | |
| $10^6$ CID | 70% isopropyl alcohol | |
| $10^6$ CID | 0.125% glutaraldehyde 0.44% phenol | |
| $10^6$ CID | 75 mg/L available iodine; iodophor | |
| $10^6$ CID | 2% glutaraldehyde, pH 8.6 | |
| Human plasma (liquid) | | |
| $10^5$ CID | 5 min, 24 °C: 1% glutaraldehyde | 19 |
| $2.0 \times 10^5$ CID | 5 min, 24 °C: 0.1% glutaraldehyde | 19 |
| $3.3 \times 10^5$ CID | 2 min, 24 °C: 80% ethyl alcohol | 19 |
| $10^5$ CID | 2 min, 98 °C | 19 |
| Surrogate viruses | | 25 |
| Duck hepatitis B virus | 5 min, 20 °C: 0.31% 5 log reduction | |

CID, chimpanzee infective doses.

*As measured by chimpanzee infectivity tests; titred inocula.

quantity of known concentration of OPA solution was added. After an exposure of 5 minutes at 20 ºC, the virus was recovered and titrated on monolayers of either duck hepatocyctes or bovine turbinate cells for infectivity. The results showed that dilute OPA (0.31%; the use concentration of OPA is 0.55%) completely inactivated both DHBV and BVDC in 5 minutes at 20 ºC.

Testing against HCV is also difficult because the virus cannot be visualized or effectively grown in tissue culture. HCV replicate in Vero cells without producing any cytopathic effects. However, BVDC has some properties similar to HCV and has been used in the blood product industry as a surrogate for HCV.29–31 Recent studies show that the enveloped nature of HCV makes it relatively susceptible to inactivation by phenolics and chlorine.[32]

### Other hepatitis viruses

The effects of physical and chemical agents on other human hepatitis viruses, HDV and HEV, have not been studied extensively but, as mentioned previously, we are aware of no evidence to suggest that any of these viruses are intrinsically more resistant to physical or chemical agents than most viruses or that the general resistance levels can even approach that of bacterial spores. Consequently, we continue to propose that the resistance levels of the human hepatitis viruses that have not been studied in great detail be considered near that of *M. tuberculosis* var. *bovis* and non-lipid viruses (e.g. poliovirus), but much less than that of bacterial spores.

## Sterilization, disinfection and housekeeping in the laboratory

Conventional sterilization procedures such as steam autoclaving, dry heat, ethylene oxide gas and hydrogen peroxide gas plasma can be relied upon to effectively inactivate all hepatitis viruses. This is also true for liquid chemical germicides used as sterilants (sporicides). Such procedures are used primarily for medical instruments that are reprocessed for use on patients in health-care facilities. It is emphasized that this class of potent chemical germicide is designed and intended for exclusive use in 'total immersion' reprocessing of certain heat-sensitive medical instruments and is not appropriate for use on environmental surfaces.

In the context of laboratory settings where liquid chemical germicides may be used to disinfect laboratory worktops or laboratory instruments directly exposed to cultured or concentrated hepatitis viruses or human or animal source specimens containing these agents, it is recommended that chemical disinfectants in their appropriate concentrations and contact times capable of producing an intermediate level of disinfection activity should be used (e.g. oxidative or phenolic chemicals: see Table 53.4).

For general housekeeping purposes such as cleaning floors, walls and other similar environmental surfaces in the laboratory area, any disinfectant-detergent product can be used according to the manufacturer's instructions. In some high-risk areas such as laboratories, haemodialysis units and other spill- or splash-prone health care environments, one is confronted with the problem of decontaminating large and small blood spills, patient care equipment that becomes contaminated with blood and frequently touched instrument surfaces such as control knobs, which may play a role in environmentally mediated transmission of hepatitis B. The strategies for applying the principles of HBV inactivation vary according to the item or surface being considered, its potential role in the risk of hepatitis virus transmission and, to a certain extent, the thermal and chemical sensitivities of the surface or instrument. For example, if a significant spill of blood occurred on the floor or a countertop in a laboratory, the objective of the procedure to inactivate HBV or other blood-borne hepatitis viruses would be one of decontamination or disinfection and not sterilization. Consequently, in such a situation we would recommend that gloves be worn and the blood spill be absorbed with disposable towels. The spill site should be cleaned of all visible blood, and then the area should be wiped down with clean towels soaked in an appropriate intermediate-level disinfectant such as a freshly made 1/100 dilution of commercially available household bleach (approximate 5–6% sodium hypochlorite depending on brand, intended minimum of 0.05% at final dilution). All soiled towels should be put in a plastic bag or other leak-proof container for disposal.

The concentration of disinfectant used depends primarily on the type of surface that is involved. For example, in the case of a direct spill on a porous surface that cannot be physically cleaned before disinfection, 0.5% sodium hypochlorite (5000 mg/L available chlorine) should be used. On the other hand, if the surface is hard and smooth and has been cleaned appropriately, then 0.05% sodium hypochlorite (500 mg/L available chlorine) is sufficient. For commercially available chemical disinfectants, the use concentrations and instructions specified by the manufacturer should be closely followed.

Other types of environmental surfaces of concern include surfaces that are touched frequently, such as control knobs or panels on laboratory instruments. Ideally, gloves should be worn and manipulated in a manner

appropriate not only to avoid skin contact with patient materials but to avoid 'finger painting' of this contamination to a variety of other frequently touched surfaces. As this ideal is seldom fully realized in a busy laboratory setting, laboratory instrument and equipment surfaces (including fixtures such as light switches and door pulls or push plates) should be routinely cleaned and disinfected. The objective here would be to reduce the level of possible contamination to such an extent that the likelihood of disease transmission is remote. In a practical sense, this could mean that a cloth soaked in either 0.05% sodium hypochlorite or a suitable proprietary disinfectant or disinfectant/detergent could be used. In this context, the element of physical cleaning is as important, if not more important, than the choice of the disinfectant. It is not necessary, cost-effective or in many cases even feasible to attempt more powerful germicidal procedures with these types of items or surfaces.

As a rule, routine daily cleaning procedures used for general microbiological laboratories can be used for laboratories in which blood specimens are processed. Obviously, special attention should be given to areas or items visibly contaminated with blood or faeces. Furthermore, cleaning personnel must be alerted to the potential hazards associated with blood, serum and faecal contamination. Floors and other housekeeping surfaces contaminated in this manner should be thoroughly cleaned of gross material and then treated with a detergent-disinfectant. Gloves should be worn by cleaning personnel doing these duties. However, in the case of large blood spills as mentioned above, this type of procedure may have to be augmented by specific site decontamination using a more potent chemical agent such as an intermediate-level disinfectant (see Table 53.4).

## Conclusions

Strategies for disinfection and sterilization used in hospitals and other health-care institutions are based on relatively conservative criteria and do not need to be changed because of concern regarding the presence of hepatitis viruses. These viruses are inactivated by a wide variety of common physical and chemical sterilization and disinfection procedures. The resistance of individual species of hepatitis viruses to heat and chemical germicides varies, but none exceeds the resistance levels of bacterial spores or *M. tuberculosis*. Consequently, conventional sterilization, disinfection or decontamination procedures can be used for processing medical devices used on patients known to have viral hepatitis infection. Extraordinary procedures or formulations are not needed, nor is there an indication for the preferential use of products with specific label claims of efficacy against specific hepatitis viruses.

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# Chapter 54
# Mechanisms of interferon resistance

Darius Moradpour, Markus H Heim, Hubert E Blum

## Introduction

Infections with the hepatitis B virus (HBV) and hepatitis C virus (HCV) are leading causes of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. Interferon-α (IFN-α) or pegylated IFN-α (PEG-IFN-α), combined with ribavirin in the case of chronic hepatitis C, are currently used to treat both forms of chronic viral hepatitis. Sustained virological response rates, however, are limited to 30-40% in chronic hepatitis B and 50-60% in chronic hepatitis C. Thus, both viruses may have evolved mechanisms to counteract the antiviral effects of IFN-α, rendering therapy ineffective in many patients. More importantly, the IFN-induced cellular antiviral response is the first line of innate defence against viral infections. Therefore, viruses must first overcome the IFN-induced mechanisms blocking viral replication in order to establish a productive infection.[1–3] Hence, mechanisms of IFN resistance may not only contribute to the resistance to IFN-α therapy observed in many patients, but may represent general escape strategies of HBV and HCV contributing to viral persistence and pathogenesis.

The mechanisms by which IFN-α inhibits replication of HBV and HCV are incompletely understood and may involve both direct antiviral and immunomodulatory effects. Investigation of these mechanisms has been hampered by the lack of efficient cell culture systems and small animal models permissive for HBV and HCV infection and replication. Only recently, model systems have been developed that make it possible to study selected aspects of the interplay between HBV and HCV and the IFN system. These include, among others, transgenic mice that replicate HBV in their livers[4] and the replicon system for HCV.[5,6] These systems have allowed investigation of the effect of IFN-α and other cytokines on HBV and HCV replication. In addition, data obtained in these and other experimental model systems indicate that both viruses have evolved strategies to counteract the antiviral effects of IFN-α.

## The interferon system

IFNs are classified into type I IFNs, which comprise IFN-α and -β, and type II IFN, or IFN-γ. Type I IFNs are produced by leukocytes, fibroblasts, epithelial cells and other cell types and type II IFN is produced by T lymphocytes and natural killer cells. In the blood, the principal IFN-α producing cells are plasmacytoid[7,8] and non-plasmacytoid dendritic cells.[9] During HBV infection IFN-α is produced in the liver by infiltrating mononuclear cells, sinusoidal cells, Kupffer cells and, to a lesser extent, by hepatocytes.[10–12]

Over the past several years, the complete signal transduction pathway from the IFN receptors to the nucleus has been identified (reviewed in Heim[13]), and viral interference with IFN-induced signalling can now be studied in detail (Fig. 54.1). Type I IFNs bind to heterodimeric type I IFN receptors consisting of IFN-α receptor I and II (IFNARI and IFNARII). Ligand binding results in activation of two cytoplasmic protein tyrosine kinases associated with IFNARI and IFNARII, Tyk2 and Jak1. The activated kinases then phosphorylate tyrosine residues of the receptors. These phosphotyrosines are consecutively bound by the SH2 domains of signal transducer and activator of transcription 1 (Stat1), Stat2 and Stat3. The STATs are then tyrosine phosphorylated and form hetero- or homodimers through mutual SH2 domain–phosphotyrosine interactions. Stat3 and Stat1 form homodimers, designated serum inducible factor A (SIF-A) and SIF-C, respectively, and a Stat1-Stat3 heterodimer, SIF-B. Stat1 can also dimerize with Stat2, and this Stat1-Stat2 heterodimer associates with a third DNA-binding protein, ISGF3γ-p48, to form ISGF3. Binding of these STAT factors to their cognate sequences in the promoter regions of target genes results in enhanced transcription of a set of genes collectively termed IFN-stimulated genes (ISGs). A number of regulatory mechanisms of the Jak-STAT signal transduction pathway have been identified. The activity of the Jak kinases is controlled by phosphatases such as SHP1, SHP2, CD45, PTP1B and TCPTP

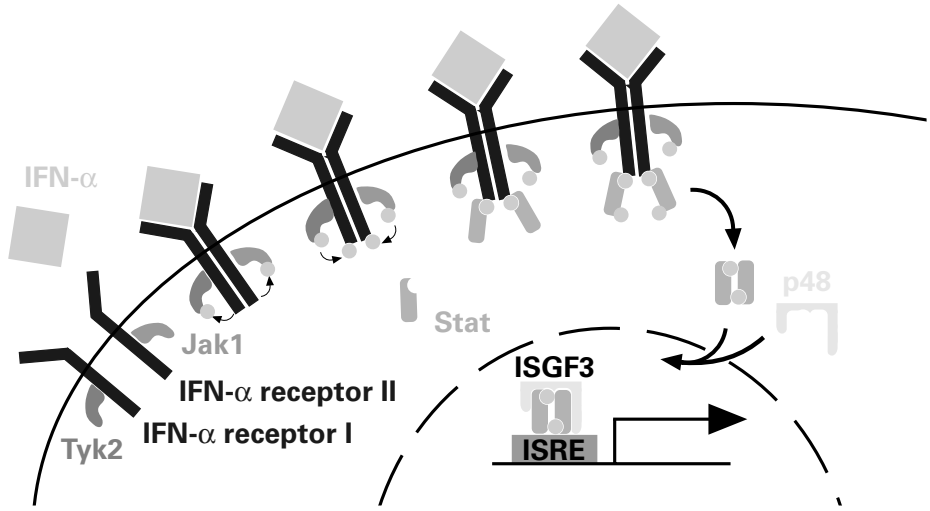


**Figure 54.1** The Jak-STAT pathway. IFN-α binds to heterodimeric type I IFN receptors consisting of IFNARI and IFNARII. Ligand binding results in activation of the cytoplasmic receptor-associated protein tyrosine kinases Tyk2 and Jak1. The activated kinases then phosphorylate tyrosine residues of the receptors. These phosphotyrosines are consecutively bound by the SH2 domains of STATs. The STATs are then tyrosine phosphorylated and form hetero- or homodimers through mutual SH2 domain–phosphotyrosine interactions. Stat1-Stat2 heterodimers associate with a third DNA-binding protein, ISGF3γ-p48, to form ISGF3. Binding of STAT factors to their cognate sequences in the promoter regions of target genes results in enhanced gene transcription.

and by suppressors of cytokine signalling (SOCS).[14] STAT proteins can be inactivated by dephosphorylation in the cytoplasm or the nucleus.[14] Furthermore, binding of Stat1 or Stat3 dimers to the response elements in the promoters of target genes can be inhibited by PIAS1 (protein inhibitor of activated Stat1) or PIAS3, respectively.[14] At any of the steps outlined above, viral proteins could interfere with the Jak-STAT pathway and inhibit induction of antiviral effector proteins.

In general, the direct intracellular antiviral effects of IFNs are mediated by several effector proteins, including double-stranded RNA-activated protein kinase (PKR), MxA GTPase, 2′-5′ oligoadenylate synthetase (2′-5′ OAS) and RNaseL.[15] In the case of HBV and HCV infection, the relevant antiviral effector systems have not yet been identified. As mentioned above, IFNs also have immunomodulatory effects. For example, they upregulate MHC class I and II expression, which leads to enhanced antigen presentation and stimulation of acquired immunity. The relative contribution of direct intracellular antiviral effects and indirect immunostimulatory effects in the elimination of HBV and HCV infections is unknown.

Interestingly, studies performed in HBV transgenic mice suggested that inflammatory cytokines released in the context of a cellular immune response, namely tumour necrosis factor (TNF)-α and IFN-γ, can block HBV replication by a non-cytolytic mechanism.[16] HBV clearance without destruction of infected hepatocytes has been documented in acutely infected chimpanzees as well, further supporting this concept.[17] The molecular mechanisms responsible for cytokine-mediated viral clearance are currently being investigated (reviewed in Guidotti and Chisari.[18]). Studies performed with the duck hepatitis B virus (DHBV), a virus closely related to HBV, have shown that recombinant duck IFN-α inhibits DHBV replication *in vitro* and *in vivo*.[19,20] This model may allow further investigation of the IFN-sensitive steps of the viral life-cycle.[21]

IFN-α inhibits HCV replication in primary human hepatocyte cultures,[22] demonstrating a direct antiviral effect *in vitro*. In addition, studies recently performed using Huh-7 cells harbouring HCV replicons demonstrated that IFN-α can efficiently inhibit viral RNA replication[5,23–27] (Fig. 54.2). These studies showed surprisingly low 50% inhibitory concentrations ($IC_{50}$) of 0.5–3 IU IFN-α/mL. However, it is presently unknown how closely the replicon system reflects the IFN sensitivity of a naturally infected hepatocyte. Interestingly, in the replicon system IFN-γ has a profound inhibitory effect as well.[28] Therefore, the replicon system is a powerful tool to analyze the antiviral activity of cytokines and to systematically dissect their mechanism of action as well as the cellular effector functions involved. In this context, it was shown that MxA is not involved in the inhibition of HCV RNA replication by IFN-α.[24] In addition, PKR does not seem to be a critical effector protein.[26] Studies investigating other IFN-α effector pathways are currently ongoing in several laboratories. The availability of additional cell lines harbouring

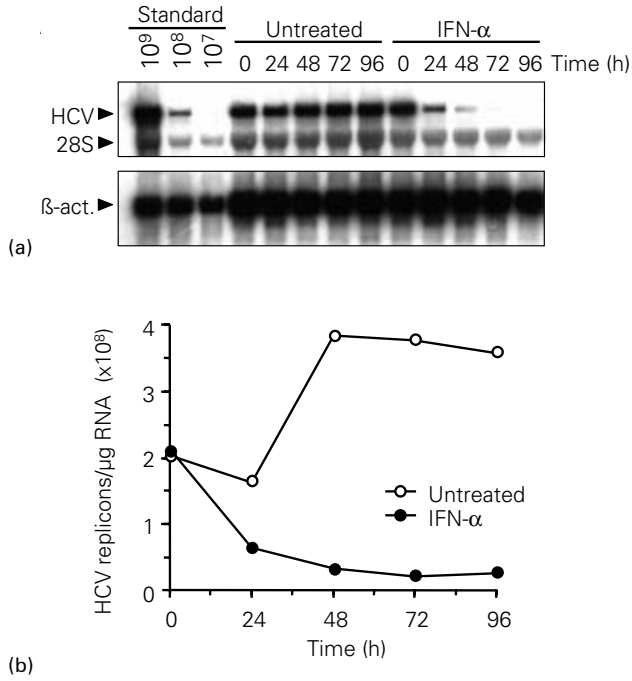


**Figure 54.2** IFN-α inhibits HCV subgenomic RNA replication. (a) Northern blot analysis of a Huh-7 cell line harbouring a subgenomic HCV replicon. Cells were incubated for the indicated times in the absence or presence of 1000 U/mL IFN-α. Total RNA was hybridized with HCV- and β-actin-specific radioactive probes. (b) Quantitative analysis of the data shown in (a). (Adapted from Frese *et al.*,[24] with permission.)

HCV replicons[29] and of replicons derived from different genotypes[30] will allow further investigation of these issues.

## Interaction of HBV with the IFN system

Only a limited number of studies have addressed a possible interference of HBV with the IFN system. In this context, expression of the HBV polymerase terminal protein has been reported to result in impaired activation of ISGF3.[31,32] Studies performed more recently in stably transfected Huh-7 human hepatoma cells suggested a selective inhibition of IFN-induced MxA protein expression by the HBV core protein.[33]

## Interaction of HCV with the IFN system

Perhaps the strongest evidence for the role of specific viral factors in determining the IFN sensitivity of HCV comes from the clinical use of IFN-α. Sustained virological response rates to PEG-IFN-α plus ribavirin combination therapy differ significantly between patients infected with HCV genotype 1 (40–50%) and genotypes 2 or 3 (~80%).[34–36] Indeed, among the viral and patient-related factors involved in determining response rates, the HCV genotype is a major factor (reviewed in Pawlotsky[37] and Zeuzem[38]). However, the biological basis for this difference is thus far unknown.

Among Flaviviridae family members, bovine viral diarrhoea virus (BVDV), a prototypical member of the pestivirus genus, was found to interfere with the IFN system to establish persistent infection.[39,40] Recent evidence suggests that the N-terminal autoprotease $N^{pro}$ of classical swine fever virus (CSFV), another pestivirus, inhibits innate immune responses.[41] With respect to members of the flavivirus genus, dengue virus non-structural protein 4B (NS4B) was recently found to inhibit IFN signalling.[42] Thus, pestiviruses and flaviviruses use different mechanisms to counteract innate immune responses, including the IFN system, and to establish productive infection.

Different mechanisms potentially underlying IFN resistance of HCV have been described. In principle, viral gene products could interfere with IFN-induced intracellular signal transduction, thereby inhibiting induction of a number of antiviral effector proteins. Alternatively, the virus could have developed defence strategies against these cellular effector mechanisms.

### Interference of HCV with IFN signalling

Numerous examples of viral interference with IFN signal transduction have been reported. Vaccinia virus, for example, encodes a soluble type I IFN receptor which neutralizes IFN before it can bind to the cellular receptor.[43] Human cytomegalovirus was reported to inhibit IFN-γ-induced Jak-STAT signalling, probably by enhancing Jak1 protein degradation.[44]

The effect of HCV on IFN-α-induced signalling through the Jak-STAT pathway was investigated using continuous human cell lines inducibly expressing HCV structural and non-structural proteins.[45] In these cells, the expression of HCV proteins can be regulated by the concentration of tetracycline in the culture medium.[46,47] As shown in Figure 54.3,[45] IFN-α-induced STAT activation, as examined by electrophoretic mobility shift assay (EMSA), was readily detectable only in cells cultured in the presence of tetracycline, i.e. in cells where viral protein expression has been repressed by tetracycline. If these cells were cultured in the absence of tetracycline, i.e. when they expressed HCV proteins, IFN-α-induced STAT activation was inhibited. Further experiments demonstrated that inhibition of Jak-STAT signalling occurred downstream of STAT tyrosine phosphorylation and resulted in reduced upregulation of IFN-α target genes,[45] as well as in an inhibition of antiviral IFN effector functions.[48] Interestingly, PKR activity was not influenced by the expression of HCV proteins in these cell lines (see below).

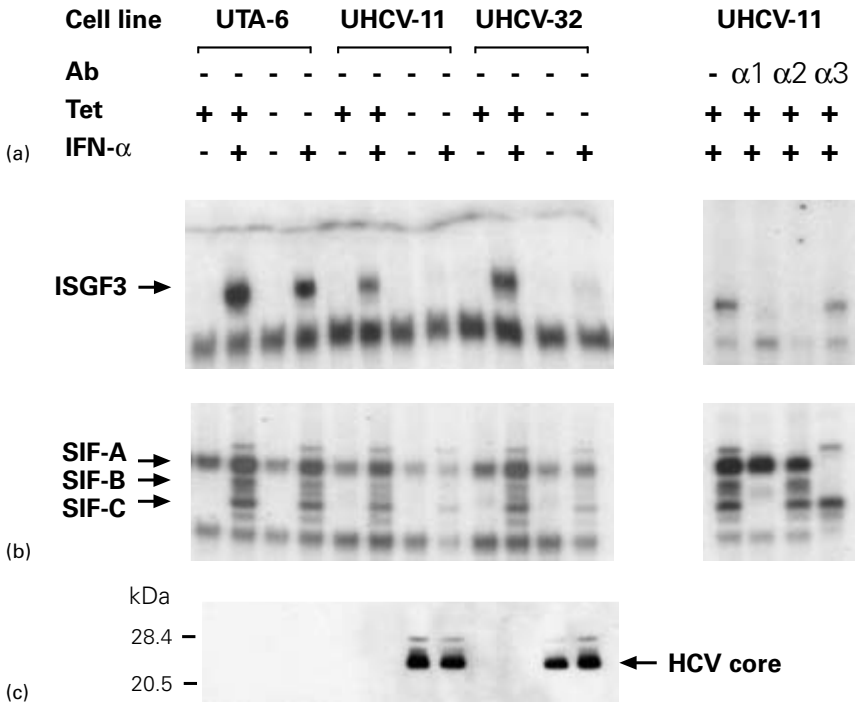


**Figure 54.3** Inhibition of IFN signalling through the Jak-STAT pathway in cells expressing HCV proteins. UTA-6, UHCV-11 and UHCV-32 cells were cultured in the presence or absence of tetracycline and then either left untreated or stimulated for 30 minutes with 500 U/mL IFN-α, as indicated. (a) Electrophoretic mobility shift assay (EMSA) with the ISRE oligonucleotide probe. The position of ISGF3 is indicated by an arrow. Supershift experiments shown in (a) on the right confirmed the identity of the induced shift as ISGF3. (b) The same nuclear extracts were tested with an m67 oligonucleotide probe. The positions of SIF-A, SIF-B and SIF-C are indicated. (c) Western blot with the monoclonal antibody C7-50 against the HCV core protein with the corresponding cytoplasmic extracts. Molecular weight markers in kDa are indicated on the left. (Adapted from Heim *et al*.,[45] with permission.)

It was recently found that transgenic mice expressing the HCV polyprotein under the transcriptional control of an α1-antitrypsin promotor had a strong inhibition of IFN-α-induced Jak-STAT signalling in their liver cells.[49] As previously observed in the inducible cell lines, inhibition occurred downstream of STAT phosphorylation. This resulted in an enhanced susceptibility of the transgenic mice to infection with a hepatotropic strain of lymphocytic choriomeningitis virus, impressively demonstrating the biological consequences of the observed inhibition of IFN signalling.

These findings were based on experiments performed in heterologous expression systems which may not appropriately reflect natural HCV infection. However, an inhibition of IFN-α-induced Jak-STAT signalling was found also in liver biopsies from patients with chronic hepatitis C.[50] As observed in the cell lines and in the transgenic mice, inhibition occurred downstream of STAT phosphorylation. In addition, it was found that protein phosphatase 2A (PP2A) is upregulated in liver extracts from HCV transgenic mice and in liver biopsies from patients with chronic hepatitis C. Upregulation of PP2A resulted in hypomethylation of Stat1, a consecutively increased association of Stat1 with its inhibitor PIAS1, and a reduced transcriptional activation of IFN-α target genes. Taken together, these results indicate that HCV may interfere with IFN-α-induced Jak-STAT signalling by a complex mechanism involving the upregulation of PP2A, a cellular phosphatase, and the increased binding of activated STAT1 by its negative regulator PIAS1.

Recently, Foy *et al*. have provided evidence for interference of HCV with a key component of the signalling pathways that activate the IFN system, namely IFN regulatory factor 3 (IRF-3).[51] IRFs are a family of transcription factors that share homology in their DNA binding domain and bind to similar DNA motifs. There are nine mammalian members of the family, termed IRF-1 to IRF-9.[52] Among them, IRF-3 and IRF-7 have been identified recently as key regulators of the induction of type I IFNs. IRF-3 is expressed constitutively in all cell types, and in uninfected cells resides in the cytoplasm. Upon virus infection, IRF-3 is activated by phosphorylation by a virus-activated kinase (VAK). Interestingly, the IκB kinase (IKK)-related kinases IKKε and TANK-binding kinase 1 (TBK1) have been identified as components of VAK.[53] This modification permits IRF-3 dimerization, nuclear translocation, and activation of the IFN-β and IFN-α1 genes (Fig. 54.4).[54] IFN-β and IFN-α1 are secreted and bind to IFN-α/β receptors in an autocrine or paracrine way. As a consequence, the Jak-STAT pathway is activated, and STAT-containing transcription factors such as ISGF3 transcriptionally activate a number of target genes, collectively called IFN-stimulated genes (ISGs). Among these ISGs is IRF-7. In the presence of a viral infection, *de novo* produced IRF-7 is again phosphorylated by VAK, translocates to the nucleus, forms a dimer with IRF-3, and transcriptionally stimulates additional members of the large family of IFN-α genes (Fig. 54.4). This feedback loop results in an enormous amplification of the signal, and leads to massive production of type I

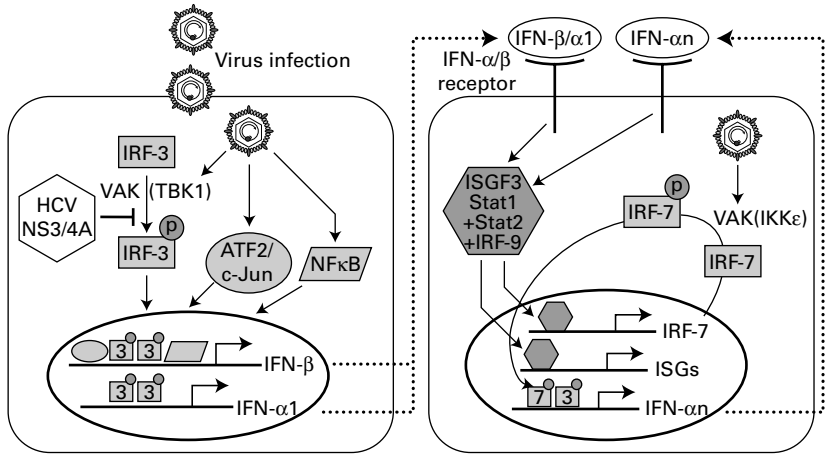


**Figure 54.4** Activation of the IFN system by viral infections. Infection of cells by viruses results in the activation of three transcription factors that are crucial for transcriptional activation of the IFN-β gene: nuclear factor κB (NFκB), ATF2/c-Jun and IRF-3. IRF-3 is activated through serine/threonine phosphorylation by virus-activated kinase (VAK). Secretion of IFN-β (and IFN-α1) and binding to IFN-α/β receptors starts an amplification mechanism through the STAT and IRF-7 dependent activation of additional IFN genes. This positive feedback system is controlled because ongoing production of IFNs requires the phosphorylation of IRF-7, and IRF-7 phosphorylation requires the activation VAK by viruses. (From Heim[54] with permission.)

IFNs. The system is controlled by three crucial features of IFR-7: expression of the IRF-7 gene is totally dependent on IFN-α/β-induced ISGF3 signalling, the IRF-7 protein has a short half-life, and IRF-7 (as well as IRF-3) must be activated by virus-induced phosphorylation.[55] These features guarantee that the activation of the IFN-induced antiviral defence system is terminated when cells are cleared from the viral infection.

Using Huh-7 cells harbouring HCV replicons and a panel of tetracycline-regulated cell lines inducibly expressing HCV proteins, Foy *et al*. mapped the inhibitory effect of HCV to the serine protease activity of the NS3-4A complex.[51] This raises the interesting possibility that novel antiviral agents targeting the HCV serine protease[56] may also restore responsiveness of the IRF-3 pathway. It will be interesting to investigate which component of the IRF-3 pathway is targeted by the HCV serine protease.

## Interference of HCV with PKR

As discussed above, HCV could also interfere with IFN effector functions. PKR has been most extensively studied in this regard. The HCV non-structural protein 5A (NS5A) and the envelope glycoprotein E2 have been reported to interfere with PKR activity. NS5A is a membrane-bound serine phosphoprotein of unknown structure and function.[57–59] A role for NS5A in modulating the IFN response was first suggested by studies performed in Japan by Enomoto *et al*. These investigators found a correlation between mutations within a discrete region of NS5A (HCV amino acid positions 2209–2248), termed IFN sensitivity determining region (ISDR), and a favourable response to IFN-α therapy[60,61] (Fig. 54.5). These studies demonstrated that strains closely matching the prototype HCV genotype 1b (HCV-J) ISDR sequence correlated with IFN resistance. These findings were largely confirmed in Japan, but not in Europe and North America (reviewed in Bréchot[62] and Herion and Hoofnagle[63]). The reasons for this discrepancy are not understood, but may involve both differences in doses and regimens of IFN treatment and the low prevalence of 'mutant type' HCV genotype 1b isolates in Western countries.[64] Even if a recent meta-analysis of published data seems to confirm an association of specific ISDR sequences with the IFN response,[65] this remains a controversial issue that has not translated into clinically applicable predictors. The same is true for other regions of NS5A that have been associated with the response to IFN therapy, such as a variable region in the C-terminal domain of NS5A termed V3[66–68] (Fig. 54.6).[5,57,60,61,69–71]

Interestingly, however, an interaction with and repression of the catalytic activity of PKR by NS5A has been found by biochemical, transfection and yeast functional analyses.[72] Mutations within the ISDR that were observed in clinically IFN-sensitive genotype 1b strains disrupted the ability of NS5A to interact with and repress PKR activity, supporting the notion that NS5A mediates HCV resistance to IFN through downregulation of PKR.[69] In addition, disruption of PKR-dependent translational control and apoptotic programmes by NS5A have been suggested to confer oncogenic potential to HCV.[73] Also, evidence has been provided for an src homology 3 (SH3) domain-dependent interaction of NS5A with growth factor receptor-bound protein 2 (Grb2) adaptor protein, which could interfere with mitogenic signal transduction pathways.[74] As growth factor signalling may be linked to IFN signalling pathways,[75] one could speculate that NS5A binding to Grb2 represents another mechanism by which HCV induces IFN resistance. Of note, this mechanism appears to be non-essential for viral replication *in vitro*, because replicons

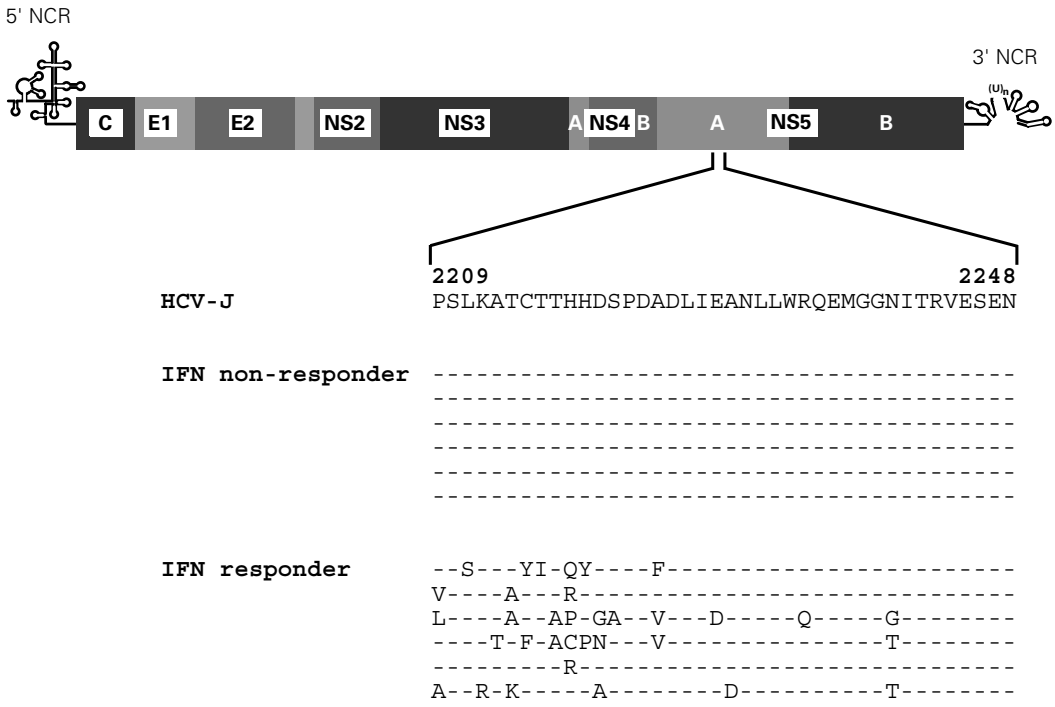


**Figure 54.5** The HCV ISDR. Examples of sequences of HCV amino acid residues 2209–2248 within the NS5A protein in genotype 1b-infected IFN-α non-responders and responders. Amino acid residues are indicated by the standard single-letter code. Dashes indicate residues identical to those in the HCV-J prototype genotype 1b sequence. Patients with 'mutant type' ISDR sequences (≥4 amino acid substitutions compared with the prototype sequence) showed a favourable response to IFN-α therapy, whereas patients with wild-type ISDR sequences did not respond.[60,61]

with a deletion of the ISDR and part of the PKR-binding domain replicate efficiently in cell culture.[5]

Expression of the NS5A protein in cultured cells resulted in a partial resistance to the antiviral effects of IFN-α against IFN-sensitive viruses, such as vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV).[73,76–80] This correlated with a partial attenuation of IFN-α- and -β-induced gene expression in cell lines expressing NS5A.[81] However, this effect was found to be independent of the ISDR sequence in three studies.[77–79] Similarly, NS5A sequences examined in the replicon system thus far do not correlate with the susceptibility to the inhibitory effect of IFN-α.[5,25] In addition, in line with observations made earlier in the context of the entire HCV polyprotein,[48] Podevin *et al.* found no effect of NS5A expression on PKR activity in Huh-7 cells consti-

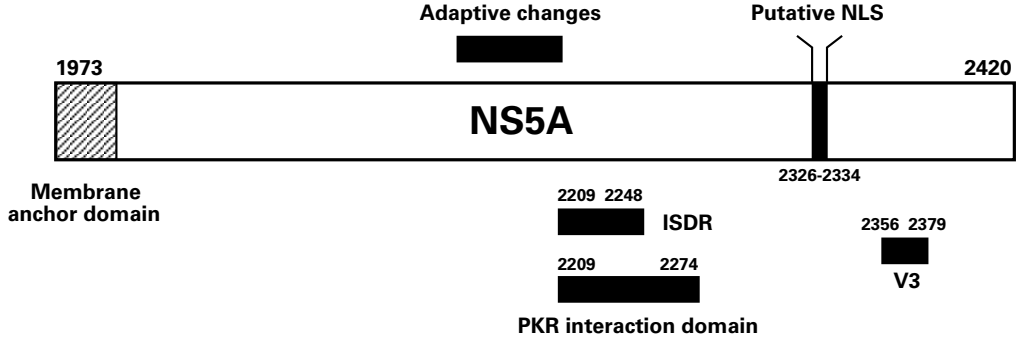


**Figure 54.6** Overview of the HCV NS5A protein. Amino acid positions are given relative to the HCV polyprotein. The N-terminal amphipathic α-helix which mediates membrane association of NS5A,[57] the region where cell culture-adaptive changes have been found to cluster in the replicon system,[5,70] the so-called IFN sensitivity-determining region (ISDR),[60,61] the double-stranded RNA-activated protein kinase (PKR) interaction domain,[69] the putative nuclear localization signal (NLS),[71] and variable region 3 (V3)[66] are highlighted.

tutively expressing different NS5A sequences.[78] Therefore, the inhibitory effect of NS5A may be mediated by PKR-independent mechanisms.

In this context, Polyak *et al*. recently reported an upregulation of the proinflammatory chemokine interleukin (IL)-8 in HeLa cells inducibly expressing NS5A.[82] These findings were subsequently confirmed by microarray studies.[83] IL-8 has earlier been shown to inhibit the antiviral actions of IFN-α *in vitro*, thus suggesting a novel mechanism to counteract the IFN system. Interestingly, pretreatment serum IL-8 levels were found to correlate with the response to IFN-α therapy in patients with chronic hepatitis C.[84]

The HCV envelope glycoprotein E2 contains a sequence identical to phosphorylation sites of PKR and the PKR target eIF2α. Starting from this observation, Taylor *et al*. described an interaction between E2 and PKR that resulted in an inhibition of the kinase activity of PKR and interference with its inhibitory effect on protein synthesis and cell growth.[85] The relevance of these observations for the natural history of HCV infection is not yet clear, but viral defence strategies targeting the effector mechanisms of IFN-induced antiviral activities could play an important role in viral pathogenesis.

## Conclusions

IFN-α is a major component of the innate immune response against viral infections including HBV and HCV. In clinical practice it is widely used for the treatment of both chronic hepatitis B and C. The mechanism of action and the IFN-α effector functions involved are only partially understood. There is evidence that HBV and HCV protect themselves against IFNs through interference with IFN production, signalling and antiviral effector systems. These and other as yet unknown mechanisms could contribute to the resistance to IFN-α therapy observed in many patients and may represent a general escape strategy of HBV and HCV contributing to viral persistence and pathogenesis of chronic liver disease. A better understanding of the interactions between these viruses and the IFN system may ultimately result in more effective therapies against HBV and HCV, which are leading causes of chronic hepatitis, liver cirrhosis and HCC worldwide.

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# Chapter 55
# New *in vitro* testing systems for hepatitis B and C viruses

David Durantel, Olivier Hantz, Christian Trepo, Fabien Zoulim

In recent years, considerable efforts have been made to develop *in vitro* testing systems for the study of hepatitis B virus (HBV) and hepatitis C virus (HCV). These *in vitro* systems are meant to contribute to the better understanding of the biology of both viruses in order to identify and utilize new molecular targets for antiviral therapy. But these systems are also meant to study and understand the viral variability and its role in pathogenesis, as well as the causes and mechanisms of viral resistance. In this chapter, some of these testing systems will be described. Molecular assays designed to quantitatively measure the viral load, determine the genotypes, or detect mutations within clinical HBV or HCV strains will not be described as they are described elsewhere in this book.

## HBV

The *in vitro* study of HBV replication has become of critical importance for the elucidation of specific steps of the viral life-cycle, including the discovery of the cellular receptors for viral entry and cellular determinants involved in covalently closed circular DNA (cccDNA) formation, as well as the determination of the yet unknown role of the HBV X-protein. Furthermore, these *in vitro* studies have become of clinical importance, with the development of new nucleoside analogues and the subsequent selection of drug-resistant mutants, to determine the replication capacity and drug susceptibility of clinical isolates.

### Infection assays

#### *Human primary hepatocytes*

Human primary hepatocytes represent a unique system that allows the study of the full HBV replication cycle, including the early steps of virus–cell interaction, viral entry, or cccDNA formation. The demonstration of a full HBV replication cycle in human primary hepatocytes of adult or fetal origin was demonstrated by several groups in the late 1980s and early 1990s.[1–4] This study system allowed the study of specific antiviral targets such as virus–cell interaction and its neutralization by anti-envelope monoclonal antibodies.[5,6] As human primary hepatocytes are more closely related to normal hepatocytes than hepatoma cell lines, this system presents some interest for the analysis of the metabolism of nucleoside analogues and the study of their anti-HBV activity.

As an example, we have evaluated the anti-HBV activity of β-L-Fd4C, an L-nucleoside analogue of deoxycytidine, in human primary hepatocytes.[7] First, we showed that this compound did not increase lactic acid production by comparison with control culture and lamivudine-treated hepatocytes, suggesting that it did not significantly affect mitochondrial metabolism, at least in our experimental conditions. This was in agreement with observations made *in vitro* in human hepatoma cell lines, primary duck hepatocytes, and in infected ducks and woodchucks, showing a good therapeutic index.[8,9] In a 'curative' protocol, where drugs were administered for 7 days starting from day 8 after virus inoculation to day 15, the analysis of intracellular viral DNA at the end of treatment showed a dose-dependent inhibition of the synthesis of viral DNA replicative intermediates by the two compounds (Fig. 55.1). In this experimental protocol, at 0.1 and 1 μM, β-L-Fd4C inhibited viral replication more significantly than lamivudine (Fig. 55.1). However, even at the highest concentration of β-L-Fd4C, viral DNA was not cleared from infected cells. These observations are consistent with the greater effect of β-L-Fd4C d4C compared with lamivudine on the hepadnavirus reverse transcriptase hepadnavirus genome replication in duck primary hepatocytes, human hepatoma cell lines and *in vivo* in animals.[8,9] The lack of viral clearance, even with long-term administration of a potent reverse transcriptase inhibitor, has been observed previously in primary duck and woodchuck hepatocytes. It emphasizes that long-term antiviral treatment is required to control

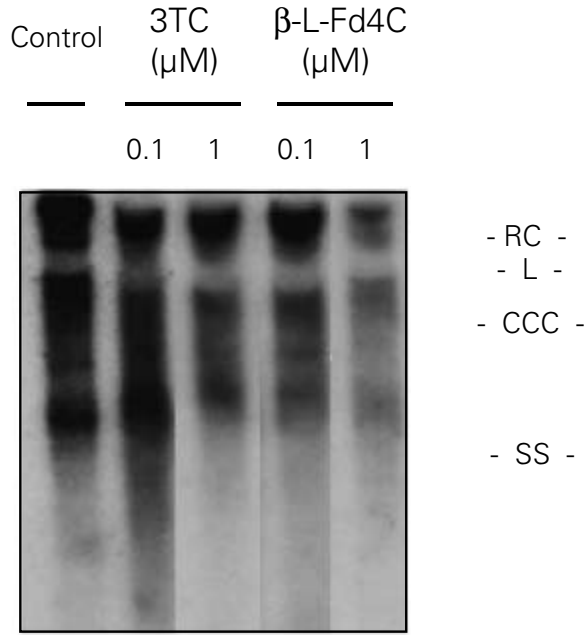



**Figure 55.1** HBV DNA synthesis in human primary hepatocytes and its inhibition by nucleoside analogues. Human primary hepatocytes were inoculated with an inoculum derived from the supernatant of the 2.2.1.5 cell line. Cells were then treated with lamivudine or elvucitabine from day 4 post-inoculation to day 12. Viral DNA replicative intermediates were analyzed after gel electrophoresis and Southern blot analysis. Results were compared with untreated control cells.

viral replication in the absence of a significant immune response directed against the residual infected cells and/or in the absence of hepatocyte turnover allowing the renewal of the infected liver with non-infected hepatocytes. The study of HBV replication when β-L-Fd4C or lamivudine were administered prior to virus inoculation until day 4, showed that β-L-Fd4C and lamivudine inhibited viral replication in a dose-dependent manner and delayed the time of detection of viral replicative intermediates by comparison with the control cultures. Interestingly, β-L-Fd4C at 1 μM inhibited viral replication more profoundly than lamivudine and its suppressive effect was maintained 12 days after cessation of treatment (until day 16 post-inoculation). Moreover, these results were confirmed by the study of hepatitis B surface antigen (HBsAg) expression and secretion in cell supernatants, which was lower in β-L-Fd4C-treated cells than in lamivudine-treated cultures. These results, in agreement with those obtained in primary duck hepatocyte cultures and in experimentally infected ducklings, suggest that neither lamivudine nor β-L-Fd4C could prevent the initial formation of cccDNA, but that the inhibition of viral replication by β-L-Fd4C resulted in a delay in viral cccDNA formation and in a decrease in viral protein expression. This suggests that although β-L-Fd4C and lamivudine would not prevent the infection of hepatocytes by the residual circulating virions during therapy, the kinetics of viral clearance may be accelerated by β-L-Fd4C treatment.

Unfortunately, there are major limitations in the use of human primary hepatocytes. These include ethical regulation of access to human liver samples for research, as well as the accessibility of liver samples of sufficient quality to perform hepatocyte cultures. The development of easier systems to cultivate human primary hepatocytes or to immortalize HBV-susceptible hepatocytes may hasten the discovery of new anti-HBV agents and their evaluation prior to clinical trials.

### *Duck, woodchuck and tupaia primary hepatocytes*

Owing to readier availability, the use of animal primary hepatocyte cultures has been and still is of great importance for the better understanding of some biological features of HBV homologue viruses, including duck (DHBV) and woodchuck (WHV) hepatitis viruses, as well as the study of antiviral agents. The demonstration of a full DHBV and WHV replication cycle in primary hepatocytes was achieved by several groups in the late 1980s.[10–12] These systems subsequently enabled: (1) the identification of the DHBV receptor[13–16] and domains of the pre-S protein involved in the interaction with this receptor,[17–19] (2) study of the entry route,[20] and (3) study of early events of the DHBV cycle.[21] Notably, duck and woodchuck primary hepatocytes have made it possible to study different antivirals, including cytokines,[22–25] antisense molecules,[26] and analogues of nucleosides/nucleotides – 3TC (lamivudine), FTC (emtricitabine), L-FMAU (clevudine), PMEA (adefovir), PMPA (tenofovir), FLG and b-L-FD4C.[7,27–36] More precisely, they have allowed the measurement of the impact of antivirals on the formation of cccDNA, synthesis of intermediate DNA, or elimination of the intranucleus pool of cccDNA. For instance, it was shown that analogues of nucleosides/nucleotides were neither able to prevent cccDNA formation nor able to clear cccDNA from infected cells, even when used in combination.[29,33]

Beside duck and woodchuck primary hepatocyte cultures, primary hepatocytes (PTH) from the asian tree shrew *Tupaia belangeri* have gained increasing interest in recent years because of their susceptibility to HBV infection itself.[37,38] This *in vitro* model offers the possibility to study the cellular and viral determinants involved in binding, uptake, upcoating, nuclear transport and cccDNA formation. It was used to confirm that the current nucleoside analogues, lamivudine and adefovir, cannot completely prevent the initial formation of human HBV cccDNA upon infection of cells,[39] thus confirming the results obtained with other animal and human primary hepatocyte systems.

Moreover, it was shown that the woolly monkey hepatitis B virus, although less infectious for human primary hepatocytes, has a higher replication competence in PTH. This may pave the way to obtain adapted mutants with enhanced replication capacity in PTH as well as *in vivo* in animals to facilitate the study of the early steps of HBV replication.

### *HepaRG cells*

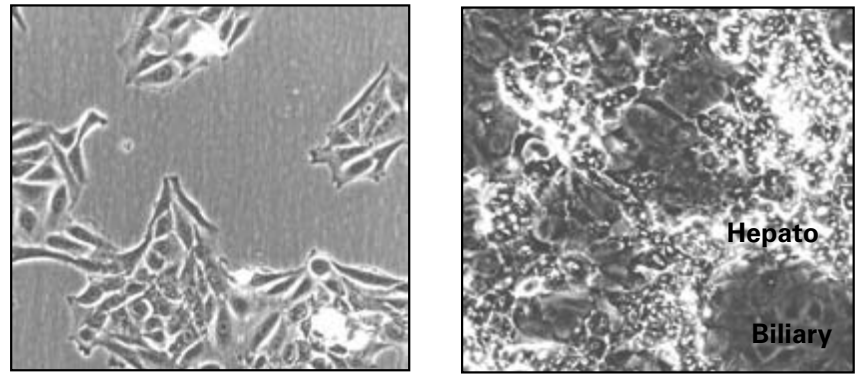
Among the numerous established human hepatoma cell lines, none were shown to be susceptible to HBV infection. Recently, a new cell line derived from a human hepatocellular carcinoma, called HepaRG, was generated.[40] This bipotent cell line is able to differentiate into two cell types after dimethyl sulphoxide-(DMSO) induced differentiation: hepatocytes and biliary-like cells (Fig. 55.2). After differentiation, the cell line exhibits liver-like organization, expresses specific hepatocyte enzymes and functions, and supports HBV infection as normal human primary hepatocyte cultures. Differentiation and infectability are maintained only when these cells are cultured in the presence of corticoids and 2% DMSO. It is worth noting that the HBV infectivity is enhanced by addition of polyethylene glycol (PEG) in the virus inoculum. All HBV replicative intermediates can be detected in infected cells, including viral cccDNA. The specificity of this HBV infection model was ascertained by both the neutralization capacity of HBV envelope protein-specific antibodies and the competition with an envelope-derived peptide. HepaRG cells therefore represent an interesting tool for analyzing further the mechanism of HBV entry, as well as the cellular and viral determinants involved in cccDNA formation and persistence during antiviral therapy. Moreover this cell line can be used to study the fitness of naturally occurring or drug-induced HBV mutants by performing competitive infection.

## Transfection and transduction assays

Although hepatoma cells, such as HepG2 and Huh7, cannot be efficiently infected by HBV, they are able to replicate and secrete the virus provided that the HBV genome is transfected or transduced into cells. Various vectors, including plasmidic and viral vectors, have been used to infect transiently or stably transformed hepatoma cells. These approaches are particularly important because the modification of HBV genome can be done by conventional molecular biology techniques. Thus, specific mutations associated with a particular phenotype *in vivo* (e.g. resistance to drug) can be transposed into these vectors and analyzed *in vitro*.

### *Classic transient transfection assays*

Until recently, the analysis of the phenotype of naturally occurring or drug-induced HBV mutants has relied either on PCR-mediated transfer of HBV genome cassettes or on site-directed mutagenesis within plasmids carrying 1.1 to 2 genome units of a well established replication-competent laboratory strain (Fig. 55.3). These plasmids contain the HBV genetic information necessary and sufficient to initiate the HBV intracellular replication cycle after transfection into cells. The synthesis of HBV pregenomic RNA can be driven either by the HBV promoter (i.e. 1.3 to 2 genome units) or a strong mammalian promoter (i.e. 1.1 genome unit). For example, the use of those genetically engineered plasmids allowed confirmation that the M204V/I ± L180M mutants selected in patients during antiviral therapy were indeed conferring resistance to lamivudine.[34,41–43] Despite its obvious utility to quickly characterize new mutations *in vitro*, the methods based on the exchange of a cassette or site-directed mutagenesis do not take into account the HBV genome variability existing in other parts of the genome. Moreover, the exchange of a cassette can cre-




(a) Undifferentiated HepaRG (b) Differentiated HepaRG

**Figure 55.2** HepaRG cell line. (a) HepaRG cells one day after seeding. (b) HepaRG cells 50 days after seeding and 30 days after the beginning of DMSO treatment. Hepatocyte-like and biliary-like cells are indicated

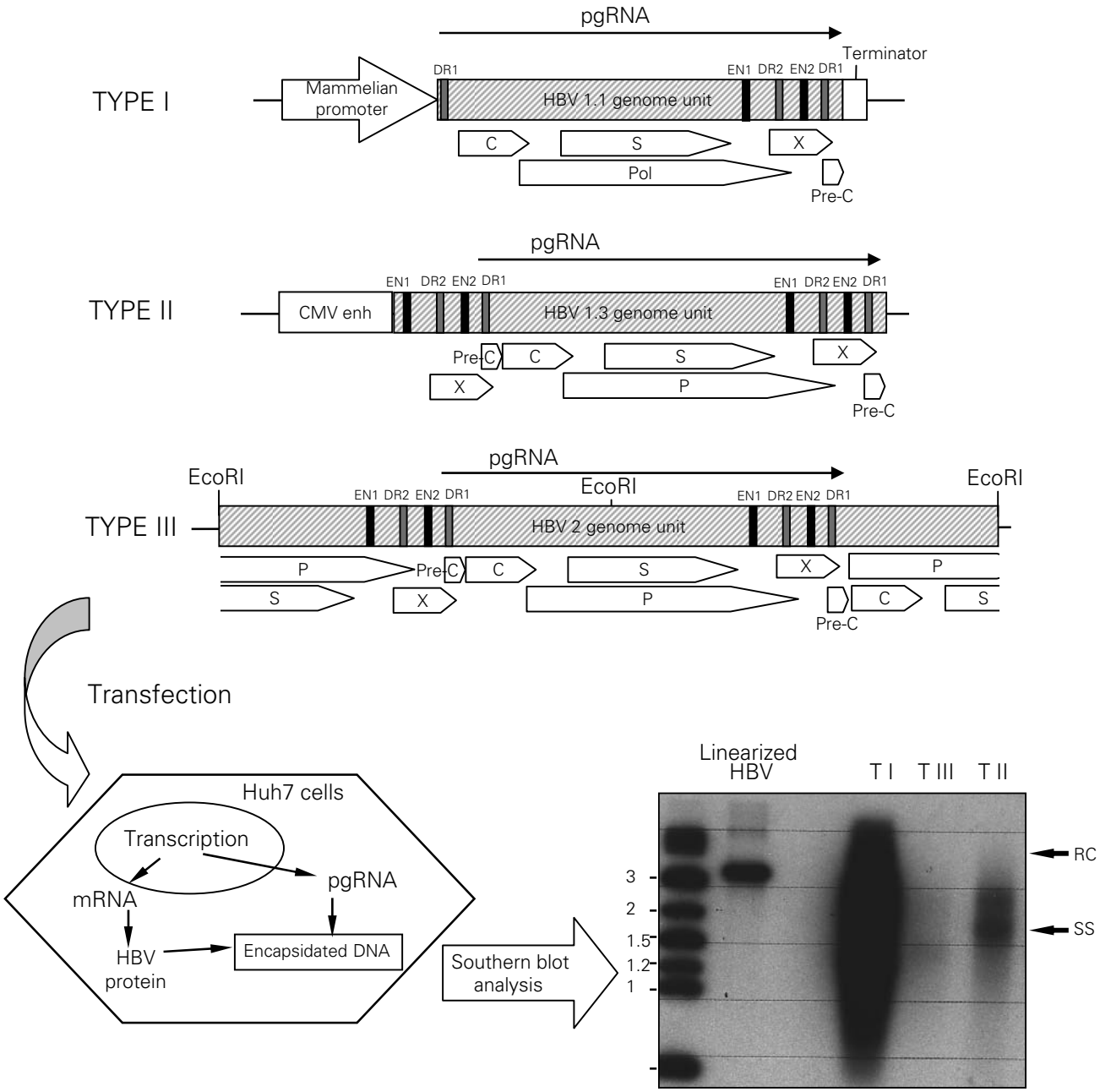



**Figure 55.3** Structure of plasmid loaded with HBV sequences and overall approach for a phenotypic study of HBV clinical isolates. Top: three types of plasmids containing 1.1, 1.3 or 2 HBV genome units are represented. Type I contains the minimal HBV length required for pgRNA synthesis. The synthesis of pgRNA is controlled by a strong mammalian promoter. Type II contains the sequence required for the synthesis of pgRNA plus the promoter of HBV. Thus, the synthesis of pgRNA is controlled by the homologous HBV promoter. Type III contains two head to tail genomes cloned using *Eco*RI restriction enzyme. Bottom: comparison of the amount of intracellular replicative HBV DNA intermediates produced after the transfection of Huh7 cells with the three type of vectors. An autoradiographic image resulting from Southern blot analysis of intracellular HBV DNA performed 4 days post-transfection is shown. A molecular marker was run on the right-hand side of the gel and radiolabelled for visualization after X-ray exposure.

ate non-natural chimeric genomes, especially when the genotype of the HBV to be studied is different from that installed in the receiver plasmid.

A vector-free method, meant to facilitate the analysis of naturally occurring HBV variants, was developed by Gunther *et al*.[44] This method relies on an original and efficient polymerase chain reaction (PCR) amplification of full-length HBV genomes isolated from patients. The amplicon obtained is then cut by a restriction enzyme that renders the extremities competent for the circular

closing of the molecules by ligation. The linear amplicon is tranfected into cells, and serves as a ccc-like DNA template for the initiation of the intracellular HBV replication after its repair and circularization by host enzyme. Depending on the nature of the HBV genome analyzed (e.g. particular mutants or genotypes), the level of replication obtained can be rather low. Therefore, this method remains interesting for fundamental research, but does not seem appropriate for clinical use as a standardized and transposable phenotypic assay, which requires solid replication levels.

### *Stably transfected cell line*

The absence of a productive HBV infection in most hepatoma cell lines is due to an inefficient entry process. Transfection of the HBV genetic information, as seen above, has made it possible to overcome this problem. However liposome-based transfection does not allow transfection of 100% of cells. To circumvent this problem, researchers have developed cell lines carrying HBV transgene in their genome that constitutively produces replicative viral DNA intermediates, mature Dane particles and high levels of viral antigens.[45,46] The first cell line obtained, called Hep G2 2.2.15, was widely used to study the effect of various antivirals, including cytokines,[47–50] analogues of nucleosides/nucleotides,[8,30,51–55] or inhibitors of morphogenesis.[56] Another cell line carrying lamivudine-resistant HBV genome was developed to study drugs potentially active against this mutant and more generally a cross-resistance phenomenon.[57] One could expect that for each new HBV mutant conferring a resistance to an antiviral, a cell line will be developed. However, the generation of such stably transfected cell lines is laborious and cannot be obtained for all cell types, as it implies most of the time the integration of the transgene into the host genome and this can be deleterious for the phenotype of the cells (e.g. HepaRG).

### *Baculovirus- and adenovirus-based assays*

Baculovirus and adenovirus vectors, which are able to penetrate very efficiently into mammalian cells including primary hepatocytes or hepatoma cells, have been used to improve the delivery of the HBV genetic material.

The antiviral properties of nucleoside analogues have been investigated *in vitro* in HepG2 cells infected with recombinant HBV baculovirus.[58] Different types of information can be obtained with the HBV baculovirus transduced HepG2 cells, as the levels of HBV replication can be significantly higher than those obtained from conventional HBV-expressing cell lines. Furthermore, the cultures can be manipulated and/or treated before or during the initiation of HBV genome replication. Interestingly, the high levels of HBV replication generated using this approach allow the rapid detection of HBV products including cccDNA from low numbers of HepG2 cells. The treatment of HBV baculovirus-infected HepG2 cells with 3TC resulted in an inhibition of HBV replication. The effect of 3TC on HBV replication was both dose- and time-dependent. As expected, levels of HBV transcripts and extracellular HBV antigens were not affected by 3TC. Importantly, the HBV baculovirus-HepG2 system made it possible to observe that HBV cccDNA levels are lower in cells treated with 3TC than in control cells. Delaney and colleagues[59] observed that the treatment of HepG2 cells prior to HBV baculovirus infection resulted in a slight increase in the efficacy of 3TC compared with treatments starting 24 hours post-infection. In this system, a detailed analysis confirmed that non-protein-associated relaxed circular HBV DNA, and particularly HBV cccDNA, are considerably more resistant to 3TC treatment than other forms of HBV DNA, including replicative intermediates and extracellular DNA. This system was then adapted to determine the cross-resistance profiles of drug-resistant HBV strains.[59] For these studies, novel recombinant HBV baculoviruses which encoded the L180M, M204I and L180M M204V drug resistance mutations were generated and used to examine the effects of these substitutions on viral sensitivity to lamivudine, penciclovir and adefovir. It was shown that (1) the L180M mutation confers resistance to penciclovir and partial resistance to lamivudine, (2) the M204I and L180M M204V confer high levels of resistance to lamivudine and penciclovir, and (3) adefovir is active against each of these mutants. The HBV baculovirus-infected HepG2 cells therefore represent an interesting study model for selected mutants. This model may be more tedious when considering the study of a large number of HBV mutants.

Adenovirus vectors have also been used to bypass the limiting receptor-mediated entry process and deliver HBV genome into hepatoma cell lines or primary hepatocyte cultures.[60] It was elegantly shown that adenovirus HBV vectors are able to transduce primary *Tupaia* hepatocytes (PTH) and lead to an infectious cycle including the formation of cccDNA.[60,61] This model may prove useful to gain insight in the cellular mechanism involved in cccDNA formation.

### *Rapid phenotypic assay*

In the case of HIV therapy, drug resistance testing is now recommended to guide the choice of new drug regimens after the first or multiple treatment failures.[62,63] In addition to genotypic assays, several phenotypic assays have been developed for HIV and are currently used in clinical practice to monitor drug resistance. Until recently, no standardized phenotypic drug susceptibility assays were available for HBV.

We have recently developed a new method for the cloning of HBV genomes isolated from patients into plasmidic vectors and defined the basis of one of the first phenotypic assays capable of assessing HBV drug susceptibility *in vitro* and evaluating new antivirals against clinical HBV strains (Durantel *et al*., submitted). The novel cloning technique enables the assembly of molecular clones, containing a 1.1 HBV genome unit, which allows the study of viral replication upon transfection of one clone or a mixture of clones into eukaryotic cells (Fig. 55.4). When transfected into Huh7 cells, these clones trigger the expression of pregenomic HBV RNA (pgRNA) under the control of heterologous promoters and therefore initiate the viral replication cycle including synthesis of intracellular viral DNA replicative intermediates. Thus, the initial synthesis of pgRNA is determined by the mammalian promoter of the vector and is identical from one vector to another irrespective of the HBV genome cloned. It ensures high levels of pgRNA synthesis and consequently a high level of viral DNA synthesis. This is particularly important in the context of a phenotypic assay which requires high levels of HBV DNA synthesis to facilitate the determination of drug susceptibility by standard procedures. This method enables the cloning of the whole HBV genome isolated from a given sample, and appears theoretically more appropriate to study complex situations, e.g. multiple mutations dispatched all over the genome or genotypically undefined phenotypes. Moreover, a multiclonal analysis (a mixture of at least 20 clones) enables the determination of the sensitivity of the viral population (i.e. quasi-species) to antiviral agents. This phenotypic assay was successfully applied to study the novel adefovir resistance (mutation N236T) associated with a viral breakthrough in a liver transplant patient.[64]

Another phenotypic assay was developed by Yang and colleagues.[65] This assay is based on a new plasmid vector that facilitates the cloning and expression of full-length HBV genomes amplified from the sera of chronic hepatitis B patients and enables efficient phenotypic analysis, i.e. drug susceptibility analysis of naturally occurring variants.

With the development of new anti-HBV molecules, these phenotypic drug susceptibility assays could become an important tool for the management of patients infected with resistant HBV isolates and for the evalua-

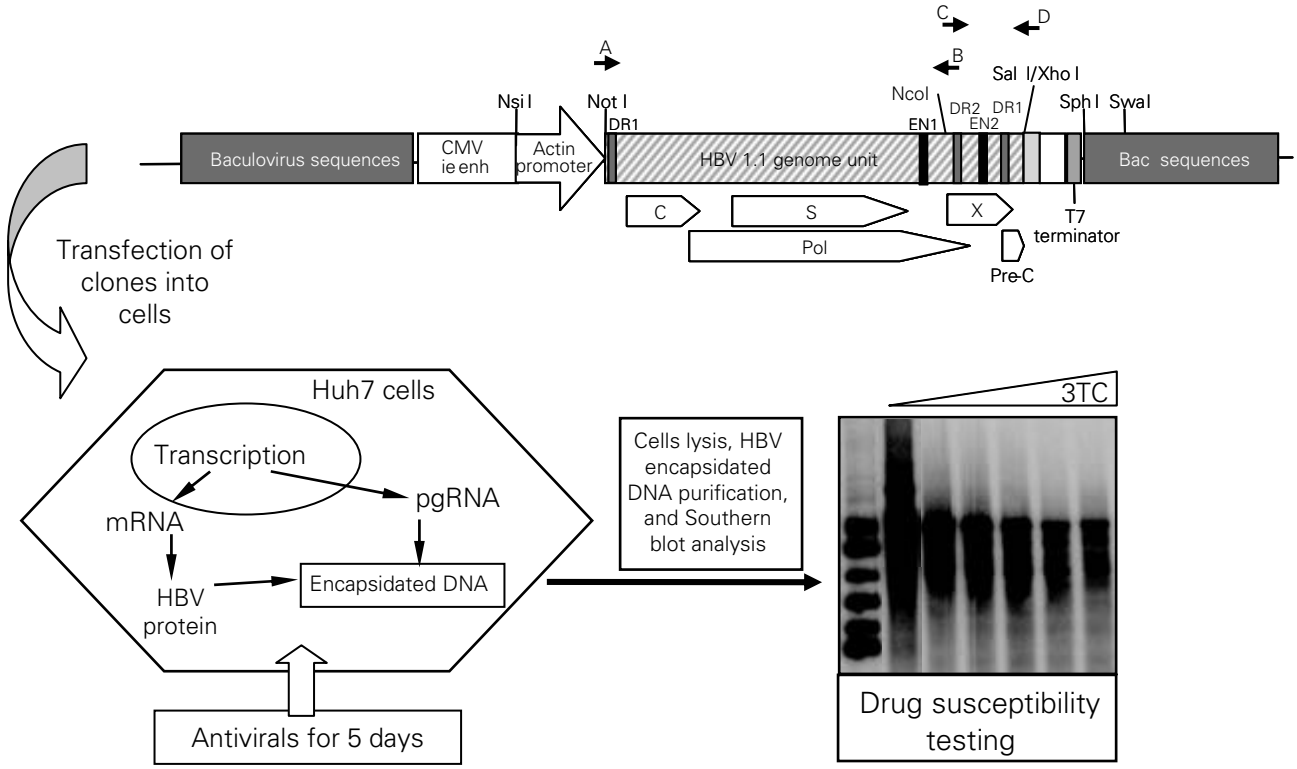



**Figure 55.4** Structure of plasmid used and overall approach for a phenotypic study (drug susceptibility testing) of HBV clinical isolates. A map of the vector pTriEX-HBV is presented at the top of the figure. The vector contains 1.1 HBV genome unit corresponding to the length of pgRNA. The primers A-B and C-D used to generate the two products which have been cloned to obtain the vector are also indicated. Vectors pTriEx-HBV were transfected into cells, in which they trigger HBV genome replication. Transfected cells were treated with drugs for 5 days before viral replicative intermediates were purified and subjected to Southern blot analysis. The $IC_{50s}$ and $IC_{90s}$ can be determined by phosphorimager analysis.

tion of new antivirals with clinical isolates circulating in the population.

### Polymerase assays

#### *DHBV polymerase assay*

The development of an *in vitro* acellular assay[66] that allowed the expression of an enzymatically active DHBV polymerase paved the way for the study of viral and cellular determinants required for reverse transcription, including (1) the formation of a complex between the 5′ epsilon structure on the pregenomic RNA, the viral polymerase and cellular chaperones, (2) the protein-primed initiation of reverse transcription, (3) the strand transfer and elongation of viral minus-strand DNA.[67–77] This system was useful to determine the mode of action of several nucleoside analogue triphosphates on the priming reaction or on the elongation of DNA synthesis.[8,34,78–80] Furthermore, the engineering of the drug-resistant mutants observed *in vivo* in HBV-infected patients in the DHBV polymerase system allowed further demonstration of their role in drug resistance and their susceptibility to new drugs.[34]

#### *HBV polymerase assay*

After the development of the DHBV reverse transcriptase assay, several attempts to develop such an assay for the human HBV polymerase were undertaken. Systems based on the expression of the enzyme in insect cells,[81,82] yeast,[83,84] bacteria,[85] or *in vitro*,[86] as well as a system based on the purification of nucleocapsid containing viral pregenomic RNA and polymerase,[87] were developed. However, these systems are not as robust as the *in vitro* system used to express DHBV polymerase, and further work is yet to be done to improve the expression of active HBV polymerase Nevertheless, the systems developed were critical to show that lamivudine-resistant polymerase mutants were indeed sensitive to adefovir diphosphate, and to determine that entecavir inhibits viral DNA synthesis through the inhibition of priming and by terminating the elongation of the DNA chain by the viral polymerase.[88]

Altogether, polymerase assays have been and still are of great use for the study of viral and cellular determinants required for reverse transcription, and the study of properties of mutant polymerases, including reverse transcription activity or drug susceptibility.

Table 55.1 summarizes the most relevant *in vitro* systems developed to study HBV.

## HCV

Since the molecular cloning of its genome in 1989,[89,90] research on HCV biology has been slowed down by the lack of an efficient cellular system able to efficiently replicate the virus. To circumvent this problem, numerous *in vitro* and *in cellulo* testing systems have been developed to enable the study of both fundamental and clinical aspects of HCV biology. The development of such systems aims at a better understanding of the replication of the virus to gain information on the hepatitis C disease, and also to study new molecular targets in order to identify new antiviral agents. With respect to clinical analysis, these assays are essentially meant to study and understand the viral variability in relation to pathogenesis, as well as the causes and mechanisms of viral resistance.

**Table 55.1** Summary of the most relevant testing systems for HBV

| System | Advantages | Limitations | Applications |
|---|---|---|---|
| Human primary hepatocyte | Full HBV replication cycle | Limited access | Receptor discovery<br>cccDNA formation |
| Tupaia hepatocytes | Full replication cycle | Low yield of replication<br>Non-human primate | Study of antiviral effects |
| HepaRG cells | Unlimited access<br>Full replication cycle | | Receptor discovery<br>cccDNA formation<br>Study of antiviral effects |
| Classic transient transfection | Post-transcriptional event | Absence of cccDNA | Role of single point mutations |
| Stably HBV transfected cell line | Post-transcriptional event | Absence of cccDNA | Study of antiviral effects |
| Baculovirus transduction | cccDNA formation | Construction of vectors | cccDNA studies<br>Study of antiviral effects<br>Drug resistance studies |
| Phenotypic assay | Rapid, polyclonal studies<br>Whole HBV genome | Absence of cccDNA | Drug resistance studies |
| Polymerase assay | Cell-free system | Difficulties of human HBV<br>Polymerase studies | Mechanism of action of inhibitors<br>Drug resistance studies |

## Infection assays

Despite considerable efforts made by all those involved in HCV research, no cellular systems capable of replicating HCV efficiently and reproducibly have been obtained to date. The development of such a system would be of great interest to better understand various aspects of the HCV life-cycle, including for instance viral entry and viral morphogenesis. An infection system would also be of great value to study biological differences between naturally occurring HCV strains in terms of infectivity, replication capacity or susceptibility to antivirals. The absence of a suitable cellular system for the replication of HCV is not due to the lack of research. Indeed, in the last decade several cell culture propagation systems, based on the infection of primary cell cultures or cell lines, have been described.

### *Primary hepatocytes*

The infection of primary hepatocytes from humans or chimpanzees has been tried extensively by several groups.[91–93] The infection is revealed by the presence of negative-stranded RNA intermediates and the production of neo-formed virions in the culture medium which were able to reinfect fresh cells. However, the level of replication is inconstant from one experiment to another and very low, as highly sensitive RT-PCR was needed for its detection. It is worth noting that such a system has nevertheless permitted the study of the antiviral effect of interferon (IFN)-α on the replication of HCV.[94] In addition to the lack of efficiency, the major drawback for the utilization of such infection systems resides in the difficulty of obtaining normal human or chimpanzee liver tissue. To overcome the latter, investigators have searched for new animal primary hepatocytes that are susceptible to HCV infection. In that respect, it was found that primary hepatocytes from the tree shrew *Tupaia belangeri* were susceptible to HCV.[95] As access to primary hepatocytes from *Tupaia* is facilitated by the rearing of this animal, this infection model may be of great interest to study HCV replication and other aspects of HCV biology.

### *Hepatoma cell lines*

Besides infection of primary hepatocytes, many attempts at infection have been undertaken with established cell lines of human origin. With respect to hepatoma cell lines, the most detailed results were obtained with the non-neoplastic cell line PH5CH.[96,97] In this cellular system, the replication of HCV is observed up to 100 days after the inoculation and is specifically inhibited by IFN-α. Moreover, a decrease in the complexity of the viral quasi-species used for the inoculation is noticed, suggesting that not all variants can replicate in these cells. Other hepatoma cell lines including WRL68, Huh7 and HepG2 were shown to be weakly susceptible to HCV infection.[98,99] Improvement of HepG2 infection was obtained by fusion of these cells to primary human hepatocytes prior to inoculation.[100] Altogether, infection of hepatoma cell lines remains inefficient and these systems are not yet really useful for many applications.

### *Other cell lines*

Although HCV is thought to be primarily a hepatotropic virus, an increasing body of evidence suggests that extrahepatic cells can be infected with the virus. Hence, it was found that several T-cell lines, including MT-2 and Molt-4, and the B-cell line Daudi, could be infected with HCV, although at a weak level.[101–105] The virus produced in these cells can reinfect fresh cells and can even be used to infect chimpanzee.[105] Another elegant attempt to replicate HCV is to cultivate *ex vivo* cells persistently infected by the virus. This has been done recently starting from non-Hodkin's B-cell lymphoma.[106] The cell line thus obtained is able to continuously produce infectious HCV virions in culture. This system may have some usefulness to produce analytical quantity of viral particles and therefore obtain information on virion structure.

All the systems described above are useful for performing some studies, such as infectivity experiments or analysis of the effect of some inhibitors/antivirals, but are not yet suitable for routine experiments and the elaboration of proper *in vitro* testing assays. To date, none of these systems has been used, for instance, to study the replication fitness or *in vitro* drug susceptibility of clinical strains of HCV, including treatment-escaping mutants. Further work has to be done to improve existing cellular systems or to identify new ones.

## Transfection and transduction assays

### *Transient transfection*

A permissive infection requires an efficient entry of the virus into cells. One could hypothesize that the lack of success with HCV infection may be due to a defect in entry. An option to circumvent entry is to transfect the viral genome into cells using liposomes or other transfectant reagents. The advantage of such a strategy is that the inoculum (e.g. cloned viral genome) is well defined and can be modified by molecular biology techniques, thus allowing genetic analysis. This strategy, which has been applied with success for many viruses including several plus-strand RNA viruses,[107] turned out to be very difficult with HCV. Nevertheless, the replication of transfected HCV genome and secretion of virions in the culture medium was obtained in the human hepatoma

cell lines Huh7 and HepG2.[108,109] Although the titre of HCV observed in the culture medium was very low, the infection could be passed to fresh cells. More recently, it was demonstrated that virions produced in this cellular system were able to infect chimpanzee.[110] Once again, the problem of this system is mainly quantitative. Indeed, the replication level is very low and can only be detected by highly sensitive RT-PCR. Then what would be a good cellular system? It should be a system where replication of the HCV genome could be detected, not by a highly sensitive RT-PCR, but by a conventional Northern blot. In fact, a higher replication level of the genome associated with a higher expression of viral protein would facilitate the study of their function during the life-cycle of HCV.

### *Baculovirus-mediated transduction*

Transfection of full-length HCV RNA into cells is not very efficient. The low level of HCV replication after transfection could be due to small numbers of transfected cells. To tackle this problem, some investigators have used other viruses to transfer the genetic information of HCV into cells. Hence, McCormick and colleagues have used baculovirus loaded with the whole HCV genome to transduce various cell lines and trigger HCV replication.[111] This transduction approach led to a higher level of RNA replication compared with transfection methods, as a Northern blot is sensitive enough to detect HCV RNA. This particular system of delivery is very interesting and deserves further attention.

### *Replicon systems*

The major breakthrough in the HCV research field has come from the development of genetically modified HCV minigenomes, called replicons, that self-amplify in cultured hepatoma cells to very high levels.[112,113] These replicons are bicistronic RNA genetic elements (Fig. 55.5a). The first traductional unit (or cistron) is composed of the 5′ UTR of HCV and the neomycin phosphotranferase gene. The *neo* gene that confers resistance to geneticyn (G418) is translated from the internal ribosomal entry site (IRES), which is included in the 5′ UTR. The second traductional unit is composed of the NS3-5B HCV coding sequence under the control of the EMCV IRES. Stably transfected cell lines resistant to G418 are obtained after transfection of replicon RNAs generated by *in vitro* transcription of the cloned replicon sequences and cell cloning by G418 selection (Fig. 55.5b).[114] Thus far, only replicons from genotype 1 have been successfully obtained. It is worth noting that replicons from genotype 1a have been obtained only with Huh7 cells that had been stably transformed first with a replicon from genotype 1b.[115] This result is somewhat surprising and so far no explanation has been proposed. The consequence of this genotypic restriction is that replicon technology cannot be applied to all HCV strains. Another limitation of this system comes from the fact that among human hepatoma cell lines only Huh7 cells have been successfully transformed with replicons. Recently, two other human cell lines, HeLa and 293, which are not of hepatic origin, have been reported to be susceptible to replicon transformation.[116,117] It was shown that cell lines maintaining subgenomic replicons express (1) high levels of negatively and positively stranded RNA easily, and (2) high levels of non-structural protein easily detectable by Northen and Western blots, respectively. More recently, adaptative mutations conferring even higher levels of RNA synthesis have been described by several groups (reviewed by Bartenschlager *et al*.[114]). These subgenomic replicon systems have become one of the most important tools to study several aspects of HCV RNA replication, pathogenesis and persistence. They will be very useful to identify host factors required for RNA translation and replication, and to study events responsible for the switch from translation to replication or vice versa. And perhaps most importantly in terms of drug development, these replicon systems have been, and still are, very useful to identify and/or study molecules interfering with the translation, the processing of the polyprotein, and the replication of the genome. Hence, various cytokines (e.g. IFN-α), inhibitors of protease, helicase and polymerase have been studied in these systems.[114,118,119] Moreover, this system has proven useful to investigate the mechanisms responsible for the IFN-induced inhibition of HCV replication.[120] Hence, it was shown that IFN-α could suppress the accumulation of viral RNA by a non-cytopathic pathway and could also induce apoptosis of virally infected cells in a concentration- and cell line-dependent fashion. In addition, it was found that functional proteasomes were required for establishment of the IFN-α response against HCV. Based on these results, a model for the mechanism by which IFN-α therapy suppresses HCV replication in chronic infections by both cytopathic and non-cytopathic means was established.[120]

After the generation of subgenomic replicons and using similar principles, full-length genomic replicons containing the whole HCV genetic information were obtained (Fig. 55.5a).[96,121] The knowledge accumulated while developing subgenomic replicons (e.g. role of adapting mutations) was useful to facilitate the establishment of these full-length replicons. A reasonable level of RNA replication was observed in a Huh7 clone stably transformed with HCV. These full-length replicons were initially devised, then developed in order to obtain production of infectious HCV particles in the cell culture medium. Unfortunately, the production of such particles was not observed, thus limiting the use of this

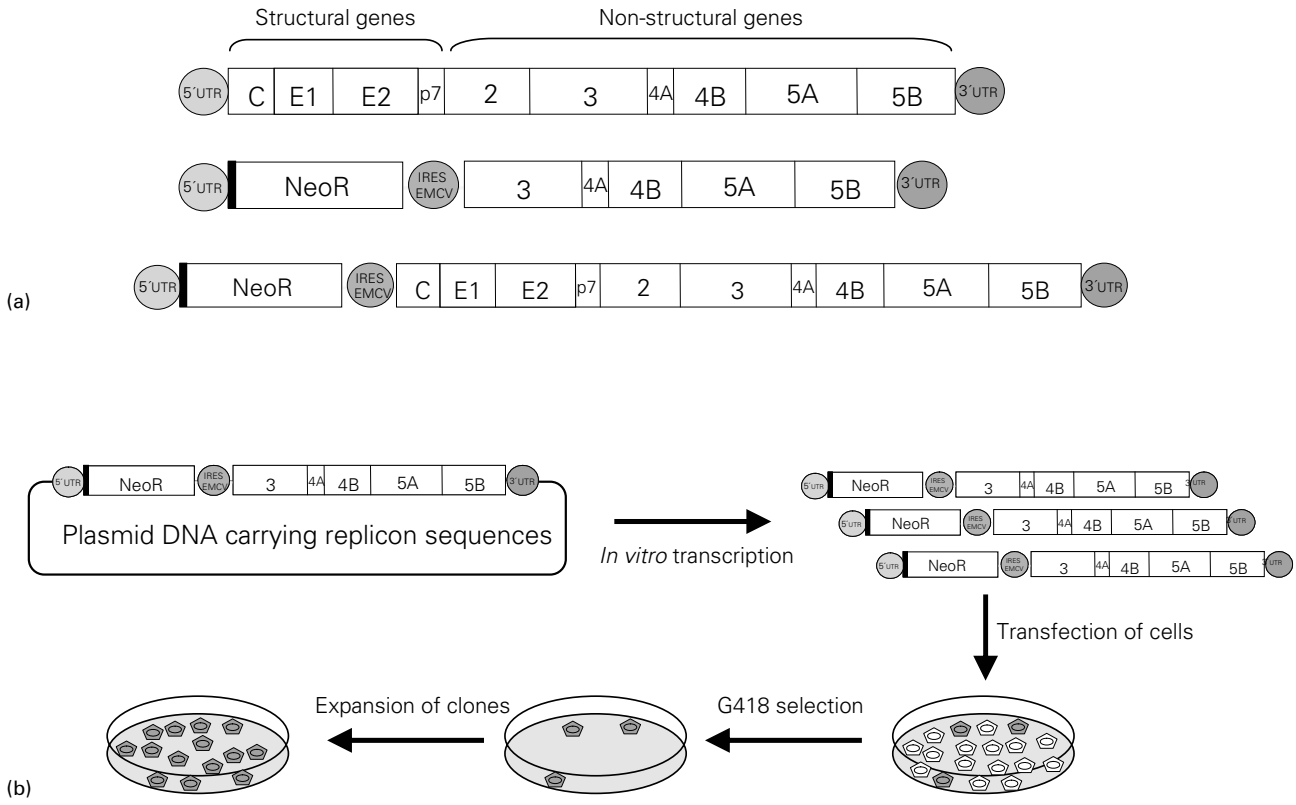



**Figure 55.5** Genetic organization of HCV and HCV replicons, and strategy of generation of cell lines carrying self-replicating HCV replicons. (a) The genetic organization of HCV genome, subgenomic and full-length replicons is presented. (b) Replicons RNA obtained by *in vitro* transcription are transfected into cells (transfected cells are in grey). If the replicon RNA self-amplifies in some cells, these cells can be selected by treatment with G418 (neomycin). The cell line is obtained by expansion of these cells under constant selection with G418. Once established, the cell line carrying self-replicating HCV replicons is cultivated with G418, but can be also be maintained without G418 for several months without loss of the replicon.

system to study, for instance, HCV morphogenesis. Further studies are required to understand why the morphogenesis is not possible in Huh7 cells.

Altogether, these replicon systems have led to tremendous progress in the knowledge of HCV biology.[114] Like *in cellulo* testing systems, they are very useful to identify, study and decipher the mechanism of action of some antivirals. One very important question remains open concerning these replicon systems. Are they going to be useful when phenotypic studies are required to analyze the drug susceptibility of HCV strains isolated from patients undergoing antiviral treatment and experiencing viral resistance? To date, only one report has described the use of replicons to analyze the drug susceptibility of an HCV mutant that appeared during ribavirin monotherapy. The sequence containing the mutation was inserted in a subgenomic replicon and a cell line was obtained. This mutant was found to be resistant to ribavirin *in vitro*, and the resistance is associated with a mutation in the polymerase gene (NS5B) at amino acid 415 (F415Y).[122] With the utilization of other antivirals targeting HCV enzymes, it is likely that other mutations will appear in the HCV genome. We have described in the HBV section how phenotypic studies could quite easily be done after the cloning of clinical strains into plasmids, subsequently used in transfection experiment. As cell lines harbouring replicons are tedious to obtain and moreover cannot be obtained for all genotypes, this replicon system does not seem appropriate for routine phenotypic assays. Further work is needed to develop other *in cellulo* systems based on either easy or non-cloning strategies that will enable phenotypic studies (e.g. replication capacity or drug susceptibility) of HCV quasispecies.

## Enzymatic assays

As infection assays and other *in cellulo* systems are not very well suited or are difficult to use, particularly to study viral resistance to antivirals, enzymatic assays have an important role to play. The HCV genome encodes a polyprotein that is proteolytically cleaved into

four structural proteins (C, gpE1, gpE2, p7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Fig. 55.4a).[123–125] It is worth noting that an additional protein, called F, has recently been identified.[126] As its function is not clear, this protein has not yet been classified among the structural or non-structural sets of proteins. Some non-structural proteins carry one or two enzymatic activities.[123–125] There are two different proteases, including a metalloproteinase (NS2/3) and a serine protease (amino-terminal domain of NS3),[127–129] one ATPase/helicase (carboxy-terminal domain of NS3),[130–132] and one RNA-dependent RNA polymerase (NS5B).[133] Except for NS2, all non-structural proteins are thought to form a complex called replicase, which is involved in the replication of the genome. Because these enzymes are crucial for the replication of the virus, they represent an ideal target for an antiviral intervention.[119,134–137] In an attempt to first, better understand and characterize the function of each enzyme and second, identify and study molecules interfering with their activity, *in vitro* systems have been developed. They are based on the expression of enzyme in prokaryotic or eukaryotic cells, the subsequent purification of a biologically active form of the protein and the setting up of the *in vitro* assay.

### *NS3 protease*

Following its cloning and expression in different systems, the structure and biochemical properties of NS3 have been extensively studied in recent years.[119,138,139] Surprisingly, it was found that the HCV NS3 protease had structural and functional differences in comparison with other serine proteases described to date. These include (1) the heterodimerization of NS3 with a co-factor (NS4A) that enhances its stability and proteolytic activity,[140] and (2) the presence of a prominent zinc-binding site that stabilizes the complex.[141,142] However, the most important discovery, relevant to antiviral drug development, was that the catalytic site of HCV NS3, unlike other serine protease, is long, shallow and flat in conformation, rendering difficult the design of molecules able to bind and inhibit its activity.[143] Retrospectively, this explained why this protein was particularly refractory to inhibition by conventional serine protease inhibitors. Most of the data gained on NS3 have been obtained thanks to the development of *in vitro* assays based on the expression of the protein in *Escherichia coli*, insect, or mammalian cells, and its biochemical purification.[119,144–147] As new information was gained on NS3, much more sophisticated assays were developed. To date, several high throughput screening (HTS) assays have been developed[148,149] and used to identify new inhibitors of the NS3-4A serine protease among chemical libraries.[119] These assays, often called cleavage assays, utilize purified NS3 protein and artificial substrate containing the cleavage sequence of NS3. The cleavage is quantitatively measured by either radiometry or fluorometry.[148,149] In principle, the NS3 protein used in these assays can be either wild-type or mutant. Thus, these assays can be used to identify new inhibitors, but also to study mutant NS3 protein (obtained by mutagenesis or cloned from patients) and determine their susceptibility to inhibitors.

### *NS3 helicase*

The carboxy-terminal 450 amino acids of NS3 have an ATPase/Rnase helicase activity.[130–132,150] The expression of this protein in bacteria followed by purification using conventional biochemical procedures has allowed the characterization of HCV helicase at molecular level. Hence, it was demonstrated that NS3 helicase is able to unwind duplex RNA (as well as duplex DNA and DNA/RNA) structures only in a 3′ to 5′ direction, by disrupting hydrogen bonds that keep the two strands together, and that this unwinding activity is associated with hydrolysis of nucleoside triphosphate (NTP). Once again from simple *in vitro* assays, more sophisticated assays have been designed and set up to screen and identify inhibitors of the enzyme. These assays utilize purified NS3 protein and artificial substrates, which can be DNA duplex. The strand displacement is measured by radiometry or fluorometry. A good example of such an assay is the Flashplate helicase assay developed by BioChem Pharma.[151]

### *NS5B RNA polymerase*

NS5B bears the RNA-dependent RNA polymerase (RdRP) activity of HCV, and is therefore responsible for the synthesis of both the negative- (i.e. RNA intermediate) and positive- (i.e. viral genome) stranded RNA. Due to its specificity and crucial role in the viral cycle, this enzyme is an excellent target for antiviral therapy. Therefore, considerable efforts have been made to express it in heterologous systems and to purify a biologically active form of the enzyme so as to establish *in vitro* testing assays. First full-length enzyme was purified and used for biochemical studies.[133,152] However, the full-length enzyme displayed a rather poor activity.[152,153] A deletion of 21 amino acids at the carboxy-terminal domain enabled researchers to obtain a soluble form of the enzyme with improved activity.[153,154] The protein was expressed in a wide range of cellular systems, including bacteria[153,154] and mammalian[155] cells. While developing these assays, major enzymologic and structural[156] data were obtained about NS5B. They have been extensively reviewed in the literature.[119] Besides their use in the study of the enzymology and the structure of NS5B, *in vitro* biochemical assays are potentially of great interest to identify new inhibitors. Thus far, no HTS assay has

**Table 55.2** Summary of the most relevant testing systems for HCV

| System | Advantages | Limitations | Applications |
|---|---|---|---|
| Human primary hepatocyte | Full HCV replication cycle | Limited access<br>Low yield of replication | Receptor discovery<br>Study of antiviral effects |
| Tupaia hepatocytes | Full HCV replication cycle | Low yield of replication<br>Non-human primate | Receptor discovery<br>Study of antiviral effects |
| Subgenomic replicons | Replication of HCV genome | Absence of morphogenesis | Study of the replication complex<br>Study of antiviral effects<br>Drug resistance studies |
| Full-length replicons | Replication of HCV genome | Absence of viral secretion | Study of the replication complex<br>Study of virus/host cell interaction |
| Baculovirus transduction | Replication of HCV genome | Construction of vectors<br>DNA delivery for RNA virus | Study of the replication complex<br>Finding of new susceptible cells |
| Enzyme assays | Cell-free system | | Discovery of new antivirals<br>Mechanism of action of inhibitors |

been set up due to the complexity of the *in vitro* systems developed. However, existing systems allow the screening of rationally selected molecules.[157]

### *Concluding remarks on enzymatic assays*

All the *in vitro* enzymatic assays described above have been of great use to better understand enzymologic and structural aspects of HCV enzymes, as well as to identify and study inhibitors of these enzymes which could be developed as antivirals. In the near future, this *in vitro* assay could also be very useful to study enzymes encoded by resistant HCV strains. Indeed, with the probable introduction of new antivirals, including anti-protease, anti-helicase, or anti-polymerase agents, it is likely that new patterns of resistance will appear.

Table 55.2 summarizes the most relevant *in vitro* systems developed to study HCV.

## Conclusions

In this chapter, we have summarized the different *in vitro* testing systems that have been developed recently to better understand HVB and HVC biology and identify new targets to develop novel antiviral therapy based on drug combinations, as well as to study clinical aspects of HBV and HCV infection, including viral resistance to antivirals and pathobiology of natural and drug-induced mutants. In the near future, the further development of *in vitro/in cellulo* testing systems, in particular for HCV, will be important to (1) discover new drugs to better combat HBV and HCV infection, (2) monitor and prevent the appearance of mutants, and (3) better understand liver disease induced by these viruses.

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# Chapter 56
# New vaccine technologies and the control of viral hepatitis

Colin R Howard

It is universally accepted that the development of vaccines against hepatitis A and B represent major milestones in the control of viral hepatitis, but the contributions these developments have made in advancing the design of products against other infectious diseases is perhaps less widely recognized. In particular, experience gained over two decades with recombinant hepatitis B vaccines, whether produced in yeast or mammalian cells, has confirmed that such recombinant products are safe and effective. Hepatitis A vaccines were quickly found to be exquisitely immunogenic in humans and deemed so promising in early clinical trials that one hepatitis A vaccine was licensed prior to efficacy trials having been completed. A vaccine against hepatitis E is now on the horizon, and again this will represent a first for vaccine research given the difficulties there have been in developing vaccines against related agents. It is not surprising, therefore, that vaccine researchers investigating new adjuvants, delivery vehicles and vectors turn to viral hepatitis as paradigms where much is known, both at the level of immunological properties but equally importantly how new vaccines can best be assessed and monitored in humans. Increasingly, much of this work is being done by smaller biotechnology companies, pushing back the boundaries and challenging previously cherished concepts in vaccine design. The paradox is the extreme difficulties experienced hitherto in developing a vaccine against hepatitis C virus (HCV), a member of the Flaviviridae family of viruses containing agents such as yellow fever and tick-borne encephalitis, against which effective vaccines have been available for many years. The way forward is most certainly cross-utilization of technology platforms informed by a better understanding of what constitutes immune protection in hepatitis C. This knowledge can be extended to improving the antiviral treatment of patients with chronic infections.

Therapeutic vaccination to complement antiviral treatment of those patients with chronic hepatitis B has become a major goal in vaccine research. Some attempts to modify and adapt existing products designed for prophylaxis have largely been unsuccessful. The considered opinion is that such products need to stimulate a different type of immune response that is focused on class I major histocompatibility complex (MHC)-mediated presentation of peptides derived from the hepatitis B virus (HBV) core antigen, as well as enhancing the level of co-stimulatory cytokines that may directly aid virus clearance from hepatocytes. However, it is likely that – once developed – their availability will facilitate the design of better preventative vaccines, especially for administration to those carriers of either HBV or HVC during the years before the development of clinical disease.

New vaccine technologies are being developed as our understanding grows of the inflammatory events leading to both innate and adaptive immune responses. In particular, vaccine design has begun to focus on the role of dendritic cells (DCs) and the need to bring these into close proximity to the antigen. For example, the oligonucleotide CpG sequences present within DNA plasmids are thought to mediate their effect in part by binding to a Toll-like receptor (TLR-9) on dendritic cells.[1] Other adjuvants may play a similar role in dendritic cell recruitment by recognizing Toll-like receptors on dendritic cell surfaces.[2] Newer adjuvants for peptides and recombinant proteins capable of providing signals for both DC maturation and cytotoxic T-lymphocyte (CTL) stimulation are likely to prove particularly effective. There are considerable hurdles, however, in bringing such technologies to the marketplace, not least of which is the lack of a suitable small animal model for either hepatitis B or hepatitis C. Although chimpanzees have proved invaluable in the past for unravelling the pathogenesis accompanying infection and for demonstrating proof of principle for vaccine candidates, their high cost, limited availability and ethical issues preclude their practical use for screening large numbers of candidate formulations and adjuvants in any meaningful way. The woodchuck and duck hepadnavirus models fill this shortcoming to some degree but are hampered, respec-

tively, by the lack of immunological marker reagents for the woodchuck and the limited nature of the immunopathology in ducks. Mice transgenic for part of or the whole HBV genome have done much to identify possible strategies for therapeutic vaccination, but as with DNA vaccination, encouraging results in rodents are not necessarily mirrored in humans.

This chapter focuses primarily on new vaccine technologies within the context of the prevention and treatment of hepatitis B and C. Hepatitis A vaccines based on more traditional technologies, with recent developments focused on delivering these vaccines in combination with other vaccines, are advances discussed elsewhere in this book. A plethora of alternative vaccine technologies have been proposed as 'third-generation' hepatitis B vaccines and these are reviewed first. Immunization against hepatitis C virus has proved exceedingly difficult, and areas where newer technologies might prove useful are touched upon where appropriate. The last section deals with the increasing interest in applying new vaccine technologies for post-exposure therapy (so-called therapeutic vaccines).

## Can existing hepatitis B vaccines be improved upon?

Despite the clear success of HBV vaccination programmes, there remains much that can be done to improve the vaccines currently in use. For example, one need is increasing the potency of licensed products for those individuals with acquired or natural immunodeficiencies who respond poorly to existing HBV vaccines. The length of time required together with the necessity of utilizing at least three doses is also a major drawback, particularly in poorer regions of the world where access to vaccine is difficult, as well as in the developed world where non-compliance, especially among younger people, is increasingly of concern. It is also possible that vaccines may require modification if HBV variants are found to become more prevalent in the face of developing herd immunity in vaccinated populations, particularly in areas such as Asia where HBV carriage is so high. In addition, the large number of doses that are required annually could cause supply difficulties, as can the need for trained personnel to deliver the vaccine. A final point is that presently licensed products do not contain the complete antigenic repertoire present on the virus particle itself. The inclusion of pre-S epitopes, for example, may enhance vaccine potency and efficacy. For these reasons, there are considerable resources devoted towards improved, 'third-generation' HBV vaccines (the first and second generations are regarded as products obtained from plasma and recombinant DNA technology, respectively).

There has recently been an upsurge in activity directed at new vaccines generally. These novel vaccine concepts offer several opportunities to develop improved HBV vaccines. These include the use of new adjuvants, additional (pre-S) components, synthetic peptides and DNA immunogens. To this list must be added non-viral vectors and mixed immunization regimes, for example, the 'prime-boost' approach,[3] although this work tends to be more focused on the development of post-exposure vaccines, particularly for the immunotherapy of chronic infections.

One concern is meeting the global demand for hepatitis B vaccines, and achieving this at a price that is affordable for less well off countries. In this context, much publicity has been given to the use of plants for the production of virus vaccines, and hepatitis B surface antigen (HBsAg) has been expressed successfully in tobacco and potatoes.[4,5] The utility of plants as alternative sources of antigen is restricted, however, by the low yields in wet weight and the resultant difficulties in purification. The concept of antigen expression in edible vegetables and fruits is driven by the theoretical possibility that such transgenic plants would open up fresh opportunities for the development of oral vaccines, but in practice control of dose, proteolytic sensitivity following digestion and the frequent lack of immunogenicity when many antigens, e.g. HBsAg, are delivered across the mucosal surface of the gut, are all major challenges. The development of systemic immunity by oral immunization requires a detailed understanding of how viral antigens can stimulate specialized lymphoid structures and the eventual expression of Th1- or Th2-dependent immune responses.[6] Unfortunately, this basic information is largely lacking for all hepatitis virus infections, and thus an oral vaccine against HBV or HBC appears unlikely in the foreseeable future.

### Inclusion of pre-S

There is a view that current hepatitis B vaccines could be improved by the inclusion of pre-S domains, the N-terminal extension to the major S proteins found predominantly on the envelope of complete HBV particles. The rationale is that the pre-S region bears the ligand essential for HBV binding to the hepatocyte receptor, and thus antibody to this ligand would induce more potent neutralizing antibodies. In the absence of an *in vitro* assay for neutralizing antibodies, this is difficult to confirm in the manner possible for other viruses, but it is a paradox that the major *a* determinant responsible for inducing a protective antibody is highly conserved across HBV genotypes yet is not thought to play a predominant role in the attachment of virus to host cells. The difficulty has been in expressing HBsAg particles

with stable pre-S sequences, partly due to its sensitivity to proteases. Whereas the complete pre-S region may be required for hepatocyte recognition, there is evidence from animal work that the pre-S2 region alone may have some benefit. For example, Schirmbeck *et al*.[7] showed enhanced clearance of HBsAg from transgenic mice injected with DNA expressing both pre-S2 and S proteins. A substantial improvement in immunogencity for mice is obtained if particles express both S and the complete pre-S sequence,[8] but attempts to mirror such an improvement in humans have proved disappointing. No significant difference in antibody or cellular responses has been found in volunteers found previously to be non-responsive to vaccine containing only S protein.[9] An important observation from the latter study was the finding that many non-responders eventually seroconverted if given one or more additional doses of an existing licensed product. Similar results have been obtained using a vaccine with an additional pre-S component.[10]

## The potential of short peptides

The use of synthetic peptides as potential vaccines is often dismissed, yet recent advances in peptide chemistry coupled to developments in delivery systems for small molecules makes the reality of a peptide vaccine that much nearer. Synthetic peptide vaccines and killed virus vaccines share the problems of poor immunogenicity and the requirement for booster doses. The use of peptides as immunogens has been achieved for some viruses, however. For example, Sarin and colleagues[11] showed that a synthetic peptide immunogen consisting of 30 amino acids from the p17 protein of HIV-1 coupled to keyhole limpet haemocyanin (KLH) and adsorbed onto alum can be delivered to humans without undue toxicity. This peptide immunogen induced a protective cell-mediated immune response as demonstrated by the transfer of vaccinated human peripheral blood mononuclear cells to severe combined immunodeficiency (SCID) mice, followed by protection against challenge with a different strain of virus. The critical issue is protection against challenge in the target species, however. This has been achieved by Langeveld *et al*.[12] who successfully protected dogs against challenge with canine parvovirus using a vaccine containing two synthetic peptides mimicking the N-terminal region of the major capsid protein VP2.

Moynihan *et al*.[13] designed and synthesized a 48-mer amino acid peptide, representing the entire *a* determinant of the HBsAg sequence (*adw2* subtype). The S121/48 peptide sequence was designed to resemble the native *a* determinant as much as possible. In the case of the S121/48 peptide, a T-helper cell epitope is known to be located between amino acids 164 and 167 and so a promiscuous epitope was not added. This sequence is thus worth considering for inclusion in a synthetic peptide vaccine candidate. The design of the S121/48 peptide is supported by previous work by Manivel *et al*.[14] and Mishra *et al*.[15] using a synthetic peptide representing amino acids 124–147 of the HBsAg sequence (*adw*) showing that the peptide contained HBsAg-related antigenic determinants, specifically the *a* determinant. This antigenicity was mostly destroyed in disulphide-reduced analogues. The peptide induced antisera recognizing different HBsAg subtypes in rabbits, and the B-cell epitopes contained within the peptide sequence were found to be dominant in humans. The region of amino acids 148–174 of the pre-S2 sequence is recognized by T cells of seven MHC haplotypes tested and is thought to contain multiple T-cell epitopes that are recognized uniquely by the different MHC haplotypes.[14] Work by the same group on a model polyepitope immunogen, a recombinant polypeptide containing pre-S1, pre-S2 and S sequences, demonstrated the immunogenicity of the polyepitope in different strains of mice and also confirmed the presence of B-cell and T-cell epitopes in HBsAg between amino acids 124 and 147.[16] The S121/48 peptide elicited a sustained anti-peptide antibody response both in BALB/c (H-$2^d$) mice and guinea pigs, when immunized with FCA. Cross-reactive, anti-HBs antibodies were induced. These antibodies were directed against a significant proportion of the conformationally restrained epitope repertoire on the native HBsAg particles. Collectively, these data demonstrate that a long synthetic peptide mimicking conformational and linear epitopes can be produced by chemical synthesis and can be used to induce significant titres of anti-HBs antibodies after a single injection.[13] Peptides are able to induce both immunity and tolerance, however. A single subcutaneous injection of a peptide in incomplete Freund's adjuvant protected mice against challenge with lymphochoriomeningitis (LCM) virus, but repeated injections of the same peptide intraperitoneally resulted in tolerance.[17]

In order to formulate a synthetic peptide immunogen, some form of adjuvant effect is normally required to compensate for the inherent low immunogenicity of small peptides. Adjuvants may also act as delivery vehicles for antigens, resulting in the targeting of antigens to immunocompetent cells. Examples include liposomes,[18–21] virosomes,[22,23] oil adjuvants, small (<10 μm) PLG microspheres and immune stimulating complexes (ISCOMs).[24,25] Compounds such as muramyl dipeptide,[26] cholera toxin,[27] MF59 (an oil-based adjuvant),[29] lipopolysaccharide and certain CpG oligonucleotides[30] act as immunostimulators. Such delivery systems can direct the immune response to synthetic peptides towards either Th1 or Th2 phenotype, dependent upon which is required. In addition, modification of an antigen, for example, coupling of synthetic peptides to KLH, tetanus toxoid[31] or palmitoylation of peptide antigens[32] may improve immunogenicity.

Biodegradable lactide polymers, such as poly(lactide co-glycolide) (PLG) microparticles, have been used extensively as vehicles for targeting antigens to antigen-

presenting cells on mucosal surfaces or after parenteral injection. The microparticles undergo degradation principally by non-enzymatic hydrolysis to lactic and glycolic acid, thus releasing encapsulated antigen. The integrity of the entrapped antigen may play a role in the induction of different IgG subclasses, as may the microparticle shape, the hydrophobicity of the microparticles and the amount of intact antigen.[33] Controlled release, single-dose vaccines can theoretically be made using PLG microparticles of differing lactide contents and injecting them together. Different lactide concentrations affect the rate of hydrolysis of the microparticles, and thus the judicious use of particles in a single injection could be used to induce a sustained immune response from a few days to as long as a year. Moynihan *et al*.[13] have shown that such preparations containing the 48-mer peptide described above (Fig. 56.1) can induce a high titre antibody response in mice directed against the major a determinants. Oligosaccharide ester derivatives of trehalose, a disaccharide, are alternatives to PLG, overcoming some of the stability problems associated with the use of PLG.[34]

## Nucleic acid vaccines

There has been much excitement over the past decade as to the potential of nucleic acid immunogens to circumvent at least some of the shortcomings associated with protein subunit and inactivated whole virus vaccines – most notably, the limited capacity of vaccines other than attenuated products to induce class I MHC responses. CTLs play a particular role in the pathogenesis of HBV and HCV, and thus there is a dogma that vaccines against these infections could be enhanced by the capacity to stimulate both antibody- and cell-mediated immune responses. This is particularly the case for the development of post-exposure therapeutic vaccines, as discussed below. In contrast to protein subunit or inactivated vaccines, the relevant epitopes are expressed intracellularly after the DNA is taken up into antigen-presenting cells. Thus, both MHC class I (CTL-inducing) and MHC class II (antibody-inducing) pathways are stimulated. But DNA immunization offers additional advantages. The response is often longer in duration and the relative titre of the immune response components can be fine-tuned by varying the route of delivery and by including co-stimulatory molecules. Other advantages include use in neonates where immunization results in a response despite the presence of maternal antibody,[35] and such vaccines also have the potential for use in immunocompromised individuals.

DNA vaccines have been found to be effective against a growing number of viral diseases, including HIV, rabies and rotaviruses.[36,37] A number of studies have looked at the potential of this approach for the development of candidate hepatitis C vaccines. Encke *et al*.[38] showed that the injection of mice with a plasmid encoding for the NS3 and NS5 proteins of hepatitis C virus elicited a good class I MHC response.

Safety continues to be a major concern, particularly with regard to the potential for plasmid DNA integration into the host chromosome and the risks associated with endotoxin contamination during the growth of plasmids in gram-negative bacteria such as *Escherichia coli*. There is the additional, albeit theoretical, risk that autoantibodies to host DNA could arise, predisposing recipients to

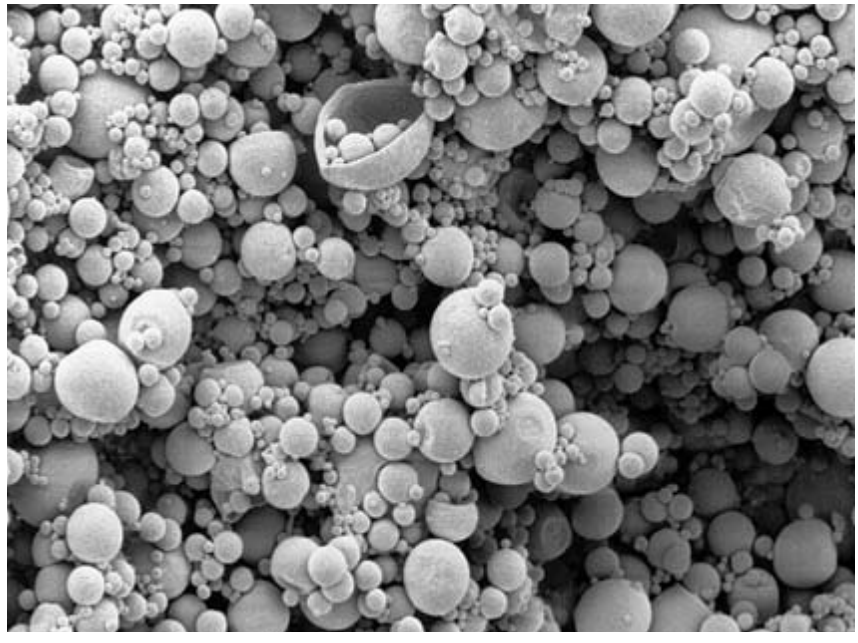

**Figure 56.1** Polylactide-polyglycolide (PLG) microparticles containing a 48-mer peptide of hepatitis B surface antigen. Immunization of mice stimulates high titre anti-HBs antibodies reactive with the major *a* determinant.[13]

autoimmune diseases such as systemic lupus erythematosus (SLE). Very small quantities of DNA can act as an adjuvant, principally due to the presence of CpG motifs, stimulating B-cell responses as well as secretion of interleukin (IL)-6, IL-12, IL-18 and interferon (IFN)-γ.[39]

Muscle cells are particularly efficient in taking up extracellular DNA, with intramuscular injection inducing predominantly a class I-type response leading to activation of virus-specific CTLs.[40] In contrast, epidermal inoculation using DNA bound to gold beads induces a predominantly class II-type response, preferentially inducing specific antibodies.[41] This difficulty can be partially overcome by inducing muscle degeneration in animals, but this is clearly not an option in humans.

Brazolot Millan *et al*.[42] demonstrated the different responses seen in young and adult mice. Mice immunized below 3 days of age with an HBsAg/alum/CpG formulation produced a mainly Th2-type response, whereas older mice produced a mixed Th1/Th2-type response. The experiments also showed the difference between different vaccine types in that immunization of young mice (<7 days) with a DNA vaccine for HBsAg produced Th0-type responses, whereas older mice stimulated the Th1 phenotype. The researchers also noted that the HBsAg/alum/CpG immunizations led to faster and higher antibody responses and stronger CTL responses than DNA immunization. Prange and Werr[43] analyzed the humoral immune response to HBV DNA injected intramuscularly and demonstrated that high levels of antibodies were induced following immunization with the S protein, or S plus pre-S1 and pre-S2 epitopes (with secretion), but not with the L protein or S plus pre-S1 and pre-S2 (without secretion). A note of caution, however, is that the nature of the immune response may differ compared with conventional vaccination as was shown by Mor and colleagues who found that a DNA immunogen coding for the malarial circumsporozoite antigen induced tolerance in neonatal mice.[44]

Immunization with an HBsAg-encoding plasmid with aluminium or calcium phosphate adjuvants has been shown to induce enhanced Th1-type responses over naked DNA.[45] DNA vaccination of woodchucks with constructs expressing the WHV core and surface antigen proteins (WHcAg and WHsAg, respectively) has been shown to partially protect woodchucks against WHV challenge.[46] Cationic polymers are a class of non-viral transfection agents which condense plasmid DNA by ionic interaction. Results generally have been disappointing, although recent studies using poly (2[dimethylamino] ethyl methacrylate, pDMAEMA) derivatives show significant promise for DNA delivery. High levels of anti-HBs antibodies were induced in mice, predominantly of the IgG2a isotype, suggestive of a Th1 type of immune response (Fig. 55.2).[47]

The greatest hurdle to developing a DNA vaccine for human use is the lack of a suitable delivery system. Over 90% of DNA injected either in saline or bound to microparticles is degraded before cellular uptake. Most work that has focused on this issue has involved the use of lipid-based liposomes to protect the DNA from extracellular nucleases.[48] By incorporating specific ligands targeted to specific cells and tissues, it is thought possible to target such DNA–liposome complexes to specific cell types, thereby increasing the efficiency of uptake. For example, the inclusion of epidermal growth factor[49] and the reovirus σ1 protein that interacts with intestinal M cells.[50] There is no reason why such liposome-based delivery systems could not be tailored to the delivery of hepatitis B or hepatitis C genes to antigen-presenting cells and/or hepatocytes.

## The use of viral vectors

The use of viral vectors for delivering immunogenic proteins confers a number of advantages, particularly for stimulating CTL responses. After injection, limited replication occurs, allowing the expression of the gene

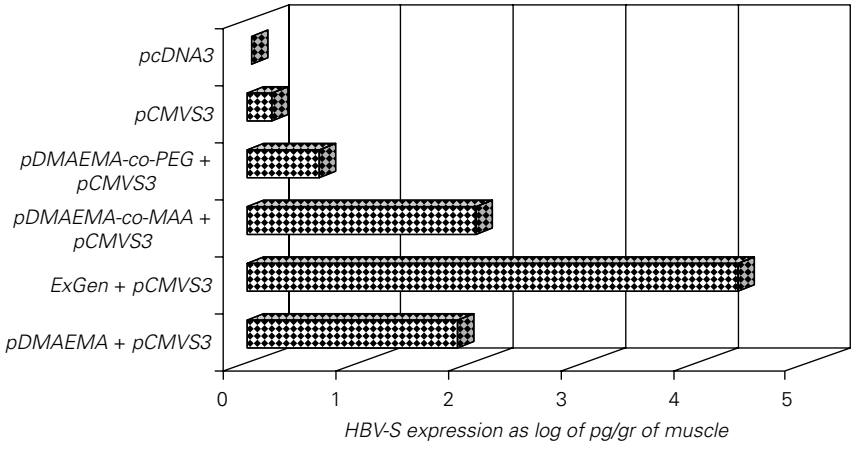


**Figure 56.2** Immune response in mice injected with a DNA construct expressing HBsAg delivered together with polymers of poly-(2-dimethylamino) ethyl methacrylate (pDMAEMA). From Bos *et al*.[47]

product to a level sufficient for the induction of both antibody and cellular responses.[51] Progress has been made with the development of a vaccinia-based recombinant HBV vaccine whereby the HBV S gene is incorporated into the genome of vaccinia virus used previously for the eradication of smallpox. Although adenovirus vectors are an alternative, these are not derived from attenuated parent viruses and thus generate safety concerns. The use of vaccinia recombinants has a number of advantages, e.g. it need be delivered only once, is easy and cheap to produce and offers the opportunity to induce both antibody- and cell-mediated immune responses. However, there are a number of disadvantages, not least of which is the risk of adverse reactions in a significant number of recipients, particularly young infants. Heightened concern as to bioterrorism has revised the perception that vaccinia constructs could be used increasingly as the level of herd immunity to vaccinia declines in the human population.

## Prospects for hepatitis C vaccines

Despite the success of yellow fever and Japanese encephalitis vaccines, hepatitis C vaccine development has proved problematical, largely due to the circumstantial evidence that immunity needs to take account of the variability between isolates, particularly in the so-called 'hypervariability 1' (HVR1) region towards the N-terminus of E2, one of two viral envelope proteins. There is evidence that CTLs can limit infection in humans[52,53] and a multi-specific response has been noted in chimpanzees with resolving disease.[54] Thus, the perception is that a successful hepatitis C vaccine should include a cross-reactive antibody response against a wide number of prevailing subtypes as well as eliciting a strong CTL response to limit the spread of virus that is not neutralized by vaccine antibody.

In contrast to hepatitis B, there appears to be little or no immunity to hepatitis C after exposure,[55] and chimpanzee studies using recombinant E1/E2 heterodimer preparation produced immunity lasting only for a few weeks.[56] Importantly, this study showed that immunity is limited even against challenge to homologous virus. Current thinking is that HCV-specific memory exists and may be at a sufficient level to impart a degree of protection, particularly if the challenging virus dose is of low titre. However, once infected, the virus rapidly disseminates and runs ahead of the host's capacity to mount an effective CTL response. Because of the heavy antigen burden, HCV-specific CTLs become functionally impaired and thus have a restricted capacity to limit or resolve the infection. It is this functional impairment that presents the greatest hurdle to overcome in the design of any therapeutic or preventative vaccine if antibody alone is insufficient to confer protection.[57] There has been very limited progress towards this end, however. DNA immunogens show most promise: Rollier *et al*. have shown that immunization of chimpanzees with a construct expressing all three major structural proteins together with NS3 altered the course of disease on challenge.[58] This study also revealed just how important the quality of the response is to individual conserved epitopes if the correct T-helper response is to be stimulated. This complexity is confounded more by the requirement to ensure the DNA coding for epitopes is of the correct length and construction for optimum expression.[59]

## Therapeutic vaccines

There remains the treatment of those individuals already persistently infected with HBV or HCV. Can the immune system of these persons be stimulated to clear virus before the onset of chronic liver disease? Recent studies in transgenic mice and patients with chronic hepatitis indeed suggest that a therapeutic vaccine delivered many years after first exposure may be effective in reducing, if not eliminating, the virus.[60] Combined antiviral and immunotherapeutic treatments – delivered simultaneously or sequentially – may yet offer an alternative strategy to controlling these diseases which represent a major drain on public health resources in many countries.

Individuals persistently infected with HBV (or HCV) have lost their ability to mount an effective immune response at the time of the acute infection, and the specificity of the missing component plays a pivotal role in recovery. The big question is: what is the relevant antigen to stimulate a class I MHC-mediated response? There is ample evidence of the importance of HBcAg specificities being expressed on the surface of infected hepatocytes, and Hilleman has argued strongly that any strategy must take into account the role of tolerance to HBeAg in maintaining the persistent state, and that progression from mild to severe clinical disease is frequently accompanied by its absence.[61] Put another way, a marker of a successful therapeutic product would be seroconversion to e antigen. Notwithstanding, there remains a view among many investigators that modifying existing prophylactic vaccines may be beneficial.

Perhaps one of the simplest approaches is to combine HBsAg particles with anti-HBs antibodies to form immune complexes, as it is well known that such complexes stimulate both arms of the immune response. HBsAg complexed with anti-HBs antibodies and immunized intranasally in BALB/c mice was found to induce IgG1 antibodies (Th2-type systemic and mucosal responses) when immunized alone or IgG2a antibodies (Th1-type response) when given with cholera toxin or CpG motifs. These results were in contrast to HBsAg delivered intranasally (without adjuvant), where no anti-HBs response

was seen.[62] Antigen–antibody complexes have been shown to be immunogenic in hepadnavirus-infected ducks,[63] and there is some evidence of benefit to patients with chronic hepatitis B.[64] Larger-scale studies are currently underway in China.

The strategy of prime-boosting involves priming the immune system with one vector and then selectively boosting the response to the target antigen by use of a second vector. Particularly powerful levels of synergy can be obtained by first using DNA followed by a vector such as vaccinia or adenovirus.[3] A strategy that exploits the priming capacity of DNA followed by a boost with a vector expressing the homologous antigen is increasingly becoming a focus of attention. There are a number of studies in mice that show priming the response using DNA targeted to hepatitis C antigens followed by peripheral injection of a recombinant vector produces sustained and high titre systemic responses to the immunizing antigen.[65,66] An indication of the utility of the prime-boost approach is the work of Pancholi and colleagues who showed in chimpanzees chronically infected with hepatitis B a >400-fold decline in the levels of HBV DNA with coincident increase in the level of IFN-γ.[67] Several human trials are currently underway focusing on the use of prime-boost strategy for malaria and HIV.[68,69]

A number of adjuvants have been shown to enhance CTL responses to injected proteins, but the magnitude of class I responses obtained in humans generally is disappointing. A number of cytokines, particularly IL-2, IL-12 and granulocyte macrophage colony-stimulating factor (GM-CSF), have been tested in patients as part of trials to evaluate anti-tumour vaccines, but such approaches are limited owing to the relatively short half-life of cytokines when given as recombinant proteins. The effects are more prolonged when the relevant gene is given together with the gene coding for the target antigen in the same plasmid construct, as was shown by Barouch and colleagues[70] who found long-lasting CD4+ and CD8+ CTL responses to SIV-gag protein in rhesus monkeys injected with a DNA construct that included the sequence for IL-2.

The best small animal models for hepatitis B are mice transgenic for the HBV genome, although the discovery that chimpanzees can recognize HLA-A2- and HLA-B7-restricted HBV epitopes gives hope for the eventual testing of potentially therapeutic vaccines for HBV in higher primates.[71] Several groups have exploited the use of transgenic mice for evaluating DNA immunogens. Both class I and class II MHC responses can be induced, but antibody alone is insufficient to achieve long-term clearance of the antigen and IFN-γ-secreting T cells are required.[72,73] Chisari and colleagues have shown that passive transfer of CTLs or their cytokines TNF-α, IFN-γ and IL-2 clear HBV DNA from the nuclei of murine hepatocytes, with a concomitant cessation of nucleocapsid formation and a reduction in viral RNA transcription.[74,75] These findings inform the process of developing therapeutic vaccines, and it seems as a result that any successful immunotherapeutic for chronic hepatitis B will need to include one or more of these cytokines, or at least the genes coding for these modulators.

Hui *et al*.[76] have demonstrated the induction of humoral and cytotoxic immune responses in 57/BL6 mice, and clearance of circulating HBsAg in transgenic mice, following immunization with a plasmid which coded for a pre-S1-derived peptide, fused to the C-terminus of HBsAg.[76] Efficient processing of an antigenic sequence for presentation by CD8+ MHC class I molecules depends upon the neighbouring residues in the protein, and the degree of protection against lethal viral disease is related directly to the amount of naturally processed antigen peptides.[77] As antibody is protective against HBV, induction of CD8+ T-cell responses is not essential for protection. However, the importance of CTL responses in clearing chronic HBV infection suggests that if a peptide were to be designed for therapeutic HBV vaccination, then inclusion of correct flanking sequences bordering the CTL epitope would be important. Data suggest that a synthetic CTL epitope can be made into a vaccine which is immunogenic and which promotes a protective Th1-type response. This has been achieved by coupling a 10-mer CTL epitope from herpes simplex virus (type 1) to a 15-mer region from β2 microglobulin through a GGG spacer. The construct is known as a ligand epitope antigen presentation system (LEAPS™) and is injected with a non-phospholipid surfactant liposomal adjuvant.[78] Two examples of the use of HBV peptides for therapeutic vaccination are the use of short, linear peptide PADRE (Pan DR helper T-cell epitopes) constructs to break CTL tolerance in a transgenic mouse model[79] and immunization with an HBV core epitope-containing lipopeptide to induce CTL responses in chronically infected humans with a variety of HLA-A2 types.[80] This construct, although stimulating a good CTL response in humans, proved to have little efficacy in patients with chronic hepatitis B.[81]

## Conclusion: the vision

The deployment of vaccines against infectious diseases is undoubtedly one of the major medical achievements of the last century. Despite the overwhelming success of hepatitis B vaccines, an extensive reservoir of infection will remain for at least the next 25 years owing to virus persistence in a small, but significant number of those infected, especially early in life. Although claims of near 100% efficiency are made for existing products, only a few per cent escaping immunization or being non-responsive is all that is required for the infection to persist within a community where HBV is endemic. Thus,

the ideal prophylactic vaccine is one that relies on fewer doses, gives rapid seroconversion against all the antigenic repertoire of the virus, and immunological memory that extends at least beyond the years where exposure is most likely to induce a chronic persistent infection. Vaccine technologies already exist that go some way to meeting these criteria, but increasingly severe regulatory hurdles are likely to prevent manufacturers from pursuing such developments, especially as the market for the products is in the main in the economically disadvantaged regions of the world. The newer technologies also rely on the bringing together of individual components or delivery vehicles each subject to separate patenting and licensing arrangements, adding further complexity to the development progress. However, the drive to use such technologies for the more economically attractive market of treating patients with chronic hepatitis is likely in the longer term to benefit the future development of preventative vaccines, especially for those individuals identified as carriers of the virus ahead of developing clinical disease. Therapeutic vaccination is an often-discussed concept but as yet has not produced significant advances in the clinic. This may be largely due to our lack of understanding of all the immunological process that triggers the disease state as much as a lack of the right technology platforms. A more realistic first goal is the reduction of the virus burden to the level where there is distinct patient benefit rather than the elimination of the virus completely. How this is achieved may depend on designing the right synergy of antiviral compound and immunotherapeutic agent. Quite apart from the scientific challenges, newer vaccines are likely to be more complex in terms of formulation, giving rise to additional regulatory hurdles, especially if different products are to be used at various times during the immunization process.

These issues are compounded by the issue of hypervariability in the instance of hepatitis C. Despite extensive efforts, progress towards hepatitis C vaccines is frustratingly slow. Among the other flaviviruses of humans, dengue shows a number of parallels in that vaccine development has been hampered by the presence of four distinct serotypes and the fear that immunity to one may exacerbate disease if a recipient is then exposed to another. Hepatitis C may well be the first infectious disease where therapeutic vaccination becomes a reality before the availability of a prophylactic vaccine: post-exposure treatment focuses on stimulating CTLs that have broader serotype reactivity than is the case for antibody responses. Advances in the design of delivery systems for drugs are likely to be increasingly relevant for combined antiviral and immunostimulatory molecules. Ultimately, the wish is for new products to be delivered orally but this dream is probably some way off as yet. Notwithstanding, the vaccine industry has undergone a dramatic revival in the last decade, with smaller companies being prepared to meet the challenges of vaccine design. It is the challenge for investors, public funding bodies and regulatory authorities to ensure that such effort is translated into the clinic.

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# Chapter 57
# Safety of hepatitis B vaccines

Arie J Zuckerman

## Introduction

Concerns about vaccine safety have increased substantially as the incidence of vaccine-preventable diseases is reduced by the increasing use of effective vaccines and the success of immunization programmes. True adverse vaccine reactions and those associated with immunization only by coincidence are reported frequently and with enthusiasm on the Internet, by the media and by anti-vaccination groups. Indeed, some scientists approach the popular media before or simultaneously with publication. If the hypothesis relates to vaccine safety, whether or not there are valid supporting data, the issue frequently becomes a topic of public debate before scientific investigation could possibly generate data which support or reject the hypothesis, and important public health programmes can be damaged. Even if the hypothesis is disproved, the public has already seen images of damaged children supposedly linked to the vaccine and the issue may be exploited by anti-vaccination groups, liability lawyers and certain elements of the media who thrive on this type of reporting.[1] When scientific data become available, the 'news worthiness' of established facts is lost and they remain unreported by the media.

Review of the literature on clinical studies and trials of hepatitis B vaccines, and the results of post-marketing surveys indicate that serious clinical adverse events directly related to immunization were recorded very rarely. Most adverse reactions reported include minor symptoms at the site of injection, temporary malaise, fatigue, headache, nausea, rash, influenza-like symptoms, dizziness, arthralgia, myalgia, urticaria, paraesthesia and drowsiness.

Nevertheless, there are allegations of at least three serious disorders resulting from, or associated with, hepatitis B vaccination:

- sudden infant death syndrome,
- chronic fatigue syndrome, and
- multiple sclerosis.

There is no evidence (reviewed below) of a causal link between immunization against hepatitis B and these conditions. The features common to recent vaccine scares have been summarized extremely well by Professor Neil Halsey, Head of the Institute for Vaccine Safety at Johns Hopkins University, Baltimore as reported by Jefferson.[2]

- A causal link is usually claimed with a disease or condition of unknown or unclear aetiology.
- The association is claimed by one investigator or a group of investigators.
- The association is not confirmed by peers or by subsequent research.
- The claims are made with no apparent concern for potential harm from public loss of confidence and refusal to vaccinate children (and adults).

In addition, the findings of subsequent studies that fail to confirm the original claim never get the publicity given to the 'original finding', and thus the public never receives a balanced view.[3]

Note that over one billion doses of hepatitis B vaccine were used between 1981 and 1998 with an outstanding record of safety and efficacy.[4]

## Hepatitis B vaccine and sudden infant death syndrome

Although allegations have been made that hepatitis B immunization of infants causes sudden infant death syndrome (SIDS), these allegations did not gain any credibility, as outlined by Harold Margolis before the US House of Representatives on 18 May 1999 as follows:

> 'Allegations have been made that hepatitis B vaccination of infants causes Sudden Infant Death Syndrome (SIDS). Because almost 10 million doses of hepatitis B vaccine are administered to infants each year, some infants will die shortly after vaccination by coincidence alone. Available scientific data do not support any causal role of vaccination in the deaths. In fact, in 1992, the first full year after the hepatitis B vaccine was first recommended universally for infants, there were 4800 SIDS deaths and hepatitis B vaccination coverage was 8%. In contrast, by 1996, when coverage had risen to 82%,,

the number of SIDS deaths had actually decreased to 3000 deaths. These data are reassuring because if hepatitis B vaccines were a major cause of SIDS, we would have expected an increase in SIDS, not a decrease. SIDS deaths have continued to decrease as a result of the effort to change infant sleep position, despite a marked increase in hepatitis B vaccination coverage'.[5]

## Hepatitis B vaccine and chronic fatigue syndrome (post-viral fatigue syndrome or benign myalgic encephalomyelitis)

In 1991, the Canadian press reported on the possible association between immunization against hepatitis B and chronic fatigue syndrome. Chronic fatigue syndrome is a non-specific condition of unknown aetiology and there is no objective test to confirm the diagnosis, which is made, in part, by excluding other diseases.

The reviews by the Bureau of Communicable Disease Epidemiology and by the Working Groups of the Laboratory Centre for Disease Control, Canada are referred to frequently. The Working Groups met in September 1989 and in November 1992 to consider a suggested possible link between hepatitis B immunization and chronic fatigue syndrome. The Working Group which met in 1992 examined the data collected on 30 self-reported cases of chronic fatigue syndrome, meeting a standard case definition, and concluded that there was no evidence of causal association between hepatitis B vaccination and chronic fatigue syndrome.[6,7]

It is understood that from August 1987 to June 1995, some 7 million doses of the recombinant yeast-derived hepatitis B vaccine (Engerix B) were distributed in the UK and Ireland, and that 10 reports of chronic fatigue, post-viral fatigue syndrome or myalgic encephalomyelitis were made in temporal association with vaccination. A causal link between the vaccine and the symptoms was not established.

There are suggestions in the literature that immune complexes are responsible for neurological symptoms during viral hepatitis, and that a similar mechanism may pertain to immune complexes which may be formed between antigen present in the vaccine and antibodies elicited by immunization. There are no published laboratory data to support this hypothesis. However, it has been noted that although hepatitis B surface antigen–antibody complexes have been found in biopsies from several organs in patients with hepatitis B with neurological symptoms, these complexes have not been found in nerve tissues. Vasculitis has been shown in the epineurium of sural nerve biopsies from such patients.[1]

A DNA sequence homology between the encephalogenic site of myelin base protein and an 8 amino acid site of hepatitis B virus DNA polymerase has been demonstrated in experimental allergic encephalitis in rabbits. But this observation may not be relevant to the higher primates which are susceptible to hepatitis B virus. A search of the recombinant surface antigen gene of hepatitis B reveals no homology between human albumin, myelin basic protein and other central nervous system (CNS) proteins.

Indeed, it was concluded that among the millions of people worldwide who acquire acute hepatitis each year, few have neurological symptoms, which suggests that access of hepatitis B virus to the central nervous system must rely on host-dependent or environmental factors.[8]

## Hepatitis B vaccine, multiple sclerosis and other demyelinating diseases

In 1996–1997, articles in the French popular press and television programmes raised concerns that hepatitis B immunization may be linked to new cases or flare-ups of multiple sclerosis (MS) or other demyelinating diseases, following a report of primary demyelinating events within 8–10 weeks of immunization against hepatitis B using a recombinant vaccine at a hospital in Paris.[9,10] On 1 October 1998, the French Ministry of Health decided to suspend routine hepatitis B immunization of adolescents in French schools, while continuing the immunization of infants and high-risk adults.

However, CNS demyelinating diseases and MS are believed to have multifunctional pathogenesis with the contribution of both genetic and environmental factors. The age distribution of the onset of disease and migration studies suggests that exposure to an environmental agent in early childhood or adolescence contributes to the pathogenesis of MS with a 10–15-year interval to the onset of disease. The geographical incidence and prevalence of hepatitis B are the opposite of those for MS, with the highest rates of MS and the lowest rates of hepatitis B in Scandinavia and northern Europe, while sub-Saharan Africa and Asia have the lowest rates of MS and the highest rates of hepatitis B virus infection. If the virus does not cause MS, it is unlikely that the vaccine which is prepared from the surface protein of the virus could do so.

Post-marketing surveillance (reports of adverse events following vaccination) from the USA were examined in 1987 and 1996, and from Canada in 1992. No increased rates of MS or other neurological diseases were found compared to the background. Similar results were obtained in France and in other countries.

The results of an official pharmacovigilance study on the neurological tolerance of all hepatitis B vaccines available in France were presented to a National Commission in December 1994 and in December 1996. The data showed that over 60 million doses of hepatitis B

vaccine were distributed in France between January 1989 and December 1996, and a total of 106 case reports of CNS and peripheral nervous system demyelinating diseases were notified in vaccine recipients. The notification rate of demyelinating diseases in temporal association with hepatitis B immunization was approximately 0.6 cases per 100 000 vaccinated subjects. This rate is significantly lower than the expected incidence of demyelinating disease in the same population which, for MS alone, is 1–3 cases per 100 000. The epidemiological patterns of the notified cases were similar to those expected in a similar non-vaccinated population in terms of age distribution, gender ratio and the nature and severity of neurological symptoms. The time interval between vaccination and the occurrence of neurological symptoms was random.[11]

Observations in other countries (Australia, Belgium, Canada, Germany, India, the UK and the USA) show similar patterns to those observed in France of 0.1–0.8 cases of demyelinating disease per 100 000 vaccinees.[11]

The findings of two more recent large-scale studies have also shown no significant association between hepatitis B vaccination and the development of multiple sclerosis. A case-crossover study in patients included in the European Database for Multiple Sclerosis who had a relapse between 1993 and 1997 showed that there was no increase in the specific short-term risk of relapse associated with hepatitis B vaccine.[12] The second study in a large population of women studied in the USA for many years also provided solid evidence that there was no relation between receiving hepatitis B vaccine and the development of multiple sclerosis.[3]

## Conclusions

No vaccine is completely safe and no system of immunization is completely free of human error.[13] Nevertheless, hepatitis B immunization of the population has been introduced into National Immunization Programmes in over 150 countries and well over 1000 million doses of hepatitis B vaccine have been used. The evidence to date is that the licensed hepatitis B vaccines are the safest vaccines ever introduced for the protection of the public.

Anecdotal, provocative and unfounded stories stressing temporal associations with immunization continue, currently in relation to hepatitis B vaccines and diabetes mellitus, rheumatoid arthritis and other autoimmune diseases. However, only in a few rare cases has autoimmune pathology been firmly associated with particular vaccines;[14] for example, polyradiculoneuritis in the 1976–1977 vaccination campaign against swine influenza, and idiopathic thrombocytopenia after the administration of the measles-mumps-rubella vaccine (MMR). It is undoubtedly becoming increasingly difficult to persuade a sceptical public (and compensation lawyers) of the safety and enormous success of immunization programmes in reducing or eradicating infectious diseases. To that end, the establishment, as a priority, by the World Health Organization of the Immunization Safety Priority Project to introduce a comprehensive system to ensure the safety of all immunizations given in national immunization programmes, is a valuable strategic development – strengthening the position that protective immunization is one of the safest of health interventions.

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# Chapter 58
# Before and since the discovery of Australia antigen: a chronological review of viral hepatitis

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## Introduction

Infectious jaundice was known to Hippocrates, and its clinical and pathological features had already been accurately described by the end of the 19th Century. However, its varied aetiology led to much confusion up to the end of the first half of the 20th Century, and full clarity has only emerged within the last 15 years with the discovery of the main agent of 'non-A, non-B' hepatitis. The turning point, though, was undoubtedly the discovery in 1965 of Australia antigen and its subsequent association with hepatitis B virus (HBV). This was an advance which, as John Enders observed, refreshed hepatitis studies 'like an unexpected shower on desert soil'. It ushered in a series of serological, virological and molecular findings that have since borne fruit in the form of effective vaccines and the first antiviral treatments against chronic hepatitis.

To understand how hard it was to investigate infectious hepatitis before Australia antigen was discovered requires an exercise of the imagination. No specific tests existed and the rapid virological advances in other fields from the 1930s to the 1960s, for example the culture of many mammalian viruses in fertile eggs and tissue culture, or on cell monolayers, all passed the human hepatitis viruses by. The very existence of hepatitis viruses was at that time inferential, and there was no evidence for there being more than two types, whereas five at least are now known. There was, nevertheless, a wealth of clinical and epidemiological data to draw on, as summarized by F.O. MacCallum in his opening address to the International Hepatitis Symposium that took place in Toronto in 1971.[1] It was MacCallum who, 25 years before, had proposed the A and B nomenclature for the two putative hepatitis viruses then recognized.

In briefly reviewing infectious hepatitis before the discovery of Australia antigen, we have taken four vantage points – 1900, 1920, 1940 and 1960 – and, using those milestones, have picked out the discoveries that show the way through that confused period. Then, in the latter part of this chapter, we describe the discovery of Australia antigen and the many developments that flowed from it. After that discovery, clinical and human volunteer experiments became less important as new opportunities, first serological and then molecular in nature, arose. These have brought about spectacular diagnostic and prophylactic successes, and they may soon lead to effective suppressive and even curative treatment for the chronic forms of viral hepatitis.

## A 1900 standpoint

In 1900, what is now understood to be viral hepatitis was seen as a single disease, 'catarrhal jaundice', said by the influential pathologist, Virchow, to be the result of inflammation at the biliary–duodenal junction. This was contrary to observations by, for instance, Richard Bright who had written as early as 1836 that jaundice could be due to 'inflammatory action of the liver [that] occurs in its substance … producing a gradual change in the texture of the liver'. Nevertheless, following the 'bacteria craze' of Koch and his followers in the 1880s,[2] jaundice was usually attributed to biliary obstruction secondary to a specific, even if not yet identified, bacterial infection. At first a salmonella was proposed as the cause, later (see below) a hepatotropic leptospira. The term 'catarrhal jaundice' reflected the idea of an obstructive process, and in the 19th Century the occurrence of catarrhal jaundice was generally regarded as sporadic. This was in spite of the large outbreaks observed during military conflicts, for instance in the American Civil War and the Franco-Prussian War. Recovery from catarrhal jaundice was usual, but acute yellow atrophy of the liver was a rare and usually fatal complication, first described by the Viennese pathologist Rokitansky, in 1842.

At the turn of the 19th Century, research in Cuba by the Reed Commission showed that yellow fever – a particular form of acute hepatitis in which other systems were also involved – had a distinct incubation period following exposure to an infected mosquito and was caused by a 'filterable agent' i.e. a virus.[3] At the time, however, no analogy seems to have been drawn between this and catarrhal jaundice, presumably because the clinical aspects of yellow fever were so acute and its mortality so much higher. The clue that catarrhal jaundice might also be caused by a filterable agent was therefore ignored. Instead, most researchers followed the Japanese-American researcher Noguchi in erroneously ascribing all forms of jaundice (including, it should be added, yellow fever) to leptospiral infection, and so accepted the rather implausible implication that leptosira could wriggle through filters that withheld other bacteria.

Other physicians, meanwhile, were reluctant to recognize that catarrhal jaundice, which they encountered mostly as a sporadic condition, was an infection at all. The idea that a proportion of most infections (not least infectious jaundice) occurred subclinically, making it hard to recognize chains of infection, had yet to develop.[4] And even when clusters of cases of jaundice were recognized, false conclusions were drawn about sources, incubation periods and routes of infection. With long incubation periods and so many non-icteric illnesses involved, successive cases of catarrhal jaundice that might point to its infectious nature were hard to recognize.

## A 1920 standpoint

In 1912, E.A. Cockayne published an article on catarrhal jaundice that reveals how what is now commonly known as infectious or viral hepatitis was conceived of at that time.[5] Cockayne was a young physician who subsequently became paediatrician to the Great Ormond Street Hospital for Children, London, and his review stemmed from work for his doctoral thesis. He was perhaps the first to use the term 'infectious hepatitis', one which did not gain general currency until the 1940s.

Cockayne distinguished between acute, generally benign, jaundice in the community and the more severe form of jaundice with nephritis first described by Weil in 1886. The causative leptospira of the latter disease were first observed microscopically by Japanese researchers in 1914, and this same group went on to culture *L. icterohaemorrhagica* in 1916.

Cockayne, in his review, drew an instructive analogy between infectious hepatitis and mumps. This shows that he had turned his back on Virchow's theory that catarrhal jaundice was, as its name would suggest, the consequence of biliary stasis. Instead, he believed that the jaundice was, as Bright and a few others had previously suggested, due to an inflammation of the liver itself. For Cockayne the analogy was clear. Just as mumps was an inflammation of the salivary glands, catarrhal jaundice was an inflammation of liver parenchyma. He commented: 'the great rarity of gastro-intestinal trouble with jaundice in epidemics and the early appearance of tenderness over the liver also suggest that the liver is attacked primarily'. Cockayne also mentioned approvingly several authorities who had speculated that catarrhal jaundice in its sporadic and epidemic form were both the same disease, and at that point made an insightful comparison with the sporadic and epidemic forms of poliomyelitis, another viral disease in which subclinical infections made clinical discovery of chains of infection difficult, if not impossible.

Plenty of room for error remained, though. Had Cockayne pursued his analogy with mumps he might have realized that the incubation period of infectious hepatitis in any of its forms is measured in weeks not days. Instead, he offered a clinical description of his own case of infectious hepatitis which he assumed that he had caught from his housemaid who had become ill two days previously. With hindsight we know that that imputation was unjust! Cockayne was also of the view, in common with most authorities up to the 1940s, that infectious hepatitis (in contrast to Weil's disease) was transmitted by the airborne route. On the other hand, he did perceive that for practical purposes patients became non-infectious once jaundice had appeared. Cockayne's peroration is worth quoting:

> 'I regard catarrhal jaundice as a specific disease due to an unknown organism endemic over a wide area and appearing not uncommonly in restricted and, very rarely, in widespread epidemics. If the view of its pathology which I have suggested is correct [i.e. the analogy with mumps] the name "infectious hepatitis" would more accurately express the condition than 'catarrhal jaundice'.

Cockayne's paper also includes a section on acute yellow atrophy of the liver which 'in nearly every instance I consider to be a sequel to [catarrhal jaundice] … Its great frequency in women is well known and it occurs in the proportion of two women to one man … due to its special incidence in pregnancy'. In addition, Cockayne refers to the association of jaundice with syphilis which was first being seen at about the time of his review. He correctly related this to intravenous anti-luetic treatment.

The First World War brought a resurgence of 'campaign' jaundice. Dr (Lt Col) C.J. Martin wrote:

> 'During Autumn 1915 troops at Gallipoli, and to a lesser extent in Egypt, suffered from a nearly non-fatal form of infectious jaundice, usually without complete obstruction of the bile passages, and not associated with any tendency to haemorrhages. [It] appeared to be markedly infectious; some units

had 25% of their strength affected and many cases occurred amongst patients in hospital for other complaints'.[6]

Martin's paper was valuable for several reasons. It disposed of the idea that infectious jaundice was due to '*Bacillus typhosus*'. It reported on the post-mortem examinations of three soldiers who died in the course of jaundice, and it challenged Willcox and Hurst's repetition of the concept of catarrhal jaundice. Martin wrote:

'it seems to me unfortunate that in the absence of definite knowledge of the aetiology of the disease these gentlemen should thus light-heartedly throw the weight of their authority in favour of one possible interpretation of its pathology and method of spread …, I submit … we are dealing with a hepatitis following a systemic infection'.

Among the reasons Martin gave for this were that: (1) jaundice is ushered in by a febrile attack like influenza; (2) jaundice does not occur for some days and is preceded by swelling and tenderness of the liver; (3) the spleen is often enlarged; (4) histologically, there is inflammation around the portal areas and necrosis of liver cells. Martin's paper also contains a lone reference to the idea that epidemic jaundice in its mild form (i.e. not Weil's disease) might be due to a pathogenic agent 'invisible like that of yellow fever', an insight offered to him in 1915 by an otherwise unidentified 'Professor Kartulis'.

## A 1940 standpoint

By 1940, many clues had been uncovered as to the diversity of infectious hepatitis, although orthodox medical opinion held fast to the view that catarrhal jaundice was a single disease. An old guard still remembered Noguchi's leptospiral theory, and not until 1939 did the technique of percutaneous liver biopsy provide proofs that catarrhal jaundice was an inflammation of the liver parenchyma and not of the biliary tract, and that it was not bacterial in nature.[7]

By 1940, also, two sets of epidemiological evidence had emerged that were in retrospect very significant. The first was due to clinical observations such as those by W.N. Pickles on catarrhal jaundice in Yorkshire, England. Pickles carefully documented incubation periods in a rural community outbreak of jaundice. During four cycles of infection in an extended family group between August and December 1929, the incubation of cases of jaundice was always close to 28 days (Table 58.1).[8] By his close observation of jaundice in the community, Pickles also confirmed Cockayne's opinion that during the latter part of the illness patients were non-infectious, adding: 'It is those who are mildly affected and able to carry on their usual avocations that are mainly responsible for spread'.

By contrast, there was a second quite different presentation of jaundice in clinics where patients were treated for syphilis by intravenous infusions or injections of arsphenamine ('salvarsan').[9] This drug was given weekly for 7, 10 or even more weeks, often with inadequately sterilized needles and syringes. The associated jaundice showed a longer and more chaotic incubation period than the 28 days observed by Pickles. The contrasting sets of observations on what were to prove different forms of viral hepatitis were later confirmed by experimental means in US Army volunteers in the mid-1940s (Fig. 58.1).

By 1940, it had also been noted that intravenous therapies and other medical interventions in which plasma or serum might be exchanged between humans carried the risk of what came to be called 'homologous serum jaundice'.[10] Measles prophylaxis with convalescent sera, vaccines against smallpox and yellow fever to which human serum had been added to stabilize them, and transfusions of blood and plasma (in particular pooled plasma), all carried the risk of jaundice. The associated wide range of incubation periods observed in homologous serum jaundice had a median of between 2 and 3 months in contrast to the regular 4-week intervals between cases of 'catarrhal' jaundice described by Pickles in a community setting.

The impact of viral hepatitis in debilitating the combatant armies during World War II is hard to exaggerate.

**Table 58.1** The incubation period of epidemic catarrhal jaundice: note the regular intervals of 4 weeks between cases reported by Pickles[8]

| Patient | Date of onset (1929) | Remarks | Interval (days) |
|---|---|---|---|
| N | 16 August | Young woman, source of infection not traced | 29 |
| O, P | 14 September | Brother and sister of N | 27 |
| Q | 11 October | Brother of N, O and P | |
| R | 12 October | Young woman, a great friend of the family | 27 |
| S | 7 November | Sister of N, O, P and Q | |
| T | 6 November | Little girl, inseparable from the family | 28 |
| U | 4 December | Fiancé of S (contact of N) | |
| V | 2 December | Two-year-old sister of T | 27 |
| W | 29 December | Father of T and V | |

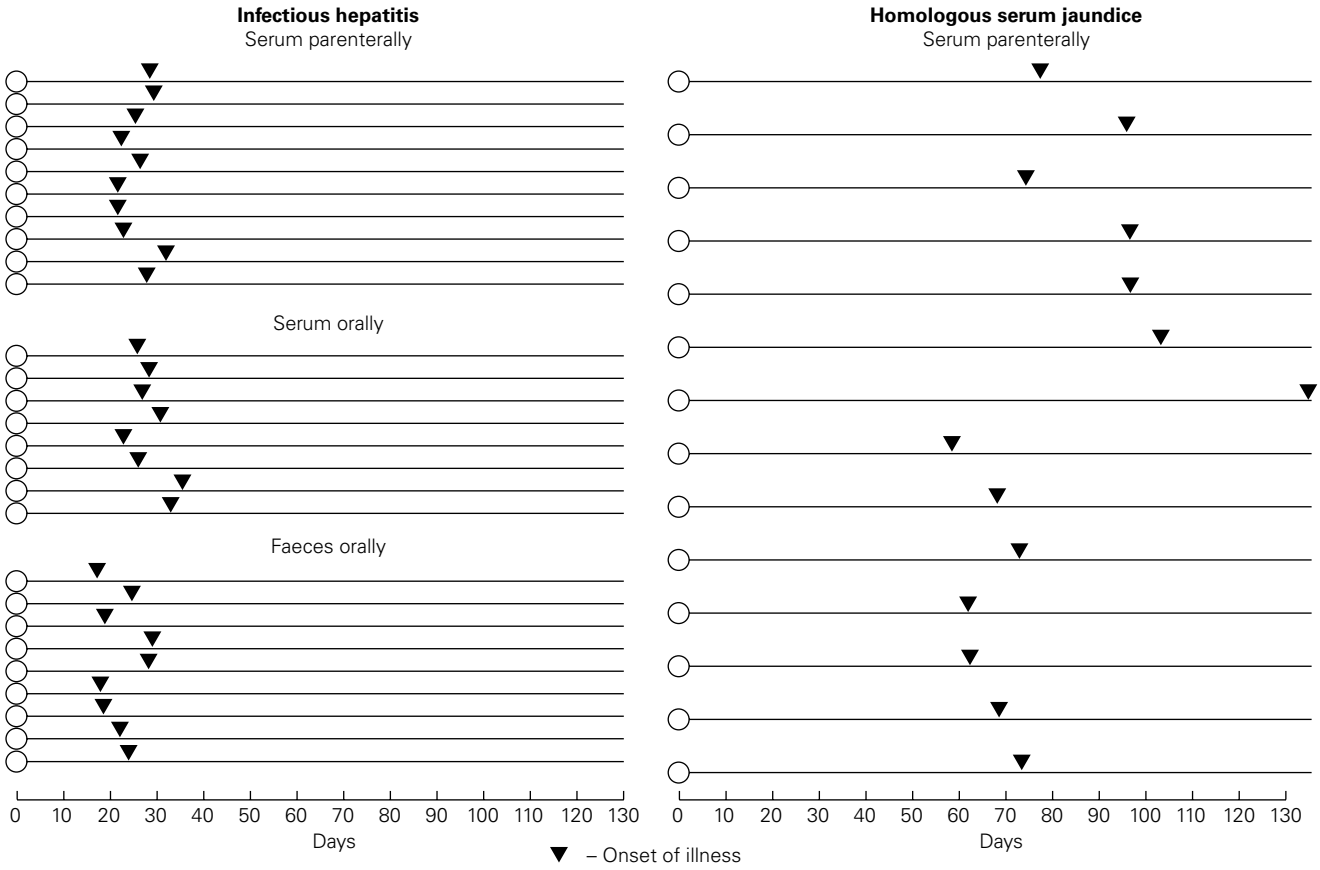


**Figure 58.1** A comparison of incubation periods to onset of hepatitis for US volunteers given either serum or faecal suspensions from cases of infectious hepatitis, or injected with serum from cases of 'homologous serum jaundice'.

Although 'campaign' jaundice had been described in successive wars since the 18th Century, and the tens of thousands of cases seen in World War I had greatly compromised battlefield effectiveness, the allied armies of World War II were ill-prepared when the same phenomenon recurred. This is what L.D. Heaton, US Surgeon General, wrote in his foreword to the official history of 'Preventive Medicine in WWII':

> 'Hepatitis furnished the most unexpected, and perhaps the most serious, problem of all the diseases discussed in this volume. Although this was an "old and ugly camp follower", neither its enormous incidence nor its serious potentialities had been foreseen by any medical authorities before the war. During 1943, in the midst of hard combat in the North African theater, medical officers were suddenly confronted with … hepatitis, the infectious variety, whose victims filled to overflowing the hospitals just to the rear of the combat area'. This came fast upon the "bombshell" of epidemic serum hepatitis, caused by the use of icterogenic human serum in certain lots of yellow fever vaccine'.[11]

It was a situation that both confirmed the existence of two main forms of viral hepatitis and greatly boosted research into them. The occurrence of nearly 50 000 cases of jaundice after the ill-conceived programme of yellow fever vaccination of US troops in early 1942,[12] plus the high incidence of jaundice in battlefield conditions, and the heavy toll of jaundice and acute yellow atrophy following pooled plasma transfusions for injuries to combat troops,[13] were the stimuli for important observational[14] and military volunteer[15,16] studies. These studies addressed various conflicting theories current at the time about the nature of infectious hepatitis:[11]

> '(1) That it was a contact disease spread by droplet infection and that measures conceivably useful in the control of respiratory disease should be considered; (2) that it was transmitted through the agency of human excrement and spread primarily through human carriers of the virus, as is typhoid fever; (3) that some unknown, extrahuman factor, such as rodents, insects, or pigs, was responsible for its spread; (4) that the disease was an example of bacterial warfare; and (5) that it was due to or made worse by malnutrition and inadequate diets'.

Together with British studies in conscientious objectors between 1943 and 1946, the studies on volunteers by the US Army medical teams finally established that

there were at least two forms of infectious hepatitis, A and B, that there was no cross-immunity between them, that the A virus was spread not by the respiratory route but by the faecal-oral route, that the B virus was present in icterogenic serum and was often carried indefinitely by individuals who were themselves usually healthy and not jaundiced, and, finally, that Cohn's recently described cold ethanol method for fractionating human plasma pools isolated an immunoglobulin fraction which, when given intramuscularly, protected fully against exposure to the A and partially against the B virus. This immunoglobulin fraction was, moreover, not itself icterogenic.

These volunteers had made an extraordinary contribution to the elucidation of hepatitis, as was graciously acknowledged: 'The contribution made by these volunteers to the welfare of humankind was great, and no discussion of their participation in human research should fail to take cognizance of their courage and generosity'.[11]

At the same time as humans were being exposed experimentally, laboratory investigations into hepatitis were intensified. Attempts to culture virus in embryonated eggs and tissue culture were renewed and (following the description in 1928 of the rhesus monkey model for yellow fever by Stokes, Bauer and Hudson[17]) transmission of putative hepatitis viruses to all manner of monkeys and other animals was attempted.[18] None of this work was successful, and it unfortunately led to the erroneous conclusion that human hepatitis viruses could not infect experimental animals, even primates. It was to be another 20 years before that potentially fruitful line of investigation was renewed through the work of Deinhardt, Purcell and others.

## A 1960 standpoint

In the years following World War II, hepatitis research again suffered from competition from those branches of virology where success was more easily won. The huge wartime epidemics of infectious hepatitis were over and the disease had mostly assumed again its sporadic character. It was a time when attempts to grow hepatitis viruses in monolayer cell cultures repeatedly failed and when the mistaken belief that these viruses could not be transmitted to primates was rarely challenged.[19]

However, a new incentive to continue the quest for hepatitis viruses emerged in the form of the growing range of medical interventions found inadvertently to transmit hepatitis (Table 58.2). Hepatitis proved to be a serious complication of multi-transfusion and of renal dialysis. Furthermore, from the late 1960s, the new blood products prepared from pooled plasma such as factor VIII concentrate almost universally transmitted hepatitis.[20] This was a phenomenon which in retrospect should have been anticipated from what had already been learnt in wartime and later of the dangers of plasma transfusion. For instance, Lahane[21] had shown that large-pool (at least 300 donations) plasma prepared before 1945 gave rise to jaundice in 11.9% of recipients, whereas plasma prepared from fewer than 10 donations gave rise to jaundice in only 1.3% of recipients. (From this study in the Liverpool region, it was possible to estimate that the hepatitis carrier rate in the donors was 0.35%.)

**Table 58.2** Medical procedures found to transmit hepatitis

| Procedure | First described |
|---|---|
| Arm to arm smallpox vaccination | 1885 |
| Syphilis therapy with arsphenamine | 1917 |
| Insulin therapy and associated glucose estimations | 1925 |
| Measles prophylaxis with convalescent serum | 1936 |
| Serum stabilization of yellow fever vaccine | 1937 |
| Mass yellow fever immunization of US servicemen | 1942 |
| Blood transfusion | 1943 |
| Mass therapy for schistosomiasis, yaws | 1950s |
| Renal dialysis | 1960s |
| Treatment with anti-haemophilic factor concentrate | 1975 |

Hepatitis was also endemic in institutional settings where faecal-oral contamination, scratching and biting might occur,[22] and any intervention seemed as difficult as the case post-transfusion jaundice. For want of specific virological tests, it was hard to study the efficacy of immunoglobulin prophylaxis in this institutional context and apparently impossible to develop vaccines. However, the empirical use of a heated MS2 serum as a vaccine by Krugman and Giles, first reported at Toronto in 1971,[23] was able to anticipate this advance. As Krugman foresaw, the first generation of hepatitis B vaccines would be in the form of a heat-inactivated clarified plasma collected from healthy carriers. Krugman's experiments were a bold initiative that was to bring great benefit, although their design and context have since become mired in ethical controversy.

Meanwhile, the wide range of epidemiological associations of HBV that is now taken for granted – sexual and mother-to-child transmission, the relationship to liver cancer – was still largely unsuspected. This was in spite of there having been in the 1940s occasional recorded instances of secondary spread, e.g. to sexual partners,[12] some evidence of hepatitis in infancy and reports of long-term risk of cirrhosis following acute hepatitis. Acute yellow atrophy of the liver or fulminant hepatitis also occurred as a feared and ill-understood phenomenon.

**Table 58.3** Early landmarks in the understanding of viral hepatitis

| |
|---|
| The Reed Commission of 1901–1902 (yellow fever due to a 'filterable agent', i.e. a virus) |
| Stokes, Bauer and Hudson's description of a model of clinical yellow fever in the rhesus monkey (1928) |
| Pickles defines the incubation period of community hepatitis (1942) |
| *In vivo* liver biopsy findings consistent with a viral aetiology for hepatitis (1943) |
| Ministry of Health memorandum describes 'homologous serum jaundice' (1943) |
| Fractionation and viral inactivation necessary for blood product safety (1940s) |
| Realization of the importance of needle and syringe hygiene (1950s) |

In summary, then, this 'dark age' of hepatitis leading up to the ground-breaking description of Australia antigen in 1965 contained unanswered questions but landmark discoveries, some of which are listed in Table 58.3. Some other observations and conclusions of that era were false and became obstacles to understanding that later had to be cleared away.

## Up to 1980 (the discovery of HBV and HAV)

That the last four decades of hepatitis research have proved so much more productive than the previous ones is ascribable to the discovery of Australia antigen, the subsequent rapid development of serological tests for hepatitis B, the use of immune electron microscopy, the many experimental transmission studies in non-human primates and, most recently, the application of molecular biology to the whole, disparate, group of human hepatitis viruses.

Before considering these advances, the immediate post-World War II period must be dealt with. It was the wartime studies in Germany, the British Mandate of Palestine, the UK and, most extensively, the US (reviewed by Zuckerman[24]) that first established the varied viral aetiology of hepatitis. The post-war studies at the Willowbrook State School in New York then greatly extended knowledge of its prophylaxis.

The number of children at the Willowbrook State School, an institution for children with learning disabilities, increased between 1949 and 1963 from 200 to over 6000, and from 1953 viral hepatitis was endemic among them. Many hundreds of cases of viral hepatitis with jaundice were observed to be transmitted in this institution by natural contact, and most of the newly admitted children contracted the infection within the first 12 months. The decision to artificially expose these children to the Willowbrook strains of hepatitis virus was reached after consideration of many factors.[25] For instance, it was almost inevitable that children would become infected with hepatitis in the institution, and induced viral hepatitis was observed to be mild in the 3–10-year age group. These studies were carried out with optimum isolation facilities to protect the inoculated children from other infectious diseases such as shigellosis and parasitic and respiratory infections that were also prevalent. Controlled exposure to hepatitis conferred immunity to infection and thus, over a period of 12 years, approximately 250 children participated in experimentally induced hepatitis with parental consent and in accordance with the contemporary ethical approval of local, state and federal agencies. The studies were also in compliance with the World Medical Association's Draft Code of Ethics on Human Experimentation.

The work at Willowbrook confirmed that there were at least two distinct epidemiological, clinical and immunological types of hepatitis circulating in the institution. One, induced by MS-1 serum, closely resembled hepatitis A, and a second, induced by MS-2 serum, resembled hepatitis B. There was no cross-immunity between MS-1 and MS-2. Persistence of infection, i.e. a carrier state, occurred in some cases. A most important observation was that the inoculation of diluted MS-2 serum heated at 98 °C for 1 minute was not infectious and provided immunity against challenge. This laid the foundation for the later production of a plasma-derived hepatitis B vaccine consisting of inactivated, highly purified, 22-nm hepatitis B surface antigen particles. The serum bank of >25 000 serum specimens obtained from both naturally acquired and experimental infections became a valuable source of material for the study of the natural history of both hepatitis A and hepatitis B.

Meanwhile, in the laboratory, hepatitis studies had reached a turning point as a result of a largely serendipitous discovery, that of hepatitis B surface antigen. It came about as follows. 'Polymorphism' is defined as the occurrence in the same habitat of two or more inherited forms of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation. In polymorphic traits, two or more of the genotypes determining variation of the trait are common in the population. Polymorphisms arise as a result of selective differences between genotypes, and they provide convenient systems for the study of discontinuous inherited biochemical variations in man. Such systems include the red blood cell antigens (ABO, MNS, P, rhesus, etc.), sickle cell haemoglobin, haptoglobin, transferrin, glucose-6-phosphate dehydrogenase deficiency and gamma globulin groups.

On this basis, in 1961, Allison and Blumberg[26] argued that as donor blood was commonly only matched for the major red blood cell antigens before transfusion, patients would be likely to receive blood containing other proteins that they had not themselves inherited. Some of these would be antigenic and lead to the development of antibodies in the transfused patients. A systematic investigation of the serum of transfused patients was therefore begun, using the two-dimensional micro-Ouchterlony immunodiffusion technique to visualize lines of precipitation of immune complexes.

After examining 13 sera from frequently transfused patients in the centre well and a panel of sera from different geographical areas in peripheral wells, one transfused patient's serum was found often to react with a precipitin present in some of the sera in the panel. It was soon demonstrated that this patient's serum acted as an antiserum identifying a system of inherited antigenic specificities of low density beta-lipoproteins, designated the Ag system. A search for additional antisera was initiated and in 1963 two sera from multiply transfused American haemophilia patients were found to give an identical precipitin line with 1 of 24 sera in a test panel. The antigen in this one serum contained little or no lipid and clearly differed from the Ag precipitin, but it stained with azocarmine, indicating that protein was a major component. The reactive serum had been obtained from an Australian Aborigine and so the antigen was named 'Australia' antigen.[27]

Subsequent studies of the distribution of Australia antigen in normal populations in different geographical areas revealed that it was rare in North American and European communities, but occurred frequently in the serum of apparently healthy people living in the tropics and south-east Asia (6–25%). Because the antigen was found frequently in the serum of patients with acute leukaemia, it was suggested in 1965 that the presence of Australia antigen might be of value in the early diagnosis of leukaemia. It was further postulated that the antigen might be related to a virus causing leukaemia. Evidence was also presented that Australia antigen was inherited, probably as a simple autosomal recessive trait. A corollary of this hypothesis was that individuals at high risk of developing leukaemia might have an increased frequency of Australia antigen. As if to corroborate this theory, up to 30% of patients with Down's syndrome, who were known to have a high risk of leukaemia, were found to carry Australia antigen.

In 1966, Blumberg observed a patient with Down's syndrome who on initial examination did not have the Australia antigen, but in whose serum the antigen was found on a subsequent test. Liver function tests and a liver biopsy revealed that this patient had developed anicteric hepatitis concurrently with the appearance of Australia antigen in his blood. Then, in April 1967, a technician working on Australia antigen in Blumberg's laboratory developed malaise, loss of appetite and dark urine. Australia antigen was detected in her blood for a single day and she subsequently developed a mild icteric hepatitis.[28] Sera of other patients with viral hepatitis were examined for Australia antigen and it was present in 10.4%.

In 1968, a strong association was observed in Japan between Australia antigen and post-transfusion hepatitis,[29] and subsequent studies established that Australia antigen was actually the lipoprotein surface antigen of hepatitis B virus (HBsAg). Thus, by 1970 it had become possible to screen blood donations for HBsAg with an immediate impact on the incidence of post-transfusion hepatitis. Within 5 years of the description of Australia antigen, huge benefit had emerged from what had begun as an academic interest in the genetics of serum precipitins.

A second line of investigation involved electron microscopy (EM). Although viruses were among the first objects to be examined by EM in the 1930s, little information had been obtained on their fine structure except in the case of bacteriophages. Then, in 1959 a negative-contrast technique for high-resolution EM was introduced. In this method particles such as viruses are suspended in a rigid electron-dense material, e.g. a uranium salt. This offers good preservation of the biological material with minimum distortion of structure. In the technique of immune electron microscopy (IEM), to increase sensitivity and specificity, virus particles are concentrated within an immune complex by the addition of an antiserum.[30] Bayer and colleagues in 1968 were the first to examine serum specimens rich in Australia antigen by IEM.[31] These specimens were obtained from three patients, one with acute myelogenous leukaemia, one with chronic reticuloendotheliosis and one with Down's syndrome and chronic hepatitis. Negative-contrast staining revealed spherical particles with a diameter of 19–21 nm and surface knob-like subunits about 3 nm in diameter. Elongated particles varying in length from 50 to 230 nm were also observed. The particles had been aggregated by the addition of rabbit antibody raised against Australia antigen. Then, in 1970, the complete HBV virion (Dane particle), measuring about 42 nm in diameter with a 28-nm core, was visualized.[32]

In 1973, IEM was also used to identify hepatitis A virus (HAV) in faecal extracts obtained before illness or during the acute phase from adult volunteers who had been infected orally or parenterally with the MS-1 strain.[33] The availability of HAV antigen in faeces collected in the pre-icteric phase also allowed the development of specific serological tests for hepatitis A, and for immunity to it. Soon afterwards, it was shown by the same group that the virus could be propagated through long incubation in monolayer cell culture.

Meanwhile, following the lead given by the Willowbrook experiments, the first hepatitis B vaccines were prepared, derived from the plasma of healthy carriers of Australia antigen (by then designated HBsAg). These vaccines, consisting of highly purified and inactivated 22-nm surface antigen particles, were licensed for general use in 1981 and 1982. Yeast-derived hepatitis B vaccine produced by recombinant DNA technology followed and was licensed in 1992. This has since been introduced into national immunization programmes in over 150 countries.

As a result of the successful propagation of HAV, as a virtually non-cytopathic infection of various monkey and semi-continuous human cell lines, inactivated hepatitis A vaccine became available in Europe in 1991, and then in the USA and elsewhere in 1995. These vaccines are whole virus preparations from HAV grown in cell culture and then formaldehyde-inactivated. The first combined inactivated hepatitis A and B vaccine was licensed in 1997.

In 1977, another nuclear antigen had been detected, by immunofluorescence, in hepatocytes of patients with chronic hepatitis B infection. This antigen resembled the core antigen of hepatitis B in its cellular localization and was always associated with hepatitis B infection, but it rarely co-existed with the core antigen. The antigen was named delta, and patients with the antigen develop specific anti-delta antibodies.[34] In 1980, delta antigen was recognized as a component of a novel defective virus that requires HBV co-infection for its replication. The delta hepatitis virus (HDV) has HBsAg as its virus coat and, as its core, delta antigen and a circular single-stranded RNA genome. In its genome structure and its replication HDV resembles certain subviral agents of plants, especially viroids.

## Since 1980

As the specific diagnosis of types of viral hepatitis increasingly became possible, it was apparent that there must be at least one other important form, unrelated to hepatitis A, B or D, that was transmitted by the parenteral route. This was referred to as non-A, non-B or NANB hepatitis. During the 1980s, this became the object of intense research efforts, both epidemiological and experimental.

Results from several carefully conducted epidemiological surveys of post-transfusion hepatitis in the United States and elsewhere provided strong evidence of the transmission of NANB hepatitis, and it was found to be universally present in a proportion of blood donors. Indeed, in the many countries where all blood donations were already by 1980 being screened for HBsAg by sensitive techniques, NANB hepatitis accounted for some 90% of cases of post-transfusion hepatitis. Outbreaks of NANB hepatitis, often subclinical, were also associated with treatment with batches of blood clotting factor VIII and IX concentrates. NANB hepatitis occurred in haemodialysis and other specialist units, among intravenous drug users, and after accidental inoculation with contaminated needles. (Mother-to-infant transmission of NANB hepatitis has only occasionally been reported.)

Some NANB cases were apparently not associated with transfusion or drug use, and these sporadic cases of NANB hepatitis accounted for up to 25% of all adult patients with clinical viral hepatitis. Thus, in many cases of NANB hepatitis the mode of transmission of this type of hepatitis could not be identified. The illness was in general mild, anicteric or even subclinical, but severe hepatitis with jaundice did also occur and NANB hepatitis accounted for some fulminant hepatitis. In many patients, the infection was followed by prolonged viraemia and a persistent carrier state.

Transmission studies in chimpanzees established that the main agent of parenterally acquired NANB hepatitis was likely to be an enveloped virus, 30–60 nm in diameter. These studies also made available a pool of plasma which contained a high titre of the main NANB agent which was cloned in 1989 and thereafter referred to as hepatitis C virus (HCV).[35] The genome of HCV resembled those of the pestiviruses. Hepatitis C virus has six closely related but distinct genotypes with differing geographical distribution. The degree of divergence apparent within the viral envelope proteins implies the absence of a broad cross-neutralizing antibody response to infection by different HCV isolates, and these may co-exist in the same patient. There is no evidence for non-pathogenic HCV variants.

The successful cloning and expression of portions of the HCV genome permitted the development of diagnostic tests.[36] The 5-1-1 antigen was the first to be detected, by antibodies in the serum of an infected patient, and it was an obvious candidate as the basis of an ELISA to detect the presence of HCV antibodies. Then a larger clone, C100, was assembled from a number of smaller overlapping clones and expressed as a fusion protein in yeasts, facilitated by the use of human superoxide dismutase sequences. The C100 fusion protein was widely used as the antigen for first-generation tests for anti-HCV. However, this first-generation ELISA was associated with a rather high rate of false-positive reactions when applied to low incidence populations, and also in the retrospective studies done on stored sera. Second- and third-generation ELISAs, which included antigens from the nucleocapsid and further non-structural regions of the HCV genome, proved more specific and sensitive, and immunoblot tests served further to assure specificity. Polymerase chain reaction (PCR) amplification confirmed the presence of HCV viraemia and quan-

tified it as a means of monitoring the success of antiviral treatment.

Epidemics of enterically transmitted non-A, non-B hepatitis have often occurred in countries in Asia and Africa where sanitation is suboptimal. Transmission is by the faecal-oral route, commonly via contaminated water supplies. A virus, hepatitis E, was first identified in 1983 in a volunteer study[37] and was subsequently cloned in 1989. The virus genome resembles most closely in sequence those of rubella virus and beet necrotic yellow vein virus. While man is the natural host of hepatitis E virus (HEV), swine strains have been identified and are able to cross the species barrier and infect humans. In endemic areas, antibodies to HEV have been found in 42–67% of domestic farm animals, and there is some evidence of infection in rodents, suggesting that HEV may be a zoonotic infection.

## Epilogue

In 2005, viral hepatitis remains a major public health problem throughout the world. Effective vaccines are available against hepatitis A and hepatitis B, and trials of a vaccine against hepatitis E are in progress. However, only modest progress is being made in developing a vaccine against hepatitis C. In all cases, antiviral therapy is proving difficult, although progress is being made, especially against hepatitis C. Meanwhile, infant vaccination against hepatitis B is having a huge impact on carrier rates of HBV in adolescents and young adults, mitigating the anticipated burden of chronic disease in the countries of south and south-east Asia and elsewhere. This is an achievement in international public health that rivals the global eradication of smallpox in 1978.

Other blood-borne viruses have recently been identified, including the GB viruses (in particular GBV-C), the Torque Teno viruses (TTV) and SEN virus and TTMV, but they are not specifically associated with hepatitis. There may be still other non-A, non-B, non-C, non-D, non-E hepatitis viruses awaiting discovery, but it appears that their pathogenicity is minor. Therefore, by a combination of vaccination and antiviral treatment, the long-term prospect of bringing viral hepatitis under control worldwide is now realizable. This goal should not be lost sight of through lack of imagination and/or parsimonious funding, as historically the clinical consequences and economic costs of viral hepatitis have made it one of the most important of all infectious diseases.

## Acknowledgement

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# Index

Note: Page numbers followed by 'f' refer to figures and those followed by 't' indicate tables.

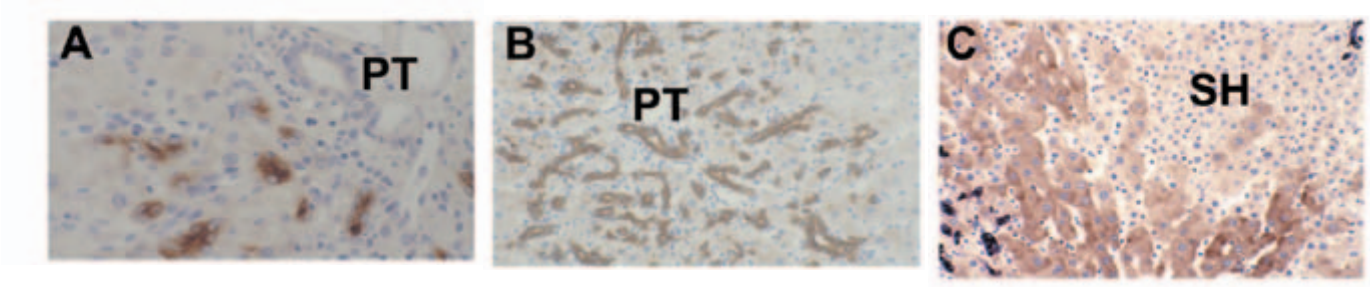



**Plate 1.1** Oval cell behaviour in the rat liver treated by the AAF/PH protocol. (A) AFP expression (IHC staining) is typically observed in the migrating oval cells; note the absence of staining in the interlobular duct in the portal tract (PT). (B) The oval cell response can be visualized by CK8 immunostaining, with cords of cells emanating from the portal tract (PT). (C) At later times, the cords of oval cells differentiate into small hepatocytes (SH) but with a notable lack of CYP immuno-expression (brown staining). Note the occasional residual oval cell ductules expressing CK19 (purple staining).

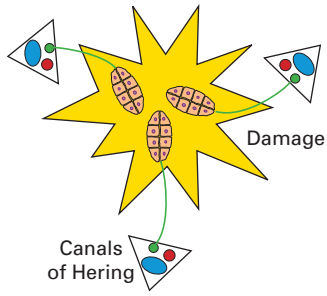



**Plate 1.2** The canals of Hering (green) extend from the portal areas into the proximate third of the hepatic lobule in the human liver (see Theise *et al*[33]), and major hepatocyte damage activates the lining cells to divide and probably differentiate into hepatocytes.

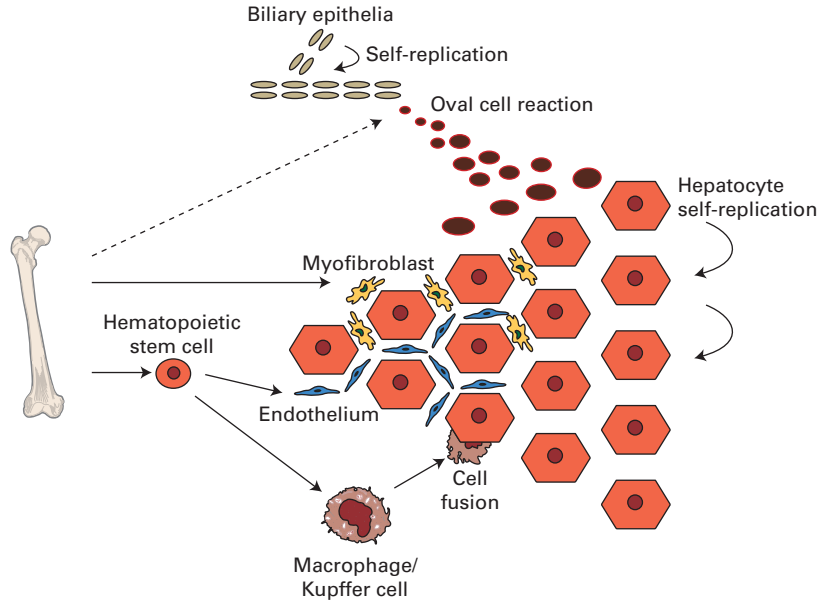



**Plate 1.3** Most hepatocyte regeneration comes from self-replication. This pathway is efficient and can replace the great majority of hepatocytes under most circumstances. Under conditions of regenerative stress such as fulminant hepatic failure or later stage chronic hepatitis, a second axis of hepatic regeneration comes into effect. The so-called oval cells or hepatic progenitor cells develop from the terminal branches of the intrahepatic biliary tree. They probably differentiate into hepatocytes by passing through an intermediate hepatocyte stage. There is currently controversy as to whether oval cells can be supplied from the bone marrow. Previous reports of bone marrow cells transdifferentiating into hepatocytes may in fact represent fusion of bone marrow-derived macrophage lineages with mature hepatocytes. The bone marrow does supply non-parenchymal cells to the liver; inflammatory cells and Kupffer cells traffic from the bone marrow to the liver. The bone marrow is also able to supply the hepatic endothelium and a proportion of the hepatic myofibroblasts.

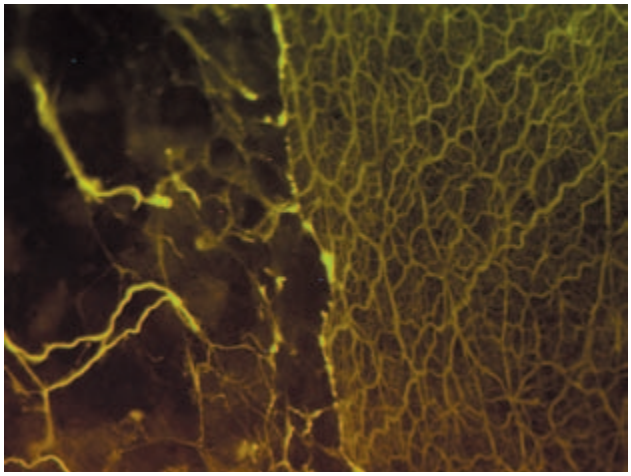
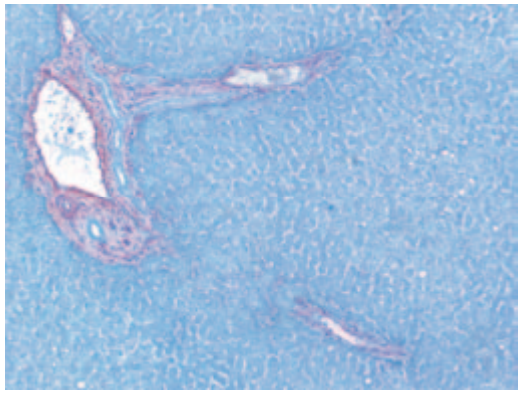


**Plate 2.1** Collagen type I investing the capsule and invaginating to support portal triads

(a)



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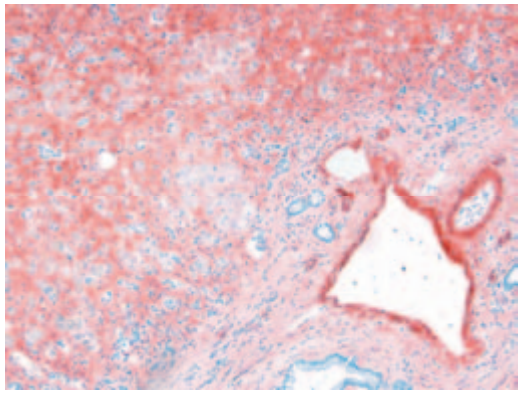
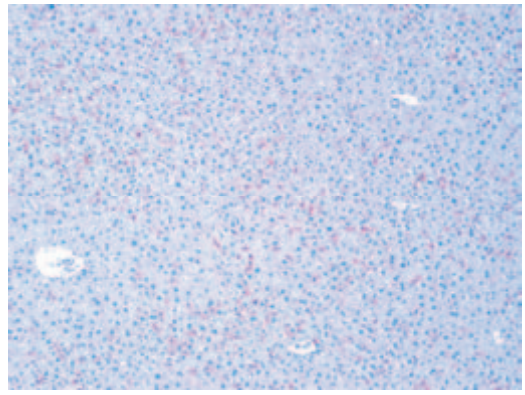


**Plate 2.2** Collagen type 4 normal (a) vs cirrhotic (b) magnification 100x ("capillarization" of the sinusoids).

(a)



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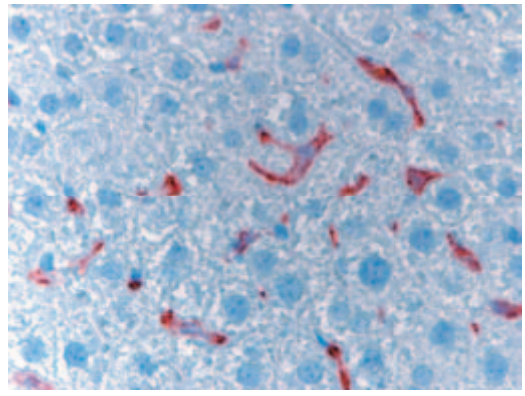
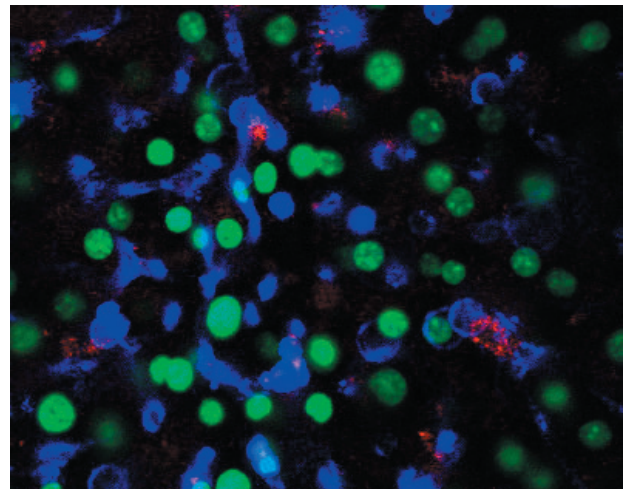


**Plate 2.3** Kupffer cells (F4/80) in mouse showing architecture: 100x (a) and 600x (b).

(a)



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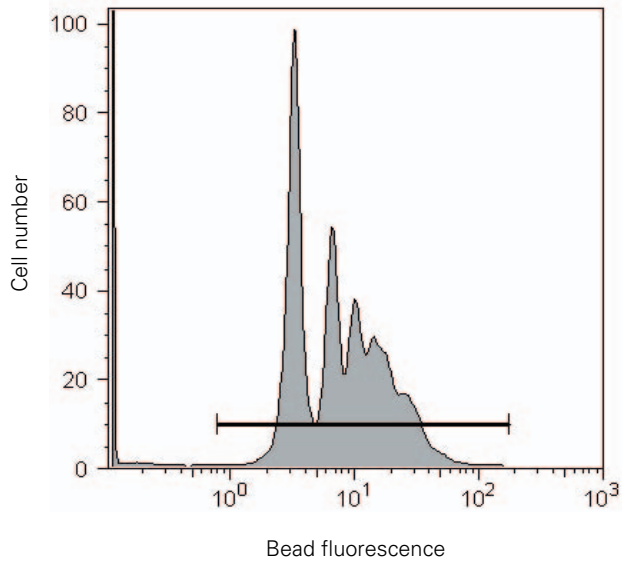



**Plate 2.4** (a) Mouse liver status/post injection of fluorescent beads (red, fluorescent nanospheres; blue, F4/80; sytox green nuclear stain). (b) Flow grapgh showing cells with phagocytosed beads.

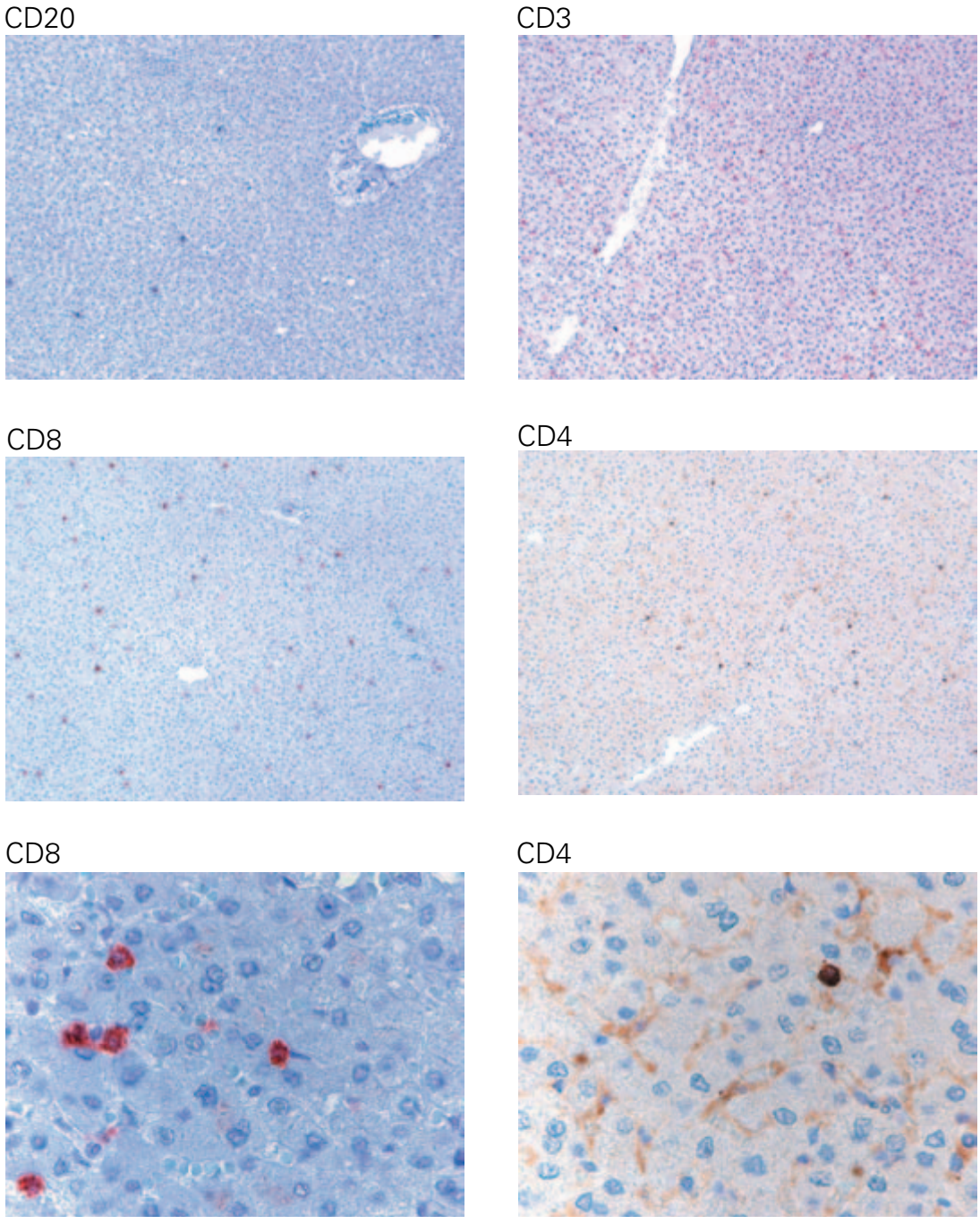



**Plate 2.5** Normal human liver immunohistochemistry. CD20, CD3, CD8 and CD4 at 100x magnification; CD8 and CD4 at 600x magnification.

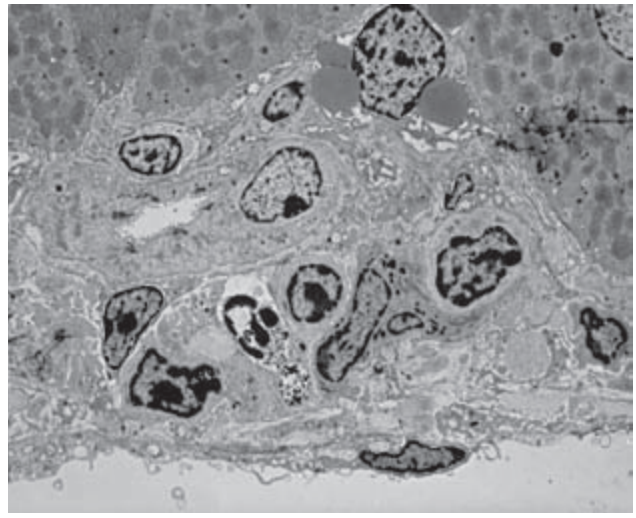


**Plate 2.6** Transmission Em of inflammatory focus (OT-1 model) in subendothelial space of Disse.

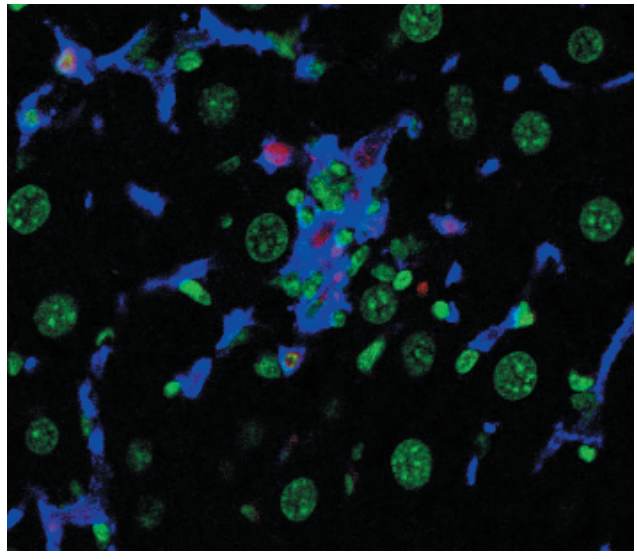
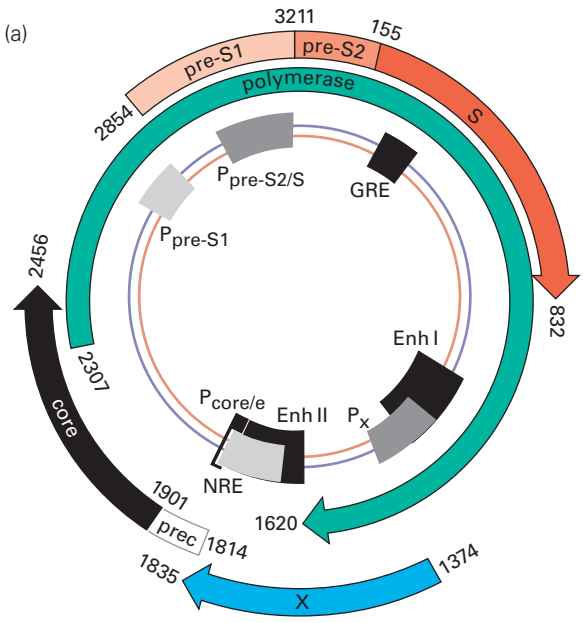
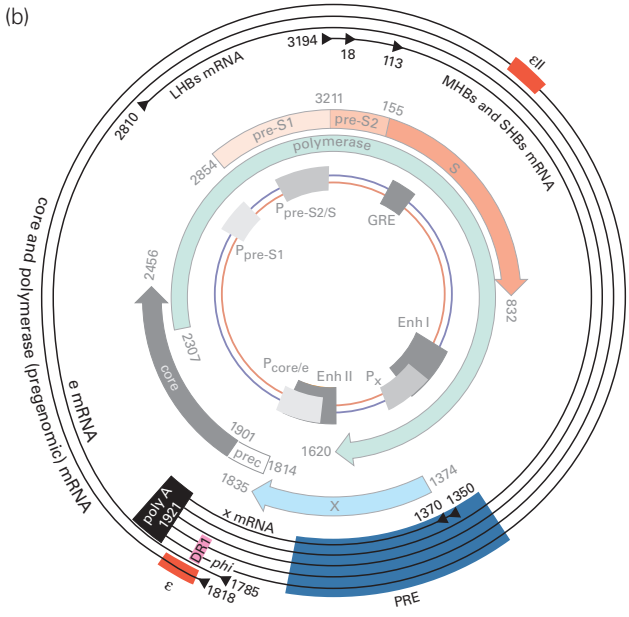
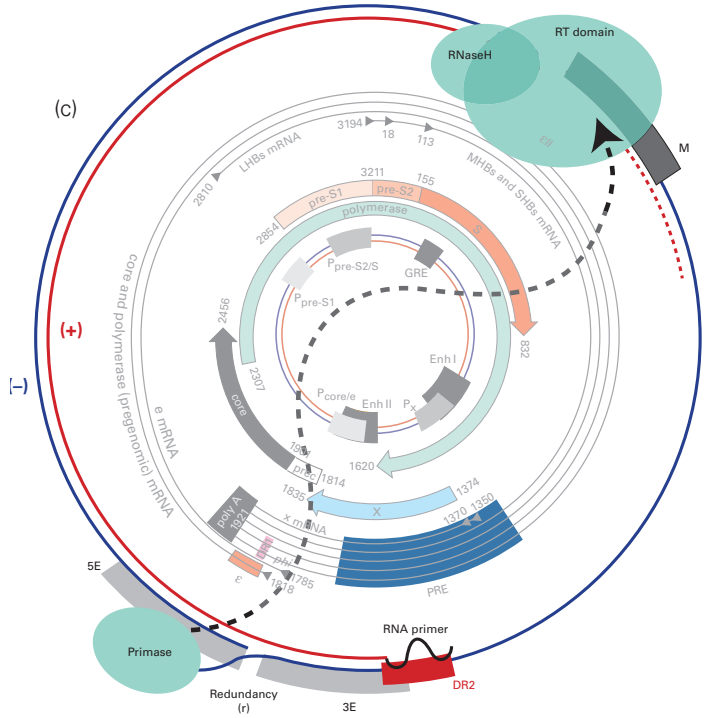


**Plate 2.7** Chronic hepatitis in association with AAV: TUNEL, F4/80, sytox green.

**Plate 10.1** (a) Genome organization and regulatory elements on HBV cccDNA. The numbering is according to the HBV isolate 991 (HBs subtype adw2, genotype A) starting at the unique EcoRI site. All known complete HBV genomes are circular and between 3181 and 3221 bases long. The negative strand is depicted as a blue line, the positive DNA strand as a red line in the centre. The ORFs shown here are defined by the first start codon of protein synthesis and the first stop codon. Some in-frame internal start codons are also shown. The longest ORF, with its four contiguous domains, encodes the polymerase polyprotein of HBV. Nonconserved ORFs or ORFs of uncertain function are not shown. The promoters are shown as grey boxes, the enhancers: the negative regulatory element (NRE), and the glucocorticoid responsive element (GRE) as black boxes. The CCAAT element overlapping the S promoter is not depicted. Liver-specific promoters are drawn in light grey; obligatorily used promoters are depicted as mid-grey boxes. Transcription of the cccHBV DNA is governed by enhancers I and II, the glucocorticoid response elements (GRE) and the promoters upstream of the mRNA start sites. The common termination signal for all mRNAs is the TATAAA box at base 1921.
(b) HBV mRNAs and their signal elements. The initiation sites of mRNA synthesis for the various HBV proteins are shown as triangles. All HBV mRNAs are polyadenylated and comprise the post-transcriptional regulatory element (PRE) that prevents splicing. Other elements, such as ε I, ε II, phi and the DR1, are only functional on the pregenomic mRNA and are required for packaging and subsequent minus-strand DNA synthesis. Plate 10.1a is included in the centre of this figure.
(c) HBV DNA as it is found in the virion. The DNA strand that encodes the viral proteins (i.e., the minus-strand) is of full length but is not covalently closed (blue line). It has a small redundancy (r) of 9 to 10 bases at the ends and the domains 5E, 3E and M required for circularisation of the genome during plus-strand DNA synthesis. The minus-strand (red line) carries at its 5′ terminus a covalently bound part of the viral polymerase, known as terminal protein or primase because it is necessary for priming of the minus-strand synthesis. As indicated by the dashed line the primase domain is only one part of the polymerase that comprises the RT domain and the RNase H domain (all domains in green), catalysing DNA synthesis of both strands and degradation of the RNA in the intermediate hybrid of minus DNA strand and RNA. The plus-strand keeps the minus-strand in a circular conformation because it bridges the discontinuity of the minus-strand. The 3′ end of the plus-strand is not at a fixed position and is still connected with the HBV DNA polymerase. Most virion-bound genomes contain a single-stranded gap of 300 to 2000 bases long. Plates 10.1a and 10.1b are included in the centre.



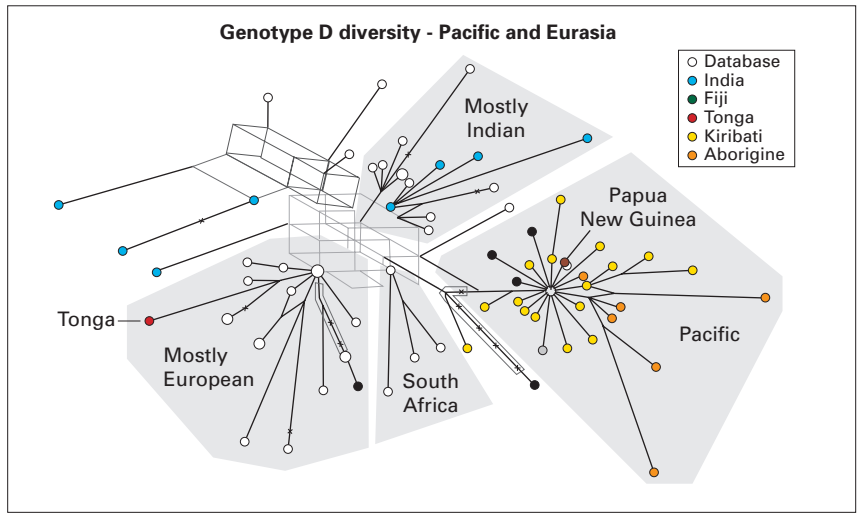



**Plate 14.1** Network analysis of D genotype S genes from a number of regions of the world, with particular emphasis on the Pacific. Note how most examples in the Pacific were found in Kiribati and that isolated examples only were found in other Pacific islands, perhaps due to subsequent movement between islands. This network is unresolved in the centre (see boxes) because of the large number of strains; the links between geographical areas are therefore unclear.

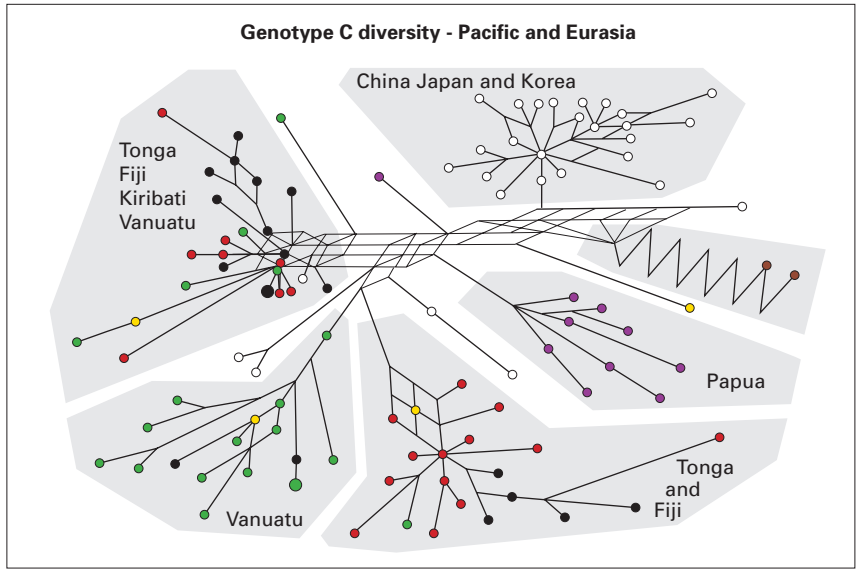



**Plate 14.2** Network analysis of C genotype S genes from a number of regions of the world, with particular emphasis on the Pacific. Note how strains are clearly localized geographically. In particular, note how Vanuatuan and Papuan strains are highly clustered, probably due to little movement to and from other islands (of those studied) in the region. On the other hand, some Fijian strains appear to have been derived from Tongan strains.


**Plate 25.1** Worldwide prevalence among blood donors.

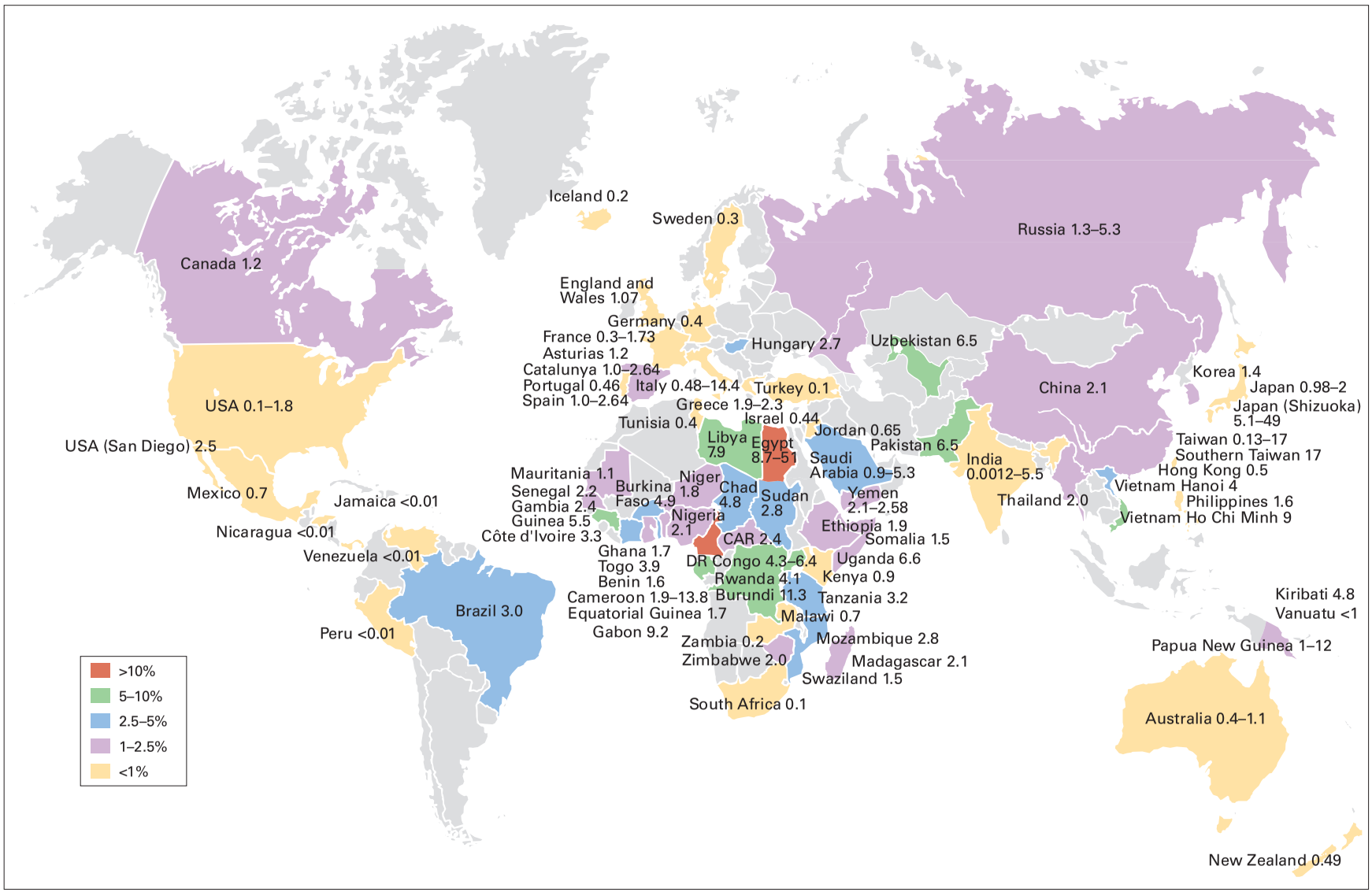



**Plate 25.2** Worldwide prevalence of HCV among general population cohorts.

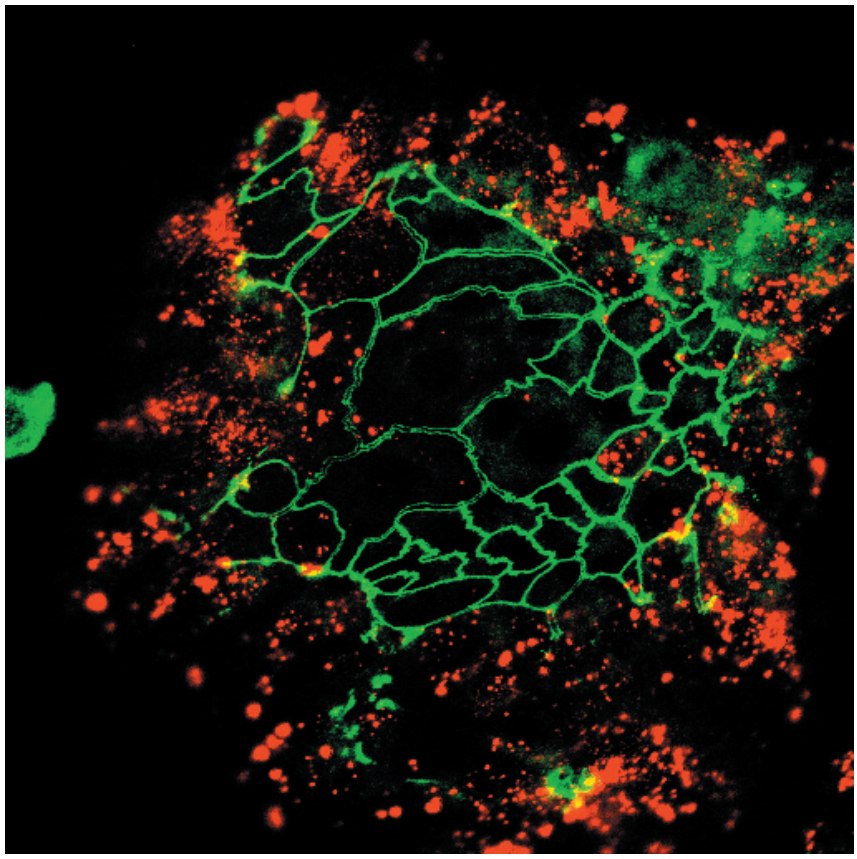


**Plate 39.1** Binding of HEV VLPs to polarized hepatocytes. A polarized subclone of HepG2 cells was grown as microcolonies on glass and incubated with 1 µg/mL HEV VLPs for 1 hour at 37 °C. Cells were fixed, stained with monoclonal antibody 2E2 to detect HEV (red) and rabbit anti-Zo1 to detect tight junctions (green), and examined by confocal microscopy.[29,42] HEV VLPs bind to cells at the periphery of colonies, where cells are not yet polarized and thus apical and basolateral membrane proteins are exposed to the medium, but are unable to bind to the polarized, cells in the centre of the colonies where the basolateral domains are in contact with the glass rather than the medium. (Photo courtesy of Michelle Snooks, Burnet Institute.)

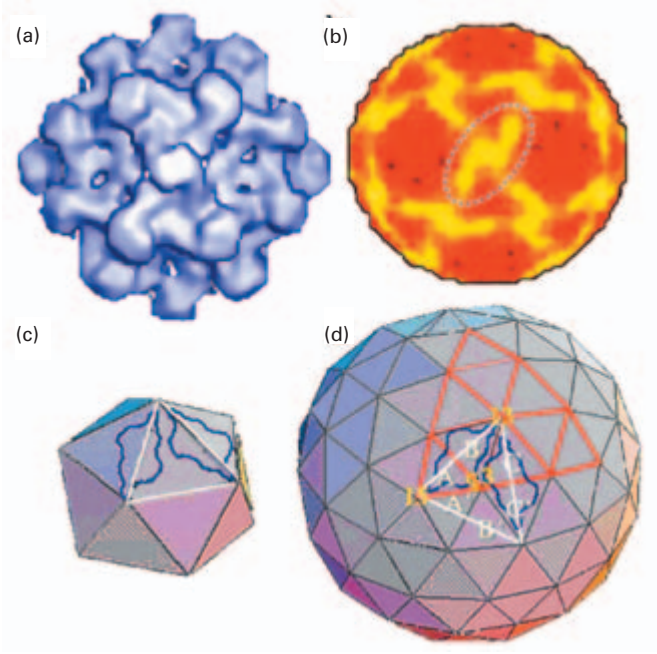
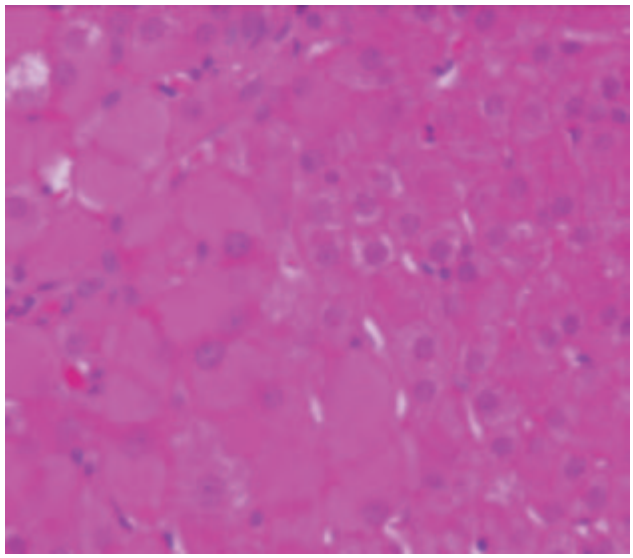
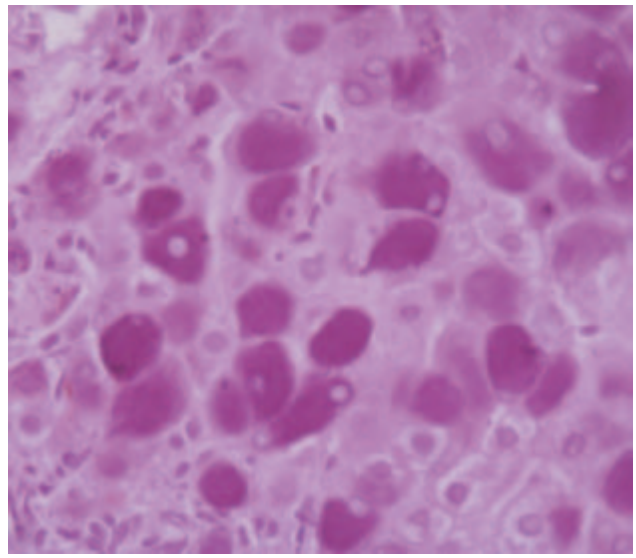


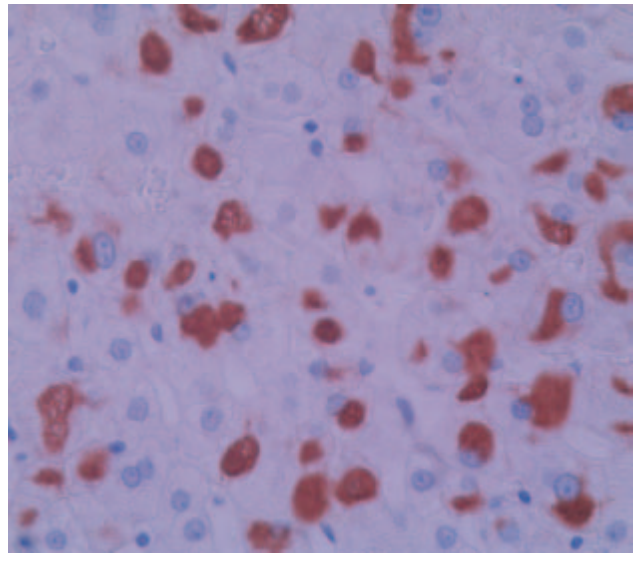

**Plate 39.2** The structure of HEV capsid, approximately 270 Å in diameter, was revealed by cryoelectron microscopy and 3D reconstruction (a).[47] The self-assembled VLP of truncated PORF2 peptide (residues 112–608), is composed with 30 predominant protrusions around the icosahedral surface. The protruding spike forms a balcony of 38.6 $nm^3$, while the thickness of the protein shell varies from 85 Å at twofold positions to 10 Å at fivefold positions. Detailed structural analysis reveals how the individual dimeric units (b, one marked by a dashed circle) establish contacts across the icosahedral twofold axes.[47] Two icosahedral surface lattices, showing the packing of 30 dimers at a strictly icosahedral twofold position in a $T = 1$ lattice of 270 Å in diameter (c) or the assembly of 90 of the same size dimers in a $T = 3$ lattice of 390 Å in diameter (d). The white triangle in the $T = 3$ lattice corresponds to one equivalent icosahedral face in the $T = 1$ lattice. The detailed organization of PORF2, the dimeric contact interface and the configurations of both N- and C-termini are still under investigation with high-resolution structural analysis (Xing, Miyazaki, Wang and Cheng, unpublished results).

(a)





(b)



(c)

**Plate 52.1** Ground-glass hepatocytes are characteristic of chronic HBV infection. On routine staining they are recognized by the very finely granular nature of their cytoplasm (a). They can be highlighted by histochemical stains, such as orcein (b) and their nature confirmed by immunohistochemical staining (c).

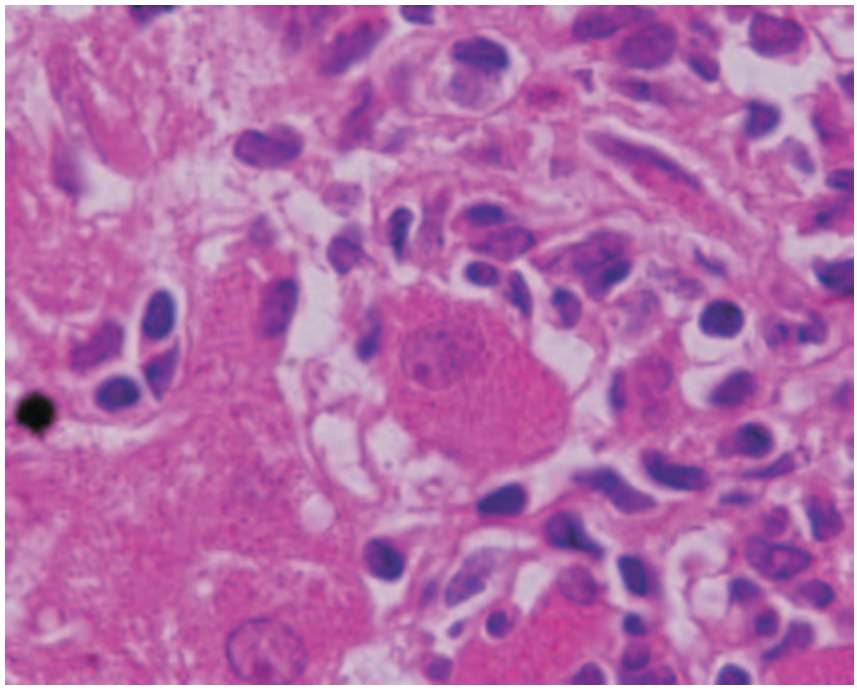
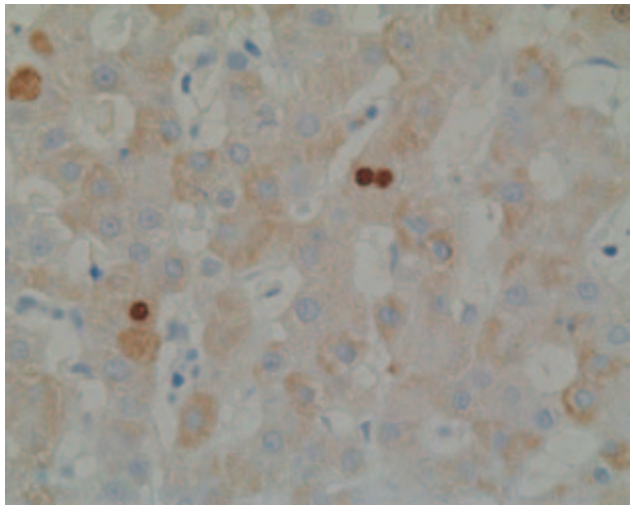
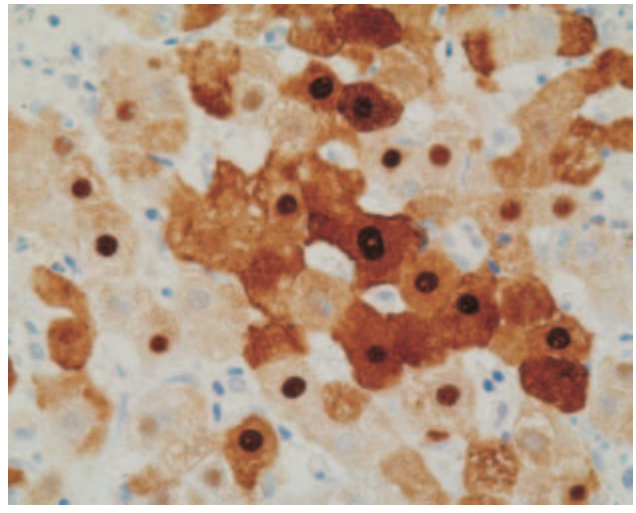


**Plate 52.2** The histological correlate of the immune-mediated damage characteristic of HBV is the presence of lymphocytes closely apposed to hepatocytes.



(a)



(b)

**Plate 52.3** Immunohistochemical staining for HBcAg can be used to assess disease activity. In (a), a case with a low level of viral replication shows a few cells with nuclear staining, while (b) is from a different case with a high level of viral replication and shows widespread nuclear and cytoplasmic staining.

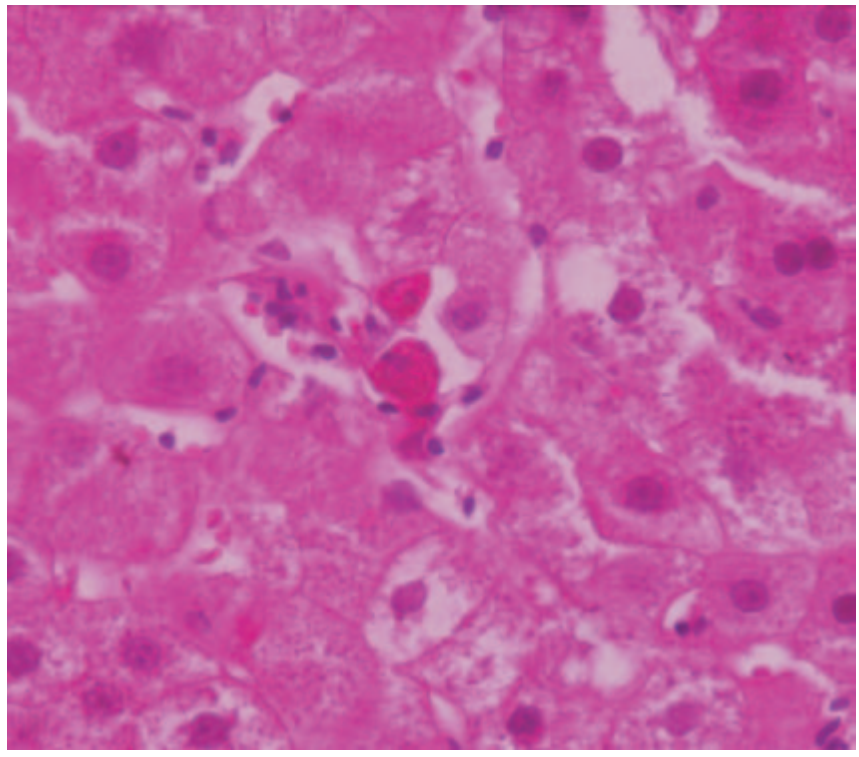


**Plate 52.4** HDV infection often produces increased lobular activity with prominent apoptotic hepatocytes.

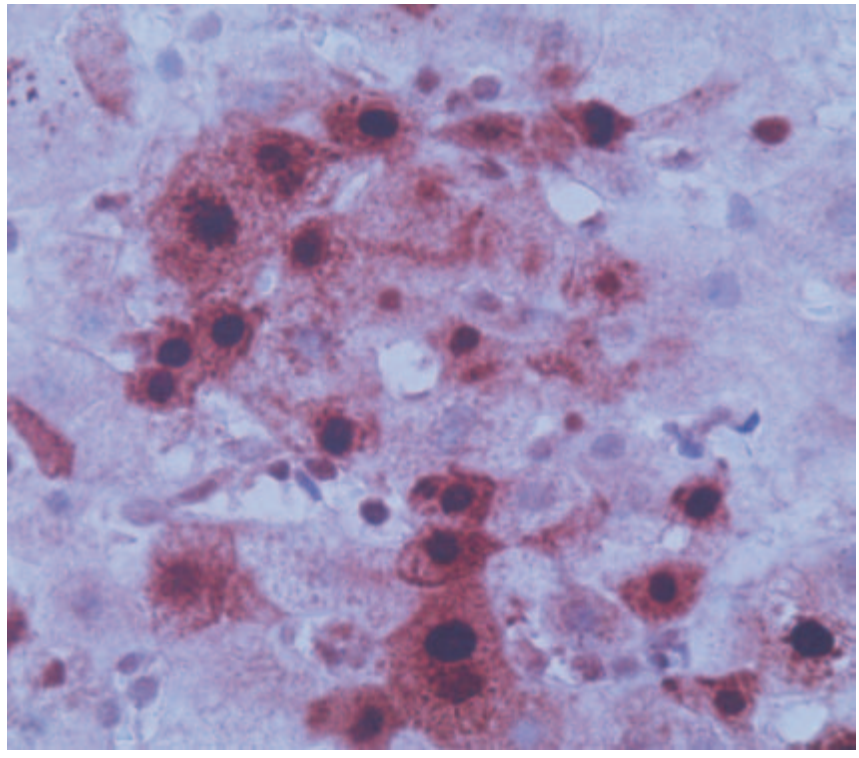
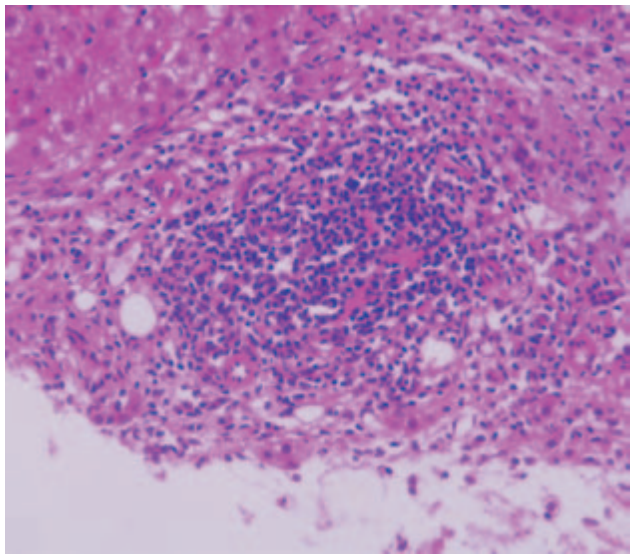
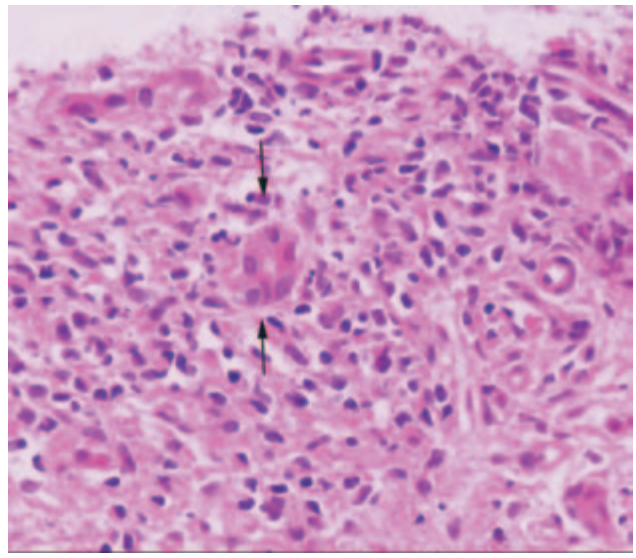


**Plate 52.5** Immunohistochemical staining is a rapid and reliable technique for making the diagnosis of HDV infection.

(a)





(b)

(c)

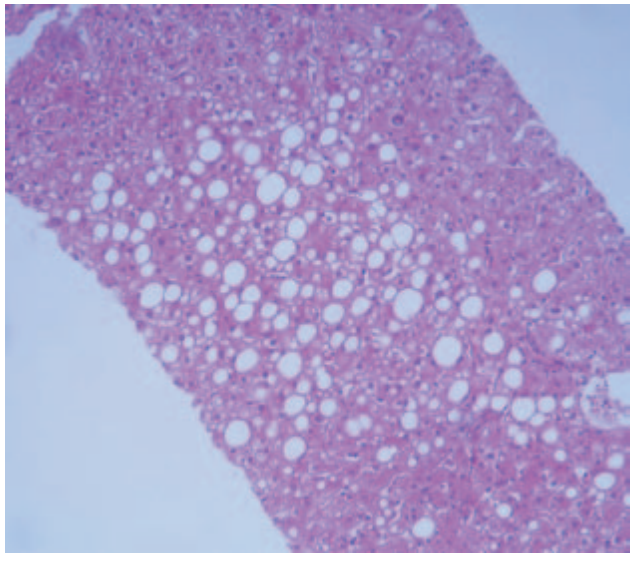


**Plate 52.6** The characteristic histological features of HCV infection are portal lymphoid aggregates (a), hepatitic bile duct damage (b) and large droplet fatty change, which is most common in cases of infection by genotype 3 (c).

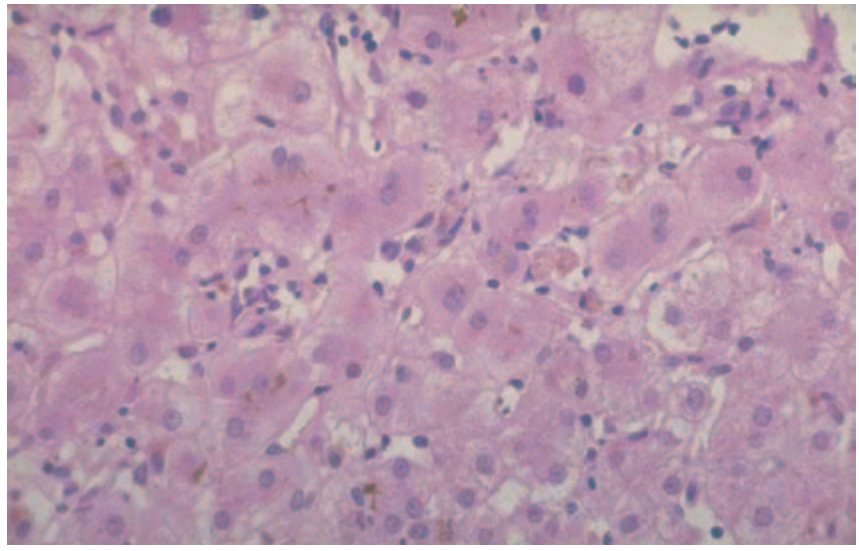


**Plate 52.7** Biopsy from a patient with HCV who became jaundiced following a course of erythromycin. Canalicular cholestasis in a biopsy from a patient with HCV always indicates a superimposed pathological process.

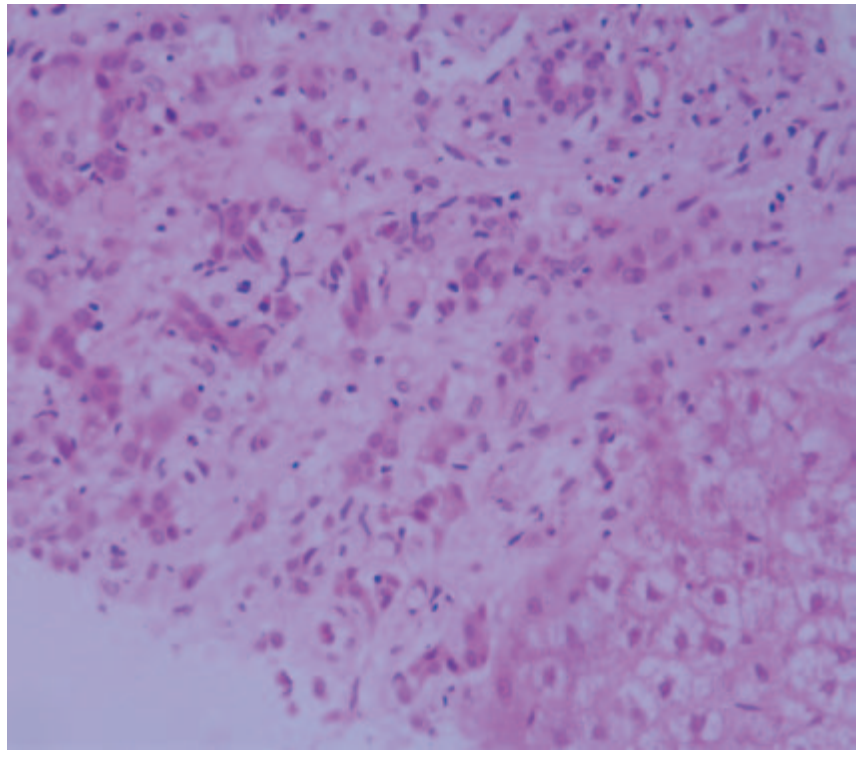
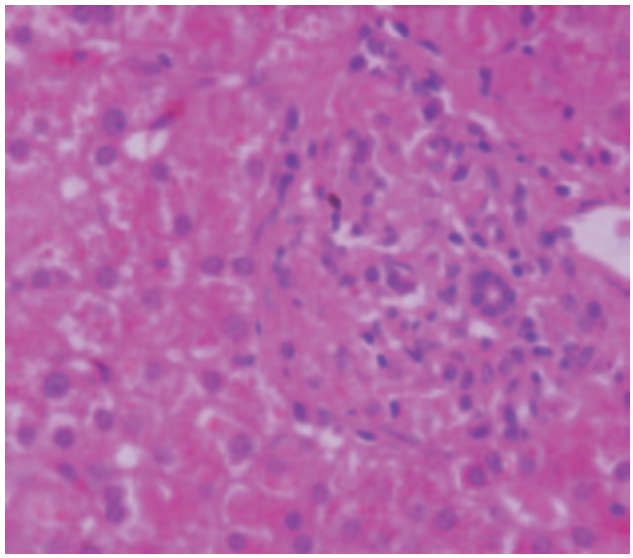
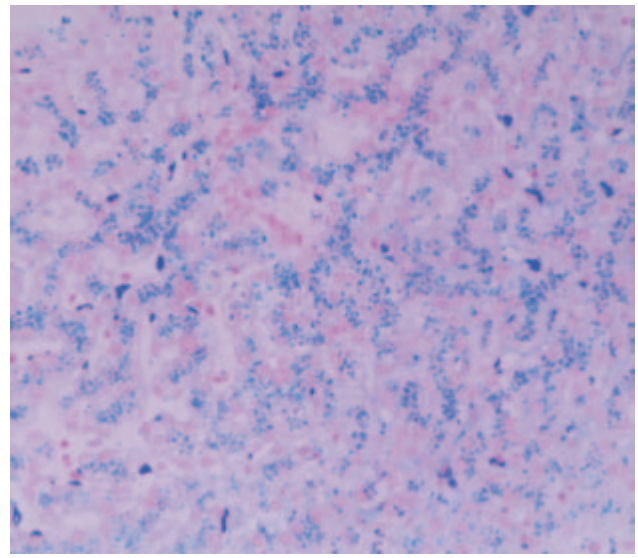


**Plate 52.8** Fibrosing cholestatic hepatitis in a patient with HCV who was immunosuppressed because of having had a renal transplant.

(a)

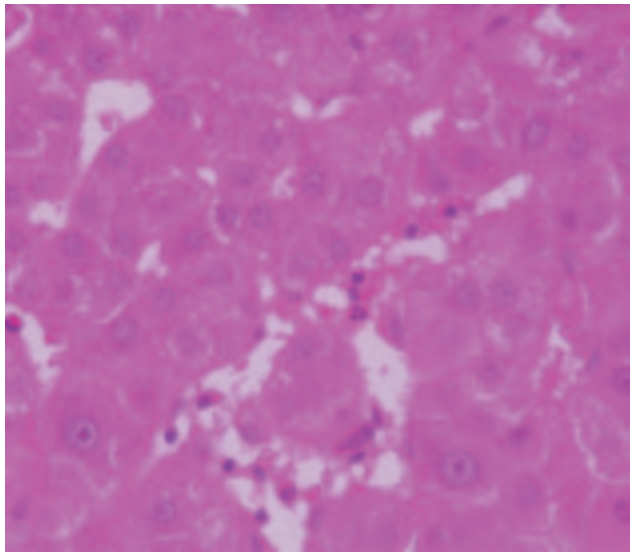


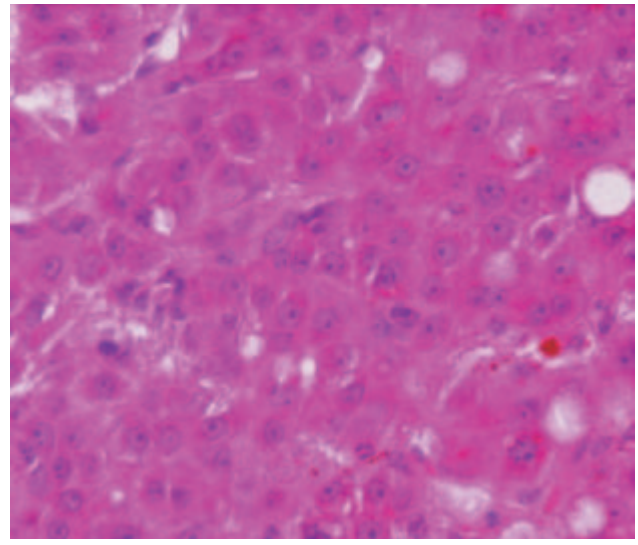


(b)

**Plate 52.9** A pretreatment liver biopsy from a patient with HCV which showed minimal inflammatory change (a) but marked parenchymal iron deposition (b) due to previously undiagnosed genetic haemochromatosis.

(a)





(b)

**Plate 52.10** (a) Large cell dysplasia (change) is characterized by cells with large nuclei but a normal nuclear:cytoplasmic ratio and is a marker for an increased risk of developing cancer. (b) In small cell dysplasia (change), which is a premalignant lesion, the nuclei are not enlarged, but the nuclear:cytoplasmic ratio is increased.