Viral Hepatitis

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Preface

Viral hepatitis remains a major public health problem throughout the world. Hepatitis A virus infects 1–90% or more of the human population, and it varies according to the socioeconomic, sanitary, and public health infrastructure of each country. Hepatitis B virus has infected one-third of the world population, with between 350 and 400 million carriers of the virus, many of whom progress to chronic liver disease and hepatocellular carcinoma. Hepatitis C virus is estimated to have infected 150–200 million people (probably a gross underestimate), with about 80% infected persistently, and this leads to serious sequelae including primary liver cancer. Infection with hepatitis D virus also occurs throughout the world and is hyperendemic in some countries, and hepatitis E is common and epidemic in a number of non-industrialized regions, with increasing evidence of zoonotic spread and sporadic infection in many countries.

Progress on all aspects of viral hepatitis is remarkably rapid, with many thousands of published accounts of original studies, and the mountain of new information is often bewildering and may be difficult to access. The pressing need for a fourth edition became clear, and the text has been revised and updated. The chapter on the history of hepatitis has been omitted (which is somewhat unfortunate because the future evolves from the past) in order to provide space for several new topics.

The fourth edition of *Viral Hepatitis* is designed to include a balanced and carefully distilled account of the more recent advances in this field written by a constellation of internationally recognized experts from many countries. We acknowledge their outstanding contributions, including those made by our two new co-editors, Professor Anna Lok and Professor Stephen Locarnini.

We hope that the book will prove useful to virologists, immunologists, specialists in infectious diseases, hepatologists, gastroenterologists, and, of course, public health and occupational health physicians and aspiring scientists. It is a book for those addressing the management and prevention of an important common infection and its associated liver diseases, which affect a large proportion of the world's population.

Howard C. Thomas
and
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Introduction to Liver Biology

Chapter 1
Liver regeneration and fibrosis

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Summary

In a healthy adult liver, the rate of cell turnover is very low. Following acute liver injury, restoration of parenchymal mass is achieved by proliferation of normally mitotically quiescent hepatocytes. However, chronic liver injury results in the loss of this proliferative capacity of the hepatocytes, as increasing numbers of cells become senescent. In this situation, there is activation of hepatic progenitor cells (HPCs) from within the intrahepatic biliary tree. These bipotential cells are capable of supplying biliary cells and hepatocytes. In animal models, there is some controversy regarding the relative contribution to parenchymal regeneration from these two compartments, but human studies are compatible with the suggestion that as the severity and chronicity of the liver injury increase, immature progenitor cells contribute more to regeneration than mature hepatocytes. We are now beginning to understand the molecular signals and niche requirements that govern their cell fate. Alongside the parenchymal regeneration in chronic liver injury, there is a stereotypical wound-healing response with activation of hepatic stellate cells (HSCs) into scar-forming myofibroblasts and deposition of collagen. This change in the extracellular matrix (ECM) affects the regenerative capacity of the liver, and excess scar tissue can impair liver regeneration from either hepatocytes or HPCs.

Introduction

Normally the liver has a low level of hepatocyte turnover, but in response to modest hepatocyte loss, a rapid regenerative response occurs from all cell types in the liver to restore organ homeostasis (comprehensively reviewed in [1, 2]). More severe liver injury, particularly chronic repetitive injury (e.g., chronic viral hepatitis), is often associated with hepatocyte replicative senescence. This activates facultative stem cells of biliary origin that give rise to cords (the "ductular reaction") of bipotential transit-amplifying cells (named oval cells [OCs] in rodents and HPCs in humans) that can differentiate into either hepatocytes or cholangiocytes. Moreover, the major primary tumors of the liver (hepatocellular carcinoma [HCC] and cholangiocarcinoma [CC]) invariably arise in a setting of chronic inflammation that is accompanied by both hepatocyte regeneration and ductular reactions, and while it seems that the founder cell of CCs is a proliferating cholangiocyte, the morphological heterogeneity often observed in HCCs suggests that these tumors can arise from bipotential HPCs as well as more mature hepatocytes. HCCs also appear to possess subpopulations of cancer stem cells, which are responsible

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for continued tumor propagation and metastasis, and a number of phenotypic markers have been proposed for their identification.

Liver turnover and regeneration

Kinetic organization

The healthy liver in adults is mitotically quiescent with levels of proliferation suggesting a turnover time for hepatocytes in excess of a year. Nevertheless, there is still considerable debate as to how the liver is organized. Most studies concur that hepatic stem cells are located in the periportal region; for example, in the mouse, bromodeoxyuridine (BrdU) pulse-chase analysis following two rounds of acetaminophen intoxication has observed so-called label-retaining cells (LRCs), considered to be slowly dividing progenitor cells, as both interlobular cholangiocytes and peribiliary hepatocytes [2]. In humans, $EpCAM^{+}NCAM^{+}$ cells in the periportally located canals of Hering have been identified as putative HPCs and it is suggested that there are eight maturational lineage stages moving from the periportal (progenitor) region to the perivenous region.

An important question remains: is the liver organized like the intestine, with a unidirectional flux of cells that are "born" in the portal area and migrate along a trajectory leading to the hepatic veins? This so-called streaming liver hypothesis was first advocated by Gershom Zajicek and colleagues (reviewed in [2]); examining the location of labeled hepatocytes in intact adult rat livers over time after a single injection of tritiated thymidine, they suggested that hepatocytes moved at a speed of over 2 μm/day from the periportal region to the central vein. A recent murine study by Furuyama and colleagues [3] (reviewed in [4]) appears to support the idea that hepatocytes migrate centrifugally from portal areas (Figure 1.1). They examined the expression of the embry-

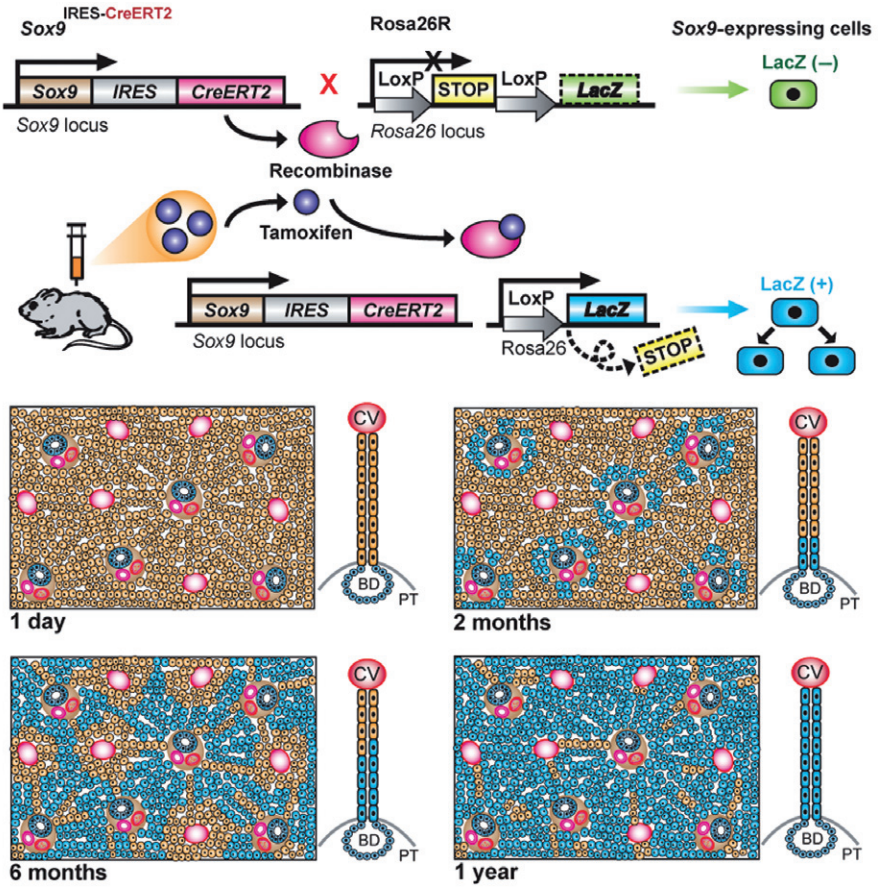

Figure 1.1 Top: Strategy of the genetic lineage-tracing study employed by Furuyama *et al.* [3] using tamoxifen-induced Cre-mediated cell tracking using Sox9IRES-CreERT2; Rosa26R mice. Bottom: Schematic illustrating the spread of X-gal staining after 8-week-old mice were injected with tamoxifen. After one day, only intrahepatic bile duct cells are labeled, but later X-gal-positive hepatocytes gradually spread from the portal tracts to the central veins, thus supporting the streaming liver hypothesis. See Alison and Lin [4] for further details. (Source: Alison and Lin. Hepatology 2011, 53: 1393–1396 [4]). (Color plate 1.1)

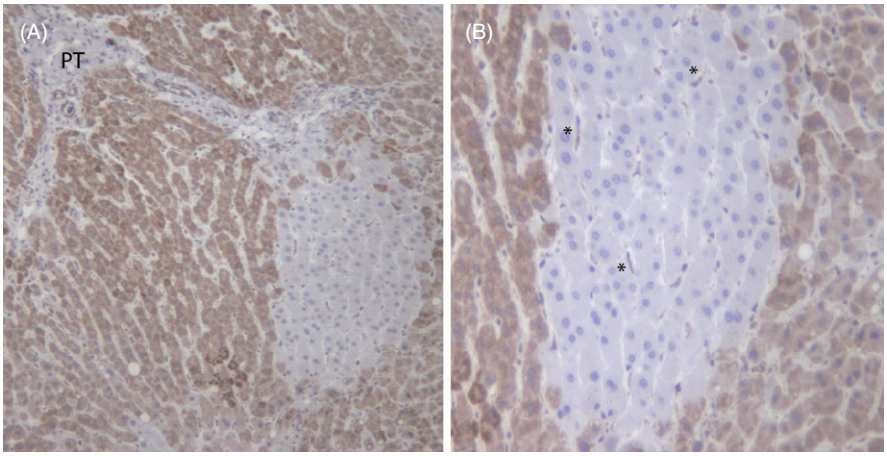

Figure 1.2 (A) A single cytochrome c oxidase (CCO)–deficient patch, appearing to emanate from the portal tract. (B) High-power magnification illustrates that within the patch there are CCO-positive sinusoid-lining cells (asterisks) indicative of different cells of origin from hepatocytes. See Fellous *et al.* [7] for further details. (Source: Fellous TG *et al.* Hepatology 2009, 49: 1655–1663 [7]). (Color plate 1.2)

onic transcription factor Sox9 in the liver. In human liver, immunohistochemistry identified interlobular bile duct cells as Sox9-expressing cells, and a similar pattern was seen in adult mice when a reporter gene, either enhanced GFP or LacZ, was knocked into the *Sox9* locus. Adopting tamoxifen-inducible genetic lineage tracing from the *Sox9* locus, detecting Sox9-lineage cells by X-gal staining, Furuyama *et al.* [3] found that X-gal positivity spread out from the portal areas toward the hepatic veins until the majority of hepatocytes were labeled within 8–12 months. Thus, the paper suggested that indeed cells "streamed," but more importantly hepatic replacement was from cytokeratin 7 (CK7)–Sox9-positive biliary cells, identifying cells within the biliary tree as drivers not only of hepatocyte replacement when regeneration from existing hepatocytes is compromised (discussed further in this chapter) but also of normal hepatocyte turnover. However, there is controversy as other studies of mice have failed to find evidence for the normal liver parenchyma being "fed" from the biliary system. Carpentier *et al.* [5] also employed lineage labeling in mice, this time from Sox9-expressing ductal plate cells in late embryonic development (E15.5), finding that these cells gave rise to interlobular bile ducts, canals of Hering, and periportal hepatocytes, and that liver homeostasis did not require a continuous supply of cells from Sox9 progenitors. Iverson *et al.* [6] have sought to quantify the dynamics of mouse liver turnover by lineage labeling following activation of an albumin–*Cre* transgene, calculating that 0.076% of hepatocytes had differentiated from albumin-naïve cells over a 4-day period.

In human liver, Fellous *et al.* [7] have identified clonal populations of hepatocytes based upon finding large patches of cells deficient in the mitochondrial DNA (mtDNA)-encoded cytochrome c oxidase (CCO) enzyme, all sharing an identical neutral mutation in the *CCO* gene indicating derivation from a single cell. Significantly, these CCO-deficient patches were all connected to portal areas and had a portal vein–to–hepatic vein orientation (Figure 1.2), suggesting a "streaming" nature but without providing information of whether they are derived from a periportal progenitor cell or an interlobular biliary cell.

Liver regeneration

The regenerative capacity of the liver is impressively demonstrated when two-thirds of the rat liver is surgically removed (a 2/3 partial hepatectomy, or 2/3 PH) and the residual liver then undergoes waves of hyperplasia and hypertrophy to restore preoperative liver mass within about 10 days [1, 2]. After a 2/3 PH in healthy adult rats, all the normally proliferatively quiescent hepatocytes leave G_0 to semisynchronously enter the cell cycle. DNA synthesis is first initiated in the periportal hepatocytes at about 15 hours after PH, with a peak in the hepatocyte DNA synthesis labeling index of ~40% at 24 hours. Midzonal and centrilobular hepatocytes enter DNA synthesis at progressively later times, but the hyperplastic response in hepatocytes is essentially complete by 96 hours, to be followed by a phase of hepatocyte hypertrophy. Elegant labeling studies have identified three groups of regenerative hepatocytes in mice, with all cells dividing at least once, but with the periportal hepatocytes that divide first dividing maybe three or more times after PH.

As might be expected, age has an adverse effect on the response; in old rats (>2 years old), a significant number of hepatocytes do not proliferate after PH,

seemingly becoming reproductively senescent. To maintain liver homeostasis, the nonparenchymal cells (cholangiocytes and endothelial cells) must also expand their numbers, and their cell cycle entry is delayed a few hours behind that of hepatocytes [2].

Molecular regulation of liver regeneration

Numerous cytokines, growth factors, and signaling pathways have been implicated in (1) the initiation (priming) of hepatocytes in order to be responsive to liver mitogens, (2) the proliferative response itself, and (3) the curtailment of the response. The "priming phase" in the first few hours after PH, which is probably instrumental in the G_0 to G_1 transition, is associated with the upregulation of many genes not expressed in the normal liver and is essentially cytokine driven [2], with activation of transcription factors such as activator protein 1 (AP1), nuclear factor kappa-light-chain-enhancer of activated B (NF-κB), and signal transducer and activator of transcription 3 (STAT3) being particularly important. The ultimate cause of cytokine accumulation is unclear, but enteric lipopolysaccharides may be the master regulator of the innate immune response, and liver injury can be associated with a defective intestinal barrier leading to exposure to lipopolysaccharides and complement fragments. Such exposure activates the NF-κB pathway in Kupffer cells, resulting in the production and secretion of interleukin 6 (IL6) that activates the JAK/STAT pathway, leading to the initiation of DNA synthesis in hepatocytes. In mice, complement activation (in particular, C3a and C5a) leads to the recruitment of natural killer T (NKT) cells and the production of IL4 by these cells [8]. IL-4 maintains IgM levels and deposition in the liver, leading to increased C3a and C5a accumulation that in turn stimulates liver macrophages to produce IL6. The cytokine interleukin 1 receptor antagonist (IL1ra) is also important in the early phase of regeneration, reducing inflammatory stress and thus promoting proliferation [9].

The proliferative response itself appears to be driven by a number of growth factors and signaling pathways, including IL6, tumor necrosis factor alpha (TNFα), hepatocyte growth factor (HGF), amphiregulin, stem cell factor (SCF), insulin-like growth factor 1 (IGF1), T3, bone morphogenetic protein 7 (BMP7), Wnt, β-catenin, Hedgehog (Hh), and phosphoinositide-3 kinase (PI3K), although no one factor or pathway appears crucial to the process [2]. Some of these signals are autocrine, and others are paracrine; for example, in mice sinusoidal endothelial cells are involved in hepatocyte regeneration with vascular endothelial growth factor receptor (VEGFR)–dependent upregulation of the transcription factor Id1 leading to the release of hepatotrophic factors such as Wnt2 and HGF [10, 11]. Moreover, it seems that endothelial progenitor cells recruited from the bone marrow after PH provide the richest source of HGF [11]. Hepatic stellate cells (HSCs) also support liver regeneration and are activated by massive upregulation of delta-like 1 homology (Dlk1) that represses *Pparγ* in stellate cells [12]. Regenerative competence in mouse and human also appears to be maintained by activation of telomerase activity in regenerating hepatocytes [13]. Micro-RNAs (miRs) are also involved in regeneration after PH; for example, in mice there is upregulation of miR-21 in the priming phase that targets a proliferation inhibitor facilitating cyclin D1 translation, and downregulation of miR-378 that targets *odc1* messenger RNA (mRNA), ornithine decarboxylase activity being essential for DNA synthesis [14]. In rats after PH, there are also dramatic changes in miRs, with upregulation of 40% of investigated miRs in the priming phase and downregulation of 70% of miRs at 24 hours after PH, presumably facilitating maximal proliferation [15].

Equally important are the molecular mechanisms that curtail the regenerative response, ensuring the liver does not overcompensate for lost mass. Transforming growth factor beta (TGFβ) produced by stellate cells inhibits hepatocyte replication, and several mechanisms are involved in its production. In mice, serotonin acts on 5-HT_{2B} receptors in stellate cells, leading to phosphorylation of JunD via ERK, resulting in recruitment of JunD to AP1 binding sites in the promoter region of the *tgfβ1* gene [16]. The multidomain matrix glycoprotein thrombospondin-1 (Tsp1) is also involved in TGFβ1 production in mice; Tsp1 is expressed by endothelial cells in response to reactive oxygen species (ROS) shortly after PH and binds to latent TGFβ1 complexes, converting them to active TGFβ1 [17]. The IL6 response is negatively regulated through transcriptional upregulation of suppressor of cytokine signaling 3 (SOCS3), but SOCS3 is not crucial for curtailing proliferation, for although SOCS3 knockout mice have higher levels of hepatocyte proliferation after PH than wild-type mice and restore preoperative liver weight 2 days earlier, proliferation stops after 4 days and liver weight does not go above normal [18]. The Hippo pathway seems particularly important for curtailing liver size; the kinases Mst1 and Mst2 (the mammalian orthologs of *Drosophila* Hippo) are responsible for phosphorylating the Yes-associated protein (Yap) at Ser127, the mammalian ortholog of *Drosophila* Yorkie, which is a transcriptional activator of cell cycle proteins such as Ki-67 and c-Myc – phosphorylation blocks its ability to translocate to the nucleus [19]. Thus, overexpression of Yap in mice leads to massive liver weight increases (25% of body weight versus 5% normally) [20], and likewise Mst1 and Mst2 double knockouts also have massive livers and eventually develop HCC [21, 22].

A second tier of regeneration: oval cells and HPCs

Massive acute liver injury, chronic liver injury, or large-scale hepatocyte senescence results in the activation of a reserve or potential progenitor cell compartment located within the intrahepatic biliary system [1, 2]. Replicative senescence can occur in conditions such as chronic hepatitis and fatty liver disease [23]. In humans and mice, the extent of the HPC response is proportional to the degree of parenchymal damage [24, 25]. HPCs are derived from interlobular biliary cells and/or the canal of Hering, and in human liver the canal of Hering extends beyond the limiting plate, even perhaps throughout the proximate third of the lobule [26].

A number of animal models have been described to activate this progenitor response. In rats, a very effective model has been to pretreat the animals with 2-acetylaminofluorene (2-AAF) before performing a 2/3 PH (the 2-AAF/PH protocol) [27]. 2-AAF is metabolized by the hepatocyte's cytochrome P450 (CYP450) system, producing metabolites that form DNA adducts, thus preventing hepatocytes from entering the cell cycle in response to PH. Under these constraints, oval cells or HPCs are activated since they lack the CYP enzymes necessary for 2-AAF metabolism. In the mouse, dietary regimes are often employed including a choline-deficient, ethionine-supplemented diet (the CDE diet) that inflicts hepatocyte damage, or a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) regime that damages cholangiocytes [28]. An oval cell response is also seen when hepatitis B surface antigen (HBsAg-tg) mice (a model of chronic liver injury) are treated with retrorsine, a pyrrolizidine alkaloid that blocks hepatocyte regeneration. This effectively abolishes hepatocyte turnover, resulting in massive oval cell–driven regeneration [29]. The exact location of stem and progenitor cells within the biliary tree is unclear, and it is also unclear if all cells in small-caliber biliary ducts and canals of Hering are capable of giving rise to oval cells, but in the mouse a small subset (3–4%) of antigenically defined biliary cells that express Sox9 give rise to most oval cells in the DDC model [30].

A wide range of markers have been used to identify ovals cells and HPCs (Table 1.1) [31]. Many factors, often produced by cells of a hepatic niche that intimately accompanies the reaction, can influence the oval cell–HPC response. Autocrine and paracrine Wnt signaling is clearly involved in the oval cell or HPC response in mice [28, 32], rats [33], and humans [28, 34, 35]. In the rat 2-AAF/PH model, oval cells display nuclear β-catenin and Wnt1 is essential for differentiation of oval cells to hepatocytes; exposure to Wnt1 small hairpin RNA (shRNA) blocked this differentiation, and oval cells generated an atypical ductular reaction – perhaps as the default position [35]. As oval cells and HPCs are bipotential, what regulates whether they become hepatocytes or cholangiocytes? Boulter and colleagues have described the mechanisms in mice governing these critical cell fate decisions [28]. After biliary cell damage with DDC, the intimate association of myofibroblasts with HPCs facilitated Notch signaling ensuring biliary differentiation in oval cells, in essence recapitulating ontogeny. On the other hand, after hepatocyte damage with the CDE diet, adjacent macrophages in response to engulfing hepatocyte debris were involved in Wnt signaling to HPCs that not only turned off Notch signaling but also specified hepatocytic differentiation in oval cells. On the other hand, in the rat 2-AAF/PH model, Notch1 may be important for hepatocytic differentiation since exposure to a γ-secretase inhibitor delayed the maturation process [36]. HGF signaling is also important for the oval cell response: genetic deletion of *c-met* from oval cells in the DDC model results in a diminished response with decreased hepatocytic differentiation [37]. Moreover, a failure to express stromal cell–derived factor 1 (SDF1) leads to less recruitment of macrophages and associated matrix metallopeptidase 9 (MMP9) secretion that is crucial for oval cell migration and liver remodeling (discussed further in this chapter). Hh signaling is another important pathway, and ligands acting through the receptor Patched (Ptc) on murine oval cells and human HPCs are required for progenitor cell survival [38]. Perhaps most

Table 1.1 Some of the markers used in the identification of oval cells and HPCs in the damaged mammalian liver.

A6 antigen (mouse marker)
ABCG2/BCRP1 (breast cancer resistance protein)
AFP (alpha fetoprotein)
Cadherin 22
CD24 and CD133
Chromogranin A
CK7 and CK19
c-Kit (CD117)
Claudin7
Connexin 43
Dlk1 (Delta-like protein 1)
DMBT1 (deleted in malignant brain tumor 1)
E-cadherin
EpCAM/TROP1 (epithelial cell adhesion molecule)
flt-3 ligand/flt-3
Fn14 (fibroblast-inducible factor 14-kDa protein; TWEAK receptor)
GGT (gamma-glutamyltranspeptidase)
GST-P (placental form of glutathione-S-transferase)
M2-PK (muscle type pyruvate kinase)
NCAM-1/CD56 (neural cell adhesion molecule-1)
PTHrP (parathyroid hormone related peptide)
TACSTD/TROP2 (tumor-associated calcium signal transducer)

Note: Many of these markers are also expressed on normal biliary epithelial cells.

significantly, inflammatory cells produce a range of cytokines and chemokines that initiate the response [2, 32]; SDF1 attracts $CXCR4^+$ T cells, and these cells express TWEAK (TNF-like weak inducer of apoptosis) that stimulates oval cell proliferation by engaging its receptor Fn14, a 14 kDa transmembrane receptor [39]. Tirnitz-Parker and colleagues employed the CDE diet and found that expression of Fn14 is markedly elevated [40]. Fn14 is not a receptor tyrosine kinase, but rather ligand occupancy activates NF-κB signaling as shown by the presence of active (nuclear) NF-κB in a progenitor cell line upon TWEAK stimulation. The early oval cell response to the CDE diet was delayed in Fn14 knockout mice, although interestingly there were comparable numbers of oval cells in wild-type and knockout mice after 3 weeks on the CDE diet. Significantly, recombinant human TWEAK (rhTWEAK) directly stimulated the *in vitro* proliferation of a progenitor cell line in a dose-dependent manner. Other components of the inflammatory response that can stimulate oval cells include lymphotoxin-β, interferon alpha (IFNα), TNFα, and histamine [41]. Resistance to the growth inhibitory effects of TGFβ may allow oval cells to proliferate under conditions inhibitory to hepatocytes [42].

In terms of negative regulators of the oval cell response, the neurofibromatosis type 2 (*Nf2*) gene product Merlin appears critically important [43]. Genetic deletion of *Nf2* leads to massive oval cell expansion and the development of CC and HCC; Merlin appears to control the availability of epidermal growth factor receptor (EGFR) and other growth factor receptors. Progenitor cells reside in a specialized supportive microenvironment known as a niche; not only do oval cells and HPCs have such a niche but also this niche seems to migrate hand-in-hand with the expansion of oval cells. For example, with the CDE diet the activation of stellate cells (upregulation of alpha smooth muscle actin [αSMA] expression) and deposition of collagen precede the oval cell response, suggesting that the extension of the niche is a prerequisite for oval cell expansion [44]. In fact, mouse and rat models of oval cell activation and HPC reactions in humans bear a striking similarity, in terms of both the deposition of ECM (particularly laminin) and cells (macrophages and αSMA^+ myofibroblasts) that accompany progenitor reactions suggestive of a stereotypical niche [45]. Further support for the idea that the ECM adjacent to oval cell reactions is not merely a passive bystander comes from studies of the oval cell reaction in mice that produce mutated collagen I that is highly resistant to MMP degradation [46]; here, a failure to remodel collagen stunts the reaction, seemingly through a failure to establish a laminin-rich progenitor niche. In the 2-AAF/PH model, blocking the activation of stellate cells with L-cysteine was a potent suppressor of the oval cell response, probably related to loss of cytokines such as TGFβ1 and the fibronectin matrix that, among other properties, can concentrate cytokines such as connective tissue growth factor (CTGF) for which oval cells have receptors [47].

Chronic viral hepatitis is, of course, invariably associated with cirrhosis and hepatocyte senescence [48–50], thus activation of HPCs in this setting is common. In the fibrous septae that surround regenerative nodules (RNs), differentiation of CK19-positive HPCs to form buds of intraseptal hepatocytes (ISHs) is often observed [51]. In cirrhosis we observed that RNs are invariably clonally derived (Figure 1.3), suggesting that they are not simply created by fibrotic dissection of the preexisting parenchyma; moreover, they are clonally related to the abutting HPCs (Figure 1.3), and thus have been derived from them [52]. Thus RNs may well represent the further expansion of buds of ISHs.

Stem cells and liver cancer (founders and propagators)

Whereas CCs are believed to arise from either established biliary ducts or HPCs, the origin of HCCs is more problematic. Clearly hepatocytes are the cell of origin of many HCCs in experimental models where tumor yield is directly related to hepatocyte proliferation or where oncogenic transgenes are driven by the albumin promoter. On the other hand, HPC activation is commonly seen in models of hepatocarcinogenesis and invariably accompanies chronic liver damage in humans, thus making it quite likely that HPCs are the founder cells of many HCCs [53]. An origin of HCCs from HPCs is often suggested because many HCCs contain an admixture of mature hepatocyte-like cells and cells resembling HPCs [1]. If tumors do arise from HPCs, then this indicates a block in HPC differentiation, a process that has been termed "stem cell maturation arrest" [54]. This hypothesis is supported by the fact that murine HCCs induced by a CDE diet have a mixture of neoplastic phenotypes recapitulating stages in normal development, suggesting intermediate states between bipotent oval cells and hepatocytes [55]. Likewise in humans, four prognostic HCC subtypes have been identified equating to liver cell maturational steps [56]. The poorest prognostic groups had a significant proportion of either $EpCAM^+AFP^+$ cells (hepatoblast-like) or $EpCAM^-AFP^+$ cells (HPC-like), whereas those with $EpCAM^-AFP^-$ cells (mature hepatocyte-like) or $EpCAM^+AFP^-$ cells (cholangiocyte-like) had a better prognosis. Gene expression profiling has identified a subset of HCCs with a profile consistent with an origin from HPCs, and these patients have a poor prognosis [57]; moreover, counting of CK19-positive cells in HCC can identify a poor-prognosis group [58] that may be related to an enhanced epithelial-mesenchymal transition (EMT) [59].

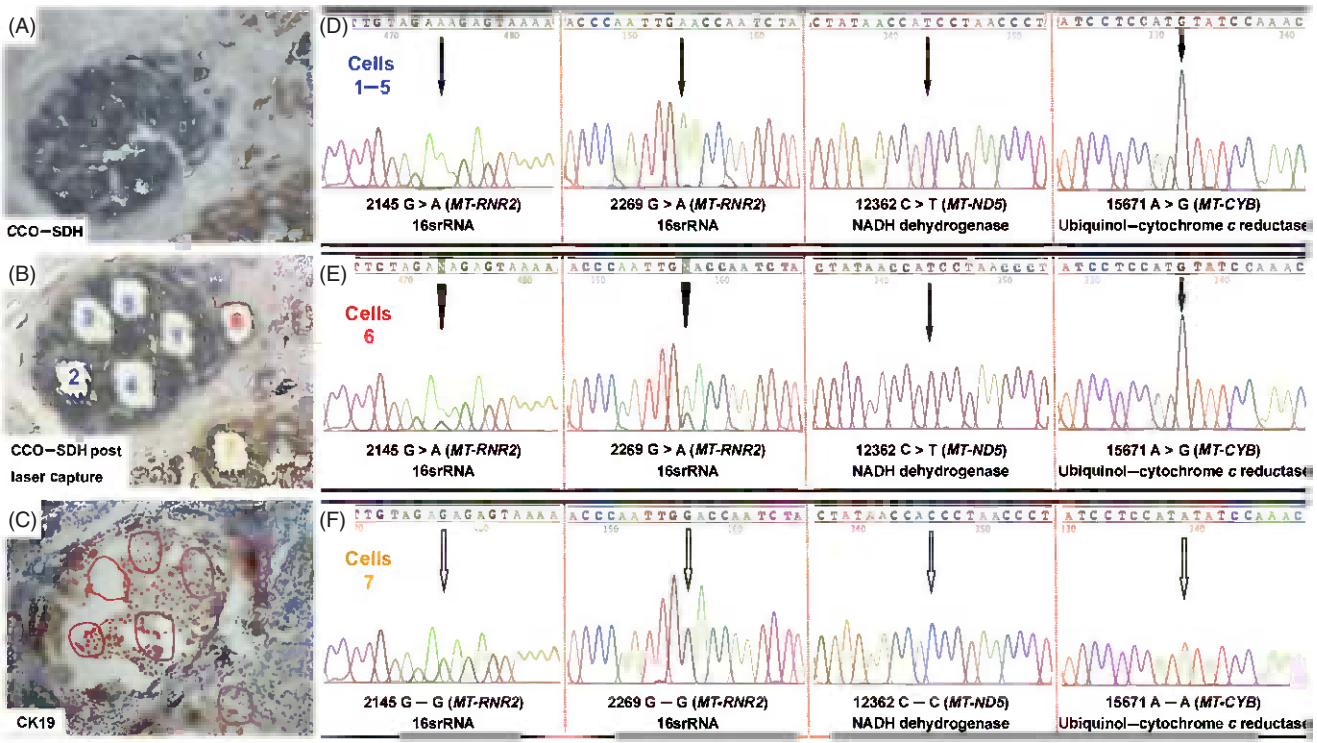

Figure 1.3 Mitochondrial DNA genotyping indicates that regenerative nodules can be derived from CK19-positive HPCs. (A) An entirely CCO-deficient nodule (stained blue for succinate dehydrogenase activity). (B) Five groups of cells (1–5) from the same CCO-deficient nodule; cells (6) from the adjacent CCO-deficient ductular reaction, confirmed by CK19 IHC on the next serial section (C, brown staining), and cells (7) from the CCO-positive nodule were laser capture-microdissected, and the entire mitochondrial genome was sequenced. (D) Cell areas 1–5 all contained four different transition mutations: 2145G>A, 2269G>A, 12362C>T, and 15671A>G (black arrows). (E) Cell area 6 from the abutting CCO-deficient ductular reaction had exactly the same mutations. Heteroplasmy was detected at locations 2145 and 2269 (arrowheads), while the mutations at locations 12362 and 15671 were homoplasmic (black arrows). (F) Cell area 7 from the CCO-positive nodule had no mutation (white arrows). See Lin *et al.* [52] for further details. (Source: Lin WR. *et al.* Hepatology 2010, 51: 1017–1026 [52]). (Color plate 1.3)

A detailed discussion of cancer stem cells (CSCs) in HCC is beyond the scope of this chapter, but a number of phenotypic markers have been proposed for their isolation including CD13, CD90, CD133, ALDH activity, and the side population [60]. As in other organs, HCC CSCs seem relatively resistant to therapy, and strategies to either reduce ABC transporter function [61–63] or induce differentiation [64] have increased CSC sensitivity. For a detailed discussion, see [65].

Liver fibrosis

Whatever the mode of chronic liver injury, a stereotypical wound-healing response occurs that results in a series of cellular and extracellular matrix changes, an increase in collagen deposition, and a disturbance in the liver architecture. In its extreme form, this results in the development of cirrhosis with gross architectural disturbance, nodule formation, heavy scarring, and vascular changes, eventually resulting in liver failure or the development of HCC.

Following liver injury there are a number of cellular responses that are key to the fibrotic response, including hepatocyte injury, and hepatic macrophages and endothelial cells are activated [66]. The cells that are primarily responsible for the deposition of ECM are the αSMA-positive myofibroblasts that are formed principally from the activation of the HSCs. Hepatic myofibroblasts are proliferative and contractile cells that directly secrete collagen matrix, and they have several important paracrine mechanisms that increase the profibrotic environment. Proliferation of hepatic myofibroblasts is stimulated by a number of mitogens, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), angiotensin II, VEGF, and thrombin. PDGF is a very potent mitogenic stimulus and is released by activated Kupffer cells, sinusoidal endothelial cells, platelets, and activated myofibroblasts

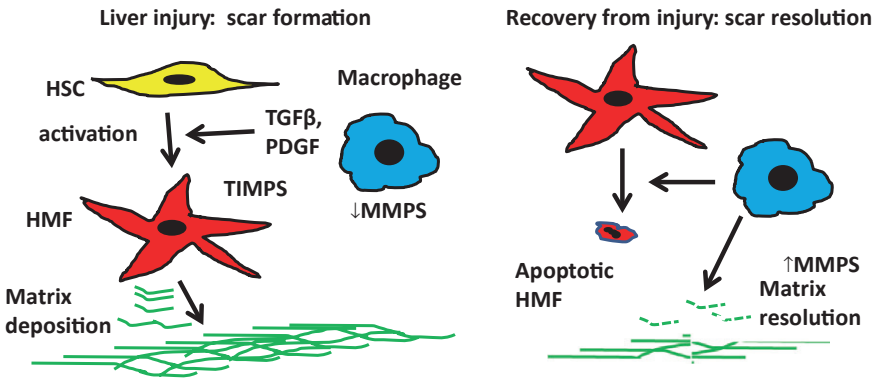

Figure 1.4 During liver injury the hepatic macrophages help, along with other factors, to stimulate quiescent fat-storing hepatic stellate cells (HSCs) into activated hepatic myofibroblasts (HMFs). The activated HMFs deposit collagen scar and secrete tissue inhibitors of metalloproteinases (TIMPS), which inhibit the degraders of scar matrix, the matrix metalloproteinases (MMPs). If the cause of liver injury is removed (e.g., viral eradication), then a recovery phase commences and, to a variable degree, the quantity of liver fibrosis lessens. In this phase, HMFs reduce their TIMP secretion, and the hepatic macrophages secrete MMPs that aid the degradation of scar and promote the apoptosis of HMFs. (Color plate 1.4)

in an autocrine manner. Importantly, myofibroblasts also secrete tissue inhibitors of metalloproteinases (TIMPs).

There has been increasing recognition that wound healing is a dynamic process involving both matrix deposition and degradation, and the balance between the factors that promote scar deposition and those that promote resolution determines the eventual degree of fibrosis within the liver. In this regard, during the formation of fibrosis there is a high level of TIMP in the liver and lower levels of MMPs. This balance reverses with the cessation of liver injury when there is active remodeling of the scar tissue in the liver. HSCs express TIMPs, which results in inhibition of matrix-degrading MMP activity. Therefore, HSCs and myofibroblasts affect the balance of matrix secretion and degradation to favor the accumulation of scar, and they are direct secretors of collagen matrix.

The hepatic macrophages are important orchestrators of the wound-healing response in the liver; they phagocytose apoptotic debris, signal to the HSCs and myofibroblasts, and secrete enzymes capable of matrix degradation. During chronic liver injury, the hepatic macrophages signal to the myofibroblasts via the secretion of TGFβ1 to promote scar deposition. Conversely, following the cessation of liver injury, when there is an active reduction in the number of activated myofibroblasts and fibrosis, the hepatic macrophages are important in promoting the degradation of scar tissue (see Figure 1.4). As mentioned in this chapter, both the myofibroblasts and hepatic macrophages are also important in the liver's regenerative response, underlining how the liver's wound response (fibrosis and its resolution) is closely linked to the epithelial regenerative response. Indeed, experimental studies have shown that the degradation of collagen scar matrix is required to enable the development of a ductular reaction. The implications are clear that strategies to minimize or even reverse liver fibrosis will likely also have an effect upon liver regeneration.

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Chapter 2
Hepatic immunology

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Summary

The immune system is an integral part of the liver as an organ. In addition to the classical roles of hepatocytes and biliary cells in metabolism and digestion, the presence of a broad range of immune cells in the liver contributes to its basic functions by sensing and reacting to external and endogenous danger signals. Several unique features characterize immune responses in the liver including the composition of immune cell types, the local tissue environment that allows close interaction between parenchymal cells and immune cells, and the gut-derived signals arriving from the portal blood. Integration of these components is pivotal for immunological homeostasis in the liver and orchestration of effective immune responses for the protection of the host.

Overview of liver immunology

The liver is a unique immunological organ due to its cellular composition and physiological function (Table 2.1). Cells of the innate and adaptive immune system actively participate in immune responses in the liver, recognizing and eliminating pathogens and other danger signals and inducing antigen-specific adaptive immune responses. Another key function of the liver is to protect the host from the presence of undesirable activated immune cells. Several factors discriminate the liver from other organs with respect to immune response. The liver is constantly exposed to gut-derived substances such as antigens, nutrients, and metabolites, as well as pathogen-derived immune activation signals from the portal circulation. The normal liver immune environment promotes immunological tolerance, which has long been recognized in the setting of liver transplantation. While the exact mechanisms for this have yet to be delineated, the presence of predominantly immature dendritic cells that induce immune tolerance instead of T cell activation and high levels of immuno-inhibitory cytokines and mediators (interleukin 10 [IL10], transforming growth factor beta [TGFß], and prostaglandin E2 [PGE2]) contribute to this phenomenon. The architecture of liver sinusoids, with slow blood flow and close proximity of liver parenchymal cells and immune cells, creates a microenvironment for prolonged interactions between these cells that may also be a factor in local immune regulation. Finally, the composition of the liver T cell and natural killer (NK)–natural killer T (NKT) cell populations is markedly different from that of the circulation and many organs, with high proportional representation of NK, NKT, and gamma delta (γδ) T cells.

Innate immunity

Innate immunity provides the first line of host defense against invading pathogens. In recent years, it was discovered that in addition to pathogens that trigger an innate immune response, the innate immune response can also recognize and respond to damaged self-molecules. A coordinated cascade of events occurs that involves recognition of exogenous or endogenous danger signals by various pattern recognition receptors. This leads to a rapid induction of intracellular signaling cascades that direct the production of pro-inflammatory

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Table 2.1 The liver is a unique immune organ.

The largest immune organ
Unique biological properties • Unique architecture and vascular structure that facilitate interaction between parenchymal and nonparenchymal cells and circulating immune cells • Constant exposure to gut-derived antigens and pathogen-derived substances from the portal circulation
Unusual composition of lymphocyte subsets • NK cells, NKT cells, and T cell receptor repertoire (γ and δ)
Promotes immune tolerance • IL10, PGE_2, and TGFß • Diversity of professional and nonprofessional antigen-presenting cells

cytokines and/or type I interferons that comprise innate immunity. Innate immunity is also critical in triggering and modifying adaptive immune responses [1].

Cell populations and mediators in the innate immune response

Monocytes, macrophages, and Kupffer cells

Monocytes and macrophages represent the major constituents of the innate immune cell population. These cells originate in the bone marrow and can be rapidly recruited to sites of inflammation due to their chemotactic and cell migration properties. Circulating monocytes differentiate into macrophages at sites of infection, injury, or inflammation in the tissues [2]. Kupffer cells are the resident macrophages in the liver; they contribute to elimination of gut-derived pathogens and play important roles in various liver diseases, including alcoholic and nonalcoholic liver diseases [3, 4].

Monocytes, macrophages, and Kupffer cells are the classical "phagocytic" immune cells that uptake pathogens or cell debris by phagocytosis, endocytosis, or pinocytosis. The phagocytic capacity of these cells also includes production of reactive oxygen species (ROS) that contribute to their antibacterial effector function and production of pro-inflammatory cytokines. Monocytes, macrophages, and Kupffer cells have overlapping functional repertoires where macrophages and Kupffer cells are most potent in pro-inflammatory cytokine and ROS production and relatively inefficient in antigen presentation compared to circulating blood monocytes [2].

Monocytes migrate from the circulation into the tissue, where they differentiate into tissue-specific macrophages, such as Kupffer cells in the liver. There are two main populations of monocytes, the classical and nonclassical subsets, which vary in phenotype, function, and morphology. The classical subset, which comprises approximately 90% of circulating monocytes, expresses high levels of CD14 (CD14++). The nonclassical subset is distinguished by expression of CD16 (Fcγ receptor III) and variable CD14 expression [5]. CD14+CD16+ monocytes have been identified as the main producers of tumor necrosis factor alpha (TNFα) [6] and secrete more IL10 in response to lipopolysaccharide (LPS) stimulation than CD14dimCD16+ or CD14+CD16– cells [5]. It has been reported that the pro-inflammatory CD14+CD16+ monocyte population is expanded in the circulation and liver of patients with chronic liver disease. In addition, the investigators report that CD14+CD16+ cells directly activate hepatic stellate cells, but CD14+CD16– cells do not [7]. Therefore, the CD14+CD16+ monocyte subset may contribute to an inflammatory and pro-fibrogenic intrahepatic microenvironment, which would affect the progression of liver disease. Another nonclassical monocyte subset, CD14–CD16+ cells, is more responsive to Toll-like receptor 8 (TLR8) stimulation than CD14+CD16– cells [8]. Phenotypically, monocytes of the classical lineage are larger and denser, capable of phagocytosis and production of ROS. In contrast, nonclassical monocytes are smaller, less dense cells, with better antigen presentation function [9–12].

Circulating monocytes are recruited into the target tissue by a coordinated sequence of signals. Chemokines regulate the expression of a number of integrins, which are cell surface receptors that interact with adhesion molecules on the endothelial cells' surface, enabling the monocyte to attach to and roll along the endothelium. Integrins are also involved in polarization of the monocyte, which allows it to extravasate into the tissue. Once inside the tissue, monocytes differentiate into dendritic cells or macrophages [13]. Since monocytes are a heterogeneous cell population, as described in this section, the stage at which they are recruited into the tissue may influence the final cell type [2].

Macrophages are an important component of immunological defense. Firstly, they act as an integral part of the innate immune response by engulfing pathogens and killing them via the release of ROS. Recognition of these pathogens also stimulates macrophages to release cytokines and chemokines, which recruit other cells to the site of infection. Macrophages also contribute to the adaptive immune response by processing and presenting antigens to activate T and B cells, components of the adaptive immune response [14]. Due to their extensive phagocytic capacity, macrophages also play an important part in the clearance of cellular debris that arises from necrosis and apoptosis [2]. Macrophages secrete a number of different classes of molecules depending on their activation status, including pro-inflammatory

cytokines such as IL1β, TNFα, and IL6; anti-inflammatory cytokines IL10 and TGFβ; chemokines; and proteolytic enzymes [15].

Classically activated macrophages (also referred to as M1 macrophages) are generated in response to Th1 cytokines, the most important activator being interferon gamma (IFNγ) [14, 16]. IFNγ activates IFN regulatory factor (IRF) transcription factors, including IRF1. IRF1 upregulates IFNα, IFNβ, and inducible nitric oxide synthase (iNOS), increasing the antiviral and antimicrobial properties of the affected cell [14]. This cell type secretes a number of inflammatory cytokines that amplify the Th1 immune response. Classical macrophages are able to kill intracellular pathogens by producing ROS and nitric oxide [17]. These cells are an important element in the innate immune response in addition to being potent mediators of inflammation [18].

Alternatively activated macrophages (also known as M2 macrophages) are generated in response to Th2 cytokines IL4 and IL13 [15]. Activation along this pathway enhances endocytic antigen uptake and presentation, eosinophil involvement, and granuloma formation that is required for an efficient response to parasitic infection or extracellular pathogens [14]. This cell type is distinct from classical macrophages in that they do not produce nitric oxide [17].

Macrophages play an important role in liver fibrosis. Macrophages produce the pro-fibrotic cytokine TGFβ [15, 17]. In addition, alternatively activated macrophages may be involved in production of the extracellular matrix [17]. However, current evidence suggests that liver macrophages act as regulators of fibrosis and fibrogenesis [19].

Kupffer cells are liver resident macrophages. They account for approximately 80% of the body's macrophage population [20], and constitute approximately 20% of the nonparenchymal cells in the liver [21]. Kupffer cells are localized to the sinusoidal vascular space in the periportal area. In this location, they are able to clear endotoxins, microorganisms, and cellular debris from the portal circulation entering the liver [20, 21]. Kupffer cells act cooperatively with neutrophils to eliminate pathogens from the blood [20, 22]. In addition, Kupffer cells are important producers of cytokines and chemokines in the liver following injury or endotoxemia [23].

Dendritic cells and antigen presentation

Dendritic cells (DCs) are the main type of antigen-presenting cells in the immune system that uptake antigens, induce antigen-specific T cell activation, and produce inflammatory and immunomodulatory cytokines. DCs are 10 times more potent at antigen presentation and T cell activation compared to monocytes and macrophages. Dendritic cells efficiently uptake and process antigens due to their rich subcellular endosomal compartments. The processed antigenic peptides are presented in the context of major histocompatibility complex class (MHC) II molecule and co-stimulatory signals to initiate activation of naïve $CD4^+$ T lymphocytes [24].

Dendritic cells can be separated into various subtypes based on their origin, cell surface marker expression, and functional capacity. Myeloid DCs are derived from the bone marrow and are present in both murine and human livers. Blood monocytes can differentiate into monocyte-derived myeloid dendritic cells upon *ex vivo* stimulation with IL4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Differentiation of circulating monocytes into dendritic cells is triggered *in vivo* by the tissue environment. Both conventional and monocyte-derived DCs (mDCs) produce the immunomodulatory cytokines IL12 and IL10 that contribute to the efficiency of their T cell activation and antigen-presenting capacity. The mDC1 (myeloid CD1c+ DC) represents the largest population of myeloid DCs (also known as conventional DCs) in the blood, which produce inflammatory cytokines and chemokines upon stimulation [25]. The mDC2 (myeloid CD141+ DC or myeloid BDCA3+ DC) represents a minor subset of blood leukocytes that have recently been identified as the human homologue of the mouse CD8+ DC subset [26, 27]. mDC2s are major producers of IL12 and cross-present antigen for CD8 class 1–restricted cytotoxic T lymphocyte (CTL) responses under TLR3 ligation [28, 29]. Plasmacytoid DCs (pDCs) represent a small population in the peripheral blood but are enriched in the liver; they are the most potent producers of IFNα in viral infections [30].

Both mDCs and pDCs are present in the liver in an immature phenotype that is characterized by a high capacity to uptake antigens but relatively low T cell activation potential. Compared to other tissues, the majority of DCs in the liver possess an immature phenotype. This phenomenon has been attributed to the state of "immune tolerance" in the liver. Pathogen-derived signals, in the presence of inflammation, rapidly induce maturation of immature DCs in the tissue. During the maturation progress, DCs change their phenotype and increase surface expression of T cell co-stimulatory molecules, resulting in their superior antigen presentation and T cell activation capacity [30, 31].

NK and NKT cells

NK and NKT cells are lymphocytes that, unlike B and T cells, do not express an antigen receptor with somatic diversification [32]. Human NK cells express CD56 and CD16 but lack CD3. NK cells constitute up to 50% of the

hepatic lymphocyte population in humans, compared to 5–20% in the peripheral circulation [31]. In addition to their increased numbers, liver-derived NK cells also exhibit enhanced cytotoxic capacity against tumor cells compared to splenic and peripheral blood NK cells derived from rodents or humans [32].

NK cell function is regulated by a balance between stimulatory and inhibitory receptors that are constitutively expressed on the cell surface. NK cells are inactivated when inhibitory receptors bind to MHC I receptors on the target cell [32]. NK cells become activated when a cell with abnormal expression of MHC I or stress-related proteins is detected. Upon activation, NK cells release granules that lyse the target cells, or they induce apoptosis via engagement of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) [31, 32].

Hepatic NK cells are important mediators of the innate immune response against tumors, viruses, intracellular bacteria, and parasites. Decreases in the number of NK cells are associated with progression of hepatocellular carcinoma [32] and chronic hepatitis C virus (HCV) infection [31]. Activated NK cells also play a role in liver injury and repair by controlling the balance between pro-inflammatory and anti-inflammatory cytokines in the liver microenvironment [21].

NKT cells express T cell markers as well as NK cell markers. Classical NKT cells (also known as invariant NKT or iNKT cells), which are CD1d dependent, are capable of producing type I and type II cytokines. Similar to NK cells, iNKT cells can induce cell lysis via perforin or the Fas ligand. Nonclassical NKT cells, which are CD1d independent, produce only type I cytokines. The number of NKT cells is enriched in the liver, comprising up to 10% of the liver lymphocyte population [32]. NKT cells recognize nonpeptide antigens such as lipid and glycolipid and, when stimulated, are able to rapidly secrete large amounts of IFNγ and IL4, influencing the balance between a pro- and anti-inflammatory microenvironment in the liver [21]. These characteristics suggest that NKT cells are involved in connecting the innate and adaptive immune responses in the liver [32]. NKT-mediated cytotoxicity has been identified as a key factor in experimental hepatitis models induced by concanavalin A and endotoxin. NKT cells are also important in protecting against liver infection. NKT- or CD1-deficient mice are more susceptible to certain viral infections, and NKT cells activated by the CD1d ligand downregulate HBV replication via induction IFNγ secretion [21].

γδ T cells are an alternative T cell type that express a γδ T cell receptor instead of the more common αβ T cell receptor [31]. These cells recognize stress proteins and nonprotein antigens. Although their number is limited in the circulation, they comprise between 15 and 25% of the liver T cell population [21]. It has been shown that γδ T cell numbers increase in patients with viral hepatitis but not in those with nonviral hepatitis [32]. It has also been reported that hepatic γδ T cells are elevated in mice with viral infection or liver tumors [31, 32], indicating that this cell type plays a role in immune surveillance in the liver.

Neutrophil leukocytes

Neutrophil leukocytes are the first line of defense in most bacterial infections and in tissue damage. Regulated expression of a cadre of adhesion molecules permits leukocytes to rapidly invade inflamed tissue from the microvessels of the circulation [33]. Neutrophils are highly chemotactic and exert direct antibacterial effects through their expression of elastase, myeloperoxidases, and ROS. Although neutrophils are primarily involved in the clearance of infection, they have also been implicated in the initiation of tissue damage in alcoholic liver disease and sepsis [23].

Cytokines, interferons, and chemokines

Cytokines, interferons, and chemokines are soluble protein "messengers" and executors of innate and adaptive immune responses. Cytokines are soluble signaling molecules that provide communication between different cells in the tissue, in the systemic circulation, and in distant organs; they have paracrine and autocrine effects. The interleukin family of cytokines can be divided into various categories of cytokines that promote inflammation including IL1α, IL1ß, IL6, and several other types of cytokines such as IL17. IL10 is an anti-inflammatory cytokine that also has negative effects on antigen-presenting functions of innate immune cells and directly inhibits T cell proliferation. Other cytokines such as TNFα, IL12, IL18, IL33, IL21, and IL22 have immunoregulatory functions [34].

The pro-inflammatory cytokine TNFα is primarily synthesized by monocytic cells, including macrophages, Kupffer cells, and microglia. There are two receptors for TNFα, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). TNFR1 is expressed on most tissues, while TNFR2 is expressed mainly on immune cells. TNFα is released during infection or trauma, and is able to generate a cytokine cascade [35]. Several studies have noted that TNFα is an important mediator in the development of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) in both humans and animals [36].

The IL1 family of cytokines includes IL1α, IL1β, IL18, IL33, and the IL1 receptor antagonist (IL1RA). These cytokines have important roles in the innate and adap-

tive immune response. The IL1 family members signal through related receptors that include an extracellular immunoglobulin domain and a cytoplasmic Toll–IL1 receptor (TIR) domain. When the ligand binds to the receptor, a second subunit is recruited; assembly of the receptor heterodimer induces signaling [37]. IL1 and IL18 require cleavage by the inflammasome complex (see section below on inflammation) to produce the biologically active form for secretion. IL1β increases the expression of adhesion markers on endothelial cells, which work in conjunction with chemokines to induce the migration of immune cells from the circulation into the target tissue [38]. Members of the IL1 family play an important role as co-stimulators of T cells. For example, IL33 enhances Th2 responses; IL18, in the presence of IL12, enhances Th1 response by producing IFNγ; however, in the absence of IL12, it enhances the Th2 response by producing IL4 [38].

Interferons are the first line of defense against viral infections in innate immunity and are produced by immune cells as well as some parenchymal cells, including hepatocytes in the liver. Type I IFNs include IFNα and IFNß; Type II IFN, IFNγ, is a major immunoregulator; and the recently discovered Type III IFNs, also called IFNλs, include IL28a, IL28b, and IL29. Type I and Type III interferons have direct antiviral effects, while IFNγ is an immunomodulator that activates and amplifies innate and adaptive immune responses [39]. Recent clinical data identified that several single-nucleotide polymorphisms (SNPs) near the *IL28* gene are strongly associated with HCV clearance during natural viral clearance as well as in response to therapy with IFNα plus ribavirin [40, 41].

The family of chemokines includes a large array of mediators that direct immune cell trafficking, recruitment, and homing to various tissues in a cell-specific manner [42]. Chemokines are separated into four families based on the pattern of cysteine residues. The CC chemokine family includes RANTES, which attracts T cells, and monocyte chemoattractant protein-1, which provides the signal for monocytes to migrate from the bloodstream into tissue to differentiate into macrophages. The CXC family includes IL8, which targets neutrophils, monocytes, and mast cells. Fractalkine is the only member of the CX3C family. The final family, XC, includes lymphotactin and SCM-2β. Chemokines exert their effects by binding to G protein–coupled receptors (GPCRs). The biological effect of the chemokine–receptor binding depends on the coupling of the different G proteins within the receptor itself [43]. Chemokines are produced by both parenchymal and immune cells in the liver; their cell-specific effect is provided by the cellular expression of the respective chemokine receptors [42].

Complement

The complement system is composed of soluble and membrane-bound proteins that associate in the form of a "cascade." Activation of the complement cascade leads to the assembly of a pore-forming structure known as the membrane attack complex (MAC) on the surface of the target cell. There are three separate pathways in the complement cascade, each of which is specific for different targets: the classical pathway, the lectin pathway, and the alternative pathway. The classical pathway recognizes antibody-bound targets, such as immune complexes and dead cells. The lectin pathway recognizes mannose-binding lectin (MBL) bound to carbohydrate molecules on bacterial cells. The alternative pathway is activated by foreign agents such as bacteria, viruses, and fungi [32, 44].

Complement proteins are involved in maintaining homeostasis through tissue repair and regeneration as well as through inflammation. Dysregulation of the complement cascade is implicated in a number of autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis. Complement proteins are involved in clearance of debris, thereby reducing exposure to potential auto-antigens. Complement also functions to maintain B cell tolerance, which reduces the production of auto-antibodies [45]. Activation of the complement cascade has been found in alcoholic liver disease; mice deficient in complement C3a or the complement receptor are partially protected in the early phase of alcoholic liver disease [46].

The complement cascade is a critical element in the immune system, and the liver plays a vital role in maintaining that system. The liver is the primary site of complement protein synthesis. Pro-inflammatory cytokines secreted during an inflammatory response such as IL6, IFNγ, and TNFα stimulate hepatocytes to produce complement proteins. Hepatocytes also synthesize the complement regulatory proteins C1 inhibitor, factor H, and factor I. C3a and C5a are critical for liver regeneration after injury. Complement can also contribute to the pathogenesis of liver disorders such as liver fibrosis and alcoholic liver disease [32, 45]. HCV has been shown to decrease C3 levels both *in vivo* and *in vitro* [47].

Inflammation in liver diseases

Inflammation is the response of the innate immune system to danger signals. Inflammation is triggered by recognition of danger signals, which induces an inflammatory response; this process is normally self-limited with resolution of the inflammation. In all chronic liver diseases, whether induced by viral hepatitis, metabolic factors, or alcohol abuse, persistent insult prevents

resolution, resulting in chronic inflammation that leads to chronic liver disease, fibrosis, and cirrhosis (Figure 2.1). The three determining stages of inflammation are recognition, response, and resolution.

Immune recognition of danger signals occurs with the help of pattern recognition receptors (PRRs) (Figure 2.2). The major families of PRRs are Toll-like receptors (TLRs); the RIG-I-like receptors (RLRs), including RIG-I and MDA5; the NOD-like receptors (NLRs), such as NALP and IPAF; as well as other intracellular sensors (Figure 2.3) [1, 48–51]. While initial discovery of TLRs was made in innate immune cells, TLRs are expressed and functionally active in virtually all cell types in the liver [52].

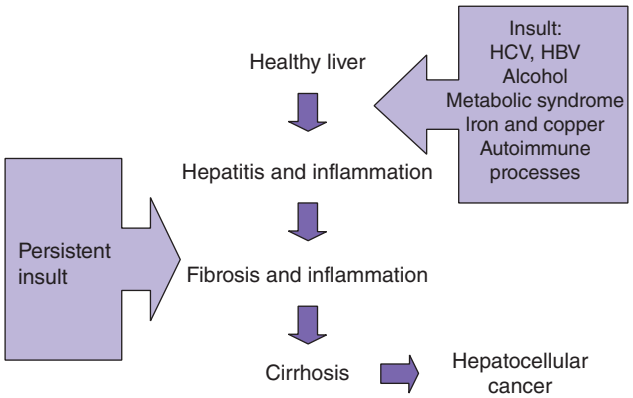

Figure 2.1 Progression of chronic liver disease. HBV: hepatitis B virus; HCV: hepatitis C virus.

Pathogen-associated molecular patterns (PAMPs) represent foreign danger signals that the host recognizes as "exogenous danger." In "sterile" inflammation associated with tissue injury, damaged host cells release damage-associated molecular patterns (DAMPs) that are recognized by the same repertoire of TLRs and PRRs as exogenous danger signals. In certain pathologies, PAMPs and DAMPs can both be involved in and amplify inflammatory responses.

TLRs are evolutionarily conserved sensors of PAMPs. Of the 13 TLRs, most are functionally active in humans. TLRs expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) recognize extracellular PAMPs, while intracellularly localized TLRs (TLR3, TLR7, TLR8, and TLR9) sense nucleic acid sequences (Figure 2.4) [1]. The cytoplasmic TIR domain of TLRs interacts with the TIR domain of adapter molecules such as the common adapter MyD88 utilized by all TLRs except for TLR3. MyD88 recruitment triggers downstream signaling via IRAK1/4 kinases and IKK kinase activation to culminate in NF-κB activation and induction of pro-inflammatory cytokine genes. TLR3 and TLR4 utilize the TRIF adapter that activates IKKε/TBK, leading to IRF3 or IRF7 phosphorylation and, after their nuclear translocation, induction of Type I IFNs [48, 53]. TLR4 recognizes endotoxin derived from Gram-negative bacteria, TLR2 senses microbial lipopeptides, while TLR1

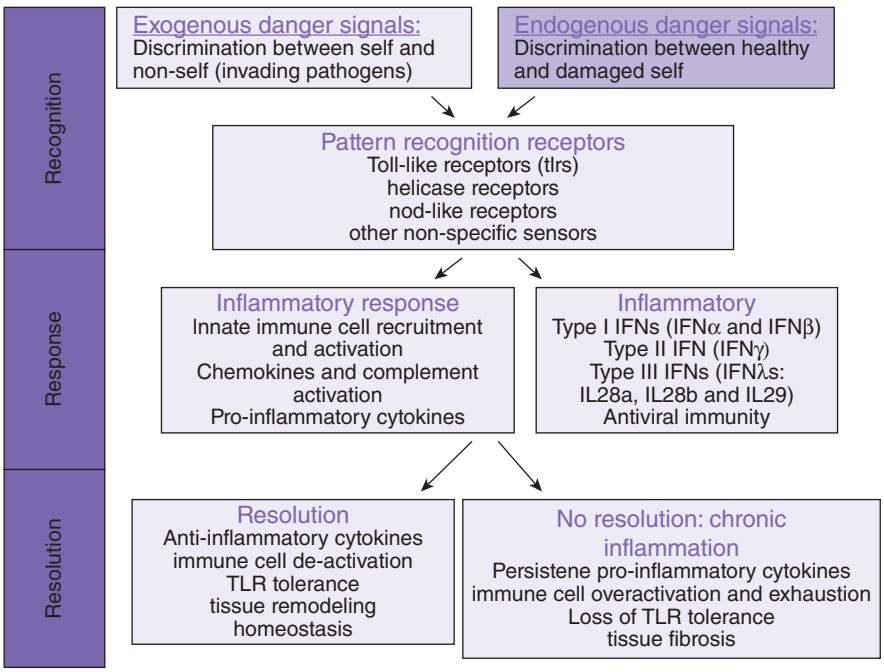

Figure 2.2 Danger signals and their recognition.

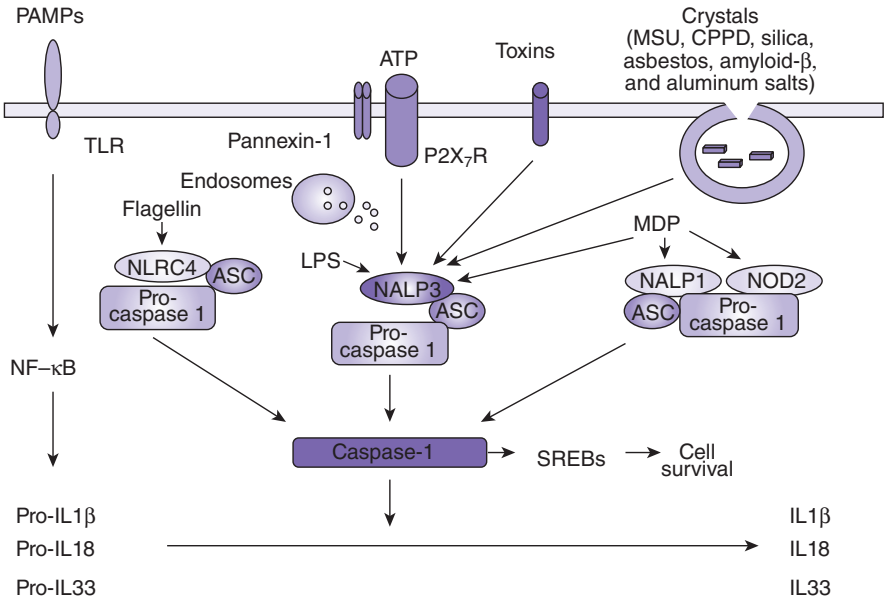

Figure 2.3 Activation of the inflammasome by PAMPs and DAMPs. PAMPs: pathogen-associated molecular patterns; ATP: adenosine triphosphate; MSU: monosodium urate; CPPD: calcium pyrophosphate dihydrate; TLR: Toll-like receptor; LPS: lipopolysaccharide; MDP: muramyl dipeptide; ASC: apoptosis-associated speck-like CARD domain-containing protein; NALP: NACHT-LRR-PYD-containing protein; NOD: nucleotide-binding oligomerization domain-containing protein; SREB: sterol regulatory element-binding protein. (Color plate 2.1)

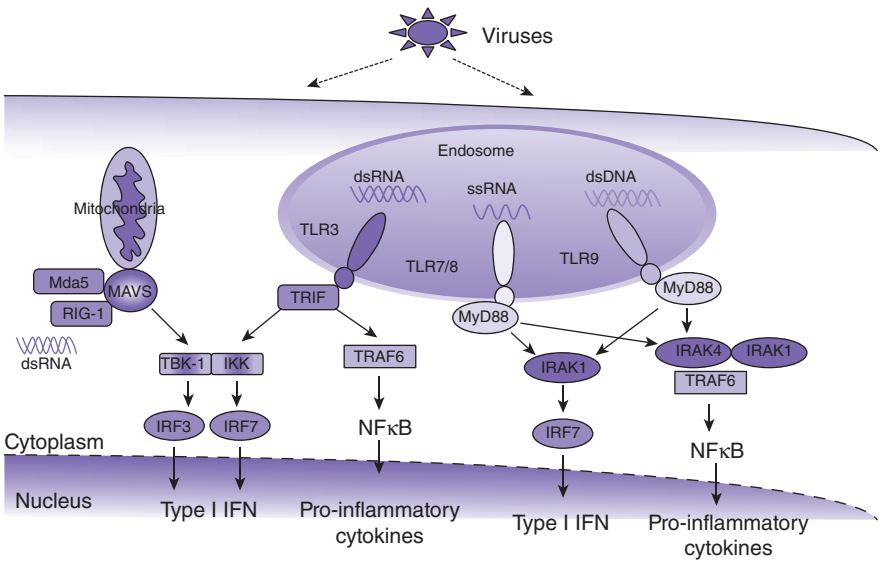

Figure 2.4 Intracellular sensors of viral infection. dsRNA: double-stranded RNA: ssRNA: single-stranded RNA; dsDNA: double-stranded DNA; MAVS: mitochondrial antiviral-signaling protein; TRIF: TIR domain-containing adapter-inducing interferon-β; MDA5: melanoma differentiation-associated protein 5; IRAK: interleukin-1 receptor-associated kinase; TRAF: TNF receptor-associated factor; IRF: interferon regulatory transcription factor; TBK: TANK binding kinase; IKK: IkappaB kinase. (Color plate 2.2)

and TLR6 combined with TLR2 distinguish between triacyl- and diacyl-lipopeptides [54]. TLR3 recognizes viral double-stranded RNA, and bacterial flagellin stimulates TLR5 [55, 56]. TLR7 and TLR8 are triggered by viral single-stranded RNA [57], and TLR9 recognizes prokaryotic CpG-rich DNA [58].

All TLRs are broadly expressed in the liver in diverse cell populations. Kupffer cells express TLR4, TLR2, TLR3, and TLR9 [59–61], and stellate cells can be activated via TLR2, TLR4, and TLR9 [62, 63]. Liver sinusoidal endothelial cells express TLR4 [64, 65], and primary cultured hepatocytes express mRNA for all Toll-like receptors of which TLR2, TLR3, TLR4, and TLR5 are expressed at low levels and show weak responses *in vivo* [66, 67]. LPS, a component of Gram-negative bacteria, is a strong activator of innate immune responses via the TLR4 complex because of its lipid A portion [68]. TLR4 cannot directly bind LPS, and the binding of LPS to the co-receptors CD14 and MD2 facilitates activation of TLR4. CD14, a GPI-anchored protein, facilitates the transfer of LPS to the TLR4–MD2 receptor complex that modulates LPS recognition [69]. MD2 associates with TLR4 and binds LPS directly to form a complex with LPS in the absence of TLRs [70]. The association between LPS and CD14 can be further facilitated by LPS-binding protein (LBP) [71].

NLRs are sensors of the inflammasome complex that, upon activation, lead to caspase-1 cleavage. The activated caspase-1 cleaves pro-IL1 to the biologically active 18 kD IL1ß [49, 51, 72, 73]. Inflammasomes are multiprotein complexes that sense intracellular danger signals via the sensor (NLR) that forms a complex with the effector molecule, pro-caspase-1, with or without the contribution of an adapter molecule, such as apoptosis-associated speck-like CARD domain-containing protein (ASC) [73–76] . Inflammasome activation leads to autoactivation of inactive pro-caspase-1 precursor into p20 and p10 subunits that form the active caspase-1 [67, 69–71] resulting in cleavage of pro-IL1β and pro-IL18 into mature forms and inactivation of IL33 [73–77]. As a pro-inflammatory cytokine, IL1ß regulates inflammation and binds to the IL1 receptor (IL1R) to exert its broad biological effects. The IL1R also recognizes IL1α and binds IL1R antagonist (IL1ra). IL1ra is a soluble protein induced by the same danger signals as pro-inflammatory cytokines, and it is a naturally occurring inhibitor of inflammation by occupying the IL1R without inducing activation [38]. IL18 activates NK cells to produce IFNγ [38, 78, 79], and IL33 is a chromatin-associated cytokine of the IL1 family that drives Th2 responses [80, 81]. The full-length active IL33 is cleaved and inactivated by caspase-1 [77].

Inflammasome activation is a multistep process where the initial signal results in upregulation of inflammasome expression; this step is mostly initiated by TLR activation. The second signal triggers functional inflammasome activation by an inflammasome ligand [75, 76]. Inflammasome ligands include both pathogen-associated (PAMPs) and endogenous danger molecules (DAMPs) [73–76]. DAMPS are released from activated, damaged, or dying cells and represent a broad range of molecules including HMGB1, fibronectin, and heat shock proteins, among others [82]. The four main prototypes of inflammasomes are NLRP1 (NALP1), NLRP3 (NALP3, or cryopyrin), NLRC4 (IPAF), and AIM2 [76]; while each of these has different ligand recognition sites and utilization of adapter molecules, all lead to caspase-1 activation.

There is increasing evidence for the involvement of the inflammasome complex in different types of liver diseases, and their potential as targets for disease modification is an emerging field in hepatitis research [83]. For example, inflammasome activation was found in acetaminophen-induced liver disease as well as in NASH [84–86].

RNA helicases such as RIG-I and MDA5 are another important intracellular pattern recognition receptor family. These receptors sense double-stranded RNA and induce Type I IFNs via the mitochondrial antiviral-signaling protein (MAVS) (also known as IPS) adaptor [49]. Translational research elegantly identified RIG-I as a target of the HCV serine protease NS3/4 [87], and now successful therapies are entering clinical practice to cure disease (Figure 2.4). Decreased expression and function of MAVS were also found in NASH, linking decreased Type I IFN production with fatty liver disease [88].

Depending on the expression profile of TLRs, other pattern recognition receptors and the components of the intracellular signaling pathways determines the response of individual cells to danger signals. Expression of pattern recognition receptors is not limited to immune cells in the liver. Essentially any of the cell types in the liver have some form of pattern recognition system that enables them to sense danger signals [32].

Resolution of inflammation is determined by the balance of pro- and anti-inflammatory cytokines and mediators elicited by the initial danger signal. The same TLR ligands that induce pro-inflammatory cytokines in the early phase of inflammation also trigger anti-inflammatory mediators such as IL10 and TGFß that downregulate the initial inflammation to establish homeostasis [34].

Adaptive immunity

$CD4^+$ T cells

Activation of T lymphocytes is largely dependent on their interactions with antigen-presenting cells (APCs)

such as dendritic cells, monocytes, and macrophages. Classically, APCs that are exposed to pathogens or other antigens will interact with naïve $CD4^+$ T cells, and the type of interaction may determine the development of the T cells into a Th1, Th2, T regulatory (Treg), or Th17 phenotype. Th1 $CD4^+$ T cells are potent producers of IFNγ and TNFα, while Th2 $CD4^+$ T cells produce IL4, IL10, and IL13. $CD4^+$ Tregs produce IL10 and TGFß, while Th17 cells secrete IL17 and IL22. Induction of the Th1 phenotype requires mature DC1 type interaction with native $CD4^+$ T cells and the presence of IL12 and IL18 as co-stimulatory molecules. Th2 cells were shown to be induced by interaction with DC2 in the presence of antigen and IL4 [89].

$CD8^+$ T cells

The majority of hepatic T cells are $CD8^+$, comprising 70% of the liver T cell population compared to 35% in peripheral blood [90]. Functions of $CD8^+$ T cells include induction of apoptosis via the Fas ligand, secretion of pro-inflammatory cytokines, and cytolysis. Activated $CD8^+$ T cells are recruited to the liver independent of their antigen specificity; however, proliferation occurs only when the specific antigen is encountered [21]. In chronic HCV infection, $CD8^+$ T cells commonly display an exhausted phenotype that includes higher expression of the inhibitory receptor PD1 [91].

Regulatory T cells

The immune system has sophisticated mechanisms in place to control overt immune activation in response to pathogens and/or antigens. Regulatory T cells (Tregs) play a central role in immune balance as mediators of peripheral immune tolerance. Tregs have a pivotal role in preventing autoimmune processes such as primary biliary cirrhosis [92], controlling rejection in liver transplantation [93], and limiting chronic immune activation and inflammation (e.g., in viral hepatitis) [94, 95]. Natural Tregs arise in the thymus while induced Tregs are generated from CD4+ T cells in the periphery in the presence of cytokines and an immunosuppressive tissue environment. Naturally occurring forkhead box P3 (Fox P3/Cd4+/CD25+ Treg) cells display a diverse T cell repertoire that is specific for self-antigens; however, Tregs are also induced or converted from activated CD25+ T cells during inflammatory processes in the peripheral tissue [96]. T regulatory 1 (T_R1) cells mediate their immunosuppressive activity via IL10, while T helper 3 (T_H3) cells produce the immunoinhibitory cytokine TGFß [97]. IL35 is a recently recgonized cytokine that is suggested to regulate Treg functions [98]. In addition to suppression by inhibitory cytokines, the basic mechanisms of Treg cell function include suppression of dendritic cells. For example, Tregs that inhibit DCs via CTLA4 could condition DCs to express indoleamine 2,3 dioxygenase (IDO), a potent regulatory molecule [99, 100].

Induction of regulatory T cells (Tregs) is typically initiated by their interaction with immature DCs in a cytokine environment enriched for TGFß or IL2. Tregs have been identified as major modulators in the immune response against HCV infection, and several studies suggest that increased number and increased activity of the different regulatory T cell populations contribute to impaired HCV clearance in chronic HCV infection [94].

Table 2.2 The balance between T helper type17 (Th17) cells and T regulatory (Treg) cells is critical in immune homeostasis.

CD4+ Th17 cells	CD4+ Treg cells
Autoimmune diseases	Prevent autoimmunity
Rheumatoid arthritis	
Colitis	Maintain tolerance
Experimental autoimmune encephalitis (EAE)	CD25+ CD4+ FoxP3+ Treg mediate spontaneous liver transplant tolerance
Liver diseases	
Acute liver injury	
Liver granulomas	
Ischemia–reperfusion	

CD17

The inflammatory Th17 T cell phenotype is induced by mature DCs with co-stimulation by IL1ß, IL6, IL23, and TGFß. The balance between Th17 and Tregs is critical in immune homeostasis (Table 2.2) [101–106]. In autoimmune diseases, as well as in certain liver disease such as acute liver injury, liver granulomas, and ischemia–reperfusion liver injury, predominance of Th17 cells contributes to disease pathology [103]. Studies suggest that administration of Tregs can improve these conditions. Consistent with this, Tregs prevent autoimmunity and maintain immune tolerance in the form of spontaneous liver transplant tolerance by CD25+CD4+FoxP3 Tregs [105].

B cells

The role of B lymphocytes is relatively poorly characterized in liver diseases compared to other immune cells. B cells comprise less than 10% of the human hepatic lymphocyte population. The majority of these cells are CD5+, a negative regulator of B cell receptor signaling. CD5+ B cells are significantly increased in the blood and liver of individuals with HCV [31].

Table 2.3 Hepatic antigen-presenting cells (APCs).

Professional APCs	Nonprofessional APCs
Monocytes	Macrophages
Dendritic cells (DCs)	• Kupffer cells
• Myeloid DCs	Liver sinusoidal endothelial
• Plasmacytoid DCs	cells (LSECs)
	Stellate cells
	Cholangiocytes
	Hepatocytes

The liver is a unique immune organ

There are several aspects to the liver that contribute to its unique status in immune tolerance. Hepatic immune tolerance has been noted in allogeneic transplantation of liver or organs together with liver as they had reduced requirement for immunosuppression post transplant. APCs play a key role in recognition of nonself and in activation of T cell responses. In the liver, there are different types of APC populations. Professional APCs include dendritic cells of both the myeloid and plasmacytoid DC lineage (Table 2.3). Liver macrophages and Kupffer cells can also perform as APCs, although their function is less superior compared to that to DCs. The majority of DCs present in the liver have a predominantly "immature" phenotype that is suboptimal in inducing potent antigenic responses by T lymphocytes. Several of the liver parenchymal and nonparenchymal cells that are not of immune origin have been shown to act as nonprofessional APCs, including liver sinusoidal endothelial cells (LSECs), stellate cells, cholangiocytes, and hepatocytes [20]. However, poor APCs promote T cell tolerance instead of antigen-specific T cell activation. Professional APCs provide coordinated signals to T cells for their full activation, including MHC II and TCR, CD80/86, and CD28 interactions, and in the absence of such signals T cell tolerance is induced instead. Nonprofessional APCs in the liver generally lack sufficient expression of some of these signals (MHC II or co-stimulatory molecules CD80/86), thereby inducing T cell tolerance instead of sufficient activation [107].

The anatomical position of the liver results in its blood supply from the portal circulation that is the collection site of all of the gut-derived venous blood. The portal blood is enriched in nutrients and in gut-derived substances including pathogen-derived compounds. The unique architecture and the vascular structure of the liver sinusoid, where blood flow slows down, facilitate interactions between parenchymal and nonparenchymal cells and circulating immune cells. A significant amount of research has focused on the role of the gut–liver axis in contributing to liver homeostasis and liver inflammation. Under normal homeostasis and with intact gut barrier and hepatocyte functions, the liver "detoxifies" the portal blood and avoids activation of the innate immune system [89, 108]. It has been shown that different types of liver diseases, including alcoholic and nonalcoholic liver disease as well as cirrhosis from any etiology, result in increased portal levels of LPS (which, as mentioned, is a component of Gram-negative bacteria), and this has been suggested to result in Kupffer cell activation and pro-inflammatory cascade activation in the liver. Sensitization of Kupffer cells to gut-derived LPS was found in animal models of alcoholic and nonalcoholic steatohepatitis [109, 110].

Additional contributors have also been proposed to promote the immunotolerant liver environment, such as the unusual composition of T cell populations characterized by the large proportion of NK, NKT cells, and γδ T cells [32, 90]. Additional factors include the relatively high expression of IL10, TGFß, and PGE2 in the liver; all are anti-inflammatory mediators that also inhibit APC function and antigen-specific T cell activation [20].

Several studies described that activated T cells tend to home to the liver, where they die by apoptosis [111, 112]. Peripheral T cell activation occurs in most infections, particularly in viral infections. For example, in cytomegalovirus (CMV), Epstein–Barr virus (EBV), and herpes simplex virus (HSV) infections, activated T cells home to the liver, where hepatocytes may suffer from "bystander" damage. Clinically, these viral infections cause hepatocyte damage and lead to increased serum transaminases that are thought to be predominantly a result of this "bystander" damage from activated T cells.

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Section II
Hepatitis A Virus

Chapter 3
Structure, molecular virology, natural history, and experimental models

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Summary

Hepatitis A virus (HAV), a small, naked, single-stranded, positive RNA virus that belongs to the *Picornaviridae*, causes acute hepatitis in humans and nonhuman primates. HAV infection typically does not induce cytopathic effect in cell culture, and disease results from an immunopathogenic process that clears the virus without leaving chronic sequelae. HAV is transmitted through the oral-fecal route, but it is unknown how it reaches the liver. The pathogenic process of HAV is poorly understood. However, recent advances in the understanding of the effects of the interaction of HAV with its cellular receptor 1 (HAVCR1), which blocks the activation of T cell receptors and shuts off regulatory T cell (Treg) functions, help explain how HAV modulates the immune response to evade detection during its long incubation period and is cleared with limited liver damage. The shut-off of Treg functions may also explain the protective effect of HAV infection in the development of atopy and autoimmunity.

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Introduction

Hepatitis A virus (HAV), a small, naked, single-stranded, positive RNA virus that belongs to the *Picornaviridae* family, is the causative agent of acute hepatitis in humans and nonhuman primates (NHPs). HAV typically does not cause cytopathic effect (CPE), and only a few fast-growing, cell culture–adapted strains are capable of inducing apoptosis. HAV is transmitted through the oral-fecal route, but it is unclear how the virus reaches the liver, its primary replication site. The immunopathogenic process by which HAV causes liver disease is poorly understood. The availability of safe and effective inactivated vaccines has significantly reduced the transmission of HAV in the developed world, but these vaccines are expensive to produce and may not confer lifelong immunity. The development of cost-effective, attenuated vaccines that confer lifelong immunity will be required to control the transmission of HAV in the developing world. Further research is required to understand the determinants of hepatotropism and hepatovirulence to develop safe and effective HAV vaccines.

Infectious hepatitis has been recognized since ancient times, with reports of epidemic jaundice described by Hippocrates in Greece in the fifth century BC. Outbreaks of infectious hepatitis in the eighteenth to twentieth centuries and epidemics of "campaign jaundice" during

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major wars, including the US Civil War and World War II, were documented in detail. In 1947, MacCallum [1] minted the terms "hepatitis A" for infectious hepatitis and "hepatitis B" for serum hepatitis. The significant work of Krugman and colleagues in the 1960s with human volunteers at the Willowbrook State School, New York, further defined the course of infection and led to the production of the MS-1 pool of sera from acute hepatitis patients that transmitted HAV infection to children [2]. The use of MS-1 inocula to transmit HAV infection to adult inmates at the Joliet Prison, Illinois, by Boggs and colleagues [3], as well as the subsequent use of the inmate plasma to infect nonhuman primates by Deinhardt and colleagues [4], paved the way for the identification by Feinstone *et al.* [5] in 1973 of the causative agent of hepatitis A and the subsequent characterization of HAV. Adaptation of the virus to cell culture by Provost and Hilleman in 1979 [6] allowed the development of inactivated HAV vaccines that were first licensed by the US Food and Drug Administration (FDA) in 1995. In the 1980s, HAV was further characterized, molecularly cloned, and sequenced, leading to the construction of infectious complementary DNAs (cDNAs) of the attenuated cell culture–adapted and wild-type (wt) strains ([7, 8] and references therein). In the 1990s, reverse genetic studies allowed the identification and characterization of genetic elements and the mutations responsible for the attenuation of HAV. Studies on virus cell entry resulted in the identification of HAV cellular receptor 1 (HAVCR1) [9], the first discovered member of the HAVCR family of glycoproteins, which have a significant role in modulating immune responses [10]. The HAV–HAVCR1 interaction plays a significant role in the immunopathogenic process of HAV [11, 12] and the protective effect of HAV infection in the development of allergies and autoimmunity [13–15]. Many comprehensive reviews on HAV have been written in the 65 years since MacCallum proposed the term "hepatitis A" (e.g., [16–18]). In this chapter, we will focus on the latest advances in HAV biology.

HAV structure and molecular virology

Because of its unique features within the *Picornaviridae* family, HAV has been classified in a separate *Hepatovirus* genus. HAV is the only species of *Hepatovirus* and exists in only one serotype, with three genotypes that circulate in humans (I–III) and three additional genotypes (IV–VI) recognized in NHPs. The slow and limited growth of HAV is an important characteristic of this virus that has very significant implications for its life cycle and pathogenic processes. However, its limited growth in cell culture complicates experimentation, and the biology of HAV has mainly been inferred from model picornaviruses, such as poliovirus (PV) and encephalomyocarditis virus (EMCV).

Genomic structure

The HAV genome consists of a single-stranded, positive-sense polycistronic RNA genome of approximately 7.5 kilobases (kb) that encodes 11 genes (Figure 3.1). A long open reading frame (ORF) coding for a polyprotein of approximately 220 kDa is flanked by a 5′ nontranslated region (NTR) of approximately 750 nucleotides (nts) and a short 3′NTR of 62 nts that ends in a poly(A) tract (Figure 3.1). The HAV genome has been divided into three regions: P1 encodes the capsid proteins, and the P2 and P3 regions encode functional proteins. By convention, the genes in each region are assigned the region number followed by consecutive letters. For instance, the 5′ end of the virion RNA is linked to a small protein of 23 amino acids encoded by the 3B gene, termed VPg ($3B^{VPg}$). VPg is attached via a Tyr residue to the 5′ terminal uridine (U) of the RNA, which functions as a primer for the RNA polymerase $3D^{pol}$. A 5′ terminal domain that forms a single stem of 41 nts is presumably involved in minus-strand synthesis and is followed by two pseudoknots. These 5′ terminal domains are separated by a short poly(U/UC) tract from a complex of several stem loops that form an internal ribosomal entry site (IRES). At the end of the IRES, there is a double initiation codon motif, AUG AAC AUG, that marks the start of the long ORF for the HAV polyprotein. HAV tolerates insertions at the N terminus of the polyprotein, and the HAV IRES can initiate dual translation of the polyprotein from AUG codons separated by 66–78 nts [19]. The HAV IRES is unique within the *Picornaviridae* because it requires all eukaryotic initiation factors, including eIF-4G, to direct the internal initiation of translation [20] and cannot compete with cellular messenger RNAs (mRNAs) for the translation machinery. Replacing the weak HAV IRES with the highly efficient EMCV IRES [21] or adding the EMCV IRES after the HAV IRES [22] did not increase viral growth, indicating that other determinants are responsible for the poor growth of HAV. The first translated region is P1, which encodes the structural proteins VP4, VP2, VP3, and VP1. The first P2 gene, 2A, does not share homology with other members of the family, behaves more like a structural protein, and remains bound to VP1. This VP1-2A protein, also known as the PX protein [23], is found in the capsid and is cleaved by cellular proteases at the junction between VP1 and 2A [24]. The 2B and 2C genes code for functional proteins that remain associated with cellular membranes. In addition to VPg, the P3 region encodes the 3A protein, which anchors $3B^{VPg}$ to membranes; the proteinase $3C^{pro}$; and the RNA-dependent RNA polymerase $3D^{pol}$. The precursors of these mature viral proteins have distinct

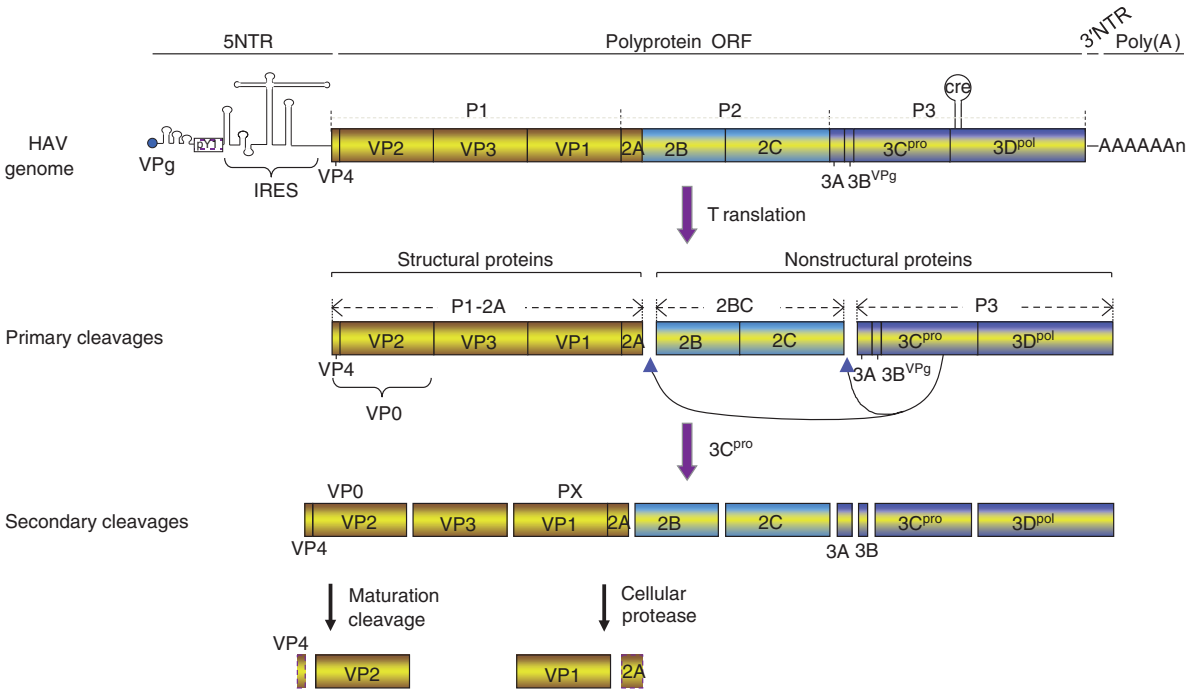

Figure 3.1 Genomic organization and cleavage of the HAV polyprotein. The HAV genome contains a 5′ nontranslated region (NTR) of approximately 750 nucleotides (nts) containing a polypyrimidine tract (pY1) of approximately 40 nts that separates the internal ribosomal entry site (IRES) from the rest of the 5′ NTR. A small viral VPg protein ($3B^{VPg}$) is covalently linked to the 5′ end. The HAV genome contains a long open reading frame (ORF) that codes for the HAV polyprotein of approximately 230 kDa. Within the $3D^{pol}$ gene, there is a *cis*-acting replication element (*cre*). A short 3′ NTR is followed by a poly(A) tract. The HAV genome has three regions: P1 encodes the capsid proteins, and P2 and P3 encode the functional proteins. The viral $3C^{pro}$ protease processes all cleavages, except for the cleavage of PX into VP1 and 2A and the cleavage of VP0 into VP2 and VP4. (Color plate 3.1)

functions. At the 3′ end, the HAV genome has a short 3′ UTR of 63 nts that is believed to be implicated in replication. Interestingly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) binds to the HAV 3′ and 5′NTRs and may allow circularization of the viral RNA and interaction of these NTRs during RNA synthesis [25]. The poly(A) tract at the 3′ end of the HAV genome is required for replication in quiescent cells but not in dividing cells, and it is restored in infectious particles, suggesting that it serves to stabilize the viral RNA and prevent its degradation in quiescent cells [26]. A stem loop of 96 nts that functions as a *cis*-acting replication element (*Cre*) has been identified within the $3D^{pol}$ gene [27].

Translation, polyprotein processing, and protein function

HAV's translation strategy differs significantly from that of the other members of the *Picornaviridae*. Translation begins at two AUG initiation sites separated by 3 nts. The second AUG is preferentially used for translation initiation of the polyprotein [28]. HAV typically grows without inhibiting cellular mRNA translation and establishes a persistent infection in cells without causing CPE. Translation initiation at the HAV IRES requires eIF-4G, an initiation factor that is cleaved by enteroviruses and aphthoviruses, to prevent cap-dependent translation of cellular mRNAs. In addition to eukaryotic canonical translation factors, several RNA-binding proteins have been identified as IRES trans-acting factors (ITAFs) that mediate binding by ribosomes and control the internal initiation of translation [29]. GAPDH [25] and La auto-antigen [30] are ITAFs that suppress HAV translation, whereas poly(C)-binding protein (PCBP) [31] and polypyrimidine tract-binding protein (PTB) [32] increase HAV translation. The poly(A)-binding protein (PABP), which binds to the poly(A) tract at the 3′ end of the HAV RNA, also binds to eIF-4G, which interacts with the IRES at the 5′NTR and enhances HAV translation [33]. The interplay and relative abundance of the ITAFs in different cell types play a significant role in HAV translation [34] and are likely to affect cell tropism and virulence. Cleavage of PTB, PCBP, and PABP by the HAV $3C^{pro}$ reduces HAV translation and controls the switch between translation and replication ([35] and

references therein). HAV also maintains a deoptimized codon usage by a poorly understood selection mechanism [36], which has a profound impact on viral growth, suggesting that translation selection plays an important role in HAV biology. Clusters of conserved rare codons in the capsid region allow the virus to use transfer RNAs (tRNAs) not commonly used by cellular messengers, which slows down the translation process but may increase the translatability of the viral RNA and allow proper folding of the viral proteins [37].

The only virally encoded protease, $3C^{pro}$, catalyzes the primary cleavage of the polyprotein into structural precursor P1-2A and nonstructural precursor 2BC-P3 (Figure 3.1). The $3C^{pro}$ also processes the secondary cleavages of the precursors except for the VP4-VP2 and VP1-2A bonds (Figure 3.1). The P1 precursor is cleaved into VP0, VP3, and VP1-2A (or PX). The cleavage of VP0 into the unusually small VP4 of 21 or 23 amino acids and VP2 occurs by an autocatalytic process driven by RNA encapsidation and termed "maturation cleavage," which is common among Picornaviruses. Cellular proteases cleave the VP1-2A junction of PX, yielding a mature VP1 with a heterogeneous C terminus [24]. While capsid proteins accumulate in infected cells and can be readily identified by biochemical and immunochemical techniques, HAV functional proteins do not accumulate and are difficult to identify in infected cells [38]. HAV-infected cells exhibit limited membrane changes associated with the presence of 2B, 2C, and precursor 2BC, which is in contrast to the profound rearrangement observed in cells infected with cytopathic Picornaviruses [39]. Cytopathic and noncytopathic strains of HAV induce similar membrane rearrangements [39].

The HAV P3 region shares a high degree of homology with other Picornaviruses and is cleaved by $3C^{pro}$ into the mature viral proteins 3A, $3B^{VPg}$, $3C^{pro}$, and $3D^{pol}$ and precursors of these mature proteins. The HAV $3C^{pro}$ structure has a highly conserved KFRDI RNA-binding site located opposite the proteolytic site, which supports the function of $3C^{pro}$ in RNA replication [40]. Both $3C^{pro}$ and 3CD bind to 3AB, which anchors $3B^{VPg}$ to membranes for the initiation of RNA synthesis and also serves as a cofactor during replication [41]. The 3ABC precursor binds with relatively high affinity to the 3′ and 5′NTRs of the HAV genome, suggesting that it plays a role in the initiation of RNA synthesis [42]. Overexpression of the HAV P3 precursors 3ABC and 3CD in cell culture disrupts type I interferon induction via cleavage of the retinoic acid-inducible gene 1 (RIGI)–MAD5 pathway protein adaptor MAVS (mitochondrial antiviral signaling protein) and the Toll-like receptor 3 (TLR3) pathway protein adaptor TRIF (TIR domain-containing, adapter-inducing interferon-beta [IFNβ]) ([43] and references therein), respectively. As in other Picornaviruses, the HAV precursors have distinct functions to enable virus growth using a small set of 11 genes.

Particle and antigen structure

HAV particles are heterogeneous and composed of 60 copies of VP1 and/or PX (VP1-2A), VP3, and VP0 and/or VP2. HAV particles isolated from cell cultures consist of a mixture of (1) mature virions, which contain viral RNA and mostly VP2; (2) provirions, which contain viral RNA and different amounts of uncleaved VP0; and (3) procapsids (empty capsids), which contain VP0 and no RNA [44]. Capsid formation begins with $3C^{pro}$-mediated cleavage of the capsid precursor P1-2A into a protomer formed by VP0, VP3, and PX. The 2A protein has no proteolytic activity and mediates protomer homo-oligomerization to form 14S pentamers (five copies of VP0–VP3–VP1-2A) [45]. Formation of the HAV capsid by 12 pentamers requires the presence of VP4 [45]. The unique morphogenic functions of HAV 2A and VP4 result in the highly efficient encapsidation of the viral RNA, which depletes the pool of RNA available for replication [46] and contributes to the protracted replication of HAV. The C terminus of PX is exposed at the surface of the viral particle [47], and cleavage of the VP1-2A junction by unknown cellular proteases releases 2A from the capsid, resulting in a heterogeneous C terminus of VP1 [24]. The autocatalytic cleavage of VP0 into VP2 and VP4 completes the maturation of the HAV virions. Most infectious HAV particles have a density of $1.325\,g/cm^3$, sediment at 156S, are stable at pH 1.0, and are highly resistant to high temperatures, losing just 2 logs of infectivity when incubated at 80 °C for 10 min. HAV is also resistant to drying and treatment with organic solvents and detergents. HAV particles have evolved to withstand harsh environmental conditions both outside and inside the host, which ensures a high degree of infectivity, explaining why HAV is one of the most common causes of hepatitis outbreaks.

Very little is known about the structure of the HAV particle. An HAV model was built based on the significant amino acid sequence homology of VP2 and VP3 (but not VP1) to other Picornaviruses, but it does not explain some mutations identified in neutralization escape mutants and has been refined to accommodate newer data [48]. Attempts to obtain the crystallographic structure of HAV particles have been unsuccessful due to the difficulty in obtaining large quantities of purified particles, the nature of the virions that tend to precipitate, and the heterogeneity of the viral particles obtained in cell culture [47]. A low-resolution structure based on cryo-electron microscopy (cryo-EM) obtained by Holland Cheng's group [18] showed the absence of a surrounding depression or "canyon" at the fivefold axes of symmetry found in many Picornaviruses and the

presence of a prominent bridge that may explain the connection between neutralizing epitopes mapped in VP1 and VP3. The HAV model predicts that the N terminus of VP2 is located at the threefold axis, suggesting that the small VP4 of only 21–23 amino acids may be found in the periphery of the pentamers, consistent with its release from the capsid after VP0 cleavage [45]. The C terminus of VP1 is also exposed at the virion surface, which makes the VP1-2A bond accessible to cleavage by cellular proteases, allowing the release of 2A from the viral particle [47].

HAV exists in only one serotype, suggesting that structural constraints are needed to maintain the viability of the virus. Pentamers and empty capsids, but not single viral proteins, induce significant levels of neutralizing antibodies [49], indicating that HAV-neutralizing epitopes are highly conformational. Sequence analysis of monoclonal antibody (mAb) neutralization escape mutants defined three HAV neutralization sites [50, 51]: an extended immunodominant neutralization site composed of residues 70, 71, and 74 of VP3 and 102, 104, 105, 171, 174, 176, 178, and 232 of VP1; a second neutralization site in residue 221 of VP1, which is also a binding site for glycophorin A, defined by monoclonal antibody (mAb) H7C27 [52]; and a third unmapped site defined by monoclonal antibody 4E7, which neutralized all escape mutants from the immunodominant and glycophorin antigenic sites. The mAbs H7C27 and 4E7 prevented the binding of polyclonal antibodies raised against the 110–121 VP3 peptide [53] to HAV, suggesting that the glycophorin and 4E7 sites are connected. Anti-HAV-neutralizing mAbs compete efficiently with polyclonal sera from HAV patients and vaccinees for binding to the virus ([51] and references therein), which further supports the notion that the neutralization sites are clustered at the virion surface. Furthermore, it has been suggested that the immunodominant site of HAV is also the binding site for HAVCR1 [54].

Growth in cell culture

In 1979, Provost and Hilleman successfully demonstrated the replication of HAV in marmoset primary liver cell cultures and fetal rhesus monkey kidney FRhK6 cells [6]. Serial passages in primate cell culture results in the accumulation of 22–30 mutations that attenuated the virus in humans and chimpanzees [8]. Mutations in two hot spots, namely, the 2B–2C region and the 5′NTR, are responsible for the adaptation of wt HAV to cell culture ([55] and references therein): mutations in the 2B and 2C genes are essential, whereas mutations in the IRES are growth enhancers and do not function autonomously. However, the growth of human wt HAV required several weeks to months in culture, and the virus was genetically unstable. A cell culture system for the stable growth of wt HAV was developed by antibiotic selection of human hepatoma Huh7 cells that supported the growth of wt HAV containing a blasticidin selectable marker (wtHAV–Bsd) [56].

Extensive passage in cell culture resulted in the emergence of cytopathic variants of cell culture–adapted HAV that replicated faster than the parental strains and induced apoptosis ([57] and references therein). It is unlikely that the rate of replication is responsible for the CPE because fast-replicating HAV variants containing mutations in the $3D^{pol}$ that enhance the rate of replication did not induce CPE in FRhK4 cells [58].

In addition to primate cells, cell culture–adapted HAV has also been adapted to grow in nonprimate cell lines ([59] and references therein), including mouse cell lines. However, mutants that grew efficiently in mouse hepatocyte cell cultures [59] did not induce hepatitis in mice (Feigelstock and Kaplan, unpublished results), suggesting that other determinants are required for HAV pathogenesis in mice.

Viral receptors and cell entry

In 1996, Kaplan *et al.* identified a novel cell surface glycoprotein in African green monkey kidney cells as an HAV receptor and termed it HAVCR1 [9]. The HAVCR1 human homolog [60] and mouse and rat orthologs [61] were identified in 1998. HAVCR1 is a class I integral membrane glycoprotein containing an N terminal immunoglobulin-like domain (IgV) extended from the cell surface by a mucin-like domain. The extracellular domain of HAVCR1 is anchored to the plasma membrane by a transmembrane domain followed by an intracellular cytoplasmic tail containing a phosphorylation site. HAVCR1 is the first identified member of the HAVCR family of receptors, which in humans has two additional members: HAVCR2 and HAVCR3 (the Human Genome Organization [HUGO] name of HAVCR3 is TIMD4). *HAVCR1* is expressed in several tissues, including the liver [61, 62]. The distribution of HAVCR1 cannot completely explain the hepatotropism of HAV, indicating that other determinants are responsible for the growth of HAV in the liver. In 2001, the mouse orthologs of the HAVCR family of receptors were shown to have T cell co-stimulatory functions, and the family was rebranded as the T cell immunoglobulin mucin (TIM) family (for a review, see [10]). TIM does not take into account the fact that HAVCR receptors are also expressed in many other cell types, including antigen-presenting cells, hepatocytes, and proximal tube epithelial cells, or the fact that the same name is used for at least two other gene families, namely, the Timeless family (TIM, UniGene Hs.118631) and the Triosephosphate isomerase family (TIM, UniGene Zm.72626). Therefore, herein, we will use the historical

and official HUGO name for the primate HAVCR1, the official Jackson name for the Havcr1 mouse ortholog, and the HAVCR name for the receptor family. It has recently been shown that mouse Havcr1 does not have HAV receptor function, in contrast to the primate HAVCR1 [12].

Evidence for the highly polymorphic nature of *HAVCR1* surfaced very early in the characterization of this receptor. Nucleotide sequence analysis of *HAVCR1* from BSC1 and Cos7 cells, two African green monkey kidney cell lines, revealed amino acid mutations in the IgV and insertions and deletions in the mucin domain, which did not impair HAV receptor function [60]. In humans, *HAVCR1* polymorphisms were identified in the mucin and noncoding regions of the gene. Polymorphisms in exon 4 of the *HAVCR1* gene containing insertions of five or six amino acids have been associated with autoimmunity susceptibility ([63] and references therein), allergies ([64] and references therein), and chronic hepatitis C and HIV viral infections ([65] and references therein). The mucin region is required for HAV receptor function ([66] and references therein), and HAVCR1 variants that contain insertions in the mucin region are better HAV receptors than variants without the insertions and are associated with increased hepatitis A severity [11].

The crystal structure of the Havcr1 IgV [67] showed that four of the conserved six cysteine residues in the IgV stabilize a pocket containing a metal ion ligand-binding site (MILBS). Primate HAVCR1 and mouse Havcr1 share approximately 60% homology at the amino acid level, and the MILBS is conserved in all HAVCR family members. Phosphatidylserine (PtdfSer), a phospholipid present in the outer membrane leaflet of apoptotic cells, binds to the MILBS of HAVCR receptors [68], which function as pattern recognition receptors (PRRs) of PtdSer, a damage-associated molecular pattern (DAMP). HAV binds to the IgV of HAVCR1 in an area superimposed with the MILBS [12, 67]. Interestingly, the binding of HAV to HAVCR1 blocks the binding of apoptotic cells to HAVCR1, thereby blocking the natural function of this receptor.

Soluble forms of HAVCR1 containing the IgV and mucin domains neutralize HAV infectivity and alter the HAV particles [66], a step required for the release of the HAV genome into the cytoplasm during infection. Therefore, a likely model for the cell entry process of HAV involves attachment of the virus to HAVCR1 at the cell surface, internalization of HAV mediated by HAVCR1, and uncoating of the HAV genome in an as yet undefined intracellular compartment. Further research will be required to fully understand the entry process of HAV mediated by HAVCR1.

Like many other viruses, HAV hemagglutinates human erythrocytes. HAV binds to glycophorin A on the surface of erythrocytes via the epitope defined by mAb H7C27 [52], but it does not infect red blood cells, and the interaction of virions with glycophorin A is minimal at a neutral pH and optimal at a pH of 5.5. The function of the interaction of HAV with glycophorin A is unknown, but it has been proposed that the interaction limits the variability of the H7C27 epitope because mutations at this site increased binding to erythrocytes and clearance of the virus from the bloodstream [37].

Human immunoglobulin A (IgA) polyclonal antibody preparations and a murine IgA neutralizing mAb mediate the inefficient infection of human and murine cells via the asialoglycoprotein receptor, which binds and internalizes IgA [69]. It has been proposed that this antibody-mediated enhancement of infection mediates reinfection of the liver and contributes to prolonged and relapsing forms of hepatitis A [70]. However, it is unclear how IgA anti-HAV antibodies induce the uncoating of the virus, which is a necessary step for the release of the HAV genome into the cytoplasm and the initiation of replication. Furthermore, IgAλ is a natural ligand of HAVCR1, and the association of IgAλ with HAVCR1 enhances the HAV–HAVCR1 interaction, suggesting that IgA also plays a role in the HAVCR1-mediated cell entry process of HAV [71].

Replication and release of viral particles

Little is known about the replication of HAV, which is mostly inferred from other *Picornaviridae* family members. The protracted growth strategy of HAV that allows the virus to establish persistent infections in cell culture and drives its immunopathogenic process is a unique characteristic within the *Picornaviridae* family. The slow and asynchronous uncoating of the viral particles that occurs between 4 and 10 h post infection [72] and the inefficient mechanism of HAV translation (discussed in this chapter) have a significant effect on HAV replication. The asynchronous growth of HAV [73] without the accumulation of functional proteins [38] and the limited cellular membrane rearrangement [39] are also likely to slow viral growth.

At its 5′ end, the HAV genome has a hairpin structure and two pseudo-knots that are involved in viral replication. The pyrimidine-rich tract pY1 that separates the hairpin and pseudo-knot structure from the HAV IRES (Figure 3.1) is not required for virus viability and pathogenesis in the marmoset model ([74] and references therein). Negative-strand HAV RNA is transcribed from the positive-strand genomic RNA. Viral proteins, including $3C^{pro}$ and $3D^{pol}$, cellular membranes, and proteins, form the replication complex that contains HAV double-stranded RNA (dsRNA) replication intermediates. The negative-sense RNA serves as a template for positive-strand RNA synthesis. A switch from translation to rep-

lication has been proposed for HAV ([35] and references therein), but it is unclear how such a switch would function during the protracted and asynchronous replication process of HAV. It has been proposed that the interaction of PABP bound to the poly(A) and cellular factors bound at the IRES bring together the 3′ and 5′NTRs, circularizing the viral RNA. The interaction of the 3′NTR, 5′NTR, and *cre* supports the positive- and negative-strand RNA synthesis catalyzed by $3D^{pol}$ and primed by urydylated VPg. Determinants that slow down HAV replication have recently been mapped outside the *cre* at nucleotides 6069 and 7027 of the HAV genome, indicating that HAV has also evolved mechanisms to control the kinetics of RNA replication [58].

HAV particles (virions, provirions, and empty capsids) are released from infected cells by an unknown mechanism. In the polarized human intestinal epithelial CaCo2 cell line, HAV uptake and release occur mainly via the apical membrane [75]. However, in a polarized clone of human hepatocyte-derived HepG2 cells, HAV was mainly released at the basolateral plasma membrane [76]. These findings suggest that HAV could be secreted into the bloodstream via the basolateral plasma membrane and into the bile via the apical membranes of hepatocytes, which form the bile ducts. In the presence of antibody, HAV complexes with IgA were transported vectorially from the apical to the basolateral compartment [70]. Further research will be required to clarify these contradictory findings and to understand the mechanism(s) by which HAV is released from cells and reaches higher titers in stools than in blood during HAV infection.

Genetic diversity

There is only one serotype of HAV [77], pointing to constraints in the antigenic variability of this virus. HAV variants are currently classified into six genotypes. Genotypes I and II have been isolated only from humans, and the rare genotype II has been suggested to cause increased incidence of fulminant hepatitis A. Genotype III was isolated from humans and owl monkeys, but it has all of the characteristics of human HAV. Genotypes I and III constitute the most common genotypes circulating in the human population. Genotypes IV, V, and VI were isolated from Old World monkeys and do not seem to cause disease in humans. The introduction of strains by travelers and imported contaminated food resulted in the cocirculation of strains from different genotypes and the emergence of mixed-genotype strains [78].

The antigenic sites of human (genotypes I–III) and monkey (genotypes IV–VI) HAV differ in amino acid residues 65 and 70 of VP3, as well as residues 102, 105, 174, 178, and 221 of VP1 [50], which form the immunodominant and glycophorin-binding (residue 221 of VP1) sites. These amino acid changes are the reason why many neutralizing mAbs raised against human HAV do not recognize monkey HAV. However, monkey HAV induced cross-protective immunity in marmosets and chimpanzees challenged with human HAV [77]. Antigenic variants containing mutations in the immunodominant site were isolated from a 2008–2009 HAV outbreak in men who have sex with men (MSM) in Catalonia, Spain, which suggested that partial immunization due to incomplete vaccination schedules or immunodeficiency could result in the selection of antigenic variants that escape neutralization by sera from vaccinated individuals [79].

The conservation of the antigenic structure of HAV may also be required to maintain the virus–receptor interaction, as suggested by the binding of HAVCR1 to the immunodominant site of the HAV [54]. It has been proposed that clusters of rare codons near the complex antigenic site also restrict the antigenic variability of HAV ([36] and references therein). Therefore, several selective forces may be responsible for maintaining the antigenic stability of HAV.

Natural history of HAV

The natural history of HAV has been reviewed extensively [16, 17]. Briefly, HAV is the causative agent of acute hepatitis A in humans and NHPs. It is a self-limiting disease that, in most cases, resolves spontaneously without inducing chronic infection or additional sequelae. HAV is mainly transmitted through the fecal-oral route by direct contact with an infected person or by the consumption of contaminated food or water. HAV is rarely transmitted by blood or blood products, although injection drug users have a higher incidence of HAV infection. Risk factors for HAV include socioeconomic status and large household size, limited access to improved water sources and sanitation facilities, travel or immigration from nonendemic to endemic regions, high-risk occupations (e.g., childcare employees), homosexuality, and intravenous drug use. HAV infection induces a lifelong immunity. It is unknown why hepatitis A is an age-dependent disease and why the majority of children younger than 6 years old develop inapparent infection. Symptomatic hepatitis A increases with age, being more common in older children and adults. The severity of hepatitis A also increases with age, with 53% of adults ≥60 years old requiring hospitalization. The case fatality rate is low, ranging from 0.1% in patients ≤15 years old to 1.8% in patients ≥50 years old, and it is higher in individuals with underlying liver disease. A low frequency of various extrahepatic manifestations of acute hepatitis A has been reported, but their etiologies are not fully understood [16–18].

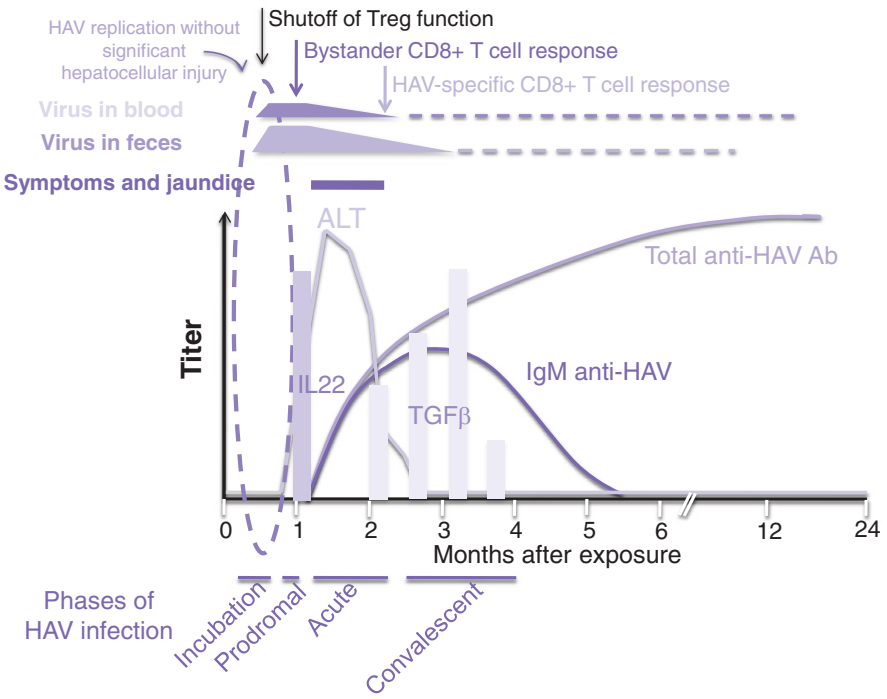

Figure 3.2 Natural history of HAV in humans. Schematic representation of biomarkers from patients with a normal course of acute HAV infection. HAV reaches peak viremia and is shed in stools during the incubation period and prodromal phase. Bystander and HAV-specific CD8+ T cells are activated at different times post infection. Alanine aminotransferase (ALT) elevation in blood indicates hepatocellular injury. Interleukins IL22 and TGFβ (histograms) are produced at different times post infection. Anti-HAV IgM and IgG antibodies appear in the blood.

Figure 3.2 shows a schematic representation of a normal course of HAV infection. After ingestion, HAV reaches the liver from the gastrointestinal (GI) tract by an unknown mechanism. During the *incubation period* of 15 to 50 days (mean 28–30 days), HAV replicates extensively in the liver. The virus grows in the infected hepatocytes without causing cell damage, as assessed by normal levels of liver enzymes in the blood. The presence of HAV antigen in Kupffer cells, the liver's resident macrophages, has been attributed to the phagocytosis of infected hepatocytes. HAV is secreted mainly into the blood and bile, from where it reaches the intestinal content and is shed with the stools. A variable amount of virus is produced during infection that can reach titers in stools of 10^7 to 10^9 genome equivalents (Geq) per gram of feces and in blood of 10^4 to 10^6 Geq/ml of plasma. The interaction of HAV with HAVCR1 impairs the function of Tregs ([12] and references therein). The peak viral levels in stools and blood occur simultaneously in the absence of significant liver damage. Patients are most infectious during the 1–4-week incubation period, especially at the peak of viral production. The appearance of dark urine due to elevated levels of bilirubin caused by a reduction in liver function marks the end of the incubation period and the start of the short pre-icteric *prodromal phase*, which lasts days to weeks. This prodromal phase is characterized by limited hepatocellular damage and nonspecific symptoms, including malaise, flulike symptoms, anorexia, and fever. Additional symptoms include myalgia, arthralgia, cough, pharyngitis, diarrhea, pruritus, and urticaria. The prodromal phase is followed by a symptomatic icteric *acute phase*, which usually lasts approximately 2–4 weeks (ranging from 7 to 87 days) and is characterized by yellowing of the skin and eyes (jaundice), transient elevation of total IgM values, hepatomegaly, and hepatic tenderness in 50% of patients. The prodromal, nonspecific symptoms tend to diminish during jaundice, and bilirubin levels rarely exceed 170 µmol/L. The hepatocellular injury during the acute phase results in the rapid elevation of serum transaminases (reaching peak levels of 500–2000 U/L), which precedes the appearance of anti-HAV IgM antibodies and, shortly afterward, IgG. Anti-HAV IgM can be detected for 3–6 months and is indicative of recent HAV infection, whereas anti-HAV IgG can persist for life. The almost concomitant peak of liver enzymes in serum and the appearance of anti-HAV IgM and IgG mark a rapid decline in the levels of virus in stools and blood, which reach basal levels in 2–3 weeks. The virus is typically

cleared between 4 and 6 months after infection. HAV RNA can be detected using sensitive polymerase chain reaction techniques in the liver, blood, and stools for approximately 1 year, but it is unclear whether this represents infectious virus. In the final *convalescent phase* of the disease, the virus is cleared, the patient recovers, and biomarkers return to normal. Cellular immunity seems to play a role in the final clearing of the virus, with a peak of cytolytic activity in peripheral blood detected in the early convalescent phase [80]. HAV infection is typically resolved within 3–6 months after infection without the establishment of chronic infection.

The majority of hepatitis A patients follow a normal course of infection, but a significant proportion can develop complications, such as relapses, a prolonged course of hepatitis A, cholestatic hepatitis A, fulminant hepatitis A, or fatal hepatitis A (for a review, see [16]). Autoimmune hepatitis can be triggered by HAV infection in individuals with a genetic predisposition to the disease (relatives with type 1 autoimmune hepatitis). Extrahepatic manifestations of HAV are rare and include transient lupus-like disease, vasculitis, cryoglobulinemia, skin rashes, arthritis, neurological complications, pancreatitis, and glomerulonephritis.

Association of HAV infection with the permanent modulation of immune responses

The inverse association between HAV infection and the development of autoimmune [13] and allergic ([14] and references therein) diseases is not surprising because polymorphisms in the *HAVCR1* receptor gene have been associated with susceptibility to rheumatoid arthritis ([63] and references therein) and asthma ([15] and references therein). Interestingly, the function of Tregs is impaired in patients with acute hepatitis A ([12] and references therein), and the interaction of HAV with HAVCR1 expressed on Tregs suppresses the function of these regulatory cells ([12] and references therein). These findings suggest that the uncontrolled expansion of allergic and autoimmune effector cells may lead to the activation of induced cell death (AICD) [81], resulting in the cleansing of pathogenic effector cells. The shutoff of Treg function during infection also serves as a mechanism to explain why HAV infection triggers autoimmune hepatitis in predisposed individuals [82] and its association with atherosclerosis [83]. Therefore, *HAVCR1* alleles and genetic predisposition to disease determine the protective or pathogenic effect of HAV infection.

Pathogenesis of HAV

The pathogenic process of HAV is poorly understood, mainly due to the lack of a small-animal model. It is currently unknown whether HAV has a primary site of replication in the GI tract. Small amounts of HAV have been detected in saliva and throat swabs in chimpanzees [84] and in crypt cells of the small intestine in monkeys [85], but it is unclear whether the virus was produced in the GI or came from the inoculum or bloodstream. In tamarins and chimpanzees, oral infection is 3200-fold less effective than intravenous inoculation [86], indicating that infection via the oral-fecal route is an inefficient mechanism. HAV may also reach the bloodstream by transcytosis via its binding to scavenger cells that sample the lumen of the GI.

In the liver, HAV induces a poorly understood, limited immune-mediated necroinflammatory process that typically lasts 4–6 months and ends with complete resolution of the disease (Figure 3.2). During the 3–4-week *incubation period*, HAV-infected cells escape immune detection and injury, as determined by the normal levels of transaminases in the blood. This limited immune response against HAV during the incubation period is paramount to the replication strategy and pathogenic process of this virus. However, it is only partially understood how HAV escapes innate immune responses. HAV infection typically does not cause cytopathic effect, cellular RNA degradation, or protein synthesis shutoff, and it inhibits apoptosis induced by dsRNA [87]. HAV also blocks IFNβ production in infected cells [87, 88], using several mechanisms to prevent the positive feedback loop responsible for the *autocrine* and *paracrine activation* of antiviral cell functions. To do so, HAV targets the activation of IFN regulatory factor 3 (IRF3) to prevent the transcription of IFNβ. It has been shown that HAV 2B protein interferes with the activation of IRF3 [89]. Also, expression in cell culture of $3C^{pro}$ protease precursors cleaves adaptor molecules in the IFNβ pathway ([43] and references therein): 3ABC intermediate cleaves the MAVS, an adaptor molecule needed for the single-stranded RNA (ssRNA)- or dsRNA-mediated activation of IRF3, and 3CD cleaves TRIF and prevents TLR3-mediated activation of IRF3. Therefore, HAV has evolved multiple mechanisms to prevent the production of IFNβ in hepatocytes. However, a significant number of HAV patients produce IFNα, another type I IFN that is secreted by leukocytes and plasmacytoid dendritic cells and signals through the same receptor as IFNβ using different adaptor molecules. The role of type I IFN as an immunomodulator in the pathogenesis of HAV is highly controversial ([90] and references therein) because HAV patients and experimentally infected chimpanzees [91] that produce low or basal levels of IFNα or β are still able to clear the HAV infection. Microarray data show low or no expression of type I IFN-induced genes (ISGs) in the liver of chimpanzees experimentally infected with HAV [91], further supporting the notion that type I IFN is not required to clear the virus infection and suggesting that other immune noncytolytic mechanisms are

involved in this process. It is also unclear why innate effector cells residing in the liver, such as natural killer (NK), natural killer T (NKT), and Kupffer cells, do not prevent the spread of the virus during the long incubation period. *In vitro*, NK cells from HAV-seropositive and -seronegative donors and patients with acute hepatitis A preferentially kill HAV-infected compared with uninfected cells ([92] and references therein), suggesting that HAV can inhibit innate effector cells from targeting HAV-infected hepatocytes during the incubation period. The interesting finding that HAV can suppress monocyte-to-macrophage maturation *in vitro* [93] further supports this hypothesis.

The pathogenic process of HAV can be described in terms of the following model based on our current understanding of the disease (Figure 3.3). Once HAV reaches the liver, it replicates in hepatocytes (A), blocking the production of IFNβ and other intracellular antiviral systems that are activated by dsRNA replication intermediates. The virus is shed in the feces and reaches the blood, inducing viremia (B). The binding of HAV to HAVCR1 blocks T cell receptor activation and causes a transitory block of Treg function ([12] and references therein), which limits *de novo* HAV immune responses while allowing the activation of preexisting responses that are no longer controlled by Tregs [12] (C). This transitory shutoff of Treg function allows the activation of antiself and preexisting responses that overwhelm the immune system, inducing a "shock and awe" effect that limits anti-HAV *de novo* responses. Indeed, HAV disrupts the homeostasis of the immune system, and patients with acute hepatitis A exhibit a transitory elevation of autoantibodies [82, 94] and the production of high levels of nonspecific IgM antibodies [2] against, for instance, bacteria present in the intestinal flora [95]. The shutoff of Treg function allows the clonal expansion of T cells, resulting in cell death by AICD and the deletion of pathogenic T cells. Such a "cleansing mechanism" could be responsible for the protective effect of HAV infection in the development of allergies and autoim-

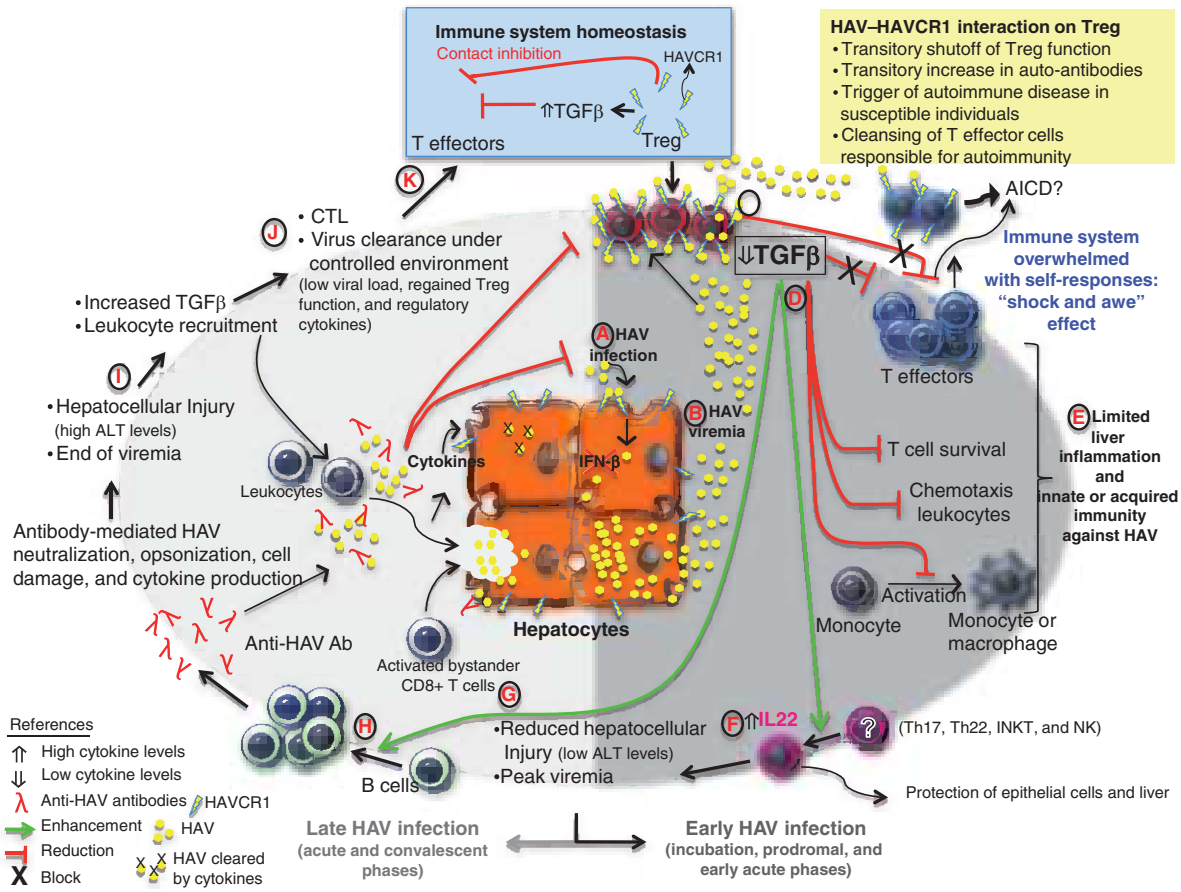

Figure 3.3 Model of the pathogenic process of HAV. Schematic representation of the cycle of immune events leading to a typical course of HAV infection characterized by an immunopathogenic process with limited inflammation that culminates in complete clearance of the virus. This cycle is described in detail in this chapter, guided by the A–K circled reference points. (Color plate 3.2)

munity. HAV infection blocks Tregs from producing TGFβ (D), which results in the reduction of T cell survival, chemotaxis of leukocytes to the site of infection, and activation of monocytes, limiting liver inflammation and innate and acquired immunity against HAV (E). The increase in the production of IL22 (F) during the viremic phase may compensate for the lack of Treg function by preventing collateral tissue damage, as IL22 protects the liver and epithelial cells from injury [96]. This particular immune milieu during early HAV infection (incubation, prodromal, and early acute phases) allows HAV to replicate in the liver without triggering hepatocellular injury. The lack of Treg function favors the activation of bystander CD8+ T cells (G) that mediate the hepatocellular damage in the early acute phase [97]. The low levels of TGFβ favor the development of a strong anti-HAV antibody response (H) that reduces viremia and prevents the HAV–HAVCR1 interaction. A gradual restoration of Treg function and production of TGFβ facilitate the recruitment of leukocytes (I) to the liver and the onset of an inflammatory process that induces hepatocellular injury. The production of cytokines by the infiltrating leukocytes promotes viral clearance via cytolytic and noncytolytic mechanisms. The activation of HAV-specific CD8+ cytotoxic T cells in the early convalescence phase [98] (J) most likely contributes to the final clearance of the virus, which allows the immune system to return to homeostasis (K).

HAV experimental models

Animal models of HAV infection have been reviewed extensively [17]. Animal models that recapitulate hepatitis A in humans are restricted to NHPs, which are susceptible to HAV infection via the oral and parenteral routes. However, hepatitis A in NHPs is milder, and there are significant differences in their susceptibility to human (genotypes I–III) and monkey (genotypes IV–VI) HAV. Chimpanzees (*Pan troglodytes*) and several species of New World monkeys, including owl (*Aotus trivirgatus*) and squirrel (*Saimiri sciureus*) monkeys, marmosets (*Callithrix jacchus*), and tamarins (*Saguinus mystax*), are susceptible to human wt HAV infection and develop hepatitis A. Cell culture–adapted human HAV that is overly attenuated for humans is also attenuated for chimpanzees and New World monkeys, indicating that the mutations accumulated during cell culture adaptation also affect pathogenicity in NHPs. Interestingly, some New World monkeys, but not chimpanzees, infected with monkey HAV develop hepatitis A, indicating that monkey HAV is restricted to lower primates.

Old World monkeys, such as stump-tailed (*Macaca arctoides*), cynamologous (*Macaca fascicularis*), rhesus (*Macaca mulatta*), and African vervet (*Cercopithecus aethiops*) monkeys, are susceptible to monkey HAV infection, recapitulate hepatitis A in humans, and represent a very good animal model for the disease. The susceptibility of Old World monkeys to human HAV is limited and controversial, with some reports indicating failure to induce hepatitis A after intravenous inoculation [17]. These data suggest that Old World monkeys infected with human HAV are not an optimal model for hepatitis A.

The primate models of hepatitis A have contributed significantly to our understanding of the natural history and pathogenesis of HAV. The pathogenic determinants of HAV vary in the different primate models. Monkey HAV is attenuated in chimpanzees and protects against challenge with human wt HAV, suggesting that monkey HAV could be used to develop attenuated vaccines for humans [77].

HAV rodent models that do not recapitulate the infection in humans and that do not support the efficient replication of HAV have been developed in recent years. Guinea pigs infected with a guinea pig cell culture–adapted HAV via the oral and intraperitoneal routes supported limited HAV replication but did not develop signs of disease [99]. Enterohepatic cycling of HAV was shown using a mouse model [70]. The development of a mouse model that recapitulates hepatitis A in humans is highly desirable and will advance the understanding of many of the open questions regarding the natural history and pathogenesis of HAV.

Conclusion

A significant effort has been devoted to HAV research, but many gaps in our understanding of this important human pathogen still remain; specifically, the immunopathogenic process is poorly understood, the basis for the restricted growth of wt HAV in cell culture has not been deciphered, and the determinants of hepatovirulence and hepatotropism have not been explored in detail. Although safe and effective inactivated vaccines have been licensed and are having a very significant impact on the control of HAV, we still do not have a detailed understanding of the antigenic structure of this virus due to the absence of a high-resolution structure of the viral particle. The modulation of the immune response via the interaction of HAV with HAVCR1 and the shutoff of Treg function provide a model with which to develop therapeutic strategies to control chronic infections, cancer, allergies, autoimmunity, and transplant rejection. Further research will be required to understand the biology of HAV and harness its potential to modulate immune responses.

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Chapter 4
Epidemiology and prevention

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Summary

Hepatitis A infects millions of humans annually, with the epidemiological patterns of infection showing distinct differences related to access to adequate sanitation and hygiene. These differences influence age at infection, which in turn largely determines the clinical course of infection. In addition to improving access to sanitation and clean drinking water, active and passive vaccination play important roles in the prevention of hepatitis A infection.

Epidemiology

The epidemiology of hepatitis A virus (HAV) may be understood in terms of the natural history of the infection, the physical properties of the virus, the relationship of these with pathogenesis in individuals, and the resulting impact on populations. HAV consists of an unenveloped, 27 nm, spherical particle (Figure 4.1) containing a linear, single-stranded, positive-sense RNA genome, and is classified in the family *Picornaviridae* genus *Hepatovirus* (see Chapter 3). The virus is more robust than other picornaviruses and may remain infectious on surfaces, in the environment, and in uncooked foods for significant periods.

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. Significant quantities of HAV persisted in a fecal suspension on the hands of volunteers after 4 h, and high levels of infectious HAV were transferred from fingers to fomites over the same period. HAV is relatively heat stable but generally inactivated by cooking temperatures. HAV has also been shown to be relatively resistant to many commonly available hard-surface disinfectants.

Millions of HAV infections occur globally annually [1]. Acutely infected human beings are the only significant natural reservoir of HAV, although a number of other primate species may be infected. HAV is secreted from the liver into the bile and is shed in high titers in the feces (reviewed in Chapter 3). No state of chronic infection with HAV has been identified, and immunity after infection is lifelong. Transmission is primarily by the fecal-oral route, most frequently person to person or via contamination of uncooked foods or water. Access to adequate hygiene and sanitation is, therefore, important in the prevention of HAV transmission, and is reflected in the variation in hepatitis A epidemiology observed in different populations, the influence of socioeconomic status, and the changing epidemiology of hepatitis A in many populations over recent decades [1–6].

Children experience subclinical infection with HAV much more frequently than adults and may excrete virus for prolonged periods [4]. 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 centers is well reported in developed countries such as the United States; however, the frequency of these outbreaks has decreased in recent years with increasing vaccination coverage [7]. In many resource-poor settings, asymptomatic seroconversion to hepatitis A in childhood is widespread, and as a consequence attributable morbidity in these populations is low [1]. In contrast, in low-prevalence countries where

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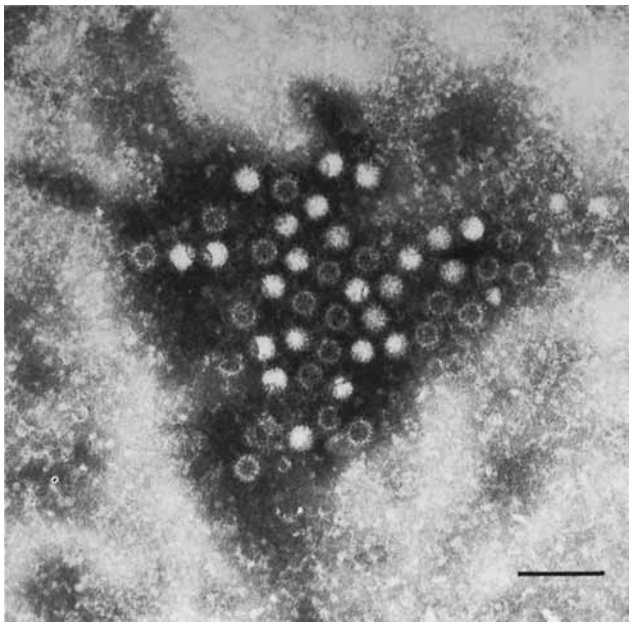


Figure 4.1 Hepatitis A virus. Bar represents 100 nm. (Electron micrograph provided by Dr John Marshall, Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia.)

the average age at infection is higher, associated morbidity and mortality are higher [3]. For reported cases of acute hepatitis A in the United States in 2007, none of the 255 patients under 15 years died, compared with 7 of the 273 (2.6%) aged over 60 years [6].

A transient viremia of moderate titer is associated with acute HAV infection, and bloodborne transmission of hepatitis A has been described experimentally and in isolated instances after blood transfusion.

Normal human immunoglobulin (NHIG) was first reported to be effective in the pre-exposure and postexposure prophylaxis of hepatitis A in 1945. Administered within 2 weeks of exposure to HAV, NHIG is 80–90% effective in preventing disease and has traditionally been the mainstay of postexposure prevention of hepatitis A [8]. Previously NHIG was also used for pre-exposure prophylaxis of hepatitis A among visitors to endemic areas; however, this role has been largely supplanted by the advent of safe and effective inactivated hepatitis A vaccines [8, 9] which have more recently also been evaluated in the postexposure context [10].

Transmission

Fecal-oral and person-to-person transmission

Direct person-to-person spread by the fecal-oral route is the most important means of transmission of HAV. High titers of virus are present in the feces from late in the incubation period, when the patient is maximally infectious, until the first week of clinical illness. Duration of fecal shedding is relatively well characterized, with data available from early studies of human transmission, animal models, and laboratory studies.

During studies at Willowbrook State School, infectivity for humans was demonstrated in pooled feces obtained from patients with infectious hepatitis 14–21 days before jaundice and was retained until 1–8 days after the onset of jaundice. Fecal samples collected during convalescence have been shown consistently not to be infectious for humans. Epidemiological investigations of HAV outbreaks have identified the period of approximately 1 week before onset of clinical symptoms as the period of maximal infectivity.

Using immune electron microscopy (IEM), HAV-like particles have been shown to be present in human feces from at least 5 days before the onset of biochemical hepatitis until the second week of illness. Duration of fecal shedding of HAV was positively related to the presence of icteric hepatitis. At the peak of shedding, feces may contain $>10^8$ infectious particles/mL.

The use of nucleic acid hybridization has demonstrated the presence of HAV RNA in the convalescent phase of illness in the feces of some patients infected with hepatitis A. Polymerase chain reaction (PCR) assays have demonstrated HAV RNA in feces of patients >1 month after clinical onset [11]. Few direct data are available regarding the potential infectivity of these patients. However, the weight of human transmission data and epidemiological data from outbreaks suggests that prolonged infectivity in the feces is unlikely to be common.

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

Of all HAV infections reported to the Viral Hepatitis Surveillance Program, Centers for Disease Control and Prevention (CDC), in 2007, 17.5% gave a history of international travel, which was the most commonly identified risk factor [6]. Other commonly identified risk factors were sexual, household, or other contact with a hepatitis A patient (16.8%); association with a daycare center (8.4%); linkage with an outbreak (6.5%); male-to-male sexual contact (5.9%); and injecting drug use (1.2%) (Figure 4.2) [6]. Transmission is usually limited to close contacts, especially to other family members. Attack rates among susceptible household contacts of cases vary between approximately 20% and 80%.

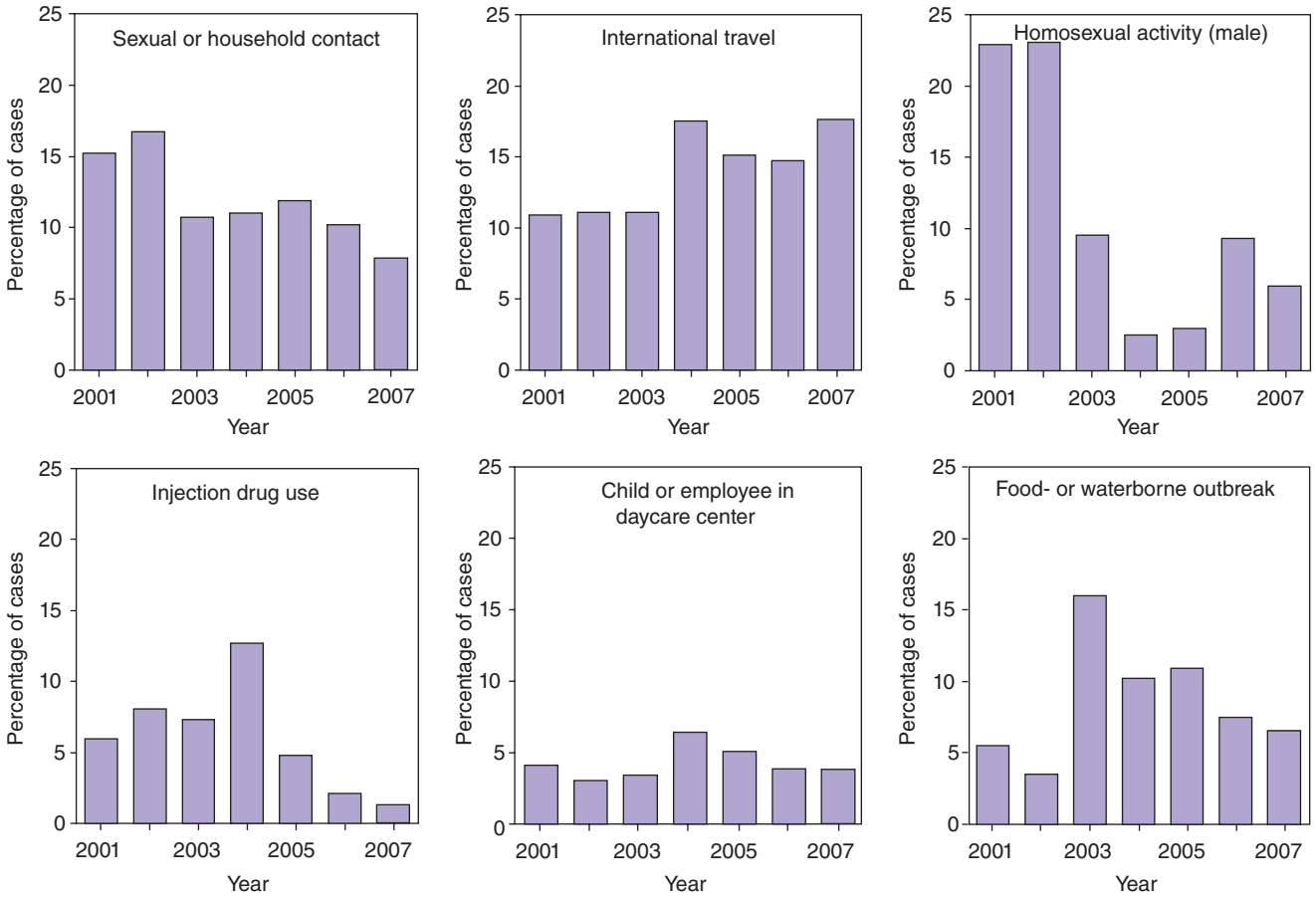


Figure 4.2 Trends in selected epidemiologic characteristics among patients with acute hepatitis A, by year – United States, 2001–2007. The percentage of cases among those reporting a specific risk factor was calculated based on the total number for whom information for that exposure was reported. Multiple risk factors may be reported for a single case. (Source: Centers for Disease Control and Prevention, Division of Viral Hepatitis, 2009; and Daniels D, Grytdal S, Wasley A. MMWR Surveill Summ. 2009;58(3):1–27 [6].)

Outbreaks of HAV attributable to direct person-to-person spread occur in settings characterized by fecal contamination, close contact, and compromise of hygienic standards such as daycare centers, schools, institutions for people with intellectual disabilities, prisons, and among military forces in the field. People with intellectual disabilities living in institutional settings have a higher prevalence of anti-HAV than age-matched controls living in the community, and prevalence of anti-HAV has been correlated with duration of institutionalization rather than with age. Many outbreaks of infectious hepatitis have been reported in this setting, and high rates of hepatitis have also been reported among staff.

Young children have a prominent role in the spread of HAV because of their relatively low hygiene standards and the frequency of subclinical infection in this age group. 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 daycare centers. The importance of daycare centers in transmission of hepatitis A began to be appreciated in the 1970s, although the frequency of such outbreaks has reduced in recent years following more widespread vaccination [7]. A large 2-year study established that outbreaks were most associated with large centers operated for profit, centers open for >15 hours per day, and centers with more than 50 children in diapers. Like other HAV outbreaks in which children play a central role, those associated with daycare centers can be prolonged and often 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 [12, 13]. 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. Virus may persist in the feces of infants and young children for longer periods than in adults.

Transmission to healthcare professionals exposed to people hospitalized with HAV infection has been relatively infrequent, probably because of the rapid decline in fecal shedding after the onset of symptoms. Cases of nosocomial HAV transmission are well described; however, usually in the context either of a patient incubating HAV and hospitalized for other reasons or of a subclinical infection in a fecally incontinent patient [14].

Men who have sex with men (MSM) have an increased prevalence of HAV-specific antibody and a relatively high incidence of new infection [15]. Many outbreaks of HAV infection among MSM have been described since the early 1980s in developed countries, including simultaneous outbreaks in 1991 in North America, England, the Netherlands, and Australia [15, 16]. These may relate to increases in frequency of sexual activities that are considered at low risk of HIV transmission but facilitate the spread of other pathogens such as HAV. For example, oral-anal contact, digital-rectal contact, and possibly oral-genital sex and handling of condoms after anal intercourse have been identified as risk factors for acquisition of HAV [17]. However, it has not always been possible to link specific high-risk sexual practices to the majority of HAV cases during outbreaks among MSM [18].

Foodborne and waterborne transmission

Transmission of HAV via fecally 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 in 2007 accounted for only 6.5% of reported hepatitis A cases in the United States [6]. Foodborne and waterborne transmissions continue to be important routes of transmission in developing countries and are particularly relevant to susceptible travelers [6].

Food may be contaminated with HAV during preparation by an infected food handler or before handling, as is typically the case with shellfish. Many outbreaks have been reported in association with consumption of raw or partially cooked bivalve mollusks, such as mussels, oysters, and clams. A dramatic example of such a shellfish-associated HAV outbreak occurred in Shanghai in 1988 [19]. More than 300 000 cases were reported, and epidemiological studies implicated consumption of raw clams in 90% of cases. HAV and HAV RNA were subsequently detected in clams.

Bivalve mollusks are frequently eaten raw, or after steaming that suffices to open the shell but does not produce internal temperatures sufficient to inactivate HAV [20]. Bivalves feed by filtering nutrients from large volumes of seawater. HAV present in fecally contaminated water is likewise filtered, and although no replication takes place within the mollusk, HAV may be concentrated 100-fold relative to the surrounding water and persist for up to 7 days [21].

Food handlers acutely infected with HAV may contaminate food during preparation if food hygiene practices are not followed. Transmission usually occurs in the late incubation period when the person is asymptomatic and fecal shedding of virus is at its peak. A variety of foodstuffs and beverages have been implicated in transmission of HAV in this manner with the implicated foods typically either uncooked or handled after cooking, as HAV is generally inactivated by normal cooking temperatures. Outbreaks may be sizable and explosive.

Waterborne transmission appears important in maintaining endemicity of HAV in resource-poor settings, where fecal contamination of drinking water can be widespread. However, accurate data are limited, particularly in view of the potentially confounding effect of hepatitis E. In well-resourced countries, contamination of water occurs but is uncommon. However, the large pool of susceptibles in low-prevalence populations means that these infrequent episodes of waterborne transmission have the potential for high attack rates.

Contaminated water is probably the most important source of HAV infection among travelers to developing countries, as a consequence of 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 [22]. Person-to-person spread, especially from young children, is another likely important source of HAV infection in travelers. Adult food handlers in endemic areas are unlikely to be shedding HAV because of the high rates of adult seropositivity in these regions. Of HAV infections reported to the CDC's Viral Hepatitis Surveillance Program in 2007, 17.5% were associated with a history of international travel [6].

Bloodborne transmission

The importance of bloodborne transmission in the epidemiology of hepatitis A remains to be definitively established. The potential infectivity of HAV by the bloodborne route is high. Experimental inoculation studies using tamarins and chimpanzees have demonstrated wild-type HAV to be $10^{4.5}$-fold more infectious intravenously than orally [23]. 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 fecal-oral transmission.

Using sensitive PCR assays, 73–100% of hepatitis A patients have been shown to be viremic at presentation [24]. Data from patients with hepatitis A have shown that the viremia is longer than previously understood, typically commencing an average of 17 days before the alanine aminotransferase (ALT) peak and persisting for between 18 and 79 days after this [25]. However, the highest viral loads and presumably, therefore, the highest risk of bloodborne transmission appear to occur prior to the ALT peak and the appearance of immunoglobulin M (IgM), being 1000-fold higher than those after the ALT peak [25]. 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.

The likelihood of transfusing a unit of blood infectious for HAV appears to be low. Furthermore, 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 posttransfusion hepatitis A. A number of prospective studies have assessed the risk of transmission of hepatitis A by single-donor blood products, and HAV was not transmitted to any recipients in any studies involving transfusion of blood or blood components. Nevertheless, isolated cases of posttransfusion hepatitis A have been described [26]. In addition, infrequent outbreaks of apparently parenterally spread HAV have occurred where transmission by multiple-donor blood products has been implicated. For example, outbreaks of HAV have been described among people with hemophilia receiving factor VIII concentrates in a number of countries [27].

Injecting drug use has had an impact on the epidemiology of HAV in many parts of the world. The prevalence of anti-HAV among IDUs was found to be several times that of the general population in Scandinavia, with outbreaks within this population also reported. Increasing numbers of outbreaks of hepatitis A among IDUs were reported in the United States during the 1980s [28]. 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 [29]. Significant outbreaks of hepatitis A have been described among IDUs in England and Australia, but needle sharing has not been clearly demonstrated as the only mode of transmission in these outbreaks, which have frequently been attributed to poor hygienic conditions [30].

Perinatal transmission

Isolated cases of apparent perinatal transmission of HAV have been described. An infant was isolated from its mother and treated with immunoglobulin when the mother developed symptomatic HAV immediately after delivery, subsequently infecting three other family members [31]. HAV RNA was detected in serum and feces 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 feces [31].

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. Our understanding of the comparative epidemiology of HAV 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. Shortcomings of HAV morbidity data are attributable to the high frequency of subclinical infection, the lack of laboratory facilities to distinguish hepatitis viruses in many regions or the failure to differentiate them in recorded data, and physician underreporting.

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 endemic areas such as Ethiopia. Three distinct patterns (A, B, and C) of age-specific HAV seroprevalence have been described (Figure 4.3) [32]. These patterns may be descriptive of the epidemiology of HAV in different countries, or in different population groups within the same country.

Seroprevalence pattern A is typical of HAV epidemiology in resource-poor settings, particularly tropical countries in which HAV may be hyperendemic, including most of Asia, the Pacific islands, Central and South America, and Africa [33]. Crowded living conditions and inadequate access to safe water and sanitation promote high levels of intrafamilial and foodborne and

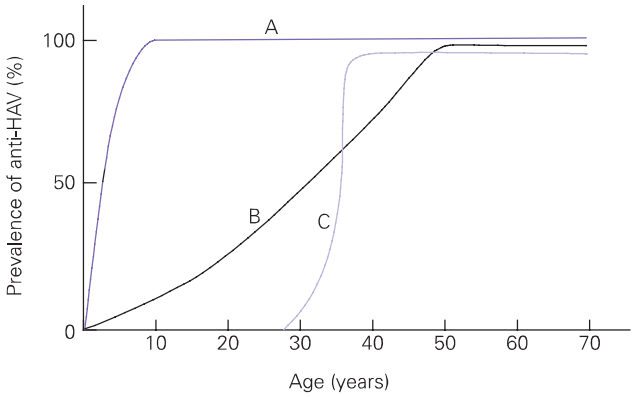


Figure 4.3 The three major patterns of age-specific prevalence of anti-HAV. (Source: Gust I. In: Vyas GN, Dienstag JL, JH H, editors. Viral Hepatitis and Liver Disease. Orlando: Grimae and Stratton; p.415–21 [32]).

waterborne transmission of HAV in these areas. Exposure to HAV before age 10 years is almost universal in these populations, and seroprevalence among adults approaches 100% [33]. Seroconversion among children is usually asymptomatic. In these countries, reported rates of HAV infection are low and outbreaks rare, with most cases of HAV among visitors from lower-prevalence areas such as tourists and voluntary workers.

Pattern B is typical of most developed countries, including Japan and the countries of Scandinavia, North America, 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 [34] among the older age group in a sigmoid-shaped curve (Figure 4.3). The increase to quite high levels of seropositivity among older age groups is probably a cohort effect, reflecting higher levels of HAV exposure present during childhood and a 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 these populations [35], and a decrease in HAV-specific antibody prevalence in sera from the same age group collected years apart in Australia, Germany, Japan, Italy, and Finland [35, 36]. For example, in Germany the HAV seroprevalence in the 20–29-year-old age group declined from 51% to 11% between 1965 and 1975 [36]. In contrast, an increase in anti-HAV seroprevalence by age group from 1998 to 2008 was recently reported from Victoria, Australia; this was hypothesized to be due to increased travel, vaccine uptake, and migration from endemic areas, as incidence of notified HAV infections fell significantly over the same period [4].

Epidemiological patterns that are intermediate between patterns A and B may occur as access to safe water and sanitation improves 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 [5]. This situation is characterized by a decline in total infection rates and an increase in the median age of exposure to HAV into older childhood or adolescence. A consequence of the increasing age at exposure to HAV may be a paradoxical increase in the rate of symptomatic hepatitis A. 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 [37].

The third seroprevalence pattern, pattern C, is typical of populations in which HAV is not endemic but has been 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 [38] and Alaska.

Demography

The differing age distribution of HAV infection between low- and high-income countries is largely a consequence of differing access to safe water and sanitation, as discussed in this chapter. In resource-poor settings, HAV infection is almost universal among children, reflecting early widespread exposure. In well-resourced populations, 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 some occupations, such as sewage work, or related to other activities such as oral-anal contact among MSM.

Socioeconomic status has been shown to be inversely associated with prevalence of HAV-specific antibody. Higher and lower HAV seroprevalences, respectively, were found in a lower socioeconomic group and in an upper socioeconomic group than in middle-class populations in the United States. A similar association of HAV seroprevalence and socioeconomic status has been demonstrated in Italy and Ethiopia. Other factors increasing the risk of HAV transmission in the Italian study were large families, crowded living conditions, and poor access to sanitation. Higher HAV seroprevalences have been observed in rural areas compared with urban areas in several countries, and a higher incidence of HAV infection has been observed among rural Thai children compared with children living in urban Bangkok [39].

Temporal patterns

Peaks of disease incidence occurred every 5–10 years between the early 1950s and the 1970s in some temperate well-resourced settings, including the United States, Canada, Australia, and Denmark. However, this cyclical pattern appears to have been interrupted as the incidence of HAV has declined in recent decades. The pattern has persisted in some population groups within the United States such as the Native American Sioux, among whom large HAV outbreaks occur approximately every 7 years, predominantly affecting the 5–9-year-old age group. A cyclical disease pattern is also seen in some countries currently of intermediate HAV endemicity, such as Mauritius and Thailand, as improving access to sanitation creates a transition phase between endemic disease and sporadic self-limited epidemics.

Similarly, seasonal peaks of HAV, which used to occur in autumn and winter in some temperate countries, are no longer seen except as reflections of travel patterns to endemic areas. For instance, Germany retains a seasonal peak of HAV in autumn, more pronounced in children than adults, which has been attributed to summer school holiday travel by children to Mediterranean countries where HAV is still endemic. 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 fecal suspensions by antigen-capture PCR [40]. 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, and VII) were recovered from human hepatitis A cases, and the remaining three genotypes (IV, V, and VI) isolated only from cases of hepatitis A–like illness among captive simian species [41]. 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 [41].

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. Genotype I strains are the most widespread in the world.

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. There is also evidence for co-circulation of imported strains with endogenous strains [41].

In the course of outbreak investigations, molecular epidemiological studies can support 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, and between viruses obtained from cases and shellfish identified as the infection source [42].

Conclusion: epidemiology

The general trend in well-resourced settings is toward declining incidence of HAV as access to hygiene and sanitation improves. In contrast, slow or minimal improvement in water supply and sanitation is likely in the foreseeable future in some resource-poor settings, and HAV will continue to be endemic in these areas. Within countries in transition toward 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 MSMs. These will remain potential foci of hepatitis A persistence and may contribute to outbreaks in an increasingly susceptible general population, as may travelers returning from countries in which hepatitis A is endemic. In addition, children in daycare centers 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 by creating a cohort of susceptible adults without altering the high-level transmission of HAV.

Improved hepatitis A surveillance and monitoring are required in both high- and low-resource settings, along with increased understanding of the duration of vaccine-induced immunity and implementation of public health measures aimed at improving sanitation and hygiene globally. In the longer term, incorporation of hepatitis A

vaccination into widespread childhood immunization protocols should facilitate eventual elimination of hepatitis A.

Prevention

Hepatitis A can be prevented by three general approaches. Because the predominant mode of HAV transmission is by the fecal-oral route, improving access to adequate hygiene and sanitation, including the appropriate disposal of human waste and provision of clean drinking water, is an important contributor to preventing HAV transmission. These considerations are particularly relevant for low-resource settings, in some parts of which improvements in socioeconomic conditions have resulted in altered age-specific prevalence of HAV infection [43]. In well-resourced countries, where transmission is usually from person to person, good hygienic practices, such as careful handwashing, can contribute to preventing transmission in settings such as daycare centers and food service establishments [44].

Another important development in hepatitis A prevention is the availability of safe and effective 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. Evidence of such reductions has been reported from the United States and other high-resource populations in which hepatitis A vaccination of children has been widely implemented [8, 45]. Because no animal reservoir has been identified and there is no chronic infection in humans, strategies of widespread vaccination, together with universal access to adequate sanitation, afford the potential to ultimately eliminate HAV transmission.

A third approach that continues to play a role in the prevention of HAV 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 (postexposure prophylaxis), such as household contacts of hepatitis A cases. However, effective postexposure 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, and there is some evidence for the role of active vaccination in this setting also [10].

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 [46]. Several years later, Binn and colleagues were the first to produce an inactivated vaccine from HAV propagated in cell culture [47].

Inactivated hepatitis A vaccines

A number of inactivated vaccines have been developed by commercial vaccine manufacturers. Two, Havrix (GlaxoSmithKline, Philadelphia, PA, USA) and Vaqta (Merck & Co. Inc., West Point, PA, USA), are approved for use in the United States as well as much of the world. Two other vaccines, Avaxim (Sanofi Pasteur, Lyon, France) and Epaxal (Berna Biotech Ltd, Bern, Switzerland), are available in Europe, Canada, and selected other countries. 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 section will focus on the two vaccines available in the United States and also used widely in Australia, Canada, and Europe (i.e., Havrix and Vaqta), 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, which was initially isolated in Costa Rica and was the first strain to be successfully cultivated *in vitro* [46]. CR326F was initially isolated in a fetal rhesus kidney line, FRhK6. After 15 passages, it was transferred to human embryonic lung diploid fibroblasts (MRC5 cells) for an additional 28 passages. Havrix contains the HM175 strain, isolated from the stool of a patient in Australia [48]. It was originally adapted to cell culture by a series of 30 passages in green monkey kidney cells followed by adaptation to MRC5 cells. Avaxim contains the GBM strain of HAV, which was isolated and propagated in human kidney cell culture for 10 passages, followed by adaptation to MRC5 cells during 20 passages [49]. Epaxal contains the RG-SB strain, grown and then harvested from disrupted MRC5 cells [50].

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. 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

Table 4.1 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

*For vaccines licensed in the United States, US-licensed age groups for pediatric 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 groups for which pediatric and adult formulations licensed vary among countries.
ELU: enzyme-linked immunosorbent assay (ELISA) units; U: units; IU: international units.

virus [51]. 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 aluminum hydroxide (alum) as an adjuvant. Epaxal uses a liposome adjuvant, immunopotentiating reconstituted influenza virosomes (IRIVs), composed of phosphatidylcholine, phosphatidylethanolamine, and hemagglutinin from an H1N1 strain of influenza virus [50].

Manufacture

All inactivated hepatitis A vaccines are produced in similar ways. Most vaccines are grown in MRC5 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 or inactivated virus is adsorbed on aluminum hydroxide, and each milliliter contains approximately 0.5 mg of aluminum as aluminum hydroxide.

Vaqta is grown in MRC5 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 aluminum hydroxide, in a concentration of approximately 0.45 mg/mL of aluminum as aluminum hydroxide.

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. 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 [47].

Preparations and dosage

Inactivated hepatitis A vaccines, with the exception of Epaxal, are available in pediatric and adult formulations (Table 4.1). Epaxal is prepared in a single formulation. A combination inactivated hepatitis A and recombinant hepatitis B vaccine (Twinrix; GlaxoSmithKline) is available in the United States for persons ≥18 years old; a pediatric formulation is available in Europe, Canada, and other parts of the world. A combined hepatitis A and typhoid vaccine (Vivaxim; Sanofi Pasteur) is also available in a number of countries for persons aged ≥16 years, though this is not currently available in the United States.

Havrix and Vaqta are licensed in the United States in two formulations, pediatric for persons aged 2–18 years, and adult for persons older than 18 years. Both formulations are given as a two-dose series. In other countries, the pediatric formulations are licensed for children as young as 1 year old. The pediatric 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 pediatric 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 pediatric 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 pediatric formulation is recommended for children aged 1–15 years old, with the second dose being given 6 months after the first; the adult formulation is for persons older than 15 years, with the second dose being given 6–12 months following the first. However, recommended schedules may be different in various countries.

The dose of Epaxal for adults and children >1 year old is 500 radioimmunoassay (RIA) units of hepatitis A antigen, associated with 10 µg of influenza hemagglutinin and 300 µg of phospholipids. 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 aluminum in the form of aluminum phosphate and aluminum hydroxide as adjuvants, and 5.0 mg of 2-phenoxyethanol as a preservative. The pediatric 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 [52].

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 [53]. 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 [54]. 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 doses [55]. 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 [56].

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. 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. Freezing destroys the vaccine, causing aggregation of the alum particles.

Immunogenicity

The inactivated hepatitis A vaccines are 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. 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. This boost in antibody concentration following the second dose is probably important for long-term protection [57].

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. 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.

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 [58]. 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. The clinical significance of these lower antibody concentrations is unclear.

Other conditions that may result in reduced immunogenicity include 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. Among men infected with HIV, higher CD+ T lymphocyte count at baseline was associated with response to vaccination. In one study, only one of nine participants with CD4 cell counts <200 at baseline responded to vaccination compared with eight of 11

participants whose baseline CD4 cell counts were ≥500 [59]. However, even among participants with CD4 cell counts ≥500, the antibody concentration after completing the two-dose vaccination series was considerably lower than those generally reported from studies of persons who do not have HIV infection. Among adults and children with chronic liver disease, seroprotection rates are similar to those observed among healthy adults, but the final antibody concentrations were substantially lower [60]. 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. Other factors, such as smoking and obesity, have not been evaluated for the currently licensed formulations [7].

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 milliliter (mIU/mL), measured in comparison to a World Health Organization (WHO) reference immunoglobulin reagent. 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 [61]. Concentrations of 10–20 mIU/mL, achieved about 1–2 months after administration of immune globulin, also are known to protect against hepatitis A. Because, as mentioned, 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.

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 [62, 63]. However, only a few studies have directly measured cellular immune response.

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, diarrhea, and vomiting occur in <5% of vaccinees. Headache has been associated with vaccination in up to 16% of adults and 2–9% of children.

Rare adverse events reported post marketing include syncope, jaundice, erythema multiforme, anaphylaxis, brachial plexus neuropathy, transverse myelitis, encephalopathy, and others. No serious adverse events among children or adults have been identified that could be definitively ascribed to hepatitis A vaccine. For events for which incidence rates are available, such as Guillain-Barré syndrome, reported rates were not higher than reported background rates. Based on limited data, vaccination of immunosuppressed persons does not appear to be associated with a greater risk of adverse events compared to that of persons with normal immune systems.

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. 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 travelers 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. 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 [64].

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 [63, 65]. Among children vaccinated during infancy, approximately two-thirds had detectable anti-HAV 4–6 years later [62]. It is estimated that protective levels of antibody following completion of the vaccination series could persist for 20 years or longer [9, 65]. 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 [57]. However, few such vaccinated cohorts are available to date. In 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 [66]. 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 [62]. 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. After 9 years of follow-up, no hepatitis A cases were reported among children vaccinated as part of a clinical efficacy study in one US community [67]. A recent systematic review concluded that hepatitis A vaccines are capable of protecting against infection for up to 15 years [9]. 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% [68]. In the vaccinated group, no cases of hepatitis A occurred with onset ≥17 days after vaccination [67].

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% CI, 79–99%) [69]. 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 ALT 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 [70]. Using IgM anti-HAV positivity as the endpoint, 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

Recent evidence suggests that hepatitis A vaccine may have some efficacy when administered after exposure to HAV, and public health agencies in several countries now recommend hepatitis A vaccine in this setting in certain circumstances. In a small randomized trial in 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 [71]. However, the CIs were wide (7–95%), and the study did not include a comparison group that received passive postexposure prophylaxis with immune globulin.

A larger randomized noninferiority trial conducted in Kazakhstan that did include comparison with passive immunization was reported in 2007 [10]. In this trial, the incidence of hepatitis A among 1090 contacts receiving either hepatitis A vaccine or immune globulin was less than 5%. Although the incidence of hepatitis A among vaccine recipients was slightly higher than among those

receiving immune globulin, the study's prespecified noninferiority criteria were met [10].

Following the publication of this trial, the Advisory Committee on Immunization Practices (ACIP) in the United States updated recommendations for postexposure prophylaxis to state that for healthy persons aged 1–40 years, hepatitis A vaccine is preferred; and for those aged under 1 year or over 40 years, and those with underlying chronic liver disease or immunodeficiency, immune globulin should be used [72].

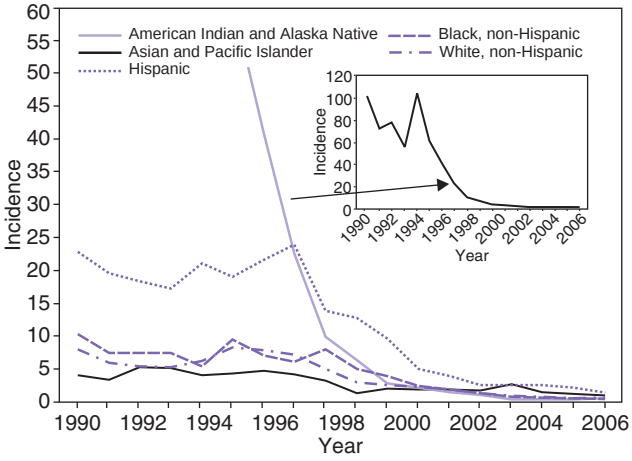
Effectiveness in populations

When reviewing evidence of the impact of hepatitis A vaccination in populations, the epidemiology of hepatitis A in the particular population in which the vaccination is being used provides an essential context. Transmission patterns are influenced by many factors, including the age-specific prevalence of immunity, access to adequate sanitation and 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 incidence 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. Historically, hepatitis A epidemics occurred 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. 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. 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 [73].

The most extensive information about the impact of ongoing hepatitis A vaccination on hepatitis A incidence in these populations over time comes from the United States. In the US vaccine efficacy study site, vaccination



* Per 100 000 population.

Figure 4.4 Incidence of acute hepatitis A per 100 000 population, by race/ethnicity and year – United States, 1990–2007. (Source: Centers for Disease Control and Prevention, Division of Viral Hepatitis, 2009; and Daniels D, Grytdal S, Wasley A. MMWR Surveill Summ. 2009;58(3):1–27 [6]).

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 disappeared, while such outbreaks continued to occur in similar neighboring communities [67].

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 incidence, surveys indicated vaccination coverage of 50–80% among preschool and school-aged Native American and Alaska Native children, suggesting that recommendations were being implemented. National surveillance data demonstrate a dramatic decrease in hepatitis A incidence (Figure 4.4) [6, 74]. By 2000, hepatitis A incidence among American Indians and Alaska Natives had declined by 97% compared with the beginning of the decade, and remains lower than the overall US rate [6, 74]. 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 Native American and Alaska Native communities.

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 southwestern areas of the United States. Periodic

community-wide epidemics can be identified in these communities, but the interepidemic period is variable and the majority of the population remains susceptible to HAV infection into at least middle age [75, 76]. 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. 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. Efforts are probably better directed toward 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 [75]. During the 5 years before the demonstration project, Butte County's average annual hepatitis A incidence 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 were the lowest number ever reported in the county since hepatitis surveillance began in 1966. Butte County's 2000 incidence of 1.9/100 000 was the lowest of any California county [75].

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. 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 [45, 75].

In the United States, vaccination of children living in the western and southwestern parts of the country has been recommended since 1999 (Table 4.2). These areas include approximately one-third of the US population, but historically accounted for approximately one-half of hepatitis A cases nationwide. Fairly low vaccination coverage of children in these areas resulted in appreciable declines in hepatitis A rates (Figure 4.5) [77]. 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 [45, 77]. Rates declined most dramatically among children 2–18 years old. These precipitous declines are reflected in overall national hepatitis A rates. Modeling 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 [45].

Table 4.2 US recommendations for routine pre-exposure use of hepatitis A vaccine*.

Group	Comments
Routine vaccination of all children aged 1 year	
International travelers§	Immune globulin may be given in addition to or instead of vaccine
Men who have sex with men	Includes adolescents
Users of injection and noninjection drugs	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	
Any person wishing to obtain immunity	

*Hepatitis A vaccine is not licensed in the United States for children <12 months of age.
§Persons traveling to Canada, Western Europe, Japan, Australia, or New Zealand are at no greater risk than in the United States.
Recommendations of the Advisory Committee on Immunization Practices, US Centers for Disease Control and Prevention, 2006 (see [7]).

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 a recommendation that vaccination against HAV be integrated into national immunization schedules for children aged ≥1 year if indicated on the basis of incidence, changing endemicity from high to intermediate prevalence, and cost-effectiveness grounds [78]. In general, hepatitis A vaccination currently has little relevance in highly endemic countries where the vast majority of the population develops HAV infection in early childhood. In lower-endemicity populations, hepatitis A vaccine is being used most widely to protect individuals at increased risk of hepatitis A or its consequences, such as travelers to areas where hepatitis

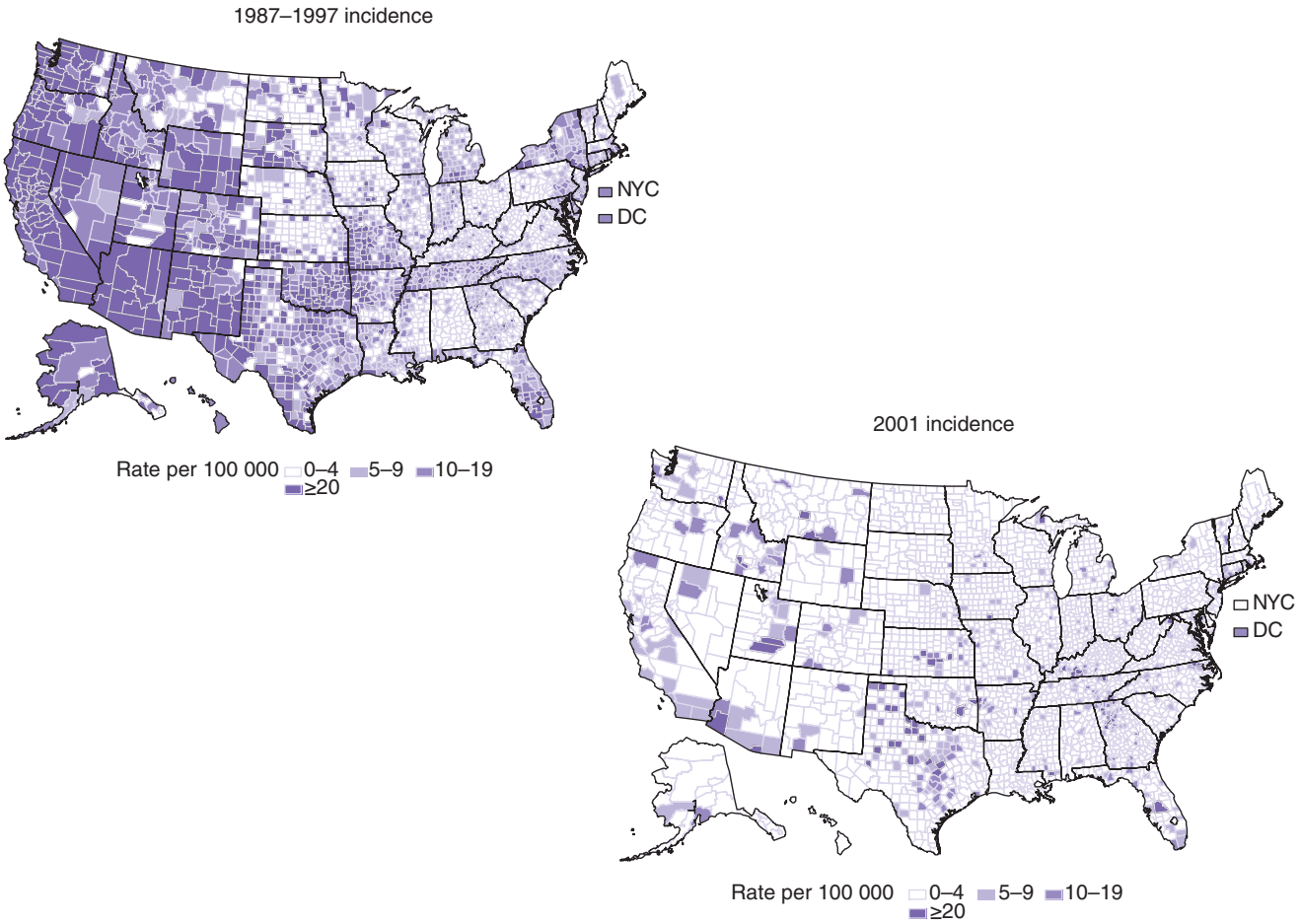


Figure 4.5 Hepatitis A incidence rates, by county, United States, 1987–1997 (top) and 2001 (bottom). (Data from Centers for Disease Control and Prevention.)

A is endemic. 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. 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 programs for children (Table 4.2) [76].

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, Australia, and a number of other countries. In the Puglia region of Italy, a program to vaccinate 15–18-month-old children with hepatitis A vaccine when they receive the measles–mumps–rubella (MMR) vaccine and 12-year-old children when they receive the hepatitis B vaccine (using the combination hepatitis A–hepatitis B vaccine) was implemented in 1997. In Israel, all 18-month-old children have been vaccinated each year since 1999. 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 4.2).

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 4.2) [57, 79]. Immune globulin can be used if hepatitis A vaccine is contraindicated or refused. A considerable proportion of these travelers are children, who may acquire hepatitis A while visiting family in the developing world, and transmit it to others when they return. Other groups at increased risk of infection for

whom hepatitis A vaccination is recommended in several countries include adolescent and adult men who have sex with men and people who inject drugs, because of recurrent hepatitis A outbreaks among these populations; members of the military; persons who work with HAV in research settings; and persons who have clotting factor disorders [79, 80]. 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.

Other groups and settings

Although hepatitis A outbreaks occur in childcare centers, 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 [7]. When outbreaks are recognized, aggressive use of immune globulin is effective in limiting transmission. In areas where routine vaccination of children is recommended, previously unvaccinated children can be vaccinated when they receive postexposure prophylaxis with immune globulin. In addition, childcare center attendees can be a readily accessible target population for ongoing routine vaccination programs.

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 [44, 81].

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, testing and vaccine costs, and the likelihood that the person will return for vaccination, should be taken into account. In the developed world, prevaccination testing of children is generally not cost-effective. Vaccination of immune people is not harmful. Postvaccination testing is not indicated because of the high probability 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. An attenuated H2 strain vaccine was developed and has been used fairly widely in China, particularly among children [82].

Antibody responses to attenuated vaccines generally have been found to be higher than those considered protective, but considerably lower than those measured in recipients of a complete course of inactivated vaccine. Whether T cell responses are also induced is unknown. Based on very limited data, the H2 vaccine appears to be immunogenic and to provide clinical protection for Chinese children [82].

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. 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.

Passive immunization

Until the licensure of hepatitis A vaccines, immune globulin was the mainstay of prevention 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 in travelers, Peace Corps volunteers, military personnel, and individuals recently in close contact with a person with hepatitis A, and to control outbreaks in childcare centers. 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 program of immune globulin every 4 months [83].

Efficacy

In general, when administered before exposure or within 2 weeks after exposure, immune globulin is >85% effective in preventing hepatitis A. 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 [84]. Similar reductions in hepatitis incidence were observed among adults in the military and children in institutional settings who received immune globulin. Results of a number of studies demonstrated 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. 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. 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, the concentration of anti-HAV achieved by the immune globulin dose, and the size of the HAV innoculum. Evidence of both phenomena has been reported.

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. One milliliter 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. 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. 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. This precipitation procedure has been shown to inactivate hepatitis B virus and HIV [85]. 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. 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.

Indications

Immune globulin is recommended for postexposure 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. Casual contacts such as school classmates or coworkers 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 childcare centers where a child or employee is diagnosed with hepatitis A and in other settings (e.g., hospitals, or facilities for developmentally disabled persons) when outbreaks occur. 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 [7]. 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 postexposure period during which immune globulin is effective will have passed.

Immune globulin also may be used for pre-exposure prophylaxis for persons who are traveling 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. Immune globulin should be considered for travelers younger than the minimum age for

which hepatitis A vaccine is 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. 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. 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. Re-administration every 5 months is necessary for extended trips, and hepatitis A vaccine, if not contraindicated, is a better choice for such travelers. 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, many argue 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 living conditions and access to sanitation. However, in countries with transitional or intermediate endemicity, a growing proportion of older children, adolescents, and adults in selected areas, such as urban centers 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 III
Hepatitis B Virus and Other *Hepadnaviridae*

Chapter 5
Structure and molecular virology

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Summary

Hepatitis B virus (HBV) displays a unique infectious cycle sharing similar features to retroviruses. Infectious virions contain relaxed circular DNA (rcDNA), which is delivered to the nucleus before the initiation of replication. Viral DNA integration into a host cellular chromosome is not mandatory for virus replication. Instead, covalently closed circular DNA (cccDNA) is generated from incoming rcDNA and serves as the template for the transcription of viral RNAs. The epigenetic control of the transcriptional activity of cccDNA by the HBx protein or cytokines may influence HBV replication and pathogenesis. In the cytoplasm, viral genome replication occurs within nucleocapsids and involves a protein-primed reverse transcription of the pregenomic RNA (pgRNA) into viral minus-strand DNA, followed by DNA-dependent DNA synthesis to generate new rcDNA. Nucleocapsids can be recycled back to the nucleus to amplify or maintain the pool of cccDNA, or be directed to the endoplasmic reticulum to be packaged by envelope proteins and secreted as infectious viruses.

HBV morphology, life cycle, and genome organization

Virion morphology: Dane particles

The identification of the Australian antigen, subsequently referred to as the hepatitis B surface antigen (HBsAg), by Baruch S. Blumberg in the mid-1960s, the characterization by electron microscopy of human HBV particles in the early 1970s by D.S. Dane and others, and the cloning and characterization of the viral genome allowed the identification of HBV as a prototypic member of the *Hepadnaviridae* family. The *Hepadnaviridae* are DNA viruses that include mammalian hepatitis viruses (among others HBV, woodchuck hepatitis virus [WHV], and ground squirrel hepatitis virus [GSHV]) and avian ones (duck hepatitis virus [DHBV] and heron hepatitis virus [HHBV]). These viruses share the same features including a small enveloped hepatotropic virion with a partially double-stranded DNA with a complete coding strand (minus (–)-strand) and an incomplete noncoding strand (plus (+)-strand) that replicate via reverse transcription in the hepatocyte. These viruses can cause chronic infection and severe liver diseases, including the development of cirrhosis and hepatocellular carcinoma (HCC). HBV virions (or Dane particles) have a diameter of 42 nm and are found in variable amounts in patient serum (up to 10^{10}/ml). The lipid envelope is composed of three surface glycoproteins that surround an icosahedral nucleocapsid (28 nm) formed by a single protein shell (240 core proteins) that contains the small (~3.2 kb) rcDNA (Figure 5.1).

Overview of the HBV life cycle

HBV binds to the surface of the hepatocyte via its envelope to a still-undefined receptor. Once the virus penetrates the hepatocyte, the viral surface proteins are

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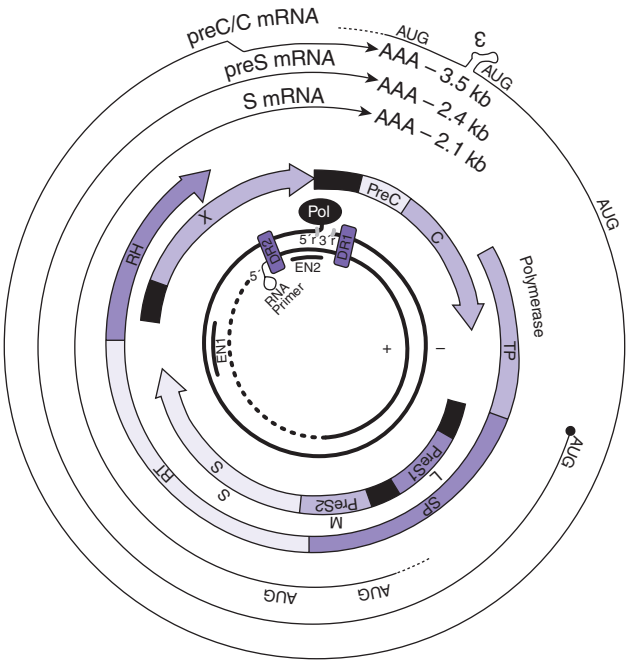

Figure 5.1 HBV DNA genome organization. The HBV genome is ~3.2 kb in size with a circular partially double-stranded DNA composed of a complete coding minus strand (–) and an incomplete noncoding positive strand (+) with variable 3′ end size. The viral polymerase (Pol) is covalently attached to the 5′ end of the (–)-strand by a phosphotyrosine bond, and two small terminal redundancy sequences are positioned at both ends (5′r and 3′r). A residual capped RNA oligomer (RNA primer), remnant of the viral synthesis, is covalently bound to the 5′ end of the (+)-strand. Two direct repeats (DRs) of 11 nucleotides (DR1 and DR2) are located at the 5′ ends of both strands. The viral genome contains four promoters (black rectangle) and two transcription enhancers (EN1 and EN2), which control the transcription of four genes (preC/C, P, preS/S, and X) with identical orientation. Three major transcripts are generated with six start codons (AUG): the 3.5 kb preC/C or pregenomic pgRNA, which contains the encapsidation sequence "ε"; the 2.4 kb preS mRNA; and the 2.1 kb S mRNA. The ~1 kb X mRNA is occasionnaly generated and is not represented here. All transcripts are polyadenylated and capped. The polymerase gene encodes four distinct proteins: the terminal protein (TP), the spacer (SP), the reverse transcriptase (RT), and the RNAse H (RH).

removed and the HBV nucleocapsid migrates from the cytoplasm to the nucleus to deliver the viral genome (rcDNA). Within the nucleus, the rcDNA is converted to cccDNA, a viral mini-chromosome that subsequently serves as a template for the transcription of four messenger RNAs (mRNAs) (3.5, 2.4, 2.1, and 1 kb). These transcripts are then transported to the cytoplasm and translated into seven viral proteins: the core protein (HBcAg); the pre-C/C protein, which is processed into the secreted HBeAg; the viral polymerase (Pol); the three envelope proteins (the large, medium, and small surface proteins bearing the HBsAg); and the X protein (HBx) (Figure 5.2).

The core proteins assemble in the cytoplasm and encapsidate both the pgRNA and the viral Pol to form nucleocapsids. Multiprotein complexes, including chaperone proteins, are also packaged into the nucleocapsid through this process. Reverse transcription of the pgRNA into (–)-strand DNA takes place within the nucleocapsid followed by the synthesis of the (+)-strand DNA. The mature nucleocapsids can either migrate back to the nucleus to deliver the viral genome and enrich the pool of viral cccDNA, or reach the endoplasmic reticulum (ER) lipid membrane together with the envelope proteins to assemble and bud into the ER lumen, to be secreted as new HBV virions through the Golgi apparatus (Figure 5.2).

Genomic structure and organization

The HBV genome has evolved structurally to a minimal compact genetic organization with a partly double-stranded DNA composed of a minus (–) and a plus (+) strand, maintained in circular configuration by their cohesive end regions that contain two direct repeats (DRs) of 11 nucleotides termed DR1 and DR2, which are situated at the 5′ end of the two strands [1]. They both play a role in the template switches during viral DNA synthesis, and they are essential for viral replication (Figure 5.1).

The full-length coding (–)-strand DNA (3.2 kb) carries the whole genome, while the incomplete noncoding (+)-strand DNA region extends to approximately two-thirds of the genome length with a variable 3′ end. The (–)-strand DNA is covalently linked by a phosphotyrosine bond to the viral polymerase. It also contains a small, 8–9-nucleotide-long terminal redundancy, termed "r," on both the 5′ and 3′ ends that is critical for the formation of rcDNA synthesis. After viral (–)-strand DNA synthesis, a residual short RNA oligomer derived from the 5′ end pgRNA remains covalently bond to the 5′ end of the (+)-strand DNA to serve as a template for (–)-strand DNA synthesis (Figure 5.1).

A small amount (5–10%) of double-stranded linear (DSL) DNA can also be found packaged into the nucleocapsid instead of rcDNA. DSL DNA molecules are produced after aberrant *in situ* priming of (+)-strand DNA synthesis, which starts at the 3′ end of the (–)-strand DNA due to the absence of RNA primer translocation from DR1 to DR2 during (+)-strand synthesis, a step required for genome circularization [2]. However, following infection, both circular and linear DNA can be converted to cccDNA in the hepatocyte nucleus. The circularization of DSL DNA by nonhomologous recombination between the two ends is called "illegitimate replication," although its role in the viral replication

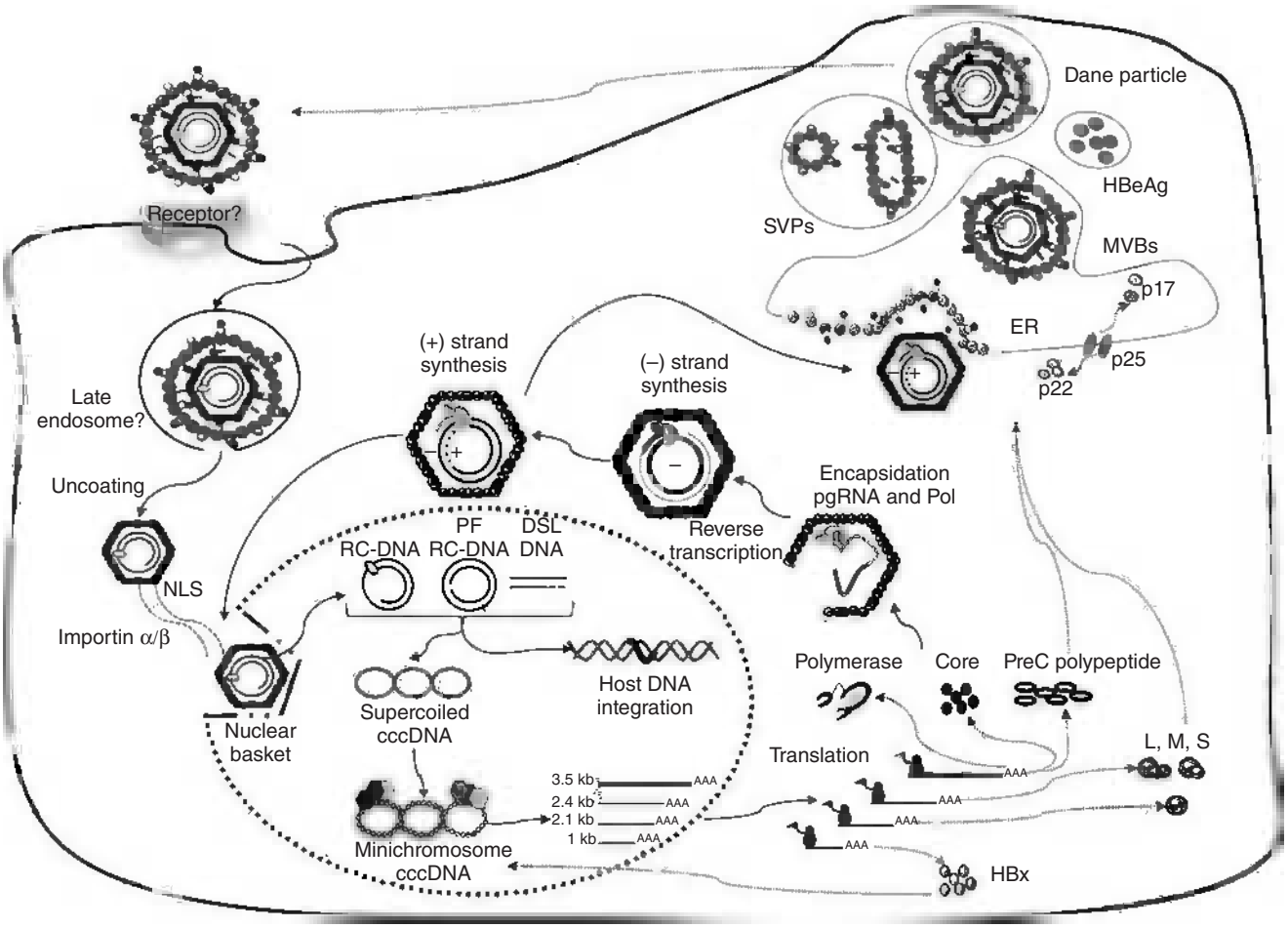

Figure 5.2 HBV life cycle: HBV virion binds to an undetermined receptor and enters into the hepatocyte to release the nucleocapsid in the cytoplasm (uncoating). The viral capsid migrates to the nuclear pore complex through the nuclear transport factors importins α and β. The nucleocapsid is arrested at the nuclear basket, where it releases the genome in the nucleus. Covalently closed circular DNA (cccDNA) is formed in majority from relaxed circular DNA (rcDNA) and, to a minor extent, by illegitimate replication from double-stranded linear (DSL) DNA. The cccDNA pool can also be enriched from protein-free (PF) rcDNA from the newly synthesized nucleocapsid, which contains a mature (+)-strand DNA without the RT polymerase. The viral genome can occasionally integrate into the host cellular gene. The cccDNA is considered as a nonintegrated mini-chromosome that uses cellular transcriptional machinery to generate the four mRNA transcripts that are translated into viral proteins after the mRNA export to the cytoplasm. The core proteins assemble and encapsidate the viral polymerase and the pgRNA from which these proteins are translated. Inside the capsid, the pgRNA is reverse transcribed by the polymerase to form the (–) and (+) DNA strands. The mature nucleocapsid is recycled to the nucleus or is directed to the secretory pathway. The surface proteins assemble at the endoplasmic reticulum (ER) membranes and may use the host cell machinery for the virus budding of vesicles into the lumen of multivesicular bodies (MVBs) before their release from the cell.

process seems to be minor but may play a role in integration.

The HBV genome contains four overlapping open reading frames (ORFs) (P, preC/C, preS/S, and X) encoded by the (–)-strand with six start codons, four promoters (preS1, preS2, core, and X), and two enhancer elements (EN1 and EN2) located upstream of the core promoter. The viral genome also contains a common 3′ polyadenylation signal in the core gene and a number of *cis*-acting signals essential for DNA replication. The major viral RNA transcripts produced are polyadenylated and capped (3.5, 2.4, 2.1, and 1 kb) and translated into seven viral proteins (HBe; core; large, medium, and small envelopes; polymerase; and HBx) (Figure 5.1).

ORF P has four domains that encode a multifunctional viral DNA polymerase (Pol, 90 kd). The first domain encodes the terminal protein (TP) situated at the N-terminus end of the protein. TP is covalently bound

to the 5′ end of the (–)-strand DNA, and a conserved tyrosine residue acts as a primer for the (–)-strand DNA synthesis [3]. The TP domain is also essential for the encapsidation of the viral pgRNA. The second domain contains a hinge region called a "spacer," which does not appear to have any enzymatic function. The third domain is the largest one and encodes the polymerase and reverse transcriptase activity that catalyzes viral genomic synthesis. The fourth domain in the C-terminal region encodes ribonuclease H (RNase H), which degrades the pgRNA during (–)-strand DNA synthesis and plays a key role in HBV genome replication [1] (Figure 5.1).

ORF preC/C partially overlaps with ORF P and encodes the precore–core protein (HBeAg), the core protein (HBc), and the pgRNA. The pgRNA is initiated five nucleotides downstream from the precore initiation codon; it serves as a template for the translation of core protein (21 kd or p21) and the viral DNA synthesis within the nucleocapsids (Figure 5.1). The preC transcript generates the precore polypeptide (p25), which is not packaged into viral nucleocapsids but contains a 5′ end signal peptide that directs the protein to the secretory pathway where two successive proteolytic cleavages to its N and C termini generate the mature secreted soluble HBeAg of ~17 kd (p17) [1] (Figure 5.3).

ORF preS/S, located within ORF P (between the spacer and the RT domains), directs the synthesis of three integral envelope glycoproteins (large L, medium M, and small S) with distinct N-terminal domains using three in-frame AUG start codons, all containing HBsAg [1].

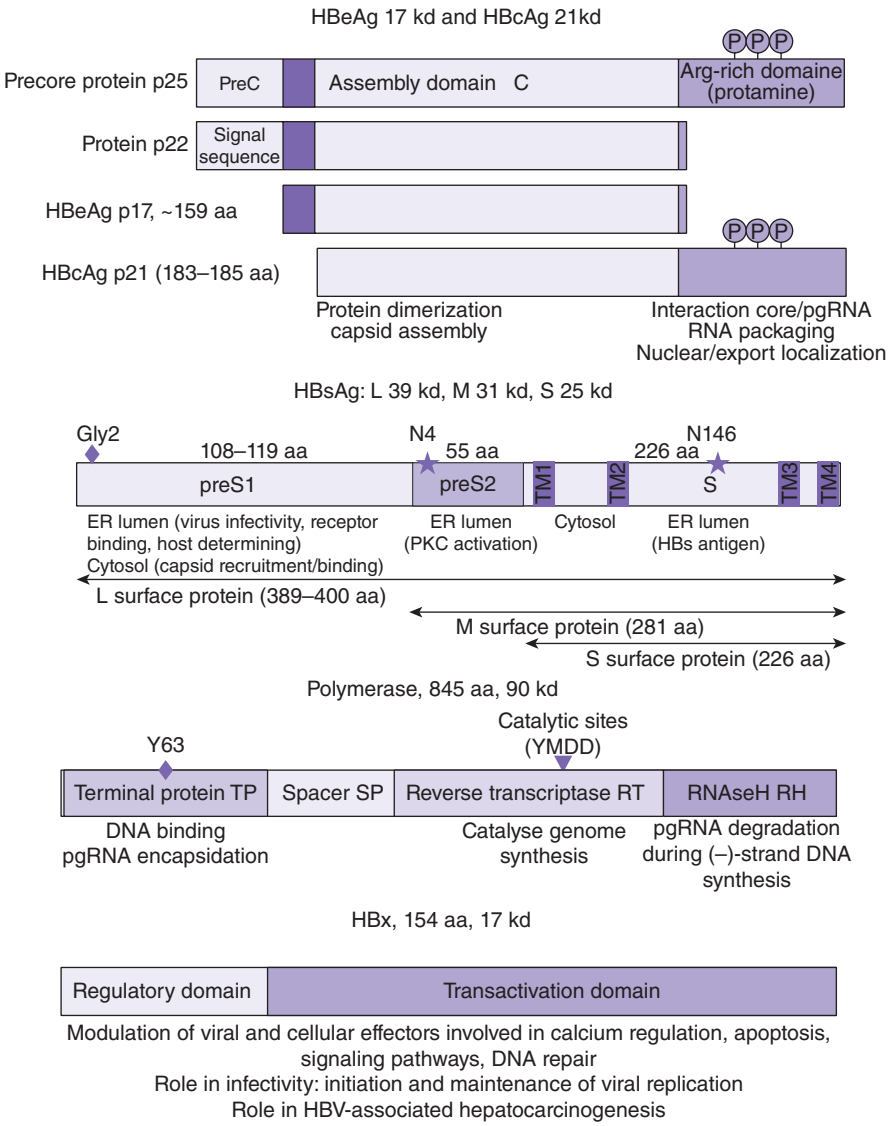

Figure 5.3 Schematic organization of HBV proteins.

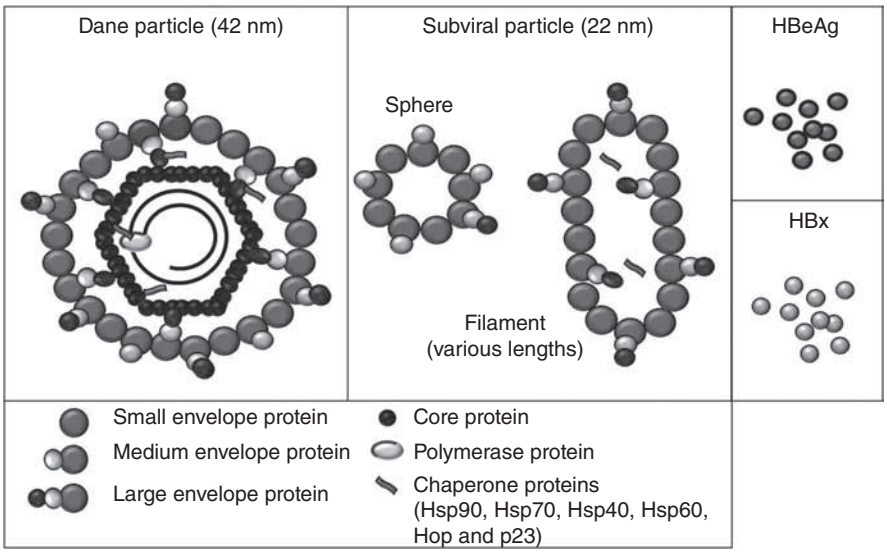

Figure 5.4 Dane particle and subviral particles. The Dane particle is composed of a lipid envelope made of three surface glycoproteins (small, medium, and large) that surround an icosahedral nucleocapsid (28 nm) formed by a single protein shell (240 core proteins) that contains the small ~3.2 kb relaxed circular DNA. The large surface protein topology is oriented inside or outside the viral envelop. Several chaperone and co-chaperone proteins are required for capsid formation and polymerase RT–ε interaction. The HbeAg and the "empty" subviral particles are secreted, whereas the X protein stays within the cell.

The smallest ORF X overlaps with ORF P. It encodes for the small protein HBx (17 kd, 154 aa) which shares no homology with any known gene. This ORF is found in all mammalian hepadnaviruses but is absent in the avian viruses.

Hepatitis B viral proteins

HBV envelope proteins and HBsAg

The surface proteins vary by their N-terminal domain length. The small hepatitis B surface protein (SHBs, 226 aa) is extended at its N-terminal end with one (medium surface protein, or MHBs) or two (large surface protein, or LHBs) extradomains that are translated within the same ORF but from different AUG start codons located upstream of the S initiation codon (Figure 5.1). The size of the L protein (p39) depends on HBV subtype *ay* or *ad* (389–400 aa). The M protein (p30) lacks the N-terminal preS1 sequence of the L protein (119 aa), whereas the S (p24) protein lacks the N-terminal preS2 sequence of the M protein (55 aa). All three envelope proteins have a glycosylated form responsible for the secretion of the viral particles. A common N-glycosylation site at residue asparagine N146 is situated in the common S domain. The M protein also displays an N-glycosylation site at N4 in the preS2 domain that is not modified in the L protein (Figure 5.3). The envelope proteins are synthesized at the ER membrane and are anchored by their S domain to form multimers stabilized by disulfide bridges between the cysteine residues in the S domain. S and M proteins contain the necessary export signals, whereas L proteins contain a retention signal controlled by its S domain; in the absence of S and M proteins, the L protein is retained in the ER membrane. The Dane particle contains a majority of S proteins and an equal amount of M and L proteins, which represent approximately one-third of the viral envelope (Figure 5.4).

The surface proteins can also assemble to generate non infectious empty subviral particles (SVPs) without the nucleocapsid and constitute HBsAg. Most vaccines against hepatitis B are based on SVPs and exhibit high immunogenicity. These particles are ~22 nm in diameter with either a spherical or a filamentous form (Figure 5.4). The sphere particles are found in great excess over virions (up to 10 000-fold) in the serum of HBV carriers (10^{13}/ml), whereas the filamentous particles are less numerous (up to 10^{11}/ml). These particles contain a majority of S proteins, which is sufficient to induce an efficient secretion of the HBsAg. Spherical particles contain mainly S and M proteins and trace amounts of L proteins, whereas filaments contain a majority of S proteins and equal amounts of M and L proteins. Their role is still unclear; they may act as immunological decoys for the host immune system by neutralizing antibodies to HBsAg, and thus support virus spread and persistence in the infected host [4].

The S protein is rich in hydrophobic amino acids with many tryptophans. It contains at least three transmembrane domains (TM1, TM2, and TM3/4) separated by two hydrophilic loops (Figure 5.3). The insertion to the ER membrane is initiated by a cotranslational translocation of TM1 (aa 8–22), which directs the N-terminus extradomain (aa 1–7) to the ER lumen. The cytoplasmic loop (aa 23–79) stays in the viral particle after budding of the HBs particles. The TM2 domain (aa 80–98) directs the translocation of the second loop (aa 99–169) in the ER lumen and forms the major HBs antigen region. This orientation allows the glycosylation of N146 by N-glycosyltransferases present in the ER lumen. The C-terminal hydrophobic region (aa 170–226) is considered as one or two transmembrane regions TM3/4 (aa 173–193 and aa 202–222) separated by a short cytosolic sequence that may insert posttranslationally into the ER membrane.

The M protein has the same S-domain topology with a cotranslational extension of the preS2 domain (55 aa). It is translocated to the ER lumen by the TM1 signal sequence that allows the N glycosylations of N4 in the preS2 domain. The preS2 domain contains no cysteine; therefore, the major epitope (aa 7–20) is not conformation dependent. Its function is still unclear as the preS2 domain is not necessary for virus infectivity.

The L-protein biosynthesis includes the addition of the preS1 sequence (aa 108–109) to the preS2 region. PreS1/S2 domains (called "preS") and part of the S domain of the large protein are initially localized in the cytosolic face of the ER with no possible N glycosylation of the N4 on the preS2 domain. In this conformation, the N-terminus end of the preS1 domain is anchored to the ER membrane by a myristylated glycine (gly2) of the preS1 domain. However, a posttranslational translocation of the preS1/2 domains into the ER lumen occurs in half of the L protein with the insertion of TM1 into the ER membrane. This leads to a dual topology of the L protein either in the cytosol (i-preS) or in the ER lumen (e-preS), which may be facilitated by chaperone binding to the preS domain such as heat shock protein hsc70/hsp40 and BiP (Figure 5.4). This dual orientation may confer several functions in the virus biology. For example, the external orientation is responsible for the host cell–virus recognition and the virus infectivity, whereas the cytosolic form recruits and binds the nucleocapsid, which subsequently leads to the budding of the nucleocapsid and the envelope assembly [4] (Figure 5.2).

The cytoplasmic localization of the preS2 domain may have a transactivator role on selected promoters. This function comes from the initial observation that some C-terminally truncated forms of LHBs and MHBs were found in chronically infected patients and suggests that they could be involved in the development of HCC. In addition, the cytosolic preS2 domain was shown to bind and activate the protein kinase C (PKC), which activates the c-raf–MEF signal transduction pathway involved in the control of viral promoters (Figure 5.3). This would subsequently lead to higher hepatocyte proliferation and increase the incidence of liver tumors in HBV animal models [5].

Hepatitis B core protein

The core protein (183–185 aa) assembles in the cytoplasm into viral capsids. The protein was detected in the cell nucleus, the cytoplasm, or both compartments depending on the serologic pattern, the liver disease, and the cell cycle. The HBc distribution in the cytoplasm was correlated with hepatic inflammation during the immune-clearance phase of patients with chronic active liver disease, whereas its nuclear localization was predominantly found in patients with high viral load and normal liver histology who are in the immune-tolerance phase. HBcAg was also found in the nucleus during the G0/G1 phases of the cell cycle and in the cytoplasm during the S phase in proliferating cells.

The nucleocapsid is composed of core homodimers bound by disulfide bridges between their homologous cysteine residues that form icosahedral T = 3 (90 dimers, 30 nm) or T = 4 (120 dimers, 34 nm) symmetry. Both forms can be found in HBV virions but with a quasi-exclusive content of T = 4 particles (~98%). The protein folding revealed an upside-down "T" structure composed of two L-shaped core monomers that form spikes on the surface of the shell. The core monomer displays two antiparallel α-helical hairpins (α3 and α4) joined by a loop at the tip of the spike, which constitutes the major immunodominant region (HBcAg). The protein dimerization forms a compact four-helix bundle that assembles into inter-subunits of five or six dimers around the five-fold and quasi-sixfold symmetry axes [6]. The capsid shell is fenestrated, which suggests the free diffusion of nucleotides into and out of the capsid lumen during the DNA synthesis or following the degradation of the pgRNA.

The core protein is composed of two structurally and functionally distinct domains (Figure 5.3). The N-terminus region (149 aa or 151 aa) is sufficient for the protein dimerization and capsid assembly. The C-terminal domain (CTD, 34 aa) is an arginine-rich domain (ARD or protamine, residues 150–183) that allows the interactions between the core protein and the pgRNA that are essential for packaging of the pgRNA–reverse transcriptase complex and for DNA synthesis [1]. In addition, the protamine domain contains not just nuclear localization sequences (NLSs) but also nuclear export signals (NESs), which allow HBc to bind to the nuclear pore complex (NPC) and shuttle backward and forward through the nucleus and the cytoplasm [7].

Three major phosphorylation sites within the protamine domain (serine 157, 164, and 172) were shown as being essential for pgRNA packaging and DNA replication [8]. The protein kinase activity identified in the viral nucleocapsid was suspected to be of cellular origin since none of the viral proteins displays kinase activity. Several enzymes have been identified such as protein kinase C, glyceraldehyde-3-phosphate dehydrogenase, a 46 kd core-associated kinase, and the SR protein-specific kinases 1 and 2 (SRPK1 and SRPK2) [5]. In addition, the capsid maturation was closely related to the phosphorylation state of the core protein as highly phosphorylated immature viral capsids were found to be progressively dephosphorylated during the DNA synthesis and nucleocapsid maturation. The progressive serine dephosphorylation of core protein may also stabilize the electrostatic interactions between the positive charges from the core basic amino acids and the negative charges that accumulate during the synthesis of (+)-strand DNA to maintain the capsid stability essential for HBV DNA synthesis and genome maturation [9].

HBe proteins

The core gene encodes two distinct proteins, the core protein (p21) and the precore polypeptide (p25, 212 aa) that generates the HBeAg (Figure 5.3). The precore sequence (29 aa) is required for the secretion of HBe protein as it contains hydrophobic residues resembling a sequence signal at its N-terminus that directs its translocation into the ER lumen. In addition, a cysteine residue (Cys7) within the 10 aa attached to the HBe protein is crucial for the quaternary structure, the antigenicity, and the production of the mature monomeric protein. The N-terminal truncation of 19 aa of the preC sequence by a signal peptidase in the ER lumen gives rise to a p22 protein that differs from the major core protein by its 10 additional terminal amino acids. The p22 form is either released into the cytoplasm to form hybrid nucleocapsids with core p21 or translocated to the ER membrane where an additional cleavage of ~34 aa occurs at the C-terminus by a furin-like endopeptidase in the trans-Golgi network before the release of the mature HBe protein (p17) [4].

HBe is not involved in the viral replication cycle nor is it required for viral infection. It may function as an immunoregulatory protein to modify the core immune response by the host cells. It can function as a T cell tolerogen to moderate HBcAg-specific liver injury during acute infection, which may result in viral persistence and chronic infection. HBc is a more potent immunogen than HBe in terms of antibody production and Th cell induction. HBc elicits preferentially a Th1-like response (interleukin 2 [IL2] and interferon gamma [IFNγ]), whereas the Th2-like response (IL4) is more specific to HBe. This suggests that HBe may downregulate the T cell antiviral response to HBc and could thus counteract the viral clearance process and favor HBV persistence. Furthermore, HBe can cross the placenta and trigger HBeAg- and HBcAg-specific T cell tolerance *in utero*; this may explain the chronicity observed after HBV vertical transmission mostly characterized by a predominance of HBeAg Th2-like cells associated with HBV persistence [10].

In clinical practice, HBeAg is used as a marker of viral replication and response to therapy; following the immune tolerant phase, the continued presence of HBeAg is associated with liver diseases, whereas its absence is associated with normalization of liver function together with the production of antibody (anti-HBe) seroconversion. However, it is not useful in patients who are infected with mutants of the precore region or the basic core promoter region; in this situation, HBV replication continues despite the absence of detectable HBeAg.

Viral polymerase (Pol)

The HBV polymerase (Pol) is a 90 kd polypeptide with distinct functions in the viral life cycle such as RNA binding, pgRNA encapsidation, protein priming of reverse transcription, RNA- and DNA-dependent DNA polymerase activity, and RNase H activity. The viral polymerase is the main target of current nucleos(t)ide analog antivirals. The YMDD sequence motif is the main part of the catalytic site of the polymerase (Figure 5.3).

The P ORF contains four distinct domains that encode the viral polymerase polypeptide (Figure 5.3). The N-terminal domain comprises the terminal protein (TP) described as a genome-linked protein and a primase essential for the priming of (–)-strand DNA synthesis. This domain is followed by a spacer sequence that is dispensable for polymerase activity but serves as a flexible connection to the C-terminal domain, which contains the reverse transcriptase and the RNase H domains required for DNA synthesis from RNA or DNA templates and for degradation of pgRNA from RNA–DNA hybrids, respectively. The reverse transcriptase and RNase H domains share homologies with other retroviral reverse transcriptases, whereas the TP domain and the spacer are unique features to P proteins of HBV.

HBV Pol binds to the pgRNA at a short 5′ end *cis*-acting sequence, which forms a stem-loop structure referred to as the encapsidation sequence, or "ε" (Figure 5.1). This complex forms a ribonucleoprotein (RNP) essential and sufficient for pregenome encapsidation and initiation of reverse transcription for (–)-strand DNA synthesis. In addition to the Pol-ε complex, the nucleotide priming activity also requires protein interactions between TP and RT/RNase H domains [1].

The HBx protein

HBx is a small multifunctional protein (17kd, 154 aa) that is well conserved among the mammalian hepadnaviruses but is absent in avian viruses, although the existence of an X-equivalent gene has been described. Its expression is under the control of the X promoter and EN1, which are autoregulated by the X protein itself. In addition, HBx exhibits an N-terminal negative regulatory domain that could repress a C-terminal transactivation domain (Figure 5.3). The X gene product can modulate the activity of a wide range of viral and cellular promoters involved in cell proliferation, cytoplasmic calcium regulation, apoptosis, signaling pathways, or inhibition of DNA repair and may play an important role in HBV-associated hepatocarcinogenesis [11].

HBx is relatively unstable as it undergoes a rapid proteolysis by interacting with the ubiquitin–proteasome pathway. Its degradation may be facilitated through binding to Id1 or indirectly by the core protein and the cellular protein p53. However, its degradation may be prevented by its binding to the DDB1 subunit of UV-damaged DNA-binding factor (UV-DDB) in the nucleus that could increase X protein stability, activate transcription, and induce cell death in culture [12]. The protein has a rapid bimodal half-life and can exert various functions depending on its localization in the cytoplasmic or the nuclear fractions. HBx nuclear localization may be due to its interaction with IkBα or its phosphorylation by MAPK, PKC, or ERK1/2. In particular, the HBx phosphorylated form was shown to interact with Pin1, which can stabilize and enhance its transcriptional and carcinogenic activity [13].

Although HBx does not appear to directly bind DNA, it has a dual role as an activator of transcription by acting on signal transduction pathways in the cytoplasm or is recruited in the nucleus onto the cellular chromatin to interact with cellular transcription factors and cofactors. HBx acting in the cytoplasm has been shown to induce multiple signaling pathways such as Src kinase, Ras–Raf–MAPK, AP1, and NFκB. The effects of HBx in the nucleus are mediated by its direct interaction with nuclear transcriptional regulators such as components of the basal transcriptional machinery (TFIIB, TFIIH, the RPB5 subunit of RNA polymerase II, and the TATA-binding protein [TBP]), and members of the basic leucine zipper family (bZIP) such as the cAMP CREB/ATF2, which is involved in liver proliferation, metabolism, and tumorigenesis. More recently, HBx has also been shown to bind to the CREB-binding protein CBP/p300, which modifies the chromatin dynamics of target genes and synergistically enhances CREB activity [12]. HBx was also shown to be recruited onto the cccDNA minichromosome together with CBP/p300, PCAF/GCN5, and the histone deacetylases HDAC1 and hSirt1, and the kinetics of HBx recruitment on cccDNA would parallel HBV replication [14].

The role of HBx in the virus life cycle still remains only partially understood. It was shown *in vivo* in the woodchuck model that X was essential for viral infection and replication [3], although residual viral replication in the absence of X protein could still be observed [15]. Further studies proposed that HBx enhances HBV transcription from cccDNA and viral replication, explaining its role in the initiation of viral replication [14, 16]. Alternatively, HBx could increase HBV replication by influencing capsid stability and the amount of core proteins through the phosphorylation of core serine proteins [17]. One other possible mechanism comes from the observation that HBx could increase the influx of cytosolic calcium through its interaction with components of the mitochondrial permeability transition pore (MPTP), which subsequently activates the Src family kinases (SrcK) [12].

HBV life cycle

Infection study models

In vitro studies of HBV biology such as the investigation of early events of infection (cell attachment, receptor recognition, internalization, and membrane fusion) have been hampered by the lack of a suitable and easy-to-use cell culture system. In addition, HBV displays a narrow host range limited to humans and closely related species, which renders the study of HBV infection biology difficult. Therefore, cell culture systems using primary cell culture and hepatocyte cell lines have been used to partially characterize the viral life cycle.

Primary human hepatocytes (PHHs) represent the typical *in vitro* infection model to study HBV infection. PHHs exhibit a low infection rate, which is usually overcome by the addition of polyethylene glycol (PEG), which significantly increases the infection process and its reproducibility. However, their limited availability and their heterogeneous quality among donors have rendered difficult the identification of a functional HBV receptor or the understanding of the mechanism underlying virus entry in human hepatocytes [18].

Primary hepatocytes from the *Tupaia belangeri* (PTHs) represent an alternative cell culture system as these cells are similarly permissive to HBV and also support the productive infection of the woolly monkey HBV but not the WHV infection. Although PTHs are less well characterized than PHHs, they may represent a suitable system for studying the early steps of infection.

The use of primary duck hepatocytes (PDHs) infected with DHBV is a convenient and reliable system. DHBV has been extensively used to decipher the viral replication cycle. It has permitted elucidation of key features

of the hepadnaviral life cycle, especially the overall replication strategy (details of reverse transcription process, and mechanisms of cccDNA formation and amplification) as well as the identification of host range determinants. In addition, this model could provide a valuable tool to study *in vitro* and *in vivo* the activity and toxicity of antiviral agents. However, it has several limitations as DHBV infection does not lead to HCC and many aspects of the mammalian biology differ from avian biology.

Woodchuck WHV infection can lead to chronic hepatitis and HCC and has become a valuable model for mechanistic considerations in HCC as well as for the preclinical evaluation of antiviral therapy. However, these animals are difficult to breed and to handle in laboratories; in addition, the hibernation cycle may also influence the experimental outcome.

The use of transgenic mice expressing viral proteins or the hydrodynamic injection of cloned viral genomes has permitted the elucidation of some *in vivo* aspects of HBV infection, but again these experimental models do not necessarily reflect a natural infection. To overcome this limitation, infection of human hepatocytes in the humanized mouse model has allowed the examination of HBV infection in long-term studies, although the animal breeding conditions are still extremely technically demanding.

The use of hepatoma cell lines such as HepG2 or Huh7 cells is another alternative to study HBV replication and secretion. However, these cells are not susceptible to HBV infection, and HBV DNA has to be transfected into these cells or be delivered by recombinant vectors (adenovirus or baculovirus). Furthermore, these transformed cell lines are not as differentiated as primary hepatocytes, and viral uptake (attachment and entry) mainly represents the limiting step [18]. In this context, the newly established, human hepatocyte-like, immortalized but nontransformed cell line, HepaRG, was reported to be susceptible and permissive to HBV infection following complete cellular differentiation [19]. In addition, these cells differ from other hepatoma cells in that they express liver-specific genes and display only "minor" chromosomal alterations, thereby enabling reproducible infection by HBV.

Attachment, membrane fusion, and entry

Attachment and infectivity

As for other viruses, the envelope proteins are the first viral factors that interact with the cell. In particular, the preS domain has been shown to be the host range prime determinant of hepadnaviral infection, as transcomplementation of mutant heron HHBV lacking its envelope proteins by the preS domain of the L protein of duck DHBV allowed HHBV infection in duck hepatocytes [20]. These results showed that the preS1 domain was essential for virus attachment to the cell membrane. In particular, the use of peptides spanning the preS1 sequence or antibodies directed against this peptide confirmed that they could block or compete for the virus attachment or infectivity (Figure 5.3). Functional mapping studies identified the preS1 amino acids 9–18 as being important for the attachment and infectivity of the virus. Notably, the preS1 QLDPAF motif shared by other viral, bacterial, and cellular proteins was shown to be the minimal epitope essential for HBV attachment [18]. The myristoylation of the preS domain was also shown to be essential for infectivity. However, several studies proposed that the hydrophobic property of the N-terminal domain was more critical for the infectious activity rather than the lipid structure of the myristic acid.

A number of potential binding partners for HBV have been investigated, but the mechanism by which HBV binds and enters the host cells is still undefined as none of these receptors has been specifically shown to be critical for HBV entry. The most likely potential receptor identified for DHBV was ascribed to a cell glycoprotein named gp180 or carboxypeptidase D (CPD) that binds with a high affinity to L protein and required the preS1 domain [21]. CPD has a rapid membrane turnover and transits between the trans-Golgi network and the cell surface. Interestingly, following infection, CPD is downregulated, which may explain the hepatocyte refractoriness to superinfection. However, other liver-specific cofactors may be required for an efficient infection since CPD expression was also found in nonpermissive cells. In addition, cells transfected with recombinant CPD allowed the virus entry but were not susceptible to DHBV. Among the proposed cofactors, glycine decarboxylase with liver specificity has been proposed and heparan sulfate proteoglycans have also been described to capture and facilitate virus attachment [18].

Membrane fusion, endocytosis, and nuclear entry

Following receptor attachment, the virus takes the endocytic route of cellular uptake. Initial studies performed in primary hepatocytes infected with DHBV showed that the infection was pH independent, with slow kinetics of internalization at neutral pH. The escape process from the endosomal compartment may be mediated by a conserved peptide translocation motif (TLM) located near the N-terminus preS1 of DHBV [22]; however, further studies with HBV showed that the corresponding TLM was dispensable for HBV infectivity in PHH. Vesicular trafficking to the late endosome and fusion to the host cell membrane may also be required since the

high hydrophobicity of the TM1 domain of L protein was shown to be essential for virus infection at the late endosome [18]. Following subsequent uncoating, the nucleocapsids are released into the cytoplasm and migrate to the nucleus through the microtubule network. The phosphorylation of the core CTD exposed this portion on the outer surface of the capsid to interact with cytosolic tubulin and migrate to the nuclear periphery. The core domain contains a nuclear localization sequence (NLS) that binds to the nuclear transport factors importins α and β (karyopherins), which mediates the migration to the nuclear pore complexes (NPCs) formed by nucleoporin proteins (Nups) [23]. The expression of NLS seems to be regulated and may depend on viral genome maturation that induces structural changes of the nucleocapsid. The capsid–importin α/β complex is retained at the nuclear basket, where it interacts first with Nup153, then importin proteins as well as the core proteins are removed to release the rcDNA from the mature capsid into the karyoplasm, where it is converted into cccDNA [24].

Formation of cccDNA

The nuclear episomal cccDNA is formed from the rcDNA genome either from incoming virions during initial infection or from the pool of progeny nucleocapsids formed in the cytoplasm during replication. A small amount of the generated HBV cccDNA is also generated by "illegitimate replication" from the DSL DNA form [2] (Figure 5.2).

The exact mechanism of the conversion of rcDNA to cccDNA is still unclear but requires repair on the terminal modifications of the genome prior to the generation of cccDNA. The conversion process may probably use cellular repair enzymes for the multiple structural modifications needed for replication such as (1) removal of the TP of DNA polymerase covalently linked to the 5′ (–)-strand DNA as a result of protein-primed (–)-strand DNA synthesis, (2) removal of the terminally redundant region "r" on the (–)-strand DNA, (3) removal of the capped RNA oligomer of ~18 nucleotides in length bound to the 5′ end of the (+)-strand DNA as a result of RNA-primed (+)-strand DNA synthesis, (4) completion of the incomplete (+)-strand DNA synthesis, and (5) covalent ligation of the 5′ and 3′ ends of both (+) and (–) DNA strands to form a plasmid-like cccDNA.

The number of cccDNA molecules in human liver biopsies and HBV-infected chimpanzees is highly variable and could reach up to 10 cccDNA copies per cell. In small-animal models, the cccDNA number can fluctuate temporally and vary from cell to cell (1–50 copies per nucleus). Very little is known about the viral and host factors that may contribute to cccDNA formation. Evidence from the duck model showed that the main source of cccDNA came from the newly synthesized nucleocapsids. The DHBV cccDNA amount was shown to greatly increase when expression of the viral surface proteins was suppressed; Summers and colleagues provided first evidence that the control of the cccDNA amount and the persistence of the infection were regulated by the DHBV large envelope protein, which could suppress cccDNA amplification [25], probably through a negative feedback regulation; when the amount of newly synthesized cccDNA reached a sufficient level in the nucleus, the subsequent increase of L-protein production could redirect the migration of the viral nucleocapsids to the secretory pathway.

The cccDNA intracellular recycling may be controlled in a virus- and cell-specific manner. Transfection of hepatoma cell lines with HBV or DHBV showed that the amount of DHBV cccDNA in the avian LMH cells was higher than the HBV cccDNA level in human hepatoma cells; in addition, DHBV cccDNA greatly increased in cells transfected with DHBV deleted of the envelope proteins. By contrast, the deletion of HBV envelope proteins showed no increase of cccDNA level, but instead led to the accumulation of an intermediate form of rcDNA and DSL DNA without the covalently attached reverse transcriptase (RT) protein (protein-free [PF]), which suggest that PF rcDNA may be a precursor form of cccDNA [26]. In addition, the PF rcDNA was mainly constituted of mature (+)-strand DNA, which suggests that the removal of the RT protein would probably occur in the mature nucleocapsids. Cross-species transfection experiments showed that the high conversion of DHBV rcDNA to cccDNA was still efficient in both human and avian hepatoma cells, suggesting that the lower conversion rate observed for HBV rcDNA was not due to the absence of enzymatic activities in the human hepatoma cell lines. Instead, the formation of HBV cccDNA may be a slower process than DHBV rcDNA conversion and may be limited by the nuclear uncoating of the packaged rcDNA, which is incompletely released from the capsid, or the removal of the covalently linked polymerase [27]. The mechanism underlying the polymerase removal is still unclear, and the formation of cccDNA may require cellular proteins; this could include the nucleolytic cleavage of a portion of the rcDNA linked to the protein or the specific hydrolysis of the phosphodiester bond between the polymerase tyrosine and the 5′ phosphoryl group of (–)-strand DNA. It has been proposed that cellular multifunctional enzymes such as Topoisomerase I, known to regulate DNA topology during transcription, could circularize the (–)-strand DNA once the terminal protein is removed [28]. As well, the existence of topoisomerase "cleavage complexes" such as the highly conserved DNA repair proteins tyrosyl–DNA phosphodiesterases (TDP2) may cleave the tyrosine and 5′ phosphotyrosyl linkage between the

topoisomerase and the DNA [29], and may thus participate in the rcDNA deproteinization process.

Hepadnaviral cccDNA is organized into a compact chromatin-like structure known as a viral mini-chromosome. It contains both histone and nonhistone proteins that undergo acetylation and methylation reactions that define the transcriptional activity state of the chromatin (e.g., HBV replication parallels the acetylation status of cccDNA-bound H3/H4 histones) [30]. The viral core protein is also a structural component of the HBV mini-chromosome and binds preferentially to the HBV dsDNA, which results in a reduction of the nucleosomal spacing of the HBV nucleoprotein [31].

Integration of HBV DNA

Unlike other retroviruses, integration of viral DNA into host chromosomes is not necessary for virus replication. However, spontaneous viral genome integration is observed in both acute and chronic infection. This can aid in the partial maintenance of the viral genome in the cell but may have oncogenic consequences as it is frequently detected in HBV-positive HCC. The viral integration into the cellular genome was initially thought to occur randomly, but several groups have shown that the human telomerase reverse transcriptase (hTERT) gene was a common site of viral integration. HBV DNA integration may affect cellular genes that encode proteins involved in the control of cell signaling, proliferation, and apoptosis [32]. *In vitro* studies performed in LMH cells transfected with replication-competent DHBV genome showed that the DSL form of hepadnavirus DNAs integrates at higher frequency in the host genome, and that the "r" regions in the 5′ and 3′ ends of the viral genome could also be preferential sites for integration [33].

Regulation of HBV DNA transcription

HBV promoters and enhancers

The HBV genome contains four promoters (core, preS1, preS2, and X) and two enhancers as *cis*-acting elements to control HBV transcription of the four 3.5 kb, 2.4 kb, 2.1 kb, and 1 kb mRNAs, which have heterogeneous 5′ ends (with the exception of preS1, which lacks a TATA box and produces transcripts with a defined 5′ end).

The transcription of HBV mRNA can be modulated by both enhancer I and II (EN1 and EN2). EN1 partially overlaps the X promoter and is approximately 200 bp long with binding sites for ubiquitous (NF1, AP1, and NFkB) and liver-specific (C/EBP, HNF4) transcription factors. EN1 is the main stimulating element and upregulates significantly preC and HBx mRNAs but has little effect on surface protein mRNAs. However, the control of transcription differs among mammalian hepadnaviruses as EN1 seems to be absent in WHV. EN1 contains three distinct domains: a 5′ modulator element, a central core domain, and a 3′ domain that overlaps with the HBx ORF. The central core domain contains the enhancer activity and binding sites for HNF3, RFX1, EFC, NF1, and a retinoic acid response element (RARE), which interacts with HNF4, RXRα/PPAR heterodimers, and COUP-TF. Interestingly, STAT3 factor may bind to NF1- and HNF3-binding sites and increase EN1 function. The 5′ modulator element and the 3′ domain have no enhancer activity, although the 5′ modulator element can interact with C/EBP and HNF1 transcription factors and increase the central core domain activity. EN2 is located upstream of the core promoter and increases preferentially the transcription of the preS1, preS2, and X promoters. EN2 contains two regions, IIA and IIB, that act concomitantly as potent enhancer elements that bind various transcription factors such as C/EBP, RXR, PPAR, HNF4, HNF3, FTF, HFL, and Sp1 [34].

Envelope protein expression is under the control of two promoters, preS1 and preS2. The preS1 promoter controls the transcription of the L mRNA (2.4 kb), whereas the preS2 promoter controls the transcription of S and M mRNAs (2.1 kb). The preS1 promoter contains a classical TATA-box sequence that binds the transcriptional factor TFIID or the TATA-binding protein (TBP). The regulation of the preS1 promoter is mediated by liver-specific transcription factors such as HNF1, HNF3, as well as NF1 and Sp1. The preS1 promoter is negatively regulated by sequences within the preS2 promoter region. The pres2 promoter contains several sequence element binding sites to NF1 and Sp1 that could positively or negatively regulate the promoter activity. This promoter has no TATA box but contains a CCAAT motif that can bind transcription factors NF-Y and CCAAT-binding factor (CBF) and stimulate the transcription of the 2.1kb mRNA or repress the production of the 2.4 kb transcript. Thus, the preS2 promoter seems to be stronger than the preS1 promoter and leads to the production of more S and M surface proteins, which is necessary for virus secretion. It has been suggested that ER stress induced by the accumulation of L protein may indirectly stimulate the production of M and S proteins through the binding of transcription factors to the preS2 promoter [35].

The 3.5 kb RNA synthesis is regulated by two partially overlapping but genetically distinct core promoters that overlap the 3′ end of the X ORF and the 5′ end of the pre-C/C ORF and direct the transcription of both preC and pregenomic RNAs. The core promoter is divided into the upper regulatory region (URR) and the basic core promoter (BCP). The URR consists of the core upstream regulatory sequences that could independently stimulate at various levels the BCP activity and

contain the EN2 element and the negative regulatory element that can suppress the core promoter activity. The BCP contains the direct repeat DR1 and the *cis*-acting elements that independently direct the transcription of preC RNAs and pgRNA. The transcription factor HNF4 has two binding sites, one located on the preC promoter TATA–like region that can interact with other transcription factors such as COUP-TF1, PPARα, RXRα, and TR2. Their effects depend on their interactions: for example, binding of HNFA and TR2 may repress preC RNA synthesis, whereas PPARα and RXRα can mediate its activation and COUP-TF1 can inhibit both preC and pgRNA synthesis. The transcription factor Sp1 has three binding sites in the core promoter and can mediate the transcription of both the precore and core mRNAs [35].

The transcription of HBx is under the control of its own promoter X to generate a ~1 kb RNA. The X promoter has no TATA box, but its sequence is located upstream of the transcription initiation site and overlaps with the EN1 3′ end. Both the X promoter and EN1 constitute the transcription regulation unit of the X gene. In particular, EN1 contain the LSR element, which contains *cis* motifs that bind transcription factors NF1, HNF3, HNF4, RXRα, COUP-TF, C/EBP, CREB/ATF, AP1, and NRF1 that activate HBx transcription, whereas the tumor suppressor gene product *p53* was shown to repress the function of the promoter [12].

Epigenetic control of cccDNA transcription

A number of transcriptional co-activators and repressors are recruited onto cccDNA to regulate the epigenetic control of cccDNA transcription and potentiate HBV replication [36]. Recent studies identified transcription factors (CREB, ATF, and STAT1/2), cellular histone acetyltransferases (CBP, p300, and PCAF/GCN5), and histone deacetylases (HDAC1 and hSirt1) that bind the cccDNA mini-chromosome to regulate HBV replication. The viral protein HBx can bind cccDNA and modulate HBV transcription by affecting the cccDNA-bound histone acetylation status [14]. HBc can also function as a transcriptional activator; its binding to the pregenomic promoter or the CpG island 2 on cccDNA was shown to be critical for the maintenance of cccDNA transcription [37]. Interestingly, antiviral cytokines such as IL6 and IFNα can also affect cccDNA transcriptional activity. IL6 can regulate the transcription of liver-enriched transcription factors HNF1α and HNF4α, which are essential to HBV promoter activity [38]. In addition, IFNα can bind to the interferon-stimulated response element (ISRE) identified in the EN1/X gene promoter region of the HBV genome and repress the transcription of genomic and subgenomic RNAs. IFNα binding to the ISRE leads to the inhibition of the recruitment of STAT1 and STAT2 onto cccDNA. In addition, IFNα can mediate hypo-acetylation of cccDNA-bound histones and components of the transcriptional repressor complex PRC2 (YY1 and Ezh2) and the recruitment of histone deacetylases HDAC1 and hSirt1 on cccDNA [39].

Termination of transcription

The termination of transcription is characterized by several RNA modifications such as the addition of methylated bases to the 5′ end (capped RNAs), the addition of a polyA tail at the 3′ end, and the export of RNA from the nucleus to the cytoplasm. A common stop polyadenylation signal TATAAA is located in the core gene and regulates the transcription of all 3′ end mRNAs. Depending on the distance between the promoters and the poly(A) site, the synthesis of HBV RNA transcripts terminates during the first or second passage of RNA polymerase II to the polyadenylation signal. In DHBV, a sequence on cccDNA, named the positive effector of transcription (PET), near the 5′ end pregenome encoding region supports the first passage of transcription complexes through the poly(A) site and suppresses a second signal sequence, the negative effector of transcription (NET), which is required for termination of transcription. Although the presence of spliced transcripts has been found *in vitro* and in chronically infected patients, the majority of avian and mammalian transcripts are unspliced, and the splice regulation and export of RNA to the cytoplasm are thought to be regulated by a posttranscriptional *cis*-regulatory element (PRE). PRE partially overlaps with EN1 and X gene and is not dependent on the produced viral proteins but contains binding sites for cellular RNA-binding proteins that mediate its posttranscriptional effect [1].

Translation

The translation of HBV proteins initiates at the first AUG codon near the 5′ end of the specific mRNA (Figure 5.1). HBe protein synthesis begins from the precore start codon and passes over the downstream core AUG start codon that is “in frame” with the precore AUG and produces an additional 29 amino acid signal sequence of the core protein that directs the polypeptide to the ER lumen. The translation of HBc protein initiates at the core AUG localized within the stem of the encapsidation signal ε from the core or pregenomic mRNA. The polymerase protein is translated from the same mRNA transcript but is not synthesized as a nucleocapsid–polymerase fusion protein by ribosomal frame shifting during translation of the overlapping region. The proposed mechanism involves instead ribosome recruitment on the capped 5′ pgRNA that avoids or bypasses, by a “leaky scanning” mechanism, the core AUG codon

to reinitiate the translation at an internal AUG codon on the pgRNA at the beginning of the pol gene [40]. HBc and polymerase proteins mostly accumulate at a constant ratio close to 200–300 to 1; however, when core proteins reach a critical concentration, the polymerase protein and the pgRNA are both encapsidated into the nucleocapsid possibly through a *cis*-recruitment of core protein to the pgRNA–polymerase complex. The envelope protein translation initiates at three in-frame start codons, AUG1, AUG2, and AUG3, within the same preS/S ORF. The L protein is generated at the start codon AUG1 from the 2.4 kb mRNA, whereas translation of M and S proteins are initiated at start codons AUG2 and AUG3, respectively, from the same 2.1 kb mRNA. The translation process starts preferentially upstream from the first AUG on 2.4 and 2.1 kb templates. In particular, ribosomes bind to the strong start sites AUG1 for LHBs and AUG3 for SHBs, which contain a consensus sequence with optimal flanking bases that favor the initiation of the translation, whereas the start codon AUG2 of MHBs seems to be weaker within the 2.1 kb transcript [40].

Genome replication and regulation

Encapsidation of pgRNA

The encapsidation process of pgRNA with the polymerase protein occurs when the amount of core protein reaches a critical amount and packaging is triggered by the binding of the polymerase to the RNA stem-loop structure ε at the 5′ end of pgRNA [40]. The formation of the ribonucleoprotein complex (RNP) together with the pgRNA packaging probably take place while the translation of core and polymerase is still proceeding, without the need of DNA synthesis per se even if these two events are intimately linked [41]. The binding of P to ε, the encapsidation signal on pgRNA, induces several conformational changes of the polymerase and alters the epsilon structure, essential for the activation of the polymerase. This P–ε binding is stabilized by a multicomponent chaperone complex involving several heat shock proteins (Hsp90, Hsp70, Hsp40, Hsp60, Hop, and p23). Therefore, the polymerase functions as part of a dynamic complex of cellular chaperone proteins and viral RNA. The polymerase protein is encapsidated together with the pgRNA at equimolar amounts together with chaperone proteins within core particles, and probably one molecule is packaged together with pgRNA [40].

Initiation of reverse transcription and minus-strand DNA synthesis

Within the nucleocapsid, the viral polymerase synthesizes the HBV (–)-strand DNA by reverse transcription of the pgRNA, which serves as a template for replication. A highly conserved motif termed T3 (EAGILYKR) within the C terminus of the terminal protein (TP) is needed for the polymerase enzymatic activity; following its exposure by the chaperones, T3 cooperates with a motif called RT-1 near the N terminus of the RT domain to form the initial RNA-binding site [42]. A tyrosine residue in the center of the TP domain covalently links HBV Pol to the 5′ end (–)-strand viral DNA and initiates *de novo* reverse transcription in a reaction called protein priming [3, 43]. The priming reaction occurs in the bulge of 5′ ε pgRNA, where a nucleotide covalently links to the tyrosine residue of the TP domain. The YMDD motif of the RT domain is required for nucleotide priming as well [3]. Three conserved hydrophobic residues of the TP domain may contribute to viral DNA synthesis (W74 and Y147) and viral RNA binding and encapsidation (Y173). An arginine residue located in the TP domain may also be critical for RNA encapsidation [44].

After the coupling of the first nucleotides, the polymerase–DNA primer complex is translocated to the pgRNA 3′ end and anneals with a complementary sequence motif in the DR1 region close to the 3′ end also named signal for reverse transcription. The mechanism of the translocation is poorly understood; however, evidence for a *cis*-acting element termed Phi (φ), which is located near the 3′ DR1 of the pgRNA and complementary to ε, has been shown to facilitate the template switch to the acceptor site. This sequence may bring in close proximity the DR1 sequence with the nucleotide primer and seems to be essential for efficient viral replication [45].

HBV Pol extends the (–)-strand and terminates the DNA synthesis at the 5′ end of pgRNA. During this step, the RNase H domain removes RNA from the pgRNA–DNA and leaves a short 5′ terminal oligoribonucleotide segment of pgRNA annealed to the 3′ end of the (–)-strand DNA. As DR1 is located within the terminal redundancy of the pgRNA, the newly synthesized (–)-strand DNA also contains the short terminal redundancy "r" in its 3′ end. The short pgRNA segment that contains DR1 will subsequently serve as a primer for (+)-strand DNA synthesis by DNA-dependent polymerase activity [40].

Plus-strand DNA synthesis

The synthesis of (+)-strand DNA can start once the (–)-strand DNA has been completed as the remaining capped RNA oligomer would serve as the template for the priming activity. The short RNA template that contains DR1 is translocated to the complementary DR2 sequence located near the 5′ end of the (–)-strand. The synthesis by HBV Pol of the (+)-strand DNA could then extend to the 5′ end of the (–)-strand DNA. Once the

(+)-strand DNA synthesis reaches the 5′ end of the (–)-strand DNA, a third template switch (termed "circularization") is needed where the 3′ end of the (+)-strand is translocated to the 3′ end of the (–)-strand by using the "r" region of the (–)-strand DNA, which becomes the template for the completion of the (+)-strand DNA to produce the 3.2 kb partially double-stranded DNA found in the mature virion. The two template switches required for (+)-strand DNA synthesis (primer translocation and circularization) are partly due to the repeated sequences that serve as donor and acceptor templates, with DR1 and DR2 for primer translocation and 5′r and 3′r for circularization, which are located near the ends of the (–)-strand DNA [40].

It is not clear how the template switches to DR2; the 5′r and 3′r on each ends of the (–)-strand DNA participate but are not sufficient for the circularization process of the viral genome. The template switch may be facilitated by the presence of three motif sequences on the (–)-strand distinct from the donor and acceptor sites that can contribute to primer translocation and circularization of rcDNA [46]. The subsequent base pairing of these sequences leads to the juxtaposition of the ends of the (–)-strand and stabilizes a secondary structure that favors the translocation and annealing of the nascent (+)-strand primer to the acceptor site on (–)-strand DNA. Moreover, the disruption of these duplexes inhibits the formation of rcDNA and produces DSL DNA, suggesting the importance of the base pairing for the circularization of rcDNA. As well, disruption of the homology between DR1 and DR2 leads to *in situ* priming as the RNA primer remains annealed to the 3′ end of the (–)-strand DNA and is extended from the site of the final cleavage. The avian hepadnaviruses contain a small DNA hairpin overlapping the 5′ end of DR1 in the (–)-strand that could inhibit *in situ* priming. In contrast to avian hepadnaviruses, which have a complete (+)-strand DNA, the orthohepadnaviruses have an incomplete (+)-strand DNA and the mechanisms underlying this premature termination of synthesis remain unclear. The integrity of the capsid structure may influence the elongation of (+)-strand DNA since C-termini truncations of core proteins showed defective (+)-strand elongation. The envelopment of core particles may also be a factor limiting the elongation of (+)-strand DNA by depriving the dNTP pool that is indispensable for the completion of DNA synthesis [40].

Amplification and stability of cccDNA

The newly formed nucleocapsids can either migrate to the nucleus and enrich the viral cccDNA pool or bind to the surface proteins to assemble and secrete new HBV virions. The amplification of the cccDNA copies is not related to the level of viral uptake, and it occurs early after the infection when the level of envelope proteins is still low and does not require surface proteins. The transport of the nucleocapsid to the nucleus is unclear; the capsid maturation might influence the nuclear transport by exposing the nuclear localization signal on the C terminus of core proteins, and this process is tightly associated with capsid phosphorylation. The pool of cccDNA in the nucleus can reach 50 copies per cell, especially in the case of avian hepadnaviruses with an undetermined half-life estimated between 3–5 days and more than 30 days in nondividing cells. However, it is not clear whether the cccDNA pool is stable in dividing cells; cccDNA is still present in the nucleus of dividing primary woodchuck hepatocytes following treatment that blocks viral DNA synthesis [47]. Moreover, it seems that cccDNA could persist in dividing cells and that its concentration is probably diluted through daughter cells in the absence of new DNA synthesis [48]. In chronically infected liver, cccDNA has been reported to be highly stable and could persist through cell division despite antiviral therapies that inhibit viral DNA synthesis.

Morphogenesis and secretion of viral particles

When the envelope proteins reach a sufficient amount, the mature core particles interact with the N-terminal domain of the large envelope protein anchored to the ER membrane; this leads to the translocation (or budding) of the core particles across the ER membrane [4] (Figure 5.2). Growing evidences suggest that intraluminal vesicles of maturing endosomes known as multivesicular bodies (MVBs) may be required for the budding of enveloped virions [49]. Proteins mediating this process interact with the endosomal sorting complexes required for transport machinery (ESCRT) that assemble sequentially to the cytosolic side of a late endosome membrane to generate MVBs. HBV core and envelope proteins colocalized with the vacuolar protein-sorting proteins VSP4A/B and Alix/AIP1, and this was shown to be essential for virus secretion [49]. Further evidence suggested that core and envelope proteins interact with a clathrin adaptor protein gamma2-adaptin and the ubiquitin ligase Nedd4 required for the assembly and regulation of the virus transport through the budding site. Other factors like thioredoxin-related transmembrane protein 2 have also been reported to mediate the early endocytic pathway [50].

The assembly of subviral particles seems to be distinct from that of enveloped HBV virions since their budding does not depend on MVBs because gamma2-adaptin interacts only with the L protein but not the S protein, the latter being the major component of the SVPs. However, the exact mechanism of their assembly remains unclear.

The secretion of Dane particles needs an excess of SHBs proteins, whereas MHBs seem to be dispensable. HBV virions migrate with HBs particles from the ER to the cell surface via the Golgi apparatus. The release of HBV virions and HBs requires the trimming of the immature N-linked glycan bound to HBs. The refolding of the preS domain of LHBs to the surface of the envelope may occur during the envelopment and the secretion process, and the low pH found in the trans-Golgi network may be suitable for this refolding [50].

Conclusion

Substantial progress has been made in understanding the regulation of HBV replication. Although HBV genome replication and viral assembly are well understood, the mechanisms of the early steps of infection (i.e., binding, uptake, and uncoating) still need to be elucidated. The use of new human hepatocyte cell lines permissive for HBV infection such as HepaRG cells may help in this understanding. The persistence of cccDNA in HBV-infected cells remains one of the main obstacles to complete eradication of the virus during chronic infection. In that respect, a better knowledge of the biochemical steps of cccDNA biosynthesis is essential. Understanding epigenetic control of cccDNA, such as DNA methylation or histone acetylation, represents a major goal to decipher the regulation of the virus transcriptional machinery. The characterization of the complex interaction between viral and host cellular proteins and/or genomes, including the role of the HBx protein, represents other research challenges that may pave the way to the identification of new treatment targets.

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Chapter 6
Epidemiology and prevention

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Summary

Infection with hepatitis B virus (HBV) continues to be a leading cause of illness and death in many areas of the world. The prevalence of chronic infection among countries varies greatly and remains high in many parts of Asia and Africa. In developed countries, the prevalence is higher among those who emigrated from high- or intermediate-prevalence countries and among those with high-risk behaviors. Since the release of a highly effective vaccine in 1981, universal infant hepatitis B vaccination programs implemented in a growing number of countries have resulted in dramatic reductions in perinatal and chronic infection. Among persons vaccinated during adolescence and adulthood, studies demonstrate persistence of immunity for at least 20 years, and revaccination (or "booster doses") is not recommended. However, among persons vaccinated at birth, some data suggest loss of immunity in a significant proportion of persons after 15 years. Studies are ongoing to examine long-term immunity among persons who received hepatitis B vaccine at birth to determine if, when, and for whom revaccination may be warranted.

Hepatitis B virus (HBV) is a significant global pathogen. Approximately one-third of the world's population has been exposed to this virus, and 240 million persons are estimated to have chronic HBV infection. In the United States, the overall prevalence of chronic HBV infection is nearly 0.3%, but it is approximately 10% among persons born in countries in which hepatitis B is endemic. In this chapter, we review the epidemiology of hepatitis B in the United States and the rest of the world, including the routes of HBV transmission, groups and settings in which the risk of HBV infection is high, and HBV co-infection with other viruses. We also discuss prevention of HBV infection with hepatitis B vaccines and their global impact on this viral infection.

Epidemiology

Prevalence

United States

From 1990 to 2009, the number of reported cases of acute hepatitis B decreased by 84%, from 21 277 in 1990 to 3371 in 2009 for an overall incidence of 1.1 cases per 100 000. Rates of acute hepatitis B decreased for all age groups; the greatest declines occurred among persons aged 20 to 39 years. The highest rates were among persons aged 30 to 39 years, and the lowest were among persons aged <19 years; the rate for males was approximately 1.6 times higher than that for females. Case rates of acute hepatitis B were lowest for Asians, Pacific Islanders, and Hispanics and highest for non-Hispanic blacks [1]. As coverage of newborns with a birth dose of hepatitis B

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vaccine remained incomplete, there were an estimated 1000 infants annually who developed chronic HBV infection via perinatal transmission in the United States during the first decade of the 21st century [2].

Although the incidence of acute HBV infection is declining in the United States, the number of people who are living with chronic HBV infection is increasing as a result of emigration from highly endemic countries. It is estimated that annually 40 000 to 45 000 people enter the United States from HBV-endemic countries [3]. Asian and Pacific Islander Americans, who make up only 4.5% of the general US population, account for more than one-half of persons in the United States who are living with chronic HBV infection. Having been born in an HBV-endemic country is the major predictor for chronic HBV infection in this population [2].

In the United States, surveillance data do not provide accurate estimates of the current burden of disease because current public health surveillance systems for hepatitis B are fragmented, poorly developed, and inconsistent among jurisdictions [2]. Therefore, serosurveillance studies, such as the National Health and Nutrition Examination Survey (NHANES), have been used most often to provide supplemental data and national prevalence estimates.

Two recent publications estimated the prevalence of chronic and resolved HBV infection in the United States using data from NHANES. For the period 1999 through 2006, one study found that overall age-adjusted prevalence of antibody to hepatitis B core antigen (anti-HBc) was 4.7% and of hepatitis B surface antigen (HBsAg) was 0.27%, which was not significantly different from their prevalence during 1988 through 1994. The authors estimated that 730 000 persons had chronic HBV infection in the United States (95% confidence interval, 550 000 to 940 000). Prevalence of anti-HBc, indicative of previous exposure to HBV, was higher among foreign-born participants (12.2%) than it was among participants born in the United States (3.5%) [4]. A second study examined NHANES data during the period 1999 through 2008 and also found an overall HBsAg prevalence of 0.27% (corresponding to approximately 704 000 persons nationwide); 4.6% were previously exposed (approximately 12 million persons) [5]. However, NHANES underreports the number of chronic hepatitis B cases in the national estimates because it excludes and underrepresents populations that have the highest prevalence of HBV infection. These populations include institutionalized and incarcerated persons, and persons of Asian and Pacific Island descent [2].

Global

Diseases caused by HBV infection have a worldwide distribution (Figure 6.1a–b). It is estimated that greater than 2 billion people worldwide have been infected with HBV, 240 million have chronic infection [6], and approximately 600 000 persons die annually from complications of HBV infection [7]. The global prevalence of HBsAg varies greatly; countries may be defined as having a high (≥8%), high-intermediate (5–7%), low-intermediate (2–4%), or low (<2%) chronic HBV infection prevalence [6]. In developed countries, the prevalence is higher among those who emigrated from high- or intermediate-prevalence countries and among those with high-risk behaviors [8]. Global endemicity of HBsAg prevalence in 2005 is shown in Figure 6.1a for children (5–9 years) and in Figure 6.1b for adults (19–49 years).

Transmission

Humans and higher primates are the sole reservoirs for HBV. Transmission occurs via percutaneous or permucosal exposure to infected blood and other body fluids, principally semen and vaginal fluid. In low-prevalence areas, most cases of HBV infection are acquired in early adulthood, when behaviors that increase the risk of HBV infection (such as injection drug use [IDU] or unprotected sexual activity) are most common. In high-prevalence areas, most HBV transmission occurs during the perinatal period from mother to infant (vertical transmission) or in early childhood. Perinatal transmission usually occurs at the time of birth; *in utero* transmission is rare and accounts for fewer than 2% of perinatal infections. The risk of perinatal transmission is highest in the presence of hepatitis B e antigen (HBeAg) in the blood of mothers infected with HBV. The risk of chronic HBV infection is in the approximate range of 70% to 90% from such mothers who are HBeAg-positive, and about 5% to 20% from those who are HBeAg-negative. There is no evidence that HBV can be spread by breastfeeding [9]. Among children in high-prevalence areas, transmission also occurs via close person-to-person contact, ostensibly from exposure of open wounds or mucosal surfaces to secretions from infected persons (horizontal transmission).

Although HBsAg has been found in multiple body fluids, only serum, semen, and saliva typically contain substantial levels of HBV and are infectious, but exposure to saliva has not been shown to transmit HBV. All persons who are positive for HBsAg are infectious, but those who are also HBeAg-positive are more infectious because of higher concentrations of HBV in their blood. As HBV is stable in the environment and can survive on inanimate surfaces for at least one week, transmission may occur even in the absence of visible blood or residue [8].

Person-to-person transmission also occurs in settings where close, nonsexual contact is continuous and frequent, as among household contacts of a person with chronic HBV infection and among developmentally

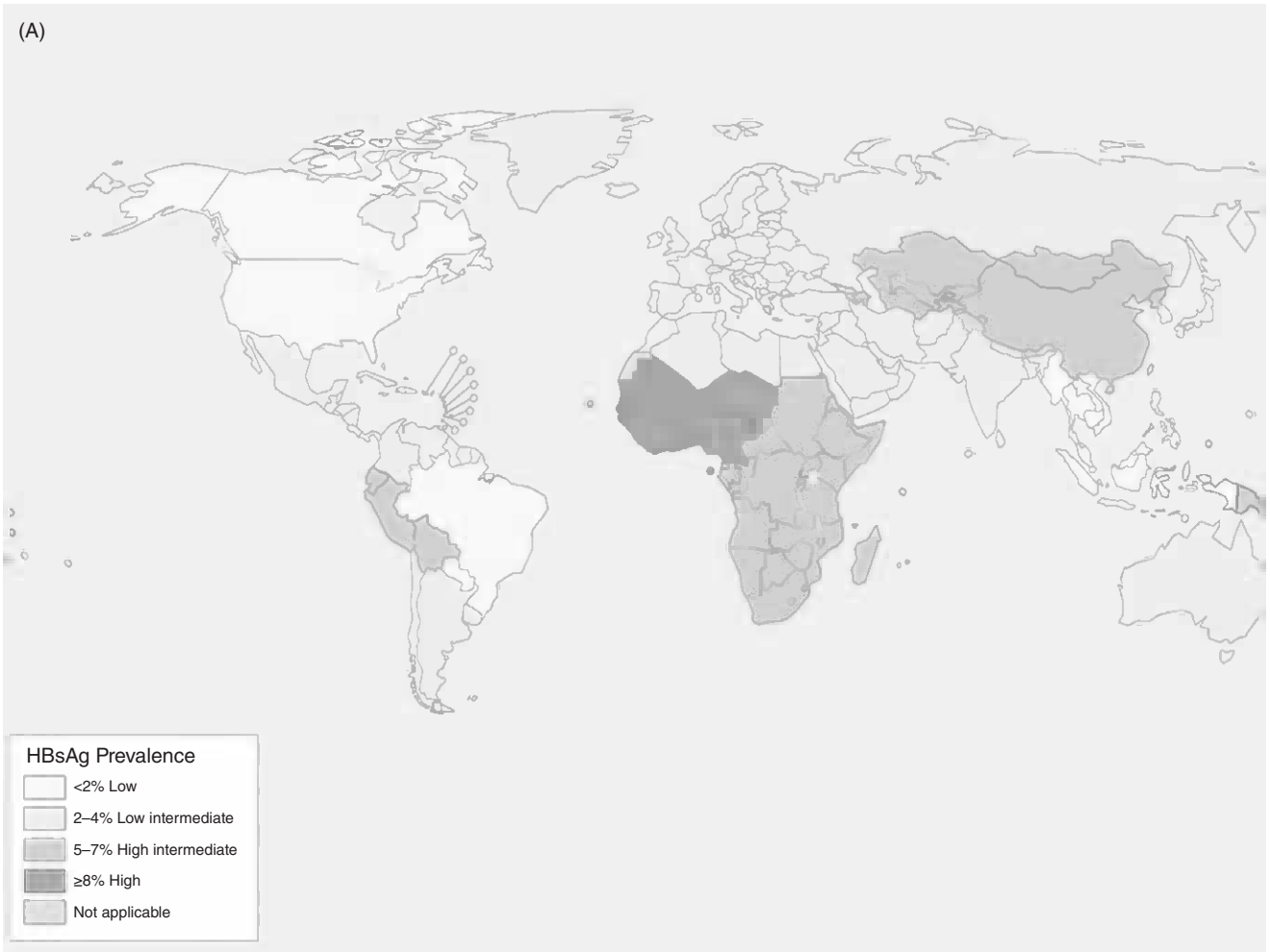

Figure 6.1 (A) Global distribution of chronic hepatitis B virus (HBV) infection, children aged 5–9 years, 2005. "High" endemicity refers to HBsAg ≥8%, "high-intermediate" 5–7%, "low intermediate" 2–4%, and "low" <2%. HBsAg prevalence may vary within countries by subpopulation and locality. (B) Global distribution of chronic HBV infection, adults aged 19–49 years, 2005. "High" endemicity refers to HBsAg ≥8%, "high intermediate" 5–7%, "low intermediate" 2–4%, and "low" <2%. HBsAg prevalence may vary within countries by subpopulation and locality. (Source: Ott JJ, Stevens GA, Groeger J *et al.* Vaccine 2012;30(9):2212–2219 [6])

disabled persons living in congregate, long-term care facilities. Persons living with HBV-infected persons are at risk for infection through indirect exposures to blood or infectious body fluids (e.g., by sharing a toothbrush or razor, through contact with exudates from dermatologic lesions or open wounds, by contact with HBsAg-contaminated surfaces, or via human bites). In countries where most infants and children have undergone hepatitis B vaccination, the risk of acquiring HBV infection in daycare centers or schools is exceeding low [3].

Transmission may occur as the result of nonadherence with infection control practices and aseptic techniques for administering injections, and with measures to prevent cross-contamination of medical equipment and devices. Globally, an estimated 21 million new HBV infections each year are due to unsafe injections in healthcare settings [2]. In recent years, clusters of acute HBV infection have been reported among unvaccinated older adults in long-term care facilities, particularly among persons with diabetes who use glucose-monitoring devices [10]. Outbreaks of HBV infection associated with tattooing and acupuncture were reported in the 1980s; in recent decades, however, reports of such exposures among patients with acute hepatitis B have been uncommon. No infections have been demonstrated in susceptible persons who had oral mucous membrane exposure to HBsAg-positive saliva [3]. Transmission of HBV from infected healthcare personnel (particularly those who perform invasive procedures or surgery) to patients has occurred rarely, and has not been documented as having occurred among HBeAg-negative providers with low HBV DNA levels.

In the United States, HBV transmission from transfusion of blood or plasma-derived clotting factor

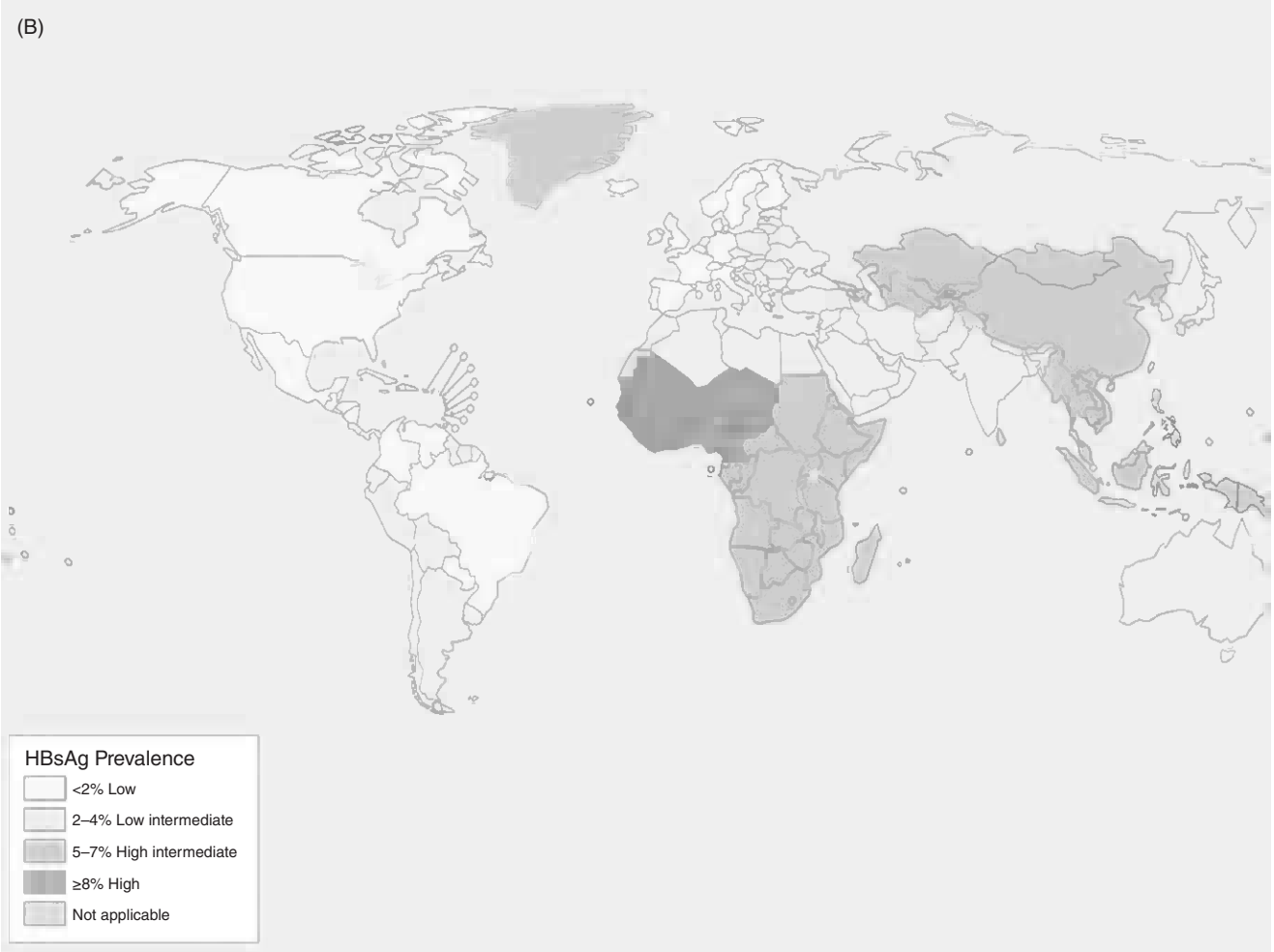

Figure 6.1 (*Continued*)

concentrates, or from organ or tissue transplant from an infectious donor, is rare and has been virtually eliminated through routine testing and selection procedures of blood donors and through viral inactivation procedures and use of recombinant clotting factor concentrates. A recent study of nucleic acid testing on nearly 4 million blood donations reported the detection of potentially infectious HBV DNA during the window period before seroconversion (including breakthrough infections among donors who had received hepatitis B vaccination), although such events occurred in 1 of 410 540 donations [11]. On the whole, however, such transmission has been extremely rare. Similar donor screening has resulted in a low risk of infection after transplantation of nonhepatic organs (kidneys, lungs, and heart) from persons with isolated antibody to anti-HBc. However, after transplantation of liver from HBsAg-negative, anti-HBc–positive donors, the risk of infection may be as high as 75% among HBV seronegative recipients [8].

Infection and its complications

Exposure to HBV is 50 to 100 times more likely to result in infection than is exposure to HIV [12]. Acute HBV infection in adults, though frequently asymptomatic, can cause severe illness and is associated with a 0.5% to 1% risk of death from liver failure [13]. The age at which a person becomes infected with HBV is the principal determinant of whether chronic infection occurs. Among newly infected, unimmunized persons, chronic infection develops in more than 90% of infants, in 25% to 50% of children aged 1 to 5 years, and in 6% to 10% of older children and adults [2]. Among children aged <5 years who become infected, fewer than 10% are symptomatic, whereas 30% to 50% of adults have symptomatic acute disease [14].

The major complications of chronic HBV infection are cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC). Between 15% and 40% of persons with chronic infection will develop liver sequelae, with

the highest risk in men [15]. Persons with chronic HBV infection have a 15% to 25% risk of premature death from hepatic decompensation and HCC [12]. In the United States, an estimated 3000 people die each year from HCC or chronic liver disease caused by HBV infection. The incidence of HCC tripled in the United States from 1975 through 2005, with the highest incidence among Asian and Pacific Islanders who have immigrated to the United States [2].

The risk factors associated with the development of cirrhosis include older age (or longer duration of infection), HBV genotype C, high levels of HBV DNA, chronic alcohol consumption, and concurrent infection with hepatitis C virus (HCV), hepatitis D virus (HDV), or HIV [16].

The estimated 5-year risk of progression to decompensation among persons with cirrhosis is approximately 20% [17]. Risk factors for decompensation include persistent viremia, age, and markers of impaired synthetic function (i.e., low albumin, low platelets, and high bilirubin). Once decompensation occurs, the estimated 5-year survival rate is 15% to 35%, compared with 80% to 85% among patients with compensated disease.

Among persons with chronic HBV infection, risk factors for the development of HCC include male gender, family history of HCC, older age, history of reversions from anti-HBe to HBeAg, presence of cirrhosis, HBV genotype C, viral load >20 000 IU/ml in persons over 30 years, core promoter mutation, exposure to aflatoxin, smoking, and co-infection with HCV. Approximately 30% to 50% of HCC cases associated with HBV infection occur in the absence of cirrhosis.

Although uncommon, several extrahepatic manifestations associated with HBV infection are likely mediated by circulating immune complexes. These include a serum sickness–like prodrome of acute HBV infection, polyarteritis nodosa, glomerulonephritis, mixed essential cryoglobulinemia, and neurologic manifestations [16].

Occult HBV infection is characterized by the presence of detectable HBV DNA in the absence of measurable HBsAg. Occult HBV infection is more common in areas of high HBV infection prevalence, in the setting of co-infection with HCV or HIV, in persons with a history of IDU, in hemophiliacs, and in patients on hemodialysis [18].

Populations at risk for infection

(See also Table 6.1 for groups at risk of HBV infection.)

Persons with HIV infection

As a result of shared modes of transmission, a high proportion of adults at risk for HIV infection are also at risk both for acute HBV and for developing chronic HBV infection thereafter. Those who develop chronic HBV co-infection have an increased risk for liver-related morbidity and mortality [20]. Chronic HBV infection has been identified in 6% to 14% of HIV-positive persons from Western Europe and the United States, but may be substantially higher in regions (e.g., Sub-Saharan Africa) of high endemicity for both viruses [3].

Table 6.1 Groups at high risk for HBV infection who should be screened and considered for hepatitis B vaccination.

Persons born in areas of high (HBsAg prevalence ≥2%) and intermediate (HBsAg prevalence 2–7%) HBV endemicity
US-born persons not vaccinated as infants whose parents were born in areas with high HBV endemicity (≥8%)*
Persons who have ever injected drugs*
Men who have sex with men*
Persons with chronically elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST)*
Patients undergoing renal dialysis*
Household, needle-sharing, or sexual contacts of HBsAg-positive persons*
Persons with multiple sexual partners or history of sexually transmitted disease*
Inmates of correctional facilities*
Individuals infected with HCV or HIV*
Persons needing immunosuppressive therapy, including chemotherapy, immunosuppression related to organ transplantation, and immunosuppression for rheumatologic or gastroenterologic disorders
Donors of blood, plasma, organs, tissue, or semen
All pregnant women
Persons who are the sources of blood or body fluids for exposures that might require postexposure prophylaxis (e.g., needlestick or sexual assault)

*Those who are seronegative should receive hepatitis B vaccine. (Source: CDC. MMWR 2007;57(RR-9):1-20 [13])

Persons with a history of IDU

In 2009, based on CDC surveillance, 16% of cases with acute HBV infection in the United States had a history of IDU [1]. The risk for transmission increases with the duration of drug use, with frequency of injection, and with sharing of related equipment (e.g., cotton and cookers), independent of syringe sharing. Among persons with a history of IDU, evidence of previous HBV infection has ranged from 50% to 81%, and increases with age. In a study of persons in a detention center with a history of drug injection, the prevalence of chronic HBV infection was 3.1% [3].

Men who have sex with men (MSM)

According to national surveillance data from the United States, transmission of HBV among MSM accounts for approximately 18% of new HBV infections among adults [1]. Serologic evidence of previous HBV infection has ranged from 10% to 25% among MSM aged <30 years. Risk factors associated with sexual transmission included having multiple sex partners, history of another sexually transmitted disease, and anal intercourse [3].

Persons with multiple sexual partners

The most common source of HBV infection among adults in the United States is sexual contact. Recent acute surveillance data indicated that heterosexual transmission accounted for approximately 32% of new HBV infections among adults [1]. Serologic evidence of HBV infection has ranged from 10% to 40% among adults seeking treatment in STD clinics. Follow-up studies identified HBV infection in 20% to 42% of susceptible heterosexual partners of persons with acute hepatitis B. Among susceptible heterosexual spouses of persons with chronic HBV infection, the seroprevalence of HBV infection has ranged from 25% to 59% [3]. Risk factors associated with sexual transmission among heterosexuals included having unprotected sex with an infected partner, having unprotected sex with more than one partner, and history of another sexually transmitted disease [3].

Household contacts of persons with chronic HBV infection

The seroprevalence of HBV infection among susceptible household contacts of persons with chronic infection has varied, ranging from 14% to 60%. The risk for infection has been highest among the sexual partners of, and children living with, a person with chronic HBV infection in a household or extended-family setting [3].

Travelers to HBV-endemic regions

Travelers to regions in which HBV infection is of high or intermediate endemicity typically are at risk for infection only through exposure to blood in medical, healthcare, or disaster assistance activities; receipt of medical care that involves parenteral exposures; or sexual activity or drug use [3].

Persons with chronic liver disease

Persons with chronic liver disease are not at increased risk for HBV infection unless they have percutaneous or mucosal exposure to infectious blood or body fluids. Furthermore, there is little evidence that acute hepatitis B increases the risk for acute liver failure among patients with chronic liver disease. However, if chronic HBV infection develops in a person previously infected with HCV, the risk for HCC is higher [3].

Hepatitis B and specific settings

Healthcare settings

Before hepatitis B vaccination was widely implemented, HBV infection was a common occupational hazard among persons exposed to blood while caring for patients or working in laboratories. Since 1981, routine hepatitis B vaccination of healthcare workers and use of standard precautions to prevent exposure to bloodborne pathogens have greatly reduced the risk of HBV infection in these populations. As a result, since the mid-1990s, the incidence of HBV infection among healthcare personnel has been lower than that among the general population. Despite a theoretical risk, the prevalence of HBV infection in occupational groups such as police officers, firefighters, and corrections officers generally does not differ from that in the general population; infection usually is associated with nonoccupational risk factors. No increased risk for occupationally acquired HBV infection has been documented in personnel exposed infrequently to blood or body fluids [3]. Reports of transmission from healthcare personnel to patients – usually providers performing invasive procedures such as dentists and surgeons – have been documented but are rare. Both the CDC and European organizations have made recommendations regarding clinical practice restrictions for healthcare workers with chronic HBV infection [19].

Hemodialysis units

Since the initiation of infection control practices and hepatitis B vaccination, the rate of HBV infection among patients undergoing hemodialysis has declined approximately 95%. However, occasional outbreaks of HBV infection among unvaccinated patients attest to the continued risk for infection in this population [3].

Long-term care facilities

The CDC has investigated multiple outbreaks of viral hepatitis among residents of long-term-care communities (e.g., senior centers, assisted living facilities, and skilled nursing facilities) that were attributed to shared devices and other breaks in infection control practices related to blood glucose monitoring. A recent review identified 33 outbreaks of HBV or HCV infection in nonhospital healthcare settings in the United States during the past 10 years. Fifteen skilled nursing or assisted living facilities in nine states accounted for out-

breaks in which 97 residents were identified as having acquired incident HBV infection; six of these residents were known to have died of their HBV infection. Approximately 1700 residents were at potential risk for exposure to HBV, and more than 900 residents (most of whom underwent regular blood glucose monitoring) were screened for HBV and other bloodborne viruses [21]. Developmentally disabled persons in residential and nonresidential facilities have had high rates of HBV infection, but the prevalence of infection has declined substantially since the implementation of routine hepatitis B vaccination in these settings [3].

Correctional settings

The Advisory Committee on Immunization Practices (ACIP) recommends hepatitis B vaccination for adults in correctional settings because of their increased risk for infection. Although the majority of HBV infections among incarcerated persons are acquired in the community, infection also may be transmitted within correctional settings through sexual activity, IDU, and other percutaneous exposures. Moreover, upon release, unvaccinated inmates are often at increased risk for infection if they resume high-risk behaviors [3].

The prevalence of serologic markers for current or past HBV infection among prison inmates has ranged from 13% to 47%, and was higher among women than among men. The prevalence of chronic HBV infection among prison inmates has been reported as 1.0% to 3.7%, two to six times the national prevalence estimate. Among correctional workers, the prevalence of HBV infection was not significantly different from that of the general population after adjusting for age and race (approximately 13%) [22].

Co-infections

Hepatitis A virus (HAV)

As the result of overlapping global endemicity, persons with chronic HBV infection frequently have serologic evidence of previous HAV infection. However, one study comparing the impact of acute HAV infection among persons with chronic HBV infection versus non-HBV-infected persons found a greater risk of fulminant hepatitis A among the chronic carriers [23]. Currently, hepatitis A vaccine is recommended only for HBV-infected persons with evidence of chronic liver disease.

HCV

Co-infection with HCV has been found in 5% to 15% of persons with chronic hepatitis B and is more common among injection drug users. Acute co-infection with HBV and HCV may shorten the duration of HBsAg in the blood and lower the peak serum aminotransferase concentrations compared with acute HBV infection alone. However, acute co-infection of HCV and HBV, and acute HCV infection with preexisting chronic HBV infection, have been reported to increase the risk of fulminant hepatitis. Persons with HBV and HCV co-infection develop cirrhosis and HCC at a higher rate than do persons infected with either virus alone [8].

HDV

HDV is a satellite virus that is dependent on HBV for the production of envelope proteins. Co-infection with HBV and HDV occurs in many but not all parts of the world where HBV is endemic, such as in the Mediterranean and the Amazon region of South America. The availability of hepatitis B vaccines and public health education on the prevention of transmission has led to a substantial decline in the prevalence of HDV infection in the past decade. HDV infection may occur in two forms: (1) co-infection of HBV and HDV, which typically results in severe acute hepatitis with a high mortality rate (though rarely results in chronic infection); and (2) superinfection of HDV in a HBV carrier, which manifests as a severe acute hepatitis syndrome in previously asymptomatic HBV carriers or as an exacerbation of previously diagnosed chronic hepatitis B (almost always resulting in chronic co-infection). A higher proportion of persons with chronic HBV–HDV co-infection develop cirrhosis, hepatic decompensation, and HCC compared to persons with chronic HBV infection alone [8].

HIV

Studies have estimated that 6% to 13% of persons infected with HIV are also co-infected with HBV. Co-infection with HIV is more common in persons from regions where both viruses are endemic, such as Sub-Saharan Africa. Persons with HBV and HIV co-infection typically have higher levels of HBV DNA, lower rates of HBeAg seroconversion, more severe liver disease, and increased rates of liver-related mortality. Severe exacerbations of hepatitis, the result of immune reconstitution, can occur after initiation of highly active antiretroviral therapy (HAART) in patients co-infected with HIV. Elevated liver enzymes in patients with HBV–HIV co-infection can be caused by factors other than HBV, including hepatitis C, HAART-related toxicity, and infections with opportunistic pathogens such as cytomegalovirus and *Mycobacterium avium*. As mentioned in this chapter, patients with HIV infection who test positive for anti-HBc can have HBV DNA present and even liver injury in the absence of measurable HBsAg (occult HBV infection). Therefore, persons infected with HIV should be tested for HBsAg and anti-HBc, and then, if either is positive, tested for HBV DNA. Persons who are negative for all HBV serologic markers should receive

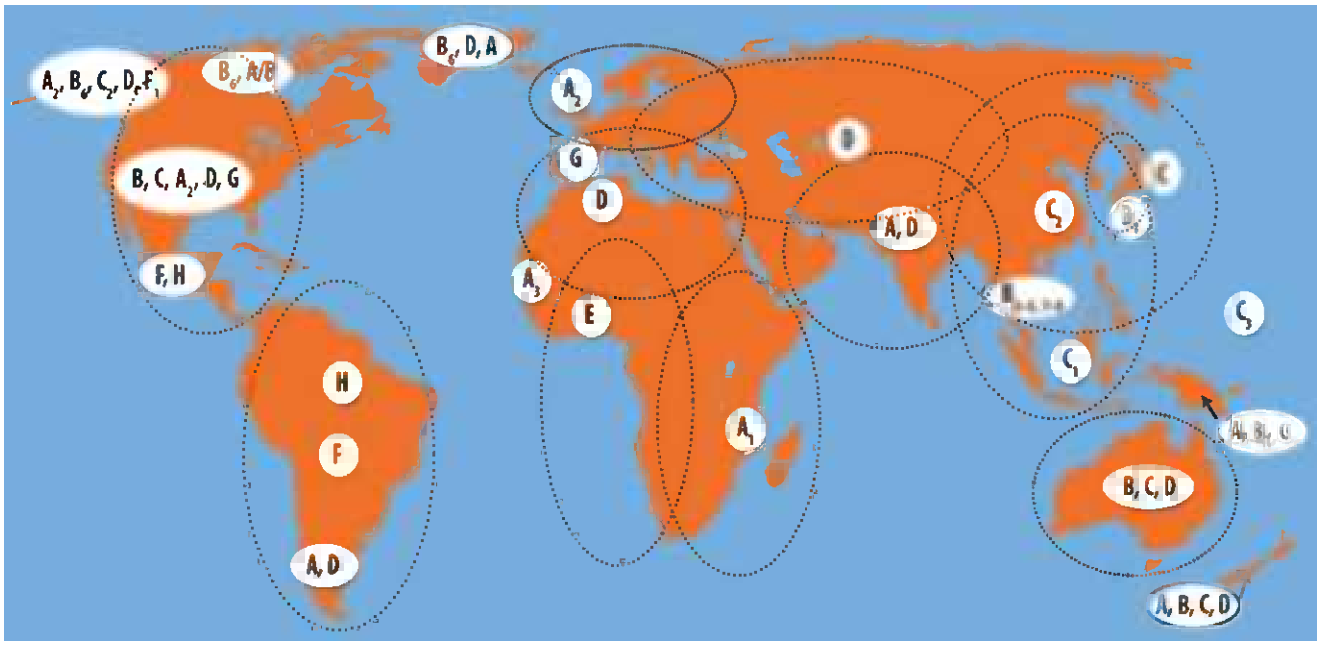

Figure 6.2 Geographic distribution of hepatitis B virus genotypes and subgenotypes. Location depicted of subgenotype C3 refers to Western Pacific islands.

hepatitis B vaccine. If feasible, hepatitis B vaccine should be given when CD4 cell counts are ≥200/ul [8].

HBV genotypes

Worldwide, at least nine genotypes of HBV (A through I) have been identified [24, 25]. These genotypes differ from each other in their complete genome sequences by more than 8%. Within a given HBV genotype, multiple subgenotypes have been identified that differ from each other after sequencing by 4% to 8%. Figure 6.2 displays a map of the distribution of these genotypes and some, but not all, of the most important subgenotypes. HBV genotype A is divided into three major subtypes. A1 is found in the central and eastern and A3 in the central and western regions of Sub-Saharan Africa. A2 is found primarily among Caucasians in Northern Europe.

HBV genotype B has two major divisions: B Japan (Bj) and B Asia (Ba). Bj is a "pure" form of genotype B, and two distinct subtypes have been identified; B1, which is found in Japan, and B6, which has been found in indigenous populations in Alaska, Canada, and Greenland. Ba has a sequence of the core region of HBV genotype C incorporated into its genome. Several Ba subtypes, B2–B5 and B7, also have been identified. HBV genotype C has at least four subtypes and is found in East Asia, the South Pacific Islands, and Western Siberia.

HBV genotype D is widely distributed throughout the world: in the Mediterranean region, North Africa, Eastern Europe and the Russian Federation, the Indian subcontinent, the Middle East, and the Arctic. Genotype E is found in western and central Sub-Saharan Africa. Genotype G is found in several parts of the world but is most commonly found in individuals together with another HBV genotype, suggesting that it survives better in an environment where it coexists with a different HBV genotype. Genotypes F and H are found in indigenous populations in Central and South America, and four subtypes, F1 through F4, have been identified. HBV genotype F is also found in Spain, possibly the result of this genotype infecting Spanish explorers to the Americas. Finally, many isolates of recombinations between one or more genotypes besides the recombination of C and B cited here have been found around the globe, but the significance of these in relation to disease outcome is unknown [25].

In the United States, HBV genotypes A2, B, C, and D are most commonly found [26] and reflect the geographic origins of immigrants, as genotypes B and C are found in persons of Asian and South Pacific Island descent and genotypes A2 and D in persons of European descent.

HBV genotypes are associated with the modes of HBV transmission (vertical vs. horizontal) and with the risk of certain outcomes of chronic infection, such as cirrhosis and HCC. Cross-sectional studies from Asia have shown that seroconversion from HBeAg to anti-HBe typically occurs approximately one decade sooner among persons infected with HBV genotype Ba than among those infected with genotype C, and

even earlier among those infected with genotype Bj [24]. In a prospective study examining HBeAg seroconversion from Alaska, where five of the eight HBV genotypes were in Eskimo populations, the median age of HBeAg to anti-HBe seroconversion was 16 to 19 years among persons infected with HBV genotypes A2, B6, D, or F, compared to age 47 years among those infected with HBV genotype C [27]. Accordingly, women infected with HBV genotype C would be HBeAg-positive throughout most of their childbearing years. In areas where HBV genotype C is common, such as East Asia, parts of the South Pacific Islands, and northwestern Alaska, perinatal transmission accounted for more than one-half of chronic HBV infections in unvaccinated infants of HBsAg-positive mothers. In contrast, in areas where genotype C is rare, such as Africa, the Middle East, Europe, and southwest Alaska, most women undergo seroconversion from HBeAg to anti-HBe before or early in their childbearing years and, as a result, only a minority of infants would acquire HBV via perinatal transmission. In these areas, most persons with chronic HBV infection would have been infected via horizontal transmission during the first 5 years of life.

Clear associations in both cross-sectional and prospective population-based cohort studies have shown that HBV genotypes influence outcomes of HBV-related liver disease. The age of onset of HCC appears to differ among the HBV genotypes (Table 6.2). In Sub-Saharan Africa, where HBV genotypes A1, A3, and E predominate, HCC occurs in young male adults. It is likely that exposure to food contaminated with aflatoxin is a cofactor for HCC in Africa [28]. In Alaska, HBV genotype F is associated with HCC in young children as well as adults younger than 30 years. In Asia as well as Alaska, HBV genotype C has been associated with a significantly higher risk of HCC than genotypes A2, Bj, Ba, and D. Furthermore, the mean age of onset of HCC among persons infected with HBV genotype C is significantly younger than among those infected with genotypes A2, B, or D (i.e., among those in their 40s and early 50s). In contrast, the onset of HCC is in the mid- to late 50s among persons infected with genotype Ba, in the early to mid-60s with genotypes A2 and D, and in the late 60s to 70s with HBV genotype B1 [24]. Thus far, no cases of HCC in the Arctic have been reported among persons infected with HBV B6. This may be due to the fact that genotype B6, like genotype B1, is a "pure" genotype B (unlike Ba) and that the population of HBV carriers in the Arctic is much younger. As more information becomes available about the incidence of HCC among the different HBV genotypes, it may be possible to design different HCC surveillance and detection strategies based on HBV genotypes.

Most of the data pertaining to HBV genotypes and the risk of cirrhosis come from Asia and consistently show that persons infected with HBV genotype C are at much higher risk of cirrhosis than those infected with genotype B [29]. The data regarding other HBV genotypes and cirrhosis are minimal.

Table 6.2 Geographic distribution and manifestation of disease associated with hepatitis B virus genotypes.

Genotype	Geographic region	Disease association
A1	Sub-Saharan Africa	HCC in young males often without cirrhosis
A2	Northern Europe	HCC and cirrhosis in older persons
A3	West Africa	High rates of HCC
B1	Japan	HCC and cirrhosis in persons >60 years old
B2–5,7	East Asia	HCC and cirrhosis occurs at younger age than B1
B6	Alaska, Arctic Canada, and Greenland	No cases of HCC yet identified
C1–4	China, Korea, Southeast Asia, Japan, and South Pacific Islands	Higher risk of HCC and cirrhosis compared with B genotypes starting at 40 years; HBeAg seroconversion occurs 1–3 decades later than in genotypes A, B, D, and F1.
D1–4	Russia, Middle East, Mediterranean, North Africa, Eastern Europe, and the Indian subcontinent	Anti-HBe chronic hepatitis B, HCC, and cirrhosis in older individuals
E	West Africa	High rates of HCC
F1	Alaska, Central America, and South America	HCC in children and young adults in Alaska
F2	Central America and the Amazon region	Unknown
G	Europe, United States, and Australia	Almost exclusively found in persons co-infected with other HBV genotypes, mainly A1; clinical significance unknown
H	Central America	Unknown

Prevention

Patients with chronic HBV infection should be counseled regarding lifestyle modifications, prevention of transmission to others, and the importance of lifelong monitoring. No specific dietary measures have been shown to affect progression of chronic hepatitis B; however, heavy use of alcohol (>20 g/d in women and 30 g/d in men) may be a risk factor for the development of cirrhosis. Susceptible household members and steady sexual partners are at increased risk of HBV infection and therefore should be vaccinated. For unvaccinated sexual partners, barrier protection methods should be employed. HBsAg-positive women who are pregnant should inform their providers so hepatitis B immune globulin (HBIG) and hepatitis B vaccine can be administered to their newborn immediately after delivery. HBIG and concurrent hepatitis B vaccine have been shown to be 95% efficacious in the prevention of perinatal transmission of HBV; the efficacy is lower (i.e., 85%) for maternal carriers with very high serum HBV DNA levels. In areas where HBIG is unavailable or in circumstances of severe maternal viremia, some studies suggest that antiviral treatment (with lamivudine and, more recently, telbivudine) during late pregnancy can safely reduce perinatal HBV transmission; however, no antiviral agent has been approved by the US Food and Drug Administration (FDA) for use in pregnancy (lamivudine is a "category C" pregnancy risk, whereas telbivudine and tenofovir are category B) [30]. Transmission of HBV from infected healthcare workers to patients also has been shown to occur in rare instances. For HBV carriers who are healthcare workers, several European countries use a threshold level varying from 200 to 20 000 IU/ml to determine if HBsAg-positive healthcare workers are allowed to perform exposure-prone procedures [8].

Primary prevention through immunization

United States

Soon after hepatitis B vaccine was licensed in 1981, ACIP recommended vaccination for adults at increased risk for HBV infection. However, the recommendations were not widely implemented, and coverage among adults at risk for HBV infection remained low. By the early 1990s, the difficulty in vaccinating adults at risk for HBV infection and the burden of disease resulting from HBV infections acquired during childhood necessitated a modification in hepatitis B vaccination strategies. In 1991, recommendations for universal hepatitis B vaccination of infants and vaccination of adults at high risk for HBV infection were promulgated by ACIP and professional medical organizations to eliminate HBV transmission in the United States [3].

Table 6.3 Adults recommended for hepatitis B vaccination.

Persons at risk for infection by sexual exposure
• Sex partners of hepatitis B surface antigen (HBsAg)–positive persons
• Sexually active persons who are not in a long-term, mutually monogamous relationship (e.g., persons with more than one sex partner during the previous 6 months)
• Persons seeking evaluation or treatment for a sexually transmitted disease
• Men who have sex with men
Persons at risk for infection by percutaneous or mucosal exposure to blood
• Current or recent injection drug users
• Household contacts of HBsAg-positive persons
• Residents and staff of facilities for developmentally disabled persons
• Healthcare and public safety workers with reasonably anticipated risk for exposure to blood or blood-contaminated body fluids
• Persons with end-stage renal disease, including predialysis, hemodialysis, peritoneal dialysis, and home dialysis patients
Others
• Persons with diabetes mellitus
• International travelers to regions with high or intermediate levels (HBsAg prevalence of >2%) of endemic HBV infection
• Persons with chronic liver disease
• Persons with HIV infection
• All other persons seeking protection from HBV infection

(Source: CDC. MMWR 2006; 55(RR16);1–25 [3])

The current national strategy for preventing new HBV infection in infants and children – including routine screening of pregnant women for HBsAg, universal infant hepatitis B immunization, and catch-up vaccination of unvaccinated children and adolescents – has resulted in a dramatic reduction in chronic HBV infection in infants and acute HBV infection in children of all ethnicities [3, 31]. In contrast, the strategy for preventing HBV transmission in adults – selective vaccination of high-risk adults (including MSM, persons who inject drugs, and correctional-facility inmates, as shown in Table 6.3) – has had limited success in reducing the incidence of acute HBV infection in US adults [2, 3].

Passive immunization

Hepatitis B immune globulin (HBIG) provides passively acquired anti-HBs and temporary protection (i.e., 3 to 6 months) when administered in standard doses. HBIG typically is used with hepatitis B vaccine for postexposure prophylaxis (PEP) to prevent HBV infection after acute exposure to blood containing HBsAg, perinatal exposure of infants born to HBsAg-positive mothers, sexual exposure to HBsAg-positive persons, and household exposure to persons with acute HBV infection. HBIG is the sole means of protection after an HBV exposure for susceptible persons who do not respond to

hepatitis B vaccination. It is also used after liver transplantation for end-stage hepatitis B to prevent disease recurrence in the transplanted liver. HBIG is prepared from the plasma of donors with high concentrations of anti-HBs. The plasma is screened to eliminate donors who are positive for HBsAg, antibodies to HIV and HCV, and HCV RNA, and manufacturing techniques for HBIG inactivate viruses (e.g., HBV, HCV, and HIV) from the final product. HBIG is administered by intramuscular (i.e., deltoid or gluteal) injection and may be administered simultaneously with hepatitis B vaccine at a different injection site [3].

Active immunization using hepatitis B vaccine

The first hepatitis B vaccine, a plasma-derived vaccine, was licensed by the FDA in 1981. A recombinant vaccine, derived from *Saccharomyces cerevisiae* (baker's yeast), was licensed by the FDA in 1986 and is the type of hepatitis B vaccine currently in global use. The recombinant hepatitis B vaccine is available as both a single- and multiantigen formulation in fixed combination with other vaccines [29]. The two single-antigen vaccines are Engerix-B® (GlaxoSmithKline Biologicals, Rixensart, Belgium) and Recombivax HB® (Merck & Co., Inc., West Point, PA, USA). There are three licensed combination vaccines: Twinrix® (GlaxoSmithKline Biologicals) is used for vaccination of adults; Comvax® (Merck & Co., Inc.) and Pediarix® (GlaxoSmithKline Biologicals) are used for vaccination of infants and young children. Twinrix contains recombinant HBsAg and inactivated HAV. Comvax contains recombinant HBsAg and *Haemophilus influenzae* type b (Hib) polyribosylribitol phosphate conjugated to *Neisseria meningitidis* outer-membrane protein complex. Pediarix contains recombinant HBsAg, diphtheria and tetanus toxoids and acellular pertussis adsorbed (DTaP), and inactivated poliovirus [3, 31].

Immunogenicity and efficacy

Primary vaccination typically consists of three intramuscular doses of hepatitis B vaccine. The three-dose vaccine series administered intramuscularly at 0, 1, and 6 months produces a protective antibody response in approximately 30% to 55% of healthy adults aged <40 years after the first dose, 75% after the second dose, and >90% after the third dose. After age 40 years, the proportion of persons who have a protective antibody response after a three-dose vaccination regimen declines to less than 90%, and by age 60 years, protective levels of antibody develop in only 75% of vaccinated persons. In addition to age, other host factors (e.g., smoking, obesity, genetic factors, and immune suppression) contribute to decreased vaccine response. Alternative vaccination schedules (e.g., 0, 1, and 4 months or 0, 2, and 4 months) have been demonstrated to elicit responses similar to those obtained on a 0-, 1-, 6-month schedule. The hepatitis B vaccine has a pre-exposure efficacy of 80% to 100% and a postexposure efficacy of 70% to 95%, depending on whether HBIG is given with the vaccine [3].

Dose and administration

Recommended vaccine doses vary by product, age of recipient, and needs of special populations. Although the antigen contents of vaccines differ, vaccines made by different manufacturers are interchangeable for all adult schedules. Hepatitis B vaccine should be administered by injection into the deltoid muscle. Concurrent administration of hepatitis B vaccine and other vaccines should be given in different injection sites. No effect on immunogenicity has been observed when minimum spacing of doses is not achieved precisely. The third dose confers the maximum level of protection (i.e., higher levels of anti-HBs) and functions primarily as a booster to provide optimal long-term protection [3].

Hepatitis B vaccine and hemodialysis patients

Compared with healthy adults, hemodialysis patients are less likely to have protective levels of antibody after vaccination with standard vaccine dosages. Better responses have been identified in patients with mild or moderate renal failure who were vaccinated before becoming dialysis dependent. After receipt of a four-dose series, seroprotection (% anti-HBs >10 IU/L after vaccination) among adult predialysis patients with serum creatinine levels under 4.0 mg/dL was 86%, compared with 37% among patients with serum creatinine levels over 4.0 mg/dL, most of whom were dialysis patients [3].

Other immunocompromised persons

Response to hepatitis B vaccination also is reduced in other immunocompromised persons (e.g., persons infected with HIV, hematopoietic stem cell transplant recipients, and patients undergoing chemotherapy). Modified dosing regimens, including doubling the standard antigen dose or administering additional doses, might increase the likelihood of response. However, limited data regarding response to these alternative vaccination schedules are available. For such patients, postvaccination testing to assess response is recommended [3]. Hepatitis B vaccines with new potent adjuvants may be available soon that may improve response in immunosuppressed persons. Adults with diabetes mellitus (type 1 or type 2) are at increased risk of HBV infection, probably because of breaches in infection control during assisted blood glucose monitoring

(e.g., reuse of single patient finger stick devices). In October 2011, ACIP recommended that all previously unvaccinated adults 19 through 59 years of age with diabetes mellitus (type 1 and type 2) be vaccinated against hepatitis B as soon as possible after a diagnosis of diabetes is made. ACIP also recommended that unvaccinated adults aged 60 years of age or older with diabetes may be vaccinated at the discretion of the treating clinician after assessing their risk and the likelihood of an adequate immune response to vaccination.

Persons with anti-HBc only

Persons who are positive for anti-HBc only and who are from a low-prevalence area with no risk factors for HBV should be given the full series of hepatitis B vaccine, because false-positive tests for anti-HBc may occur [8].

Postexposure prophylaxis

Both passive-active PEP using HBIG and hepatitis B vaccine and active PEP using hepatitis B vaccine alone are highly effective in preventing infection after exposure to HBV. Data are limited on the maximum interval after exposure during which PEP is effective, but the interval is estimated to be less than 1 week for needlestick exposures and less than 2 weeks for sexual exposures. Immunocompetent persons who have had postvaccination testing and are known to have responded to vaccination with anti-HBs concentrations of ≥10 IU/L do not require additional passive or active immunization after an exposure to HBV [3].

Vaccine safety and adverse events

Hepatitis B vaccines have an outstanding record of safety and effectiveness. Since 1982, over 1 billion doses of hepatitis B vaccine have been used worldwide [7]. The most frequently reported side effects in persons receiving hepatitis B vaccine are pain at the injection site and low-grade fever [3]. In the United States, the CDC and FDA continually assess the safety of hepatitis B vaccine by the continual monitoring of data from the Vaccine Safety Datalink (VSD) project, the Vaccine Adverse Events Reporting System (VAERS), and other surveillance systems. Hepatitis B vaccination is contraindicated for persons with a history of hypersensitivity to yeast or any vaccine component. Pregnancy is not a contraindication to vaccination because available vaccines contain noninfectious HBsAg and should not cause infection in the fetus [3].

Postvaccination testing

Although serologic testing for immunity is not necessary after routine vaccination of adults, postvaccination testing is recommended for persons "whose subsequent clinical management depends on knowledge of their immune status" [3], including healthcare personnel, chronic hemodialysis patients, persons infected with HIV and other immunocompromised persons, and sex or needle-sharing partners of HBsAg-positive persons. Among persons who do not respond to a primary three-dose vaccine series, 25% to 50% respond to an additional vaccine dose, and over 50% respond to a three-dose revaccination series. An improved response to revaccination occurs in persons who have measurable but low (<10 IU/L) levels of antibody after the initial series. Increased vaccine doses (e.g., double the standard dose) have not consistently demonstrated improved revaccination response rates. Persons who do not have protective levels of anti-HBs 1–2 months after revaccination either are primary nonresponders or are infected with HBV [3].

Duration of protection of hepatitis B vaccine

As mentioned in this chapter, the greatest risk of developing chronic HBV infection after acute HBV exposure occurs at birth and early childhood [13]. Thus, long-term protection from hepatitis B vaccine not only must protect through early childhood to prevent chronicity but also should persist through adulthood to protect against acute symptomatic hepatitis. In countries with low endemicity for HBV, most infection occurs in adolescence and adulthood when risky behavior such as multiple sexual partners or IDU occurs. Long-term studies of the duration of protection from hepatitis B vaccine can be divided into two categories: (1) studies of persons vaccinated as children older than 1 year or as adults, and (2) studies that follow persons vaccinated as infants. Studies to date have only been able to examine humoral immunity with accuracy and to look for clinical evidence of loss of protection. Three methods have been used to evaluate protection: (1) the duration of persistence of anti-HBs, (2) the response to booster doses in persons who have lost anti-HBs or whose levels have fallen below 10 IU/L (the reputed long-term protection level) to demonstrate humoral immunity, and (3) clinical observation for failure of vaccination to prevent the HBV carrier state or acute icteric hepatitis B in a vaccinated individual. Regarding studies examining vaccination in child and adulthood, there is strong evidence that protection in these age groups lasts for at least 22 years after the initial immunization series. The longest study to date is from Alaska, where over 1500 HBV seronegative children and adults received a dose of plasma-derived hepatitis B vaccine in 1981–1982 and have been followed regularly since then. After 22 years of follow-up, 61% still had anti-HBs levels ≥10 IU/L; those below this level were offered a booster dose of recombinant vaccine

[32]. Thirty days after the booster dose, 82% responded and the overall protective efficacy (those with anti-HBs ≥10 IU/L plus those with lower levels who demonstrated a booster response) was 94%. Further evidence of the long-term protection afforded by hepatitis B vaccine is found in the absence of symptomatic acute hepatitis B or the development of chronic HBV infection in persons successfully vaccinated. In most studies conducted in HBV-endemic regions, the occurrence of "breakthrough" infections, demonstrated by the appearance of anti-HBc, has been reported as anti-HBs levels wane. In the Alaska study, for example, 23 breakthrough infections were found, all evident by the appearance of anti-HBc among persons who were previously negative for this seromarker. In five of these persons, HBV DNA was transiently present, and in one of these individuals, a "vaccine escape mutant" was found transiently. None of the persons on further follow-up became persistently positive for HBsAg or HBV DNA. In addition, interviews of participants and review of medical records uncovered no instances of any of the vaccinated persons contracting acute symptomatic hepatitis B. Thus, for 22 years, hepatitis B vaccine prevented symptomatic hepatitis, the development of the chronic carrier state, and the complications of infection with HBV.

Similar results have been found for children who were immunized during infancy. However, the persistence of anti-HBs varies widely depending on when the first dose of vaccine was administered, the number of doses administered, the HBV status of the infant's mother, and whether or not HBV is endemic in the family or community. In general, infants born of HBsAg mothers who are HBeAg-positive and are subsequently protected by vaccination starting at birth tend to have a high probability of having a level of anti-HBs >10 IU/L at 10 years of age, likely due to "natural boosting" from contact with their HBsAg-positive mother and other chronically infected household and community contacts. Children living in endemic communities can also maintain higher levels of anti-HBs due to this phenomenon. In addition, when vaccination either starts at age 2 months or has a fourth dose at age 12 months that is given to those who started the three-dose series at birth, persistence of anti-HBs appears to last longer. Unfortunately, randomized controlled trials using different vaccination schedules in infants in different communities with long-term follow-up of anti-HBs levels are not available and all of the information regarding persistence comes from many individual studies that, taken together, are heterogeneous for the HBV status of the community, mother's HBsAg status, and vaccine schedule. One concerning finding is that infants of HBsAg-negative mothers immunized with a three-dose series starting at birth who are also born in low-endemicity communities rapidly lose anti-HBs and less than 10% have levels of anti-HBs >10 IU/L by 10 years of age. Some small studies have shown that up to 50% of these children fail to boost at late adolescence [33]. However, while breakthrough infections and the transient presence of HBV DNA have been documented among persons in high-prevalence areas who were vaccinated at birth, neither acute symptomatic hepatitis B nor the development of chronic HBV infection has been observed. Thus there is good evidence that children vaccinated at birth have long-term protection lasting for at least 20 years [34]. It is even possible that protection from the adverse effects of HBV after the initial vaccination series may be lifelong. The reasoning for this speculation is that HBV has a long incubation period lasting 4 to 8 weeks. If a person whose anti-HBs level has fallen to a very low level or is undetectable gets exposed to HBV, an infection could occur but cellular immunity may be present to mount an immune response to protect against disease or chronic infection. Unfortunately, efforts to measure cellular immunity in persons who received a complete series of hepatitis B vaccine in the past have not, thus far, been fruitful. If reproducible assays to measure long-term cellular immunity after hepatitis B vaccine become available, then a more accurate assessment of the duration of immunity may be possible. Based on current data, which provide strong evidence that protection after hepatitis B vaccination lasts for at least 22 years in those vaccinated as older children and adults, and for 20 years in those immunized during infancy, US policy makers (ACIP) and European experts (the Viral Hepatitis Prevention Board) have stated that booster doses are not recommended at this time.

Global vaccination programs

In 1992, the World Health Organization (WHO) recommended that countries with a high hepatitis B disease burden introduce hepatitis B vaccine in their routine immunization programs by 1995 and that all countries do so by 1997. However, uptake of the vaccine was slow because most low-income countries were unable to secure the funds needed to introduce the vaccine. In 2000, the Global Alliance for Vaccines and Immunizations (GAVI) began work with manufacturers to ensure that hepatitis B vaccine and safe injection equipment were available and affordable. By the end of 2010, 179 countries, including parts of India and Sudan, had introduced hepatitis B vaccine into their routine vaccination programs; 93 (52%) of these countries recommended administering the first dose of the vaccine within 24 hours of birth to prevent perinatal transmission of the virus. Worldwide coverage with three doses of hepatitis B vaccine was 75%; coverage ranged from 52% (Southeast Asia region) to 91% (Western Pacific region) [7].

Currently, the WHO recommends that all infants receive their first dose of hepatitis B vaccine as soon as possible after birth, preferably within 24 hours to prevent perinatal and early-childhood transmission. Even in countries where there is intermediate or low endemicity, a substantial proportion of chronic infections is acquired through early transmission. Delivery of hepatitis B vaccine within 24 hours of birth is a performance indicator for all immunization programs. The birth dose is followed by two or three doses to complete the primary series, either as (1) a three-dose schedule of hepatitis B vaccine, with the first dose (monovalent) being given at birth and the second and third (monovalent or combined vaccine) given at the same time as the first and third doses of DTP vaccine; or (2) a four-dose regimen, where a monovalent birth dose is followed by three monovalent or combined vaccine doses, usually given with other routine infant vaccines [12].

Catch-up vaccination is considered for cohorts of children with low coverage as a way to increase the number of protected children, especially in countries where there is high endemicity. For this reason, catch-up vaccination of older children and adults is less important and is best considered after an infant immunization program has been established and high coverage of hepatitis B vaccination among infants and young children has been achieved [12].

Clinical and epidemiological impact of hepatitis B vaccination

Incidence of acute icteric hepatitis B

The introduction of programs for screening pregnant women for HBsAg and administration of prophylaxis began in Taiwan and Alaska in the early 1980s and was followed shortly after by universal infant vaccination programs in Taiwan and universal infant and catch-up immunization in all age groups in Alaska [34, 35]. In the late 1980s, New Zealand began universal child vaccination of indigenous populations. The 1990s heralded the introduction of universal infant vaccination in the United States and several other countries, and by the late 1990s the WHO recommended universal infant vaccination worldwide. Currently vaccination programs for infants are being conducted in 179 countries, and many countries have added catch-up vaccination programs for children. The impact of these programs has been immense. In the United States, the incidence of the reported cases of acute hepatitis B has declined dramatically [1]. In endemic regions with comprehensive vaccination programs, the incidence of acute hepatitis B in children has dropped significantly in Taiwan and Alaska to 0/100 000 [34, 35].

Chronic hepatitis B prevalence

The prevalence of HBsAg in children has also fallen dramatically in areas where infant immunization has been ongoing for at least a decade, such as Taiwan and Alaska where in 2008 there were no children under age 20 years who were HBsAg-positive [35]. In areas where most infants receive their first dose of vaccine at birth, the subsequent prevalence of HBsAg in children will fall below 1% and approach zero. However, in endemic areas where a birth dose of hepatitis B vaccine is not administered and the first dose occurs after 6 weeks of age at the time of other childhood immunizations, the prevalence of HBsAg is unlikely to reach below 1–2%. Therefore, eradication of HBV depends on the effectiveness of starting vaccination at birth.

Incidence and prevalence of HCC

When hepatitis B vaccine was first developed and licensed, it was hailed as the first vaccine developed against a form of cancer. However, the proof of this depended on demonstrating a fall in the incidence of HCC among vaccinated populations. In Taiwan, the incidence of HCC in children was shown to decrease significantly 10 years after universal infant vaccination [36]. Similar findings have been reported from Thailand. While both of these regions are areas with a high incidence of HBV-associated HCC, the incidence in both regions was below 1/100 000 in children. In Alaska, where the incidence of HCC was high in children, 3/100 000, no cases of HCC in children have been reported since the mid-1990s, 15 years after the introduction of universal infant and catch-up vaccination programs [33]. Thus, hepatitis B vaccine truly prevents HCC. However, the impact of this vaccine on the incidence of HCC in most HBV-endemic regions will not occur until approximately 40 years after the introduction of infant vaccination because the incidence of HCC does not start to rise exponentially until HBV-infected persons reach the age of 40 years. This latent period may be shorter in Africa, where HCC rates begin to rise in the third decade of life, and in regions where childhood and adult catch-up programs have been implemented.

Conclusion

HBV infection remains highly endemic in certain areas of the world; however, global expansion of universal infant hepatitis B vaccination programs has resulted in dramatic reductions in hepatitis B incidence. Continued follow-up and assessment of vaccinated persons, especially those vaccinated in infancy, are necessary to ensure the maintenance of long-term immunity. Among persons with chronic infection, the prevention of cirrhosis and HCC with antiviral treatment remains an ongoing challenge.

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Chapter 7
Other *Hepadnaviridae* (*Avihepadnaviridae* (DHBV) and *Orthohepadnaviridae* (WHV))

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Summary

All members of the family *Hepadnaviridae* are primarily hepatotropic viruses that contain double-stranded DNA genomes that are replicated via reverse transcription of a pregenomic RNA template. There are two subgroups within this family: mammalian and avian. The avian members include duck hepatitis B virus (DHBV), heron hepatitis B virus, Ross goose hepatitis B virus, stork hepatitis B virus, and the recently identified parrot hepatitis B virus. The DHBV model was essential in determining the novel and complex replication strategy of Hepadnaviruses and was a very useful model in the assessment and development of potential direct-acting antiviral agents. The detection of endogenous avian hepadnavirus DNA integrated into the genomes of zebra finches has revealed the deep evolutionary origin of Hepadnaviruses. The animal nonhuman primate members of the *Hepadnaviridae* include the woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus, and arctic squirrel virus. The WHV and its host, the eastern woodchuck (*Marmota marmot*), have also comprised a very valuable model system for studying hepatitis B virus infection in humans. Its mode of replication and its pathogenesis resemble very much acute and chronic hepatitis B infection. Since the establishment of immunological tools to characterize the immune response in this model, woodchucks can now be used to test new antiviral drugs and also develop new vaccines, especially immunomodulatory approaches to treat chronic hepadnaviral infections. Combining antiviral treatment with nucleos(t)ide analogs and therapeutic vaccines, including prime boost regimens, has been shown to induce a potent T cell response in chronic WHV carrier woodchucks. This combination therapy seems to be a promising approach for the treatment of chronic hepatitis B to suppress viral replication and even eliminate HBV in the human host.

Hepadnaviridae: Introduction and comparative virology

There are two genera within the Hepadnavirus family: the *Orthohepadnavirus* genus that includes viruses infecting mammals, and the *Avihepadnavirus* genus that includes viruses infecting avian species. The *Orthohepadnavirus* genus includes the human hepatitis B virus (HBV), as well as specific strains from nonhuman primates, including chimpanzees (ChHBV), gorillas (GoHBV), orangutans (OuHBV), gibbons (GiHBV), and the woolly monkey (WMHBV). In addition to these human and primate strains of HBV, the *Orthohepadnavirus* genus includes the woodchuck hepatitis virus (WHV) from eastern woodchucks (discussed in this chapter), ground squirrel hepatitis virus (GSHV) from

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Table 7.1 Comparison of the different members of the *Hepadnaviridae* family.

	Hepadnaviral members			
Physical characteristics	HBV	WHV	GSHV	DHBV
Virion				
Diameter (nm)	42	45	47	40–45
Buoyant density in CsCl (g/mL)	1.24	1.225	1.24	1.16
Nucleocapsid				
Diameter (nm)	27	27	30	27
Buoyant density in CsCl (g/mL)	1.34	1.34	1.34	1.34 (with spikes)
Surface antigen particles				
Spheres (nm)	22	20–25	15–25	40–60
Buoyant density in CsCl (g/mL)	1.19–1.20	1.18	1.18	1.14
Filaments (22 nm diameter)	Present	Present	Present (long and abundant)	Absent
Genome				
Size (nt)	3182–3188	3308–3320	3311	3021–3027
Sequence homology with HBV	100	70	55	40
Number of ORFs	4	4	4	4
Number of coded amino acids or ORFs				
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

CsCl: cesium chloride; ORF: open reading frame; nm: nanometers; nt: nucleos(t)ides.
(Source: Modified from Jilbert AR, Locarnini S. *Avihepadnaviridae*. Third Edition Viral Hepatitis 2005:193–209 [7]) .

Beechy ground squirrels, and arctic squirrel hepatitis virus (ASHV). The *Orthohepadnavirus* genus is phylogenetically classified into 10 HBV genotypes, A–J, with an overall sequence divergence of 8% or more. The nonprimate strains, WHV, GSHV, and ASHV, are all assigned as separate species and share 70%, 55%, and 63% genome homology, respectively, with HBV.

The *Avihepadnavirus* genus includes the duck hepatitis B virus (DHBV) isolated from Pekin ducks, heron hepatitis B virus (HHBV) from gray herons, Ross goose hepatitis virus (RGHV) from Ross geese, stork hepatitis B virus (STHBV) from white storks, and hepatitis B viruses from the snow goose (SGHBV), Australian maned duck (MDHBV) and gray teal (GTHBV). Very recently, a new avihepadnavirus was isolated from *Psittacula krameri*, the ring-necked parrot, in Poland [1]. The parrot virus (PHBV) genome shares 76% sequence identity with other avihepadnavirus isolates and phylogenetically is most closely related to the HHBV and STHBV isolates.

Members of the *Hepadnaviridae* family are primarily hepatotrophic, contain a relaxed circular (RC) double-stranded DNA (dsDNA) genome, and have a unique method of replication that involves reverse transcription of a greater-than-genome-length RNA, the pregenomic RNA. 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 7.1. HBV and DHBV share only 40% nucleotide similarity, while, as explained here, genomic nucleotide sequences are more similar among the members of each genus. All Hepadnaviruses have very similar genomic organizations and open reading frames (ORFs). The X-like ORF of the avihepadnavirus PHBV is similar to those of other avihepadnaviruses with an identifiable start site of the ORF (HHBV, STHBV, SGHBV, and RGHBV), while the DHBV strains lack a putative translation start site [2]. Unlike the X protein of the orthohepadnaviruses, however, the X-like ORF of DHBV was found to be nonessential for replication *in vivo*. Expression of the X-like ORF in cell culture does lead to transcriptional activation of several promoters that are heterologous to those of the HBV X protein; however, since knockout mutants of the DHBV X-like ORF do not alter its ability to replicate *in vitro*, the overall significance of the avian HBV X-like ORF remains unknown.

Differences in virion structure and the production of noninfectious 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 7.1). There are also antigenic differences between the two genera. The mammalian viruses show cross-reactivity between epitopes on their core and envelope proteins [3, 4], but not with epitopes of the avian viruses. All hepadnaviruses have a narrow host range, which is usually limited to each species. For example, HBV only infects higher primates, including humans, chimpanzees, orangutans, and gibbons. DHBV infection has been found to occur naturally in domestic flocks of ducks and geese but not in other anseriforme species. DHBV has also been experimentally transmitted to geese.

As HBV does not infect nonprimate 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 and provided important preclinical data for subsequent clinical development of antiviral agents, especially nucleos(t)ide analogs.

In summary, orthohepadnaviruses and avihepadnaviruses are distinguished by the following criteria: (1) low nucleotide sequence identity, (2) differences in genome size (about 3.2 kb for orthohepadnaviruses and 3.0 kb for avihepadnaviruses), (3) larger core proteins and no M surface protein for the avihepadnaviruses, and (4) host range restricted to either mammals or birds, respectively.

Avihepadnaviridae: Classification

The avihepadnaviruses have been classified phylogenetically into "Chinese" and "Western" DHBV strains as well as four highly distinct lineages: SGHBV, RGHV, STHBV, and HHBV. Nucleotide sequence divergence within the Chinese and Western strains is 5.99 and 3.35%, while divergence between the strains is 9.8%. The SGHBV is the next closest member of the genus to DHBV, varying by 11–13%, while RGHV varies by 17.3–19.1% and STHBV and HHBV vary by 22.2–23.6%. There is an infection barrier dividing the STHBV and HHBV from the rest of the natural hosts of the other avihepadnaviruses. Thus, the larger evolutionary distance between HHBV and DHBV may reflect this distinct host range, since HHBV infects only gray herons and not ducks, and may have resulted from co-evolution of each virus in its respective host. Phylogenetic analysis of the recently discovered PHBV places it in the same clade as the HHBV and STHBV.

Avihepadnaviridae: The exogenous replication strategy

The DHBV model was essential in determining that hepadnaviruses replicate via reverse transcription of an RNA intermediate [5] and has also proved valuable in the study of the early events of the hepadnavirus life cycle. These data and their relevance to human HBV have been reviewed and updated in Chapter 5 and will not be considered further.

Development of antihepadnaviral agents: The experimental model of DHBV

The primary goal of antiviral therapy is to control active HBV replication and eventually eradicate HBV infection from the host, thereby preventing the consequences of persistent infection, namely, chronic liver disease and its further clinical progression to cirrhosis and hepatocellular carcinoma (HCC). 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 IFNα) and antiviral agents (such as nucleos(t)ide analogs). More recently, these agents are also being trialed 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 analogs such as ganciclovir, penciclovir, adefovir (PMEA), entecavir (ETV), and FLG, as well as the pyrimidine analogs including emtricitabine (FTC), an agent very similar in antiviral profile to lamivudine (reviewed in [6]). Such detailed studies in the duck model have highlighted the value of and reinforce the need for extensive preclinical testing of an agent before it enters phase I or II clinical trials. In this way, both predictable and unpredictable toxicity can be avoided. The previous edition of this chapter extensively reviewed the *in vitro* and *in vivo* evaluations performed in the duck HBV model over the last 15 years and will not be reconsidered here [7].

Future challenges for the development of therapies for chronic HBV: The role of covalently closed circular DNA (cccDNA) inhibitors and animal model testing

From the results of preclinical as well as clinical testing of HBV antiviral drugs, the most important observation

found to date is that conventional antiviral therapy has a minimal effect on intrahepatic viral cccDNA levels as well as viral RNA production [8, 9]. Complete inhibition of viral polymerase activity by a nucleoside analog 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 nucleos(t)ide analogs 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 minichromosomes in hepatocyte nuclei is essential for viral replication and establishment of persistence. Therefore, their complete elimination is necessary in order to effect a lasting cure. Because it is replicated via an RNA intermediate, viral cccDNA is not directly affected by deoxynucleos(t)ide analog 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 viruses will emerge well before cccDNA is completely depleted (see Chapter 5 for further discussion).

Endogenous avian hepadnaviruses: Deep evolutionary origins

Viruses lack a true fossil record, but key aspects of the pattern, process, and time scale of their evolution can be inferred from an analysis of the endogenous viral elements that are present in most eukaryotic genomes [10]. The best example is the retroviruses that integrate into the host genome as part of their replication cycle, and occasionally invade and integrate into the cellular genome of the germ line, thereby ensuring that they are vertically inherited as "endogenous" elements of host DNA; endogenous retroviruses comprise over 8% of the human genome. Recently, endogenous hepadnaviruses have been discovered in avian species, originally in the genome of the zebra finch. These elements have been designated endogenous zebra finch HBVs (eZHBVs) [11]. Even more integrants have been detected in genomes from birds within the order Passeriformes, including other finches (*Taeniopygia guttata*, *Poephila cincta*, *Lonchura punctulata*, and *Chloebia gouldiae*, family Estrildidae), the olive sunbird (*Cyanomitra olivacea*, family Nectariniidae), and the dark-eyed junco (*Junco hyemalis*, family Emberizidae) [11]. Phylogenetic analysis of these endogenous sequences has revealed that Hepadnaviruses integrated into these bird genomes at least 19 million [10, 11] and possibly over 40 million years ago. These findings have been independently confirmed and extended into budgerigar genomes (order Psittaciformes); these endogenous hepadnaviral sequences have been designated eBHBV [12]. Comparative phylogenetic analysis of the endogenous and existing exogenous avian Hepadnaviruses has revealed that the former are far more divergent than the latter with clear separation between the endogenous and exogenous Hepadnaviruses, forming their own distinct lineages. This indicates multiple or recurrent genomic integration events. These seminal observations require a re-interpretation and re-evaluation of the current concepts of the evolutionary origin of Hepadnaviruses, both ortho- and avi-. For example, based on the viral mutation rate alone, most standard estimates place ortho-hepadnaviruses at between 3000 and 6000 years of age in their respective hosts. Similarly, the role of extrahepatic HBV replication in the context of viral persistence and pathogenesis, linked with the pattern and profile of hepadnavirus DNA integrations in germ line as well as hepatocyte lineages, also requires re-evaluation.

Extrahepatic DHBV infection: Role of germ line infection and integration

In general, hepadnaviruses primarily replicate in the liver, but a large number of studies have provided evidence for DHBV infection in extrahepatic sites as well. Despite these studies, the significance of extrahepatic viral replication with respect to infection outcome has remained unclear. Although integration into the host genome is not required for the replication of hepadnaviruses, integrated HBV genomic fragments are commonly observed in liver cells of individuals persistently infected with HBV. These are also strongly associated with the development of HCC [13]. Also, while Hepadnavirus replication is mainly considered to occur within hepatocytes, its tropism has been shown to extend to other tissue and cell types, including germ cells. In the case of the avihepadnaviruses, DHBV replication has been demonstrated in the yolk sac of the developing duck embryo [14], while HBV DNA has been demonstrated in spermatozoa and ovaries, and HBV has been found as a chromosomal integrant in spermatozoa [15–17], demonstrating that infiltration of HBV into the germ line is possible. Further studies are clearly needed.

Intrahepatic pathogenesis of hepadnaviral infection: Insights from animal models

Within the liver, DHBV replication has 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, including cccDNA. DHBV replication has been convincingly demonstrated using primary cultures of intrahepatic bile duct epithelial (IBDE) cells isolated from ducklings. The primary IBDE cells formed ductlike structures of multilayered IBDE cells, which were identified as bile duct cells using the antikeratin 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 [18]. 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.

Thus, 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. 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 requires turnover of infected cells.

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 material resistant to periodic acid–Schiff diastase (PAS-D). 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. 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 A 2012, personal communication).

Chronic HBV (CHB) 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 HCC. 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 [19, 20]. This type of infection is similar to the low- or nonreplicative phase of CHB. 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 [19–21]. 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 [22], 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 tumors [23–26]. HCC has been found to occur only in ducks from a single province in China [20, 27, 28], where environmental aflatoxin exposure may have been prevalent. In summary, in animal models at least, 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 can also appear to play an important role, particularly in the development of HCC.

Orthohepadnaviridae: The woodchuck model

The eastern woodchuck (*Marmota marmota*) is naturally infected by woodchuck hepatitis virus, which was discovered in 1978 [29]. WHV was found to be closely related to HBV and classified as a member of the genus *Orthohepadnavirus*, family *Hepadnaviridae*.

Many questions of hepadnaviral infection have been addressed in this model (e.g. natural course of infection, immunpathogenesis, host and viral factors associated with development of chronicity, and development of HCC). From a medical point of view, the woodchuck model has been used to develop new strategies for prevention of infection and therapy of chronic hepadnaviral infection, including nucleosidanalogs, nonnucleosidanalogs, therapeutic vaccination, and gene therapeutic approaches for treatment of HCC [30]. Even liver transplantation has been performed to study early events in re-infection and adoptive immune transfer.

Very recent studies using microarray analysis revealed that WHV does not induce significant intrahepatic gene expression changes during the early-acute stage of infection (week 8). At week 14 of infection, resolution was associated with induction markers of activated cytotoxic T cells. At this time point, high-level expression of program death-1 (PD1) and various other inhibitory T cell receptors, which likely act to minimize liver damage by cytotoxic T cells during viral clearance, was detected. The IFNγ signaling response was comparable to that in chronically infected animals [31].

In chronic WHV infection, similar to HBV a limited intrahepatic IFNα response, intrahepatic markers associated with T cell exhaustion, elevated levels of suppressor of cytokine signaling 3 (SOCS3) in the liver, and intrahepatic accumulation of neutrophils were observed. In addition, WHV-induced HCC shares characteristics with human HCC. These data indicate that immune pathways play a major role in either the persistence of HBV infection or the sequelae of CHB [32].

The woodchuck is a preclinical model for new therapeutic strategies in CHB

Significant progress has been made in recent years in this model of hepadnavirus natural infection that demonstrates that noncytotoxic and cytolytic antiviral immune responses are needed for elimination of the virus from hepatocytes or to at least control viral replication.

For more than 10 years, several approaches have been undertaken to develop a therapeutic vaccine for patients with CHB infection [30, 33]. Conventional HBV vaccines used in the early clinical trials induced a specific but transient immune response to HBV; however, they failed to control HBV replication in CHB patients. So far, very limited information is available on clinical trials using new therapeutic vaccines to downregulate replication by stimulating the T cell response.

The woodchuck is an ideal model to evaluate therapeutic vaccines as WHV infection in woodchucks results in outcomes of infection similar to those of HBV infection in humans, ranging from a subclinical or acute transient infection to chronic infection progressing to HCC. Unfortunately, no inbred animals are available so far to standardize infection experiments. For understanding of the underlying mechanisms responsible for different outcomes of infection, detailed *in vivo* studies on humoral and cellular immune response to WHV are required. Assays for antigen and antibody detection have been available for many years.

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, like antigen processing (TAP1 and TAP2) and presentation (e.g., MHC class I and class II); proteins involved in signal transduction; and cell surface markers like CD3, CD54, and CD8 present in cells of the immune system have been cloned and sequenced.

The complete sequences of tumor necrosis factor alpha (TNFα), interferons alpha (IFNα) and gamma (IFNγ), interleukin 6 (IL6), IL10, IL12, IL15, granulocyte–macrophage colony-stimulating factor (GMCSF), and lymphotoxins alpha (LTα) and beta (LTβ) were obtained. Partial sequences of IL2, IL4, Fas ligand, and others were cloned. 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 [34].

TNFα, IFNα, IFNγ, IL12, and LTα have been tested for their biological activities. The entire ORF of IFNα was cloned under control of the cytomegalovirus (CMV) promoter into the expression vector PVIJ [35]. The biological action of IFNα was also demonstrated by induction of expression at MXA protein. Finally, the biological activity of IFNα was tested in WHV-infected woodchuck hepatocytes. Woodchuck IFNα reduced WHV surface antigen expression in a dose-dependent fashion [35]. The biological activity of TNFα and LTα was tested in a cytotoxicity assay using woodchuck A2 cells. The specific activities for both woodchuck cytokines TNFα and LTα were significant higher in woodchuck cells, as in mouse L9 to 9B and human HepG2 cells.

Recombinant woodchuck IFNγ (wIFNγ) was expressed in *Escherichia coli* and mammalian cells. wIFNγ protected woodchuck cells against infection with murine encephalomyocarditis virus in a species-specific manner. It upregulates the messenger RNA (mRNA) level of the woodchuck major histocompatibility complex class I (MamoI) heavy chain in permanent woodchuck WH12/6 cells and differentially regulates the gene expression [34]. 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 wIFNγ per ml or with a combination of wIFNγ and woodchuck TNFα (wTNFα). Rabbit antibodies to wIFNγ were able to block biological activity of IFNγ.

The complete coding region of CD4 has a length of 1365 bp encoding a protein of 455aa. The cDNAs of woodchuck CD28 and cytotoxic T lymphocyte–associated antigen 4 (CTLA4) have been cloned and sequenced. These two molecules are known to play important roles for the regulation of T cell activation by delivering the co-stimulation signals. Woodchuck CD28 showed a similarity of 70–80% to its mammalian homologs according to the deduced amino acid sequences. Woodchuck CTLA4 has a higher similarity of 74% to the corresponding mammalian CTLA4 molecules. The strict conservation of critical amino acid residues like cystein and asparagine residues in woodchuck CD28 and CTLA4 suggests that both molecules may structurally resemble their human or mouse homologs. A hexapeptide motif MYPPPY, which has been supposed to be essential for the interaction with CD80, is present in both woodchuck CD28 and CTLA4 [36].

Sequence information of woodchuck CD3, CD4, and CD8 has been used to determine the kinetics of influx of T cells into the liver. Sequences of wIFNα and wTNFα were suitable to determine cytokine expression during the incubation period and acute or chronic WHV infection. In week 2 post infection, an influx of CD3+ lymphocytes could be observed and reached higher levels prior to and during the recovery phase. The peak level of CD4+ and CD8+ T cells coincided with recovery. Mono- or polyclonal antibodies to CD3+, CD4+, and CD8+ of woodchuck are not available yet. One monoclonal antibody to a conserved region of CD3 from swine was found to cross-react to 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 of the total number of liver cells [37].

Development of immunological tools in the woodchuck model

In recent years, a number of immunological tools to determine T cell responses in WHV infection have been introduced, allowing the evaluation of therapeutic vaccines against hepadnaviral infections in this animal model [38, 39].

It was demonstrated that cellular immune responses to viral proteins induced liver injury in the woodchuck model, and were crucial for viral clearance. In several studies, antigen-specific T helper lymphocyte responses were detected using a [2–3H]-adenine-based proliferation assay. It was shown that T helper cell responses to WHV proteins are associated with viral clearance, whereas the absence of T helper responses is related to the development of persistent infection [39].

The determination of cytotoxic T lymphocyte (CTL) responses against WHV proteins was enabled for the first time by the recently established CD107a degranulation assay [38]. This newly established assay is of great value for studies on immunopathogenesis of hepadnaviral infections. Additionally, this technique is a powerful tool for monitoring immune therapies in chronically WHV-infected woodchucks.

In subsequent studies, the immunomodulatory approaches using therapeutic vaccines could now be directly tested in chronic WHV carriers. Up to date, several studies of therapeutic vaccinations have been carried out in woodchucks. Diverse therapeutic vaccines containing WHV core antigens (WHcAg) or surface antigens (WHsAg) in combination with a helper peptide, with adjuvant-like monophosphoryl lipid A or alum, or with antiviral treatment (i.e., clevudine [L-FMAU] and lamivudine) were tested experimentally in chronic WHV carriers [40].

Combination of antiviral treatment and vaccination

It is generally assumed that a reduction of viral load by antiviral treatments might enhance the efficacy of therapeutic vaccines. This idea is supported by Boni *et al.* reporting that the T cell response to HBV was successfully restored in patients treated with lamivudine [41].

The potent drug clevudine (previously called L-FMAU) was administered orally to chronic WHV carriers at 10 mg/kg/day for 32 weeks starting at 1–2 years of age [40]. Half of the drug-treated woodchucks and half of the placebo recipients received four doses of an alum-adsorbed WHsAg vaccine. Combination treatment with clevudine and vaccine resulted in remarkable reductions in serum WHV DNA (>6–8 logs) and WHsAg (>50–500-fold) that were sustained in most woodchucks throughout the following 104-week study period. Vaccination alone induced low-level anti-WHs antibody responses in most woodchucks, but did not affect the levels of viremia and antigenemia compared with the placebo controls.

The development of HCC in clevudine-treated woodchucks was delayed significantly, and long-term survival after 4 years likewise was increased significantly compared to placebo-treated woodchucks [40]. Lu *et al.* combined lamivudine treatment with DNA vaccination and found a significant reduction of viral load and antigenemia [42]. These results indicated that the combination of antiviral treatment and therapeutic vaccination is more effective to induce specific T cell responses in chronic carriers.

Furthermore, the protocol was modified by using the potent antiviral drug ETV and increasing the number of immunizations to six times [43]. Woodchucks chronically infected with WHV were pretreated with ETV and then received DNA vaccines expressing WHsAg or WHcAg or DNA vaccines plus protein preparations of WHsAg or WHcAg. A significant decrease of serum WHV DNA and WHsAg concentrations was measured after the start of ETV treatment and enhanced further by immunizations. After the cessation of ETV treatment, a rebound of WHV replication took place immediately in woodchucks treated with ETV alone, while a significant delay of viral rebound was observed in vaccinated woodchucks. Thus, a combined treatment with a potent antiviral drug like ETV against WHV may lead to a prolonged suppression of viral replication; however, a rebound of viremia occurred in the majority of animals.

A more potent therapeutic T cell vaccination strategy may be a DNA prime adenovirus boost immunization. A new DNA plasmid and recombinant adenoviral vectors (AdV) with high expression levels of WHcAg were constructed. These vaccines could elicit a significantly stronger CD8+ T cell response compared to

the previously used immunization strategies in naïve mice [44].

In a subsequent study, the new DNA prime–AdV boost immunization was tested to determine whether it halts the tolerance against WHV antigens in transgenic mice and in chronically WHV-infected woodchucks [45].

Immunization of WHV-transgenic mice by the DNA prime–AdV boost regimen elicited a potent and functional WHcAg-specific CD8+ T cell response that consequently resulted in undetectable WHV load in more than 70% of animals. DNA prime–AdV boost immunization in combination with ETV treatment was then evaluated in chronic WHV carriers. WHsAg- and WHcAg-specific proliferative responses and degranulation responses occurred in chronic WHV carriers that received immunizations and ETV, but not in controls treated with ETV only. Carriers from the combination therapy group showed a prolonged suppression of WHV replication, lower WHsAg levels, and decreased liver inflammation compared to controls. Moreover, two of four immunized carriers remained WHV-negative after the end of ETV treatment and developed anti-WHs antibodies. These results demonstrate that the DNA prime–AdV boost strategy leads to sustained control of chronic hepadnaviral infection [45].

Electroporation: A new strategy to improve immune response in therapeutic vaccination

An alternative strategy to improve T cell immune response after therapeutic vaccination is the application of plasmids by electroporation (EP). The efficiency of the EP-based delivery of plasmid DNA (pDNA) expressing various reporter genes was first evaluated in normal woodchucks, and the expression of reporter genes was greatly increased when the cellular uptake of pDNA was facilitated by EP [46]. The EP of WHsAG–pDNA resulted in enhanced, dose-dependent antibody and T cell responses compared to those of the conventional hypodermic needle injection of WHsAg–pDNA. This EP vaccination may be used in patients in the future as the efficacy of intramuscular plasmid vaccine in larger species like monkeys or humans is not very efficient.

New strategies for treatment of chronic hepadnaviral infection

It has been shown in other infectious disease models that the degree of exhaustion of T cells is dependent on the duration of infection, the antigenic load, the presence of CD4 help, innate immunity, regulatory T cells, the type of antigen-presenting cells, and the ratio of co-stimulatory molecules to inhibitor signals. Therefore, it raises the question of whether the substantial reduction of viral load by antiviral therapy with nucleoside analogs may influence the functionality of CD4 and CD8 cells in chronic HBV or WHV infection [47]. Several mechanisms have been identified that are involved in the downregulation of effector functions, including the PD1–programmed cell death 1 ligand 1 (PDL1) and CTLA4–B72 systems and regulatory T cells. The PD1–PDL1 system and the CTLA4–B72 system play an important role for the maintenance of peripheral tolerance to self-antigens. Exhausted virus-specific CD8+ cells upregulate the inhibitory PD1 during chronic infection. This upregulation of PD1 expression during chronic infection has been observed for HIV, HBV, and hepatitis C virus in humans [48] and in lymphocytic choriomeningitis (LCMV) infection in mice [49]. The expression of PDL1 in patients with chronic HBV infection and HCC has been investigated. A very high expression of PDL1 was observed in HCC tissue [50].

From previous studies, it is obvious that monotherapy of chronic HBV infection with interferons, nucleoside analogs, or therapeutic vaccines (e.g., a DNA vaccine to stimulate specific CD8 T cell response to WHV core and S proteins) did not result in a reduction of viremia or a loss of the virus during follow-up. Therefore, a combination of these interventions (e.g., by nucleoside analogs, therapeutic vaccination, and blockade of PD1–PDL1) may have a synergistic effect to rejuvenate T cells with a gain of function with respect to cytokine production and cytotoxic activity. IFNγ and nucleoside analogs may downregulate WHV replication, and functional CTLs may cure or eliminate WHV-infected hepatocytes.

Woodchuck PDL1 and PD1 were cloned and sequenced. The woodchuck PDL1 shows a high homology of 82% to human PDL1 and is expressed in WHV-infected woodchuck livers [51]. It is likely that the PD1–PDL1 interaction plays a role in persistent WHV infection. Preliminary experiments showed that a blockage of PDL1–PD1 interaction could enhance the functions of T cells from chronically infected woodchucks [51].

PDL1 expression could be induced by incubating primary woodchuck hepatocytes with IFNα and IFNγ, poly IC, and lipopolysaccharide (LPS). IFNα, IFNγ, and LPS induced a 10-fold increase of PDL1 expression, while poly IC induced a 50-fold increase of PDL1 expression on primary woodchuck hepatocytes. In a recent study, the PD1 expression on virus-specific CD8 cells in chronic WHV infection was investigated. Increased expression was found that may lead to an interaction of PD1 and PDL1 that impairs the function of specific CD8 cells in the liver. Blocking PD1–PDL1 interaction *in vitro* resulted in an increased function of virus-specific T cells [52].

This blockade was evaluated *in vivo* as a novel strategy for enhancing the efficacy of therapeutic vaccines in chronic WHV infection. Blocking PD1–PDL1 inhibitory

signals on exhausted CD8+ T cells in woodchucks chronically infected with WHV, in combination with antiviral treatment and therapeutic vaccination, synergistically enhanced functional CD8+ T cell responses and improved viral control. Half of the animals completely eliminated WHV after this triple treatment [52]. Such an approach will be useful for the design of immunotherapeutic strategies in patients with CHB.

Vaccination against hepatitis D virus (HDV) infections

Superinfection of HBV carriers by HDV causes the most severe form of hepatitis in humans. About 90% of these cases develop chronic HDV infection and progress rapidly into liver cirrhosis and HCC [53]. 15–20 million of HBV carriers worldwide are infected with HDV. Therapeutic options for HBV–HDV carriers are limited. Only about 30% of patients respond to IFNα therapy. Simultaneous HBV–HDV infection can be prevented by vaccination against HBV; however, HBV carriers are at risk to be superinfected with HDV. Therefore, a vaccine protecting HBV carriers from HDV superinfection would be important.

Specific CD8 T cell response upon vaccination with either DNA vaccine or viral vectors like adenovirus 5 (Ad5) that contain the only HDV protein, p27, have been investigated in mice and woodchucks. The CD8 T cells showed an increased IFNγ production after *in vitro* stimulation with HDV peptides, and the cytotoxic activity of both functions will be needed for an effective vaccine against HDV in humans [44, 54].

In the woodchuck model, several strategies have been followed to induce a protective immune response against HDV superinfection. Synthetic peptides; incomplete or complete HDAg expressed in *E. coli*, yeast, or baculovirus; vaccinia virus expressing the small or the large HDAg; and DNA immunization by gene gun have been studied [53].

So far, all vaccinations of woodchucks that are chronic carriers of WHV by proteins, DNA vaccine, or recombinant viruses failed as all animals developed HDV superinfection that in most cases resulted in a chronic course of HDV infection. The vaccinated animals, however, showed a reduced viral load during the acute infection as compared to nonvaccinated control animals [54].

To evaluate HDV plasmid constructs and recombinant Ad5 vectors prior to vaccination of woodchucks, mice were immunized three times with plasmid expressing either HDAg p27 or WHV core protein. However, the CD8 T cell response was not very strong as compared to DNA vaccine against WHV core protein.

Recent DNA prime–viral vector boost (Ad5/35) immunizations of naïve woodchucks and subsequent challenge with WHV and HDV (simultaneous infection) showed that the animals were protected from HDV infection; however, all animals were infected with WHV [54]. Seven naïve woodchucks were immunized in a prime–boost regimen (3×DNA 2×Ad5) to further enhance the immune response. After priming three times with DNA vaccine, Ad5 and Ad35 recombinant vectors expressing HDAg were administered. Immunized animals and four naïve controls were challenged with 109 copies of WHV and 105 copies of HDV. After the WHV–HDV challenge, WHV DNA was detected for up to 25 weeks in all woodchucks. HDV RNA became positive in four out of four controls; however, five of seven HDV-immunized woodchucks were protected from HDV infection [54]. In the two animals with the breakthrough, HDV RNA became detectable later than in the controls. The WHV-specific immune response showed a typical course seen before in acute WHV infection.

For the first time, protection of woodchucks from simultaneous WHV and HDV has been achieved by vaccination. This improved vaccination scheme will be tested in chronically WHV-infected woodchucks superinfected with HDV.

What can we learn from the woodchuck model?

The studies done in the woodchuck model have proven the feasibility of therapeutic vaccination against chronic hepadnaviral infection. It has become clear that the induction of antibodies to WHsAg can be achieved in chronically infected individuals, whereas the control of viral replication needs additional branches of immune responses, especially that of the T cells. Particularly, prime boost immunizations in combination with antiviral drugs appear to be an effective method to lower viral load and to stimulate antibody responses and T cell response with antiviral efficacy. For future studies, several promising options are available. Blocking the negative regulation mechanisms of immune responses by regulatory T cells, PD1–PDL1, or CT2A4–B7 interaction and downregulation of protein expression by RNA interference may be some options to reactivate virus-specific T cells. A DNA prime–adenovirus (AdV) or modified vaccinia Ankara (MVA) boost vaccination protocol may be in the future an alternative treatment for patients with chronic HBV infection (Figure 7.1). EP may be used in patients to enhance immune response. For a vaccine against HDV infection, some progress was achieved; however, a vaccine for prevention of superinfection or a therapeutic vaccine for chronic HDV infection is not yet on the horizon.

Thus, future investigations will take the major advantages in the woodchuck model as an authentic infection model. As compared with the woodchuck model, no other animal model is available that mimics the chronic course of hepadnaviral infection and presents the fea-

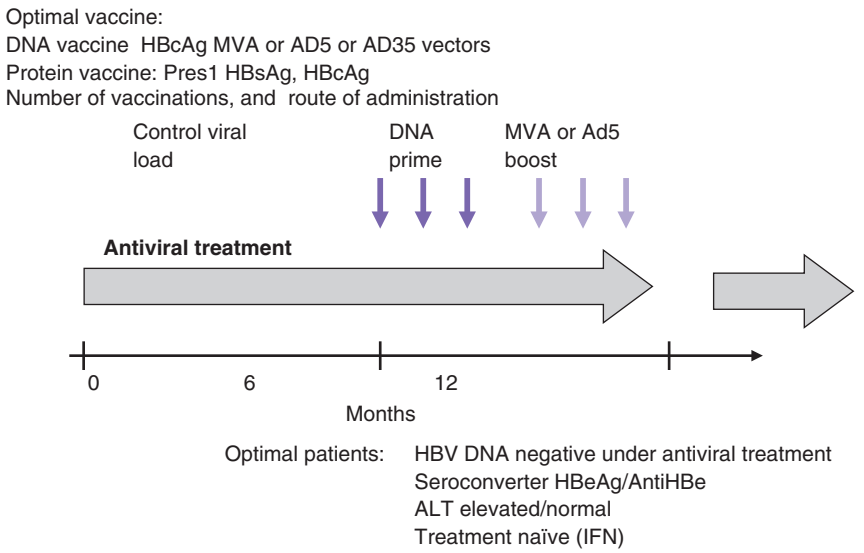

Figure 7.1 Strategies for therapeutic vaccination in chronic hepatitis B.

tures in pathogenesis and virus–host interaction therapeutic approaches in a satisfactory way.

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Chapter 8
Molecular variants of hepatitis B surface antigen (HBsAg)

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Summary

The division of hepatitis B virus (HBV) isolated from different geographical regions into genotypes and subtypes has long been recognized. This division is based on amino acid variation in the hepatitis B surface antigen (HBsAg) encoding region and, more specifically, the hydrophilic region that constitutes the *a* antigenic determinant. This same region constitutes the major neutralizing epitope recognized by natural infection- and vaccine-induced antibodies. However, a number of mutations have been described in different clinical settings that allow the virus to evade the neutralizing immune response. This chapter aims to review the current level of knowledge regarding HBsAg variants.

Introduction

Viruses are under constant selection pressure and as a consequence undergo continual sequence variation. This process has had a number of effects on many HBV genes as described here and in Chapter 9, and some of them are clinically relevant. The anti-HBs (antibody to HBsAg) response following natural infection or after immunization involves antibodies that recognize the major hydrophilic region (MHR) of the protein, which involves amino acids (aa) 99–160 that constitute the group-specific *a* determinant. Isolated cases of infection with HBV variants bearing substitutions in this region, which are predicted to escape neutralization by antibody, have been described elsewhere. There have also been cases of infection that have been missed because of failure of current serological assays to detect certain variant forms of HBsAg. Variants that escape neutralization by monoclonal and polyclonal anti-HBs have been selected during immunoprophylaxis against HBV for liver transplantation and in vaccinees receiving passive or active immunization after birth to HBV-infected mothers. Moreover, in recent years, variants resistant to nucleos(t)ide analogs as a result of mutations in the polymerase encoding gene have been described. Such variants may affect the antigenicity of the HBsAg encoded by the S gene that is overlapped completely by the polymerase open reading frame (ORF). Here, we describe HBs antigenic variation, its possible geographical differences and variation under human-induced selection pressures, and its relevance to diagnosis, vaccination, and treatment.

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The surface gene and subtypes

A single ORF that occupies more than one-third of the HBV genome encodes the three HBsAg-containing polypeptides. At the aa level, these proteins are mainly highly conserved but there are some regions of variability [1]. The domain within pre-S1 (aa 21–47) that is involved in binding to the hepatocyte [2] is highly conserved (see Chapter 5). Based on antibody–peptide binding and chimpanzee inoculation experiments as well as *in vitro* mutagenesis [3, 4], a model has been proposed [5] of the antigenic region of the small envelope protein that contains two loops formed by disulfide bridges between cysteines 124/137 and 139/147 (Figure 8.1 and Figure 8.2) [6, 7]. It is well documented that the antigenicity of HBsAg is dependent on this 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], and if the former peptide is cyclized between the terminal cysteines, affinity is increased [6]. This whole region, and the second loop in particular (aa 139–147), is commonly described as the major B cell epitope, which is highly conserved (Figure 8.2). 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 (Figure 8.2) [8, 9]. 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. These are referred to as subtypes of HBsAg, are defined using appropriate monospecific antisera (monoclonal antibodies are also available), and all contain the common *a* determinant. Geographical variability has most likely come about by population pressure over the millennia, probably under the influence of major histocompatibility complex (MHC) antigen types and other immunogenetic factors [12]. 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 only four major ones (*adw*, *ayw*, *adr*, and *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 (K) or arginine (R) at aa position 122 confers *d/y* specificity: *adw* and *adr* subtypes have lysine at this position, whereas *ayw* and *ayr* have arginine (Figure 8.1).

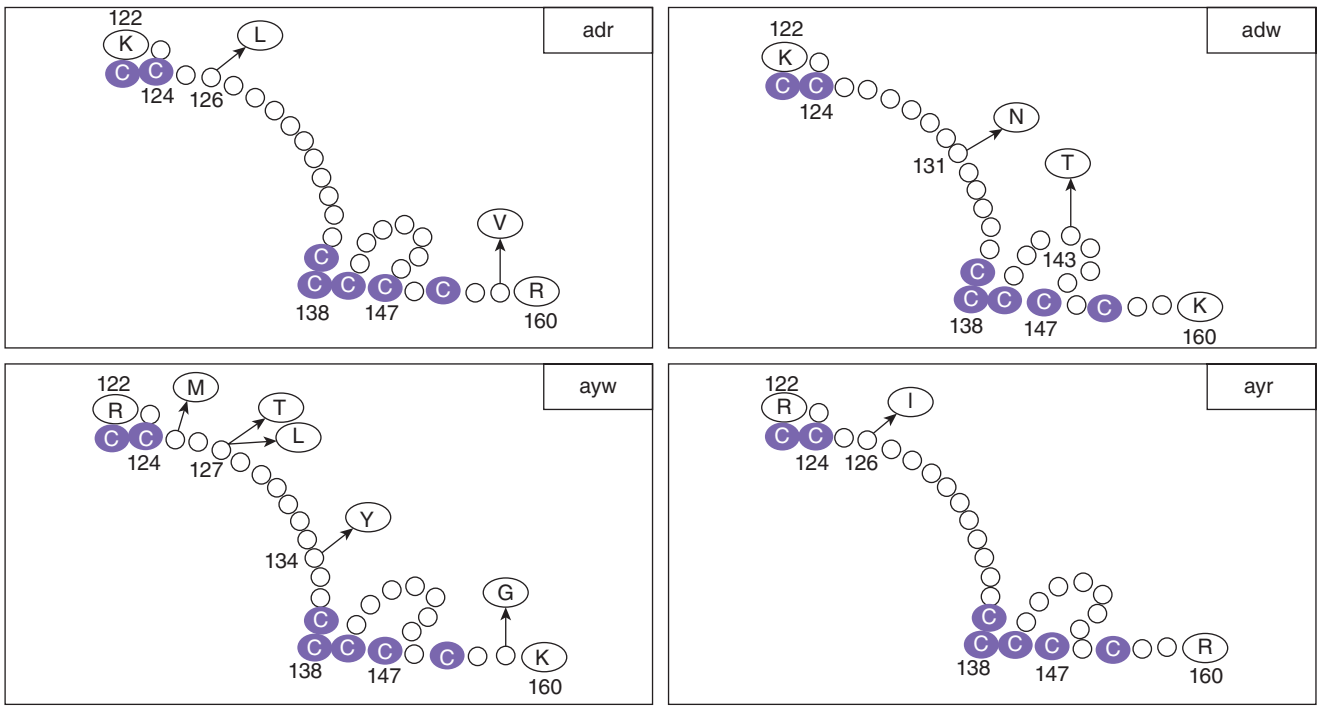

Figure 8.1 Variability within the four standard subtypes. The two loops of the new model are shown in an expanded format. Well-described variability, which distinguishes each subtype, is indicated.

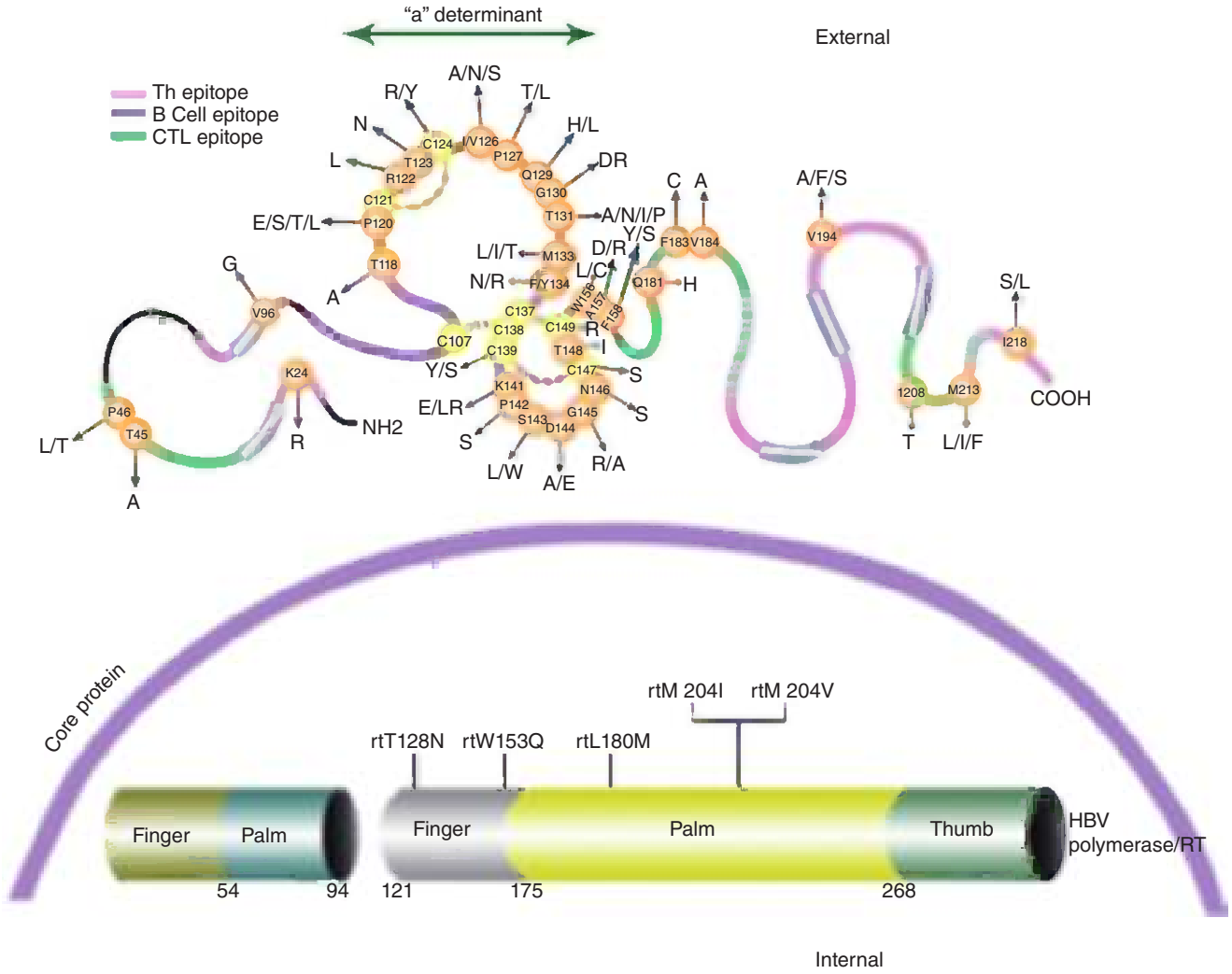

Figure 8.2 Schematic of the HBsAg immune epitopes and MHR (including *a* determinant). Epitope numbering based on the proposed antigenic epitopes dedicated from Table 8.5. Cysteine residues are shown as clear circles, and disulfide bridges are indicated as dashed lines. The changes selected by antibodies (natural, used in assays, and vaccine induced) that occur within, upstream, and downstream of the *a* determinant are indicated. The alpha helices are shown as cylindrical structures. (Color plate 8.1)

Similarly, residue 160 seems to be central to *w/r* specificity: *adw* and *ayw* have K at this position, whereas *adr* and *ayr* have R (Figure 8.1). 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 (T) and *r* isolates have isoleucine (I) at this position (Figure 8.1). 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 (see the "Diagnostic issues" section). 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 (Figure 8.2). Subtype-related variation can alter binding of antibodies to regions linearly distant. Because of this interleaved and complex nature of the antigenicity of HBsAg, we will not just refer to the *a* determinant herein but also consider the MHR in its entirety. Of course, subtypes have become less important with the advent of cheap large-scale sequencing. Genotypes are now the primary classification of HBV variability; nevertheless, as shown in Figure 8.3, some subtypes can be found within more than one genotype. Genotypes of HBV are discussed in more detail in many reviews.

Genotype	Subtype	Amino acid sequences 120					180
A	adw2	P C K T C T T P A Q G	N S M F P S C C C T	K P T D G N C T C I	P I P S S W A F A K	Y L W E W A S V R F	S W L S L L V P F V
	ayw1	– – R – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –
B1	adw2	– – – – – – – – – – –	T – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –
2	ayw1	– – R – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –
C	adr	– – K – – – I – – – –	T – – – – – – – – –	– – S – – – – – – –	– – – – – – – – – R	F – – – – – – – – –	– – – – – – – – – –
	ayr	– – R – – – – – – – –	– – – – – – – – – –	– – S – – – – – – –	– – – – – – – – – R	F – – – – – – – – –	– – – – – – – – – –
	adrq-	– – K – – – – – – – –	– – – – – – – – – –	– – S – – – – – – –	– – – – – – – – V R	F – – – – – – – – –	– – – – – – A – – –
D	ayw2	– – R – – – – – – – –	T – – Y – – – – – –	– – S – – – – – – –	– – – – – – – – G –	F – – – – – – A – –	– – – – – – – – – –
	ayw3	– – – – – M – T – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – G –	F – – – – – – A – –	– – – – – – – – – –
E	ayw4	– – R – – – – L – – –	T – – – – – – – – S	– – S – – – – – – –	– – – – – – – – G –	F – – – – – – A – –	– – – – – – – – – –
F	adw4	– – K – – – – L – – –	T – – – – – – – – S	– – S – – – – – – –	– – – – – – – L G –	F – – – – – – A – –	– – – – – – – Q – –
G	adw2	– – – – – – – – – – –	– – – Y – – – – – –	– – S – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –
H	adw3	– – K – – – – T – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –	– – – – – – – – – –

↓ d/y (residue K/R, position 122) ↓ w/r (residue K/R, position 160)

Figure 8.3 Amino acid residues 120 to 180 of HBsAg, specifying HBV subtypes. Arrows indicate d/y and w/r determinant. Residues for different subtype specifications are underlined.

Surface variants

The World Health Organization (WHO) aims at controlling HBV infection worldwide and reducing the burden of HBV-related chronic liver disease, cirrhosis, and hepatocellular carcinoma (HCC) by integrating hepatitis B vaccination into routine infant (and possibly adolescent) immunization programs. Hepatitis B vaccination is safe and effective, although breakthrough infections occasionally occur in vaccinees [5, 16, 17]. Following natural infection, the appearance of anti-HBs neutralizing antibodies is usually associated with patient recovery.

The G145R mutation, as explained in this chapter, and other less prevalent *a* determinant mutations that allow for infection in successfully vaccinated persons have been reported with various frequencies from countries that implemented Expanded Programs on Immunization (EPIs) (Tables 8.1 and 8.2). Geographical differences, the time interval that has elapsed since launching the immunization program, and particular vaccination strategies may play a role in the discrepancies between results. For example, the prevalence of HBsAg *a* determinant variants in Singapore was reported to be 39% [18], compared to 22% in Taiwan [19], 12% in the United Kingdom [20], and 0% in South Africa (Tables 8.1 and 8.2) [21–26].

In addition to HBV vaccination of infants born to HBsAg-positive mothers, immunoprophylaxis in liver transplantation recipients exerts a strong selective pressure on HBV, which in some cases induces escape mutations within the *a* determinant region [27–32]. Similar escape mutations in the *a* determinant also arise in chronic patients who have not been immunized but have cleared the virus spontaneously; these patients exhibit active viral replication and liver disease after seroconversion from HBsAg to anti-HBs (discussed further in this chapter) [33–35].

Variants associated with active immunization

The emergence of variants of HBV can be due to selection pressure associated with extensive immunization in endemic areas, 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 [36]. The first published study of a vaccine escape variant 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 was at the time >5% [37]. 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) amplification and sequencing of the MHR [5]. One case was a child of an HBeAg-positive mother who was given HBIG 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

Table 8.1 Prevalence of *a* determinant mutations following EPI worldwide.

Author	Country	Number of samples	HBsAg prevalence	% "a" determinant mutants
Basuni *et al.* [21]	Pacific	784	55 (7%)	0.12
Carman *et al.* [5]	Italy	1590*	44 (2.8%)	0.06
Daavalkhan *et al.* [78]	Mongolia	593	59 (9.94%)†	1.68
Dong *et al.* [177]	China	5407	82 (1.5%)	13.7
Fortuin *et al.* [178]	Gambia	358	32 (9%)††	0.27
Ho *et al.* [114]	Taiwan	1812	64 (3.5%)	0.05
Ho *et al.* [42]	Taiwan	2305	101 (4.3%)	0.56
Hino *et al.* [26]	South Africa	1213	4 (0.3%)†††	0
Huang *et al.* [22]	China	80 000	15 (0.018%)	0.006
Hsu *et al.* [45]	Taiwan	1200®	115 (9.6%)	0.67
		1134	52 (4.6%)	0.88
		1515	20 (1.3%)	0.59
		1357	9 (0.7%)	0.22
		7234	38 (0.5%)	0.1
He *et al.* [43]	China	340	42 (12.3%)	1.17
Ijaz *et al.* [179]	Mongolia	369	38 (9%)††	0.27
Kato *et al.* [180]	Japan	251*	251	0
Lee *et al.* [19]	Taiwan	455*	27 (5.9%)	1.31
McMahon *et al.* [181]	United States	1578	16 (1%)	0.19
Mele *et al.* [182]	Italy	522*	17 (3.3%)	0.19
Mu *et al.* [183]	Taiwan	84***	4 (4.8%)	1.19
Ngui *et al.* [20]	United Kingdom	488*	20 of 321 (6%)	0.62
Oon *et al.* [18]	Singapore	345*	41 (12%)	4.6
Su *et al.* [23]	China	461*	7 (1.5%)	0
Theamboonlers *et al.* [24]	Thailand	2229	51(2.2%)	0.49
Tsatsralt-Od [25]	Mongolia	581	47 (8%)	1.7
Xu *et al.* [184]	China	2919	60 (2.1%)	0.13

*Born to HBsAg-positive mothers.
®Before HBV vaccination began.
***18 (21.4%) born to HBsAg-positive parents.
†11 (19.3%) born to HBsAg-positive mothers.
††Anti-HBc-positive.
†††HBsAg-negative but HBV DNA-positive.

a third. The sample taken from the child at 5 years had a sG145R substitution, and the mother had the wild-type sequence (sG145). sG145R and other mutants are discussed further in this chapter.

In many studies, mutations in vaccinated children were preferentially located in the *a* determinant [38, 39], in contrast to the more randomly located mutations in unvaccinated children [26]. In general, aa substitution in the second *a* determinant loop, including positions 144 and 145, is more often associated with anti-HBs immune pressure than mutations in other epitopic domains of the MHR [40]. On the other hand, mutations in the second *a* determinant loop are unusual in the absence of anti-HBs pressure [34]. Variants may emerge *de novo* or are present as a minor quasi-species population in maternal serum, and after transmission to the newborn, they are selected under the immune pressure generated by the postexposure prophylaxis with HBIG and hepatitis B vaccine. In this scenario, *in utero* transmission and high serum levels of HBV DNA in pregnant women could be more important in failure of postexposure hepatitis B immunization [41–43], together with host genetic factors [44]; improper administration of HBV vaccine; timing of the vaccine, particularly that at birth [5]; type of vaccine (as stated in this chapter, the prevalence of *a* determinant mutants is significantly higher in children who received plasma-derived vaccines than those who received recombinant vaccines) [45]; and a break in the vaccine cold chain [46].

There is little evidence at this stage that transmission is occurring horizontally, especially among vaccinated individuals. Further, experiments in chimpanzees reveal that the "standard" vaccine is protective against an sG145R inoculum [47]. Other mutations located beyond the MHR (Tables 8.2 and 8.3) 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 [21, 48–50]. Chen and Oon [51] described a close association of vaccine-related HBsAg mutants with the *adw* subtype in Singapore compared with subtypes *adr*, *ayw*, *and ayr*. Collectively, data from different studies

Table 8.2 Prevalence of MHR escape mutants found in expanded programs of immunization (EPIs) with (purple color) and without HBIG (white color).

Country [Reference]	Number of Samples	Total number of escape mutants	Number of G145R mutations	Number of mutations in first *a* determinant loop	Number of mutations in second *a* determinant loop	Number of mutations outside *a* determinant	HBIG	Type of Vaccine
Italy [5]	1590*	1	1	—	—	—	+	P
Taiwan [114]	*1812*	*1*	*1*	—	—	—	+	*NA*
Taiwan [42]	*2305*	*13*	*5*	*9*	*3*	—	+	*P*
Taiwan [185]	1134	10	3	3	1	1	+	NA
Taiwan [186]	1515	9	1	2	1	—	+	P
Taiwan [187]	1357	3	2	1	—	—	+	P&R
Taiwan [188]	7234	7	2	1	1	1	+	NA
Taiwan [121]	22*	5	2	2			+	P
Japan [180]	251*	1	—	—	—	3	+	P &R
Taiwan [19]	455*	6	4	2	1		+	P&R
Japan [189]	29*	1	1	—		1	+	P
Italy [182]	522*	1	—	2			+	P&R
Taiwan [183]	84***	4	1	1	1	5	+	R
United States [190]	1092	22	17	1	4	2	+	NA
United Kingdom [20]	488*	2	—	2	2	—	+	R
Singapore [18, 191]	345*	5	5	—		5	+	P
Singapore [115]	8*	4	4	—		—	+	P
Thailand [192]	11*	7	1	3	1	2	+	R
China [23]	461*		—	No		2	+	NA
Iran [123]	75*	10	10	—		—	+	R
Pacific islands [21]	784	8	—	1		9	—	P
Mongolia[78]	593**	17	—	4	3	10	—	P+
China [177]	5407	6	3	2		1	—	NA
Gambia[178]	358	1	—		1	—	—	P
South Africa [26]	1213	1	—	—		1	—	P
China [22]	80 000	5	1	4		—	—	NA
China [43]	340*	4	1	2	1		—	P
Mongolia [179]	369	1	1	—		—	—	P&R
Thailand [24]	2229	4	1	1	3	3	—	NA
Mongolia [25]	581	17	—	5	1	10	—	P&R
India [193]	68*	2	—	5	3	—	—	NA
China [184]	2919†	4†	3	1		11	—	P
China [194]	404	1	1	—		—	—	P
United States [181]	1578	3	1	1	1	1	—	P

P: Plasma-derived vaccine; **R**: recombinant vaccine; **NA**: not identified in the study;
*: born to HBsAg-positive mothers;
**: 11 (19.3%) born to HBsAg-positive mothers;
***: 18 (21.4%) born to HBsAg-positive parents;
†:124 were HBsAg – ve, with sequencing done on these isolates.

indicated that the presence of vaccine escape mutants can be considered rather negligible with respect to vaccination programs worldwide.

Variants associated with passive immunization

Vaccine setting

For high-risk infants born to HBsAg-positive mothers, the maternal-neonatal transmission of HBV and the subsequent development of chronic hepatitis B in infected children plummeted when HBIG was given to them with the first dose of the HBV vaccine within 24 hours after birth, a regimen that is 85% to 95% effective in preventing HBV infection and the chronic carrier state [52, 53].

The role of HBIG as the main selective pressure for *a* determinant variants can be inferred from several studies that have demonstrated the occurrence of these variants in different parts of the world when both HBIG and vaccine were given (Table 8.2), as opposed to vaccine alone ($p < 0.0001$). Mutants are selected in these infants

Table 8.3 The most frequent medically selected amino acid changes in HBsAg.

Amino acid position	Wild-type	Mutant	Cause
4	I	L	V
24	K	R	V
45	T	A	V
46	P	L/T	V
76	C	F	V
96	V	G	V
118	T	A	V
120	P	E/S/T/L	V-L-HBIG
122	R	L	V
123	T	N	HBIG
124	C	R/Y	HBIG
126	I/T	A/N/S	V-HBIG
127	P	T/L	V
129	Q	H/L	V-HBIG
130	G	D/R	L-HBIG
131	T	A/N/I/P	V
133	M	L/I/T	V-HBIG
134	F/Y	N/R	HBIG
139	C	Y/S	V-HBIG
141	K	E/I/R	V
142	P	S	V
143	S	L/W	V-HBIG
144	D	A/E	V-HBIG
145	G	R/A	V-L-HBIG
146	N	S	V
147	C	S	V-HBIG
148	T	I	V
149	C	R	V
156	W	L/C	V-HBIG
157	A	D/R	V-L
158	F	Y/S	V-L
161	F	H/L	ENT
164	E	D	L/LdT/Fam
172	W	*/L/F	ADV/TDF
173	L	F	ADV/TDF
175	L	S/F	L/ENT
176	L	V/*	ENT
179	F	Y	L
181	Q	H	V
182	W	*	L/ ADV/TDF
183	F	C	V
184	V	A	V/ADF
193	S	F/L	L/ENT
194	V	A/F/S	V/ENT
195	I	M	L/LdT
196	W	S/L	L/LdT
198	M	I	L
204	N	S	V
207	Q	R/S	L
208	I	T	V
213	M	L/I/F	V
218	I	S/L	V

The causal selection pressures are as follows. V: vaccine; L: lamivudine (as antiviral therapy for either chronic patients or liver transplantation); LdT: telbivudine; ADF: adefovir; TDF: tenofovir; ENT: entecavir; Fam: famciclovir; HBIG: hepatitis B immune globulin;
*: stop codon mutation.

because of the ongoing elimination of the dominant wild-type virus population through HBIG action or the development of vaccine-induced antibodies.

Despite active and passive immunoprophylaxis soon after birth, in high-risk infants born to mothers with very high levels of viremia there is still a high HBV carrier rate of 5–10% [54]. This failure has been most frequently linked to inadequate neutralization effects of HBIG against HBV (wild-type infection) or the emergence of viral immune escape variants of HBV carrying mutations in the *a* determinant [31, 55, 56]. In the latter case, most of the mutations involve substitutions of one or more aa in the *a* determinant (Figure 8.2). Some of these mutations result in decreased binding to anti-HBs and may therefore escape neutralization by HBIG. However, there are no data to support the emergence of such variants after HBIG administration alone in children (without vaccine administration), and also injection of HBIG in carrier mothers did not increase the mutation rate in infants who received the HBV vaccine [57]. Some of these mutations arose after HBIG administration alone (aa 123 and 134), whereas in a majority of studies, combinations of variations within the surface protein have been reported in the presence of either HBIG–vaccine or HBIG–antivirals (in liver transplantation settings) (Table 8.3) [58]. In liver transplantation cases, it is not clear yet if the emergence of mutations is directly related to HBIG dosage. Most studies report *a* determinant mutations even after prophylaxis with high-dose HBIG [59–61].

Transplantation setting

Patients with end-stage liver disease attributable to HBV often undergo transplantation and are treated with human monoclonal anti-HBs or HBIG 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 [28, 29, 40, 61–64]. In one reported series of transplantation patients treated with monoclonal antibody [65], three of six patients exhibited reactivation of HBV in the graft. sG145R was seen alone in one patient and as a mixture with sG145 in a second patient; other mutants (sTl40S and sC124Y) were also present simultaneously as a quasi-species in the latter patient. In the third patient, sC137Y was observed to emerge. It is interesting that loss of cysteines has also been observed after vaccination and HBIG therapy (both discussed in this chapter). There are no natural strains of HBV without these cysteines that are proposed to be involved in the loop structure of the MHR.

That monoclonal antibody can select HBsAg mutants is not surprising. However, polyclonal antibody in the form of HBIG can also lead to selection of different

strains after therapy [28, 29, 31, 66–69] (Table 8.3). On the withdrawal of hyperimmune globulin, the mutants are often lost and the wild type again becomes dominant [40]. Only one study has controlled for immunosuppression in the absence of HBIG pressure. The sG145R strain was detected in four of these five studies, but not in all cases. HBIG does put selection pressure on HBsAg. In posttransplant patients who received HBIG, mutants persisted despite the withdrawal of HBIG administration for 4–5 years [62]. The variants were not present as a minority species before therapy, and not all the mutations were within the MHR (although the majority were). 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 HBIG therapy [70], implying that either these strains are less replication efficient or they are viral dead ends.

Antigenic characterization

Immune escape at the B cell level

The direct escape from protective anti-HBs antibodies is caused by mutations in B cell epitopes that diminish or abolish viral recognition by anti-HBs [16]. The mutant virus replicates efficiently, implying that the amino acid substitution does not alter the binding of virions to the liver cell.

a determinant (aa 100–124)

The importance of mutations in residues 100–117 is unknown, but amino acid substitution at these codons lies within the B cell epitope region (Figure 8.2). However, such mutations appear reactive in different diagnostic assays, implying that these residues are not essential for antibody binding (Table 8.4) [71–73]. The region between aa 118 and 123 was identified as a hot spot for insertions by investigators, illustrating its immunological importance [8, 74–76]. Changes in this region may alter the conformation of the *a* determinant and abolish binding to anti-HBs [75]. Antigenicity profiling showed an appreciable decrease in hydrophilicity due to a threonine-to-alanine conversion at residue 118. This in turn may abolish anti-HBs binding leading to inability to detect HBsAg [77]. Codon 122, the *d* and *y* subtype determinant, is rarely mutated after HBIG administration. Mutations have been detected after immunization with vaccine alone (Table 8.3) [25, 78]. Monoclonal mapping studies have shown that loss of proline at aa 120 affects the antibody-binding pattern through alteration of the three-dimensional epitope presented on the second loop (aa 139–147) of the *a* determinant [79, 80].

a determinant (first loop, aa 124–138)

Replacement of C121 or C124 with serine results in mutant proteins that show diminished binding to both monoclonal antibodies and a polyclonal serum, indicating that a structural change had taken place [81]. Amino acid substitutions at position 129 represent an altered hydrophobicity profile, which would be expected to affect the secondary and tertiary structures of the antigen, of which Arg 129 showed the lowest binding capacities [82]. T126 seems important for the *w* subdeterminant (Figure 8.3) and affects the recognition properties of the surface antigen [10]. This residue may similarly contribute to the stable establishment of disulfide bonds, which are crucial to the formation of the double-loop structure of the *a* determinant [56]. However, amino acid substitution of this residue caused minimal changes in antigenicity [83]. Residue 127 is essential for subtype specificity (Figure 8.3), and mutation at this site may lower the reactivity to the anti-HBs induced by wild-type HBsAg [79, 80].

a determinant (second loop, aa 139–148)

In peptide competition assays, the domain between aa 141 and 145 was shown to be essential for the binding of antibodies [84, 85]. The amino acid changes at positions 147 and 149 involve cysteine residues considered to be essential for intramolecular disulfide bridging (Figure 8.2). Codon 148 is within residues 140–149, which have been shown to have the greatest local hydrophilicity [86], contain the predominantly antigenic *a* determinant, and may constitute a major neutralizing epitope [10]. The most relevant variants that have been identified to emerge during vaccine and HBIG therapy include C139/SY, S143L/W, D/E144G/A, G145R/A, and C147S (Table 8.3 and Figure 8.2). D144G and sG145K were previously described as a cause of reinfection after liver transplantation in single patients [31]. The replacement of lysine 141 by glutamic acid in the variant strain results in a residue with negative polarity, which may have a significant effect on the secondary structure of the protein [87].

The nucleotide sequence at codon 144 (nucleotide number 432) can be GAT or GAC depending on the HBV genotype or subtype. A change from GGA to AGA at codon 145 that results in a glycine-to-arginine substitution will create a premature stop codon (TAG) in the polymerase if the third nucleotide at codon 144 is T [5]. The glycine-to-arginine substitution at codon 145 usually accompanies GAT at codon 144 [29]. Thus, in mutational analysis, the detected G145R mutation would not result

in premature termination of the P gene. Therefore, the worldwide detection of the 145 glycine-to-arginine mutation may be explained by the development of compensatory changes that permit its occurrence in all HBV genotypes, in contrast to the precore stop codon mutation (A1896), which is restricted to geographical areas where the predominant HBV genotypes have T at nucleotide 1858 [88, 89].

In general, replacement in the presumed second *a* determinant loop, including amino acid positions 144 and 145, is more often associated with anti-HBs immune pressure than mutations in other epitopic domains of the MHR. Mutations at aa positions 120, 143, 144, and 145 can result in poor reactivity with *d/y* subtyping monoclonal antibodies (Table 8.4) [15, 80]. 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. Subtype-related variation can also alter binding of antibodies to regions linearly distant [15, 80]. According to Ogura *et al.* [90], mutations within the *a* determinant during the natural course of infection are predominantly observed within the first loop (aa 127–138), whereas those induced under immune pressure due to active and/or passive immunization are more frequently observed within the second loop (aa 139–147).

Changes outside the *a* determinant

Initially, changes occurring outside the *a* determinant region were reported in immunized infants born to HBV-carrier mothers [91–93] (Tables 8.2 and 8.3 and

Table 8.4 Variation in sensitivity of HBsAg detection by various assays; results obtained with serum samples and/or recombinant constructs.

AA position	Reactivity (%)	Number of HBsAg assays	Reference
T118A	19 (100%)	19	[71, 74]
P120G/S/L/T	17 (68%)	25	[71, 73, 110, 195, 196]
C121S	1 (14.2%)	7	[196]
K/E/R122I	21 (67.7%)	31	[107, 196, 197]
T123N	23 (63.8%)	36	[100, 107, 196–199]
T123A	3 (100%)	3	[200]
C124R/Y	6 (60%)	10	[107, 197, 201]
T125M	3 (100%)	3	[73]
T126S/N	35 (100%)	35	[71, 116, 195, 198, 199, 202]
P127T/L	4 (80%)	5	[73, 203]
A128T	WEAK	3	[73]
G/Q129H	39 (97.5%)	40	[71, 116, 198–200, 202]
G130D/R/N	6 (60%)	10	[99, 195]
T131A/N/I/P	28 (84.8%)	33	[71, 74, 99, 107, 201]
M133I/L/T	49 (92.4%)	53	[71, 100, 108, 116, 195, 198, 199, 202, 203]
Y134S/L/N	9 (100%)	9	[107, 195]
P135S	7 (77.7%)	9	[71]
C137W	3 (75%)	4	[107]
C138R	1 (50%)	2	[201]
C139Y	3 (75%)	4	[107]
T/L/K140I/Q/E	11 (73.3%)	15	[71, 107, 201]
P142L/S	19 (76%)	25	[71, 107, 203]
143L	2 (%) / 1 weak	3	[195]
D144E/A/G	49 (80%) / 2 weak	61	[63, 71, 74, 100, 107, 116, 197–199, 202]
G145R	37 (54.4%) / 10 weak	68	[71, 99, 100, 116, 195, 196, 198–202, 204, 205]
G145A	9 (75%)	12	[71, 203]
G145K	6 (75%)	8	[108, 199]
C147S	3 (75%)	4	[107]
T123N/T143S	3 (60%)	5	[198]
T126S+ G145R	1 (20%) / 4 weak	5	[110]
T131N, G145R	0	4	[99]
G130D, G145R	0	4	[99]
P142L/S, G145R	48 (76%) / 8 weak	63	[71, 107, 116, 197, 198, 200, 202]
N/D,E144A, G145R	9 (42.8%) / 3 weak	21	[71, 116, 198]
T126S, G145R	12 (66.6%)	18	[71, 198, 202]
Y100C, P120T	1 (14.2%) / 3 weak	7	[100]
I195M	2 (100%)	2	[206]
W196 Stop	1 (50%)	2	[206]

Figure 8.2). Interestingly, some of these mutant proteins had reduced binding to monoclonal antibodies against the *a* determinant (Table 8.4), and some were most likely transmitted vertically as they were isolated from both infant and maternal serum. Their role in vaccination failure is currently unknown. Likewise, multiple aa changes in surface-exposed regions of HBsAg, including mutations and/or deletions upstream and downstream of the *a* determinant region (Table 8.3 and Figure 8.2) [94–96], abolished reactivity between the monoclonal and polyclonal anti-HBs diagnostic antibodies and the *a* loop epitope cluster. The amino acid changes were also not recognized by vaccinee sera (Table 8.4) [74, 76, 97–99]. Some of those variants were within the major hydrophilic loop of HBsAg, but mutations at positions 183 and 184 were also found; a phenylalanine-to-cysteine mutation at position 183 (F183C, outside the major loop) decreased the binding of mutant HBsAg to *a* determinant–specific monoclonal antibody (Table 8.3) [93].

Immune escape at the T cell level

The majority of mutations upstream and downstream of the *a* determinant that were found to be potentially responsible for vaccine breakthrough [92–94, 99–102] tended to cluster within known Th and/or CTL epitopes of the surface protein [103–106] (Figure 8.2 and Table 8.5). At least four regions within HBsAg contain epitopes for MHC class II–restricted CD4+ T cells (Table 8.5) [104, 105]. Further, aa substitutions have been found in residues downstream (38 to 98) and upstream (between 164 and 215) of the MHR region of native and/or recombinant surface proteins with different binding capacity to antibodies (Table 8.4) [100, 107–110].

The effects of HBsAg T cell epitope variants on the cellular immune response have been studied in individuals vaccinated against HBV by Bauer *et al.* [111]. 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; several class I–restricted CTL epitopes have been identified across the surface protein (Table 8.5). Schirmbeck *et al.* [112] showed in H-2b mice that 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. These surface gene changes outside the region of the *a* determinant deserve further investigation.

Table 8.5 Proposed antigenic epitopes within HBsAg.

Sequence	Cell subsets	HLA restriction	Reference
100–160	B	Not HLA restriction	[207]
19–28	Th	Class II	[104]
28–51	CTL	Class I HLA-A2	[106]
80–98	Th	Class II	[104]
171–179	CTL	Class I	[103]
175–184	CTL	Class I HLA-A2	[105]
186–197	Th	Class II	[105]
206–215	CTL	Class I HLA-A2	[105]
215–223	Th	Class II	[104]

HBsAg mutants and clinical outcomes

Two concerns arise from the escape phenomenon: (1) mutant HBV may infect individuals who are anti-HBs-positive after immunization (vaccine escape) and permit subsequent horizontal transmission [113–115], and (2) there may be failure in HBsAg detection (diagnostic escape) [55, 116], leading to transmission through donated blood or organs.

HBsAg mutants in chronic carriers

Little is known about the natural history of HBsAg variants. Some reports suggest that patients with pure or mixed *a* epitope variants have lower alanine aminotransferase (ALT) levels compared with those with wild-type HBV DNA [117]. Asian-Indian patients with HBsAg variants had a prolonged illness in the form of anicteric chronic hepatitis. These patients had a higher frequency of asymptomatic cirrhosis and HCC than those with wild-type HBV. However, in some reports, S-gene mutants have been associated with an aggressive or worsening clinical course [118, 119]. Further studies are needed to establish whether specific variants are associated with more severe disease or HCC.

A number of escape-variant viruses with *a* determinant mutations have been reported to be replicating in spite of the presence of anti-HBs during the course of chronic infection [5, 27–29, 31–34, 43, 83, 120–123]. This phenomenon may result from a variety of reasons, including:

1. The vaccinated individual had a compromised immune response in early childhood resulting in low titer antibody.

2. Infection *in utero* has rendered postnatal vaccination ineffective.

3. Infection with HBV mutants or simultaneous infection with wild-type and mutant viruses: These mutants may represent a small percentage with a predominantly wild-type strain either before the onset of antibody-mediated immune pressure, and thus selected, or emerging under conditions unfavorable for wild-type virus, which could not be detected by the use of sequencing analysis.

4. Mutations within the surface gene are responsible for HBsAg detection failure in the mother (diagnostic-escape mutants).

5. Nonneutralizing anti-HBs is directed solely against epitopes outside the *a* determinant, such as d/y or w/r subtype determinants [83].
6. Low expression of HBsAg is below the sensitivity of standard serologic assays [113].
Chronic infection of children may go undetected for decades but result in a risk of liver disease and 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 favor of wild-type 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). The major question that arises is whether HBsAg variants cause HCC. Although some of the HBsAg mutants (including G145R) may play a role in the development of HCC [121], Singapore children who carried the vaccine escape HBsAg mutations appeared normal 13 years after these mutants were detected, with normal liver function and alpha-fetoprotein level [91]. Further molecular studies in large cohorts of patients with long-term clinical and laboratory follow-up are warranted to better understand the biological basis and significance of such mutations and to clarify the possible role exerted by these infections in the outcome of liver disease and the development of HCC.

Transmission

Past 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 or 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 [115] and D144A [124]. Amino acid 144 replacement is frequently encountered with or without G145R [31, 125, 126].

Apart from vaccinated individuals, HBsAg variants have also been reported in blood donors (positive for anti-HBc alone) and after interferon (IFN) therapy [38]. 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.

The safety issues related to blood or tissue donation from an "HBsAg-negative" donor and the risk of transmission are of great concern. There is fear that escape mutants may be spread by blood transfusion, since HBV DNA detection by nucleic acid amplification technology (NAT) is not mandatory for blood donor screening in many countries, especially in those areas where the prevalence of escape mutants is expected to be high. Earlier studies clearly showed that transfusion of blood containing HBsAg was associated with the development of posttransfusion hepatitis B [127–129], and cases of HBV infection were identified after transfusion of HBsAg-negative blood [130–132]. Moreover, it is well known that mutants of HBsAg are able to cause infection and horizontal transmission despite the presence of anti- HBs [71, 133–136].

Studies in animals and humans have proven that the G145R mutant can infect the host without the help of wild-type virus [47, 137]. This suggests that the native conformation of the second loop is not essential for at least the entrance of the virus in the cell. As a consequence it may be argued that second loop–related antibodies may not be protective on their own, although they may lower the viral load in the circulation by the formation of aggregates that are easily opsonized [134].

HBsAg mutants in liver transplantation settings

Despite the use of HBIG as prophylaxis in transplant patients for more than two decades, the mechanisms by which HBIG protects the liver transplantation against HBV re-infection, as well as its failure against HBV recurrence after liver transplantation, are not fully understood. Two types of recurrence have been proposed in these patients. Early recurrence (less than 8weeks) is usually due to wild-type virus and probably indicates an initial inadequate binding of anti-HBs to large antigenic loads in patients with active replication (HBeAg and HBV DNA positivity) [138]. Late recurrences are usually caused by the emergence of mutations caused by the selection pressure of HBIG on the surface protein. Immunosuppression plus HBIG after liver transplantation result in a high selection pressure for variants with combinations of envelope and polymerase gene mutations [29, 31, 139]. However, the advent of nucleos(t)ide analogs that can be used in combination with HBIG, has resulted in the elimination of the recurrences and risks of selection of surface variants.

Diagnostic issues

As documented in a number of reports, HBsAg variants could not be detected by some diagnostic assays (diagnostic escape) [100, 116, 140]. Different studies assessed the performance of commercial assays using native

and/or recombinant proteins with mutated positions upstream and downstream, as well as within the *a* determinant (Table 8.4). Interestingly, the majority of HBsAg tests were able to detect the mutants. However, the performance of most assays was impaired significantly by amino acid changes in residues 120–124, 130–133, 142, and 144–145 (Table 8.4). The sG145R has also been shown to exhibit various degrees of altered binding in different commercial assays [74, 141, 142]. Accordingly, constructing HBsAg variants, particularly the sG145R, in yeast led to markedly reduced binding by monoclonal and polyclonal antibodies [143, 144], 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 [100, 145], albeit at reduced sensitivity (Table 8.4). 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.

Surprisingly, mutations up- and downstream of the MHR in different studies have been found to be potentially responsible for HBsAg undetectability [74, 76, 94, 98, 123]. Such mutations were found to be located within known Th or CTL (or both) epitopes of the surface protein (Tables 8.4 and 8.5) [103–106].

Most companies have either assessed their current diagnostic assays or produced new versions in order to better detect a range of variants. 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 each assay's 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. Several studies have compared the sensitivity of modern assays for the detection of HBsAg variants, as shown in Table 8.4.

A wide variety of commercial assays are available for the detection of HBsAg. Some use polyclonal anti-HBs, and others use multiple monoclonal antibodies. There are accumulating data from Europe, Asia, and Africa about HBsAg variants that are not recognized by either monoclonal or polyclonal antibodies specific for wild-type group *a* determinant, but are positive by PCR [22, 80, 146–149]. Although numerous studies have examined "occult HBV infection" in immunocompetent patients, the precise prevalence of this clinical entity remains difficult to define. The frequency of diagnosis depends on the relative sensitivity of both HBsAg and HBV DNA assays as well as on the prevalence of HBV infection in the study population. An example of this situation has been obtained from a recent study from Iran, a low-HBsAg-endemicity area. Twenty-one out of 75 (28%) sera from HBsAg-negative children born to HBsAg-positive mothers who received a complete regimen of vaccine and HBIG immunoprophylaxis and had adequate levels of anti-HBs (and some isolated anti-HBc) have been shown to be positive for HBV DNA by real-time PCR [123]. Clearly, there are two possible explanations for such lack of HBsAg positivity: a very low level of antigen (all had HBV DNA levels lower than 10^4 copies/mL), or an antigenic variant (10 of 14 available sera contained the sG145R variant).

Other studies have shown that *a* determinant variants were present in "anti-HBc-only"–positive individuals [125, 142, 149], or patients who were HBsAg- and anti-HBs-positive simultaneously. A good example of the last situation [150, 151] is an sD144N mutant, with additional changes at aa 126, 131, and 133. The patient also had a large deletion and other mutations in the pre-S1. Another series of cases with coexisting HBsAg and anti-HBs came from Japan [34]. Five cases had sR145, sN126, or an eight amino acid insertion in the small 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. Interestingly, the presence of both HBsAg and anti-HBs positivity has been reported in 10–25% of HBeAg-positive patients [152].

However, not all series of HBsAg- and 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 [37]. Patients who have cleared their infection by classical criteria (anti-HBc and anti-HBs-positive) [153], usually do not have viremia by PCR. An exception is within the first year after successful interferon (IFN) therapy [153]. 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 described in this chapter, 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 [33]. 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 one. sR145 as well as an insertion of N and T between aa 123 and 124 were found. Similar insertions at the same site have been seen

in Chinese and Japanese patients [34, 75]. Thus, all the insertions have been found in Far Eastern people and all within the first loop of the *a* determinant (Figure 8.2). 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 been described in an agammaglobulinemic patient, who had in addition substituted the subtype determining amino acid at position 122 with isoleucine [154].

Variants associated with antiviral therapy

Due to the overlapping nature of the polymerase and surface protein–encoding regions, reciprocal influences caused by compensatory mutations are inevitable; thus, some of them are associated with alterations in neutralization epitopes within the HBsAg [155, 156]. The overlapped areas are encompassed within the distal part of the A–B interdomain as well as the B domain of the reverse transcriptase (RT) (residues 80–236), and residues 72–228 of the surface proteins. In this regard, common surface escape mutations have been reported to occur between amino acid residues 120 and 208 (Figure 8.2) [90, 156, 157]. However, amino acids 157–207 of HBsAg contain most of the S-gene mutations observed in the presence of lamivudine (Table 8.3). The classic lamivudine resistance mutation 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 (Figure 8.2). These YMDD mutations also result in amino acid substitutions in HBsAg. Moreover, drug-resistant variants have been found to be present along with surface protein substitutions (as compensatory mutations, in the distal part) with other nucleos(t)ide analog antiviral agents such as adefovir, telbivudine, famciclovir, entecavir, and tenofovir (Table 8.3). Torresi *et al.* investigated the role of lamivudine in selecting HBV mutants with antigenically altered HBsAg using pooled individual vaccine sera. The most common variant, sG145R, was found to enhance the replication capacity of the YMDD mutant, because the nucleotide change responsible for the sG145R mutation generated a W153Q mutation in the polymerase gene [135]. Similarly, combinations of envelope (sG145R or sP120T) and polymerase (rtL180M plus M204V) mutations showed increased *in vitro* viral DNA levels in both intracellular HBV core particles and extracellular virions during sequential HBIG and lamivudine therapy in the post-transplant setting [158]. Therefore, mutations in the surface region following HBIG–nucleoside analog combination therapy may cause mutations in the polymerase that may reduce the efficacy of antivirals, and, conversely, mutations in the polymerase occurring after HBV prophylaxis for liver transplantation or other nucleos(t)ide analog treatment may cause mutations in the S gene implying simultaneous selection of polymerase and surface escape mutants that alter HBIG binding to HBsAg [159, 160]. Torresi *et al.* found that vaccine sera had reduced binding to HBsAg mutants selected during lamivudine treatment to a varying degree. In an EIA, binding of HBsAg mutants was reduced relative to binding to the wild-type HBsAg [135]. However, the use of entecavir and tenofovir in nucleos(t)ide-naïve patients, which have a high genetic barrier, has restricted the chances of emergence of such variants to virtually nil.

Pre-S and S variants and chronic hepatitis

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 [70, 161–166]. A plethora of nucleotide substitutions and deletions have been observed in this region. There is evidence to suggest that these mutants are more frequently found in genotypes C and/or B than in other genotypes [166, 167]. Serum HBV DNA is not always detected by nucleic acid hybridization. A deletion of 61 amino acid residues from the carboxy-terminal half of the pre-S1 domain and of the promoter for the messenger RNA (mRNA) for the middle and small envelope proteins has been reported. 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 [168]. 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 transcomplemented by co-transfection of the S ORF, has been observed. Retention of S protein–containing particles in vesicles and relative overexpression of pre-S1 occur [169]. Cytopathic effects of pre-S1 mutants have been shown in several studies with "ground glass–like" hepatocytes in transgenic mice and in ducks [170, 171]. However, a ground glass–like appearance also has been shown in samples with pre-S2 mutants [172]. Cells transfected with plasmid constructs bearing these deletion mutations were often destroyed, implying that the effects of these mutations were directly cytotoxic. There is also an assumption that pre-S1 regulates viral cccDNA levels via negative feedback [173]

redirecting the nucleocapsid into the virion formation pathway [174]. 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 viremia can have pre-S2 deletions as well as precore mutations. Which appears first and whether there is any special functional significance or interaction are not clear. In Koreans, who seroconverted to anti-HBe, precore and pre-S variants appeared almost simultaneously [175]. Deletions in the pre-S2 region often include the pre-S2 initiation codon, thus preventing M protein synthesis [166, 176]. Internal deletions 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 and reduce 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 [164].

Conclusion

HBV has evolved in humans into genotypes, antigenic subtypes, and other variants, some antigenically important, but 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. There is good evidence that these variants are important causes of vaccine failure in infants born to HBeAg-positive mothers and that larnivudine can occasionally lead to variants that are not detected in HBsAg assays. Variants have also been detected in the transplantation setting with use of HBIG and during chronic infection. Most manufacturers have altered their assays to better detect common mutants of HBsAg. Escape mutations have not always been found in the *a* determinant, suggesting that other regions of HBsAg may have important roles in antigenicity. Alternatively, because the evidence for antigenic effects of aa substitutions is inconclusive, a replication advantage of the variant over the wild-type virus may explain selection of new strains.

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Chapter 9
Molecular variants of the precore, core, and core promoter regions of hepatitis B virus, and their clinical significance

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Summary

Hepatitis B virus (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 precore/core gene and basal core promoter (BCP) variants, which reduce or abrogate HBeAg production, usually appear at the beginning of the seroconversion phase from HBeAg to anti-HBe. The gradual removal of the tolerogenic effect of HBeAg leads to the awakening of the immune response. Precore and basal core promoter variants have been associated with fulminant hepatitis, but host factors are also implicated. Resolution of the mechanisms by which these variants are selected, their effects on HBV replication, and their pathogenicity have been hampered by the absence of a reliable and robust cell culture system, and the use of viral strains with additional sequence changes in many studies.

Introduction

Amplification of viral nucleic acids using the polymerase chain reaction (PCR) and their sequencing, either directly or after cloning, has revolutionized our understanding of virus genetics by adding significantly to established sequence databases, thus permitting detailed genomic and proteomic analyses to be carried out. In the case of 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 precore and the BCP variants resulting in the hepatitis B e antigen (HBeAg)-negative phenotype; others affect the enhancer II (EnII) region; and there are 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 hepatitis B surface antibodies (anti-HBs), and treatment with nucleoside analogs are reviewed in Chapters 10 and 14 respectively.

Virus biology and evolution

HBV isolates exhibit significant variation that may involve up to 12% of the virus nucleotide sequence.

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Natural variants exist, which give rise to the well-recognized serological subtypes and genotypes of the virus. 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 because they have a high rate of nucleotide misincorporation 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]) that is reverse transcribed, thus rendering it liable to a high mutation rate [1]. As a consequence, HBV exhibits a higher mutation rate than would otherwise be expected for a DNA virus, amounting to 2×10^{-4} base substitutions per site per year. However, the potential for variation is constrained by the compact genomic organization of the virus, featuring partially or totally 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. The major force for evolutionary change within a virus population is "positive selection" by the host immune response, a topic beyond the scope of this chapter.

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 (Figure 9.1), while the second initiation codon is utilized for the synthesis of the nucleocapsid or core protein (HBcAg). The two proteins are synthesized from different transcripts known as the precore messenger RNA (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 [2]. 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 [3]. 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 nonresponsiveness at the T-helper (Th) level. HBeAg-induced tolerance results in T cell nonresponse to HBcAg, as well as HBeAg-derived epitopes, so that elimination of HBV-infected cells does not occur. In transgenic mice, this nonresponse 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.

HBcAg forms the nucleocapsid of the virus, which has icosahedral symmetry, as demonstrated by cryoelectron microscopy [4]. 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 (ε) (Figure 9.2), with strict requirements of structure and sequence [5]. 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.

Biology of the core promoter region

The core promoter (CP, nt1575–1849) of the viral genome has a pivotal role in the replication and morphogenesis of the virus. It directs initiation of transcription for the

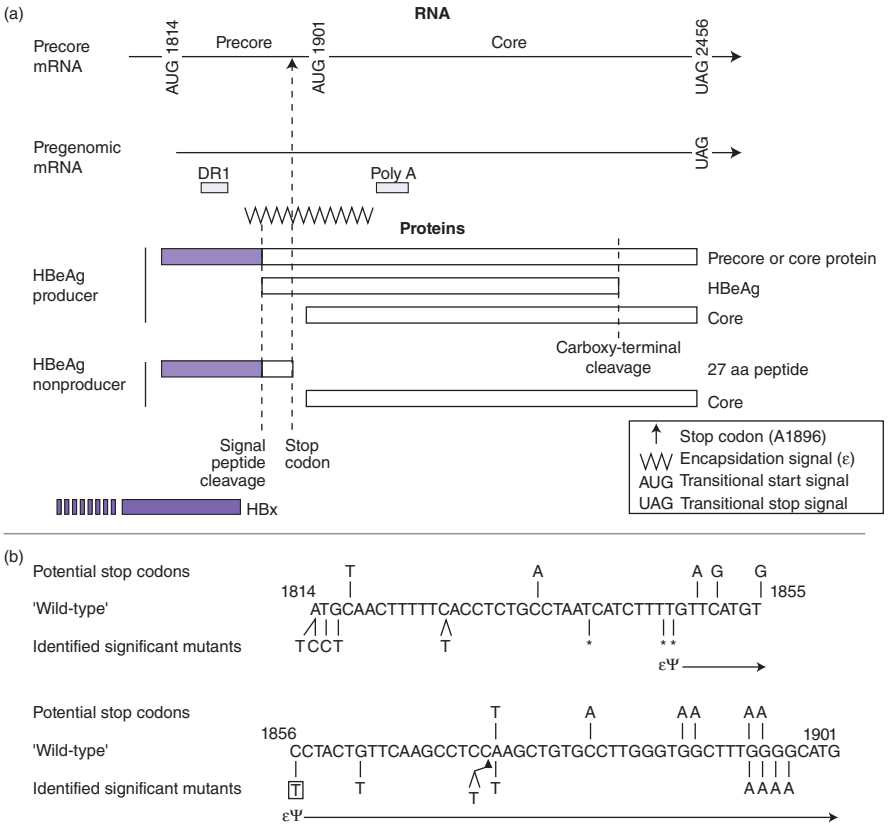

Figure 9.1 Precore/core biology and nucleotide variation. (a) There are two in-frame translation initiation codons (AUG) and one termination codon (UAG) in this gene. The precore/core protein translated from the first initiation codon is the precursor of HBeAg. This is generated by proteolytic cleavage of the first 19 amino-terminal amino acids (to remove the signal peptide) and the last 34 carboxy-terminal ones (codon 149 onward). 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.

synthesis of both the precore mRNA and pgRNA. The CP consists of the BCP (nt 1743–1849) and the upper regulatory region (URR, nt 1613–1742), the latter containing both positive and negative regulatory elements that modulate promoter activity [6]. 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 (Figure 9.3) [7]. The pgRNA starts 3′ i.e. 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 [1]. The precore mRNA, 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 described in this chapter.

The enhancer II (EnII, nt 1627–1774) element regulates the activity of the CP and partially overlaps the BCP and URR. The URR consists of the negative regulatory element (NRE, nt 1613–1636) and the core upstream regulatory sequence (CURS, nt 1637–1742). This whole region contains nucleotide motifs that constitute transcription factor binding sites, imparting at the same time liver cell specificity for optimal function of these elements. 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 coordinately or differentially regulate the transcription of the two mRNAs [7]. Moreover,

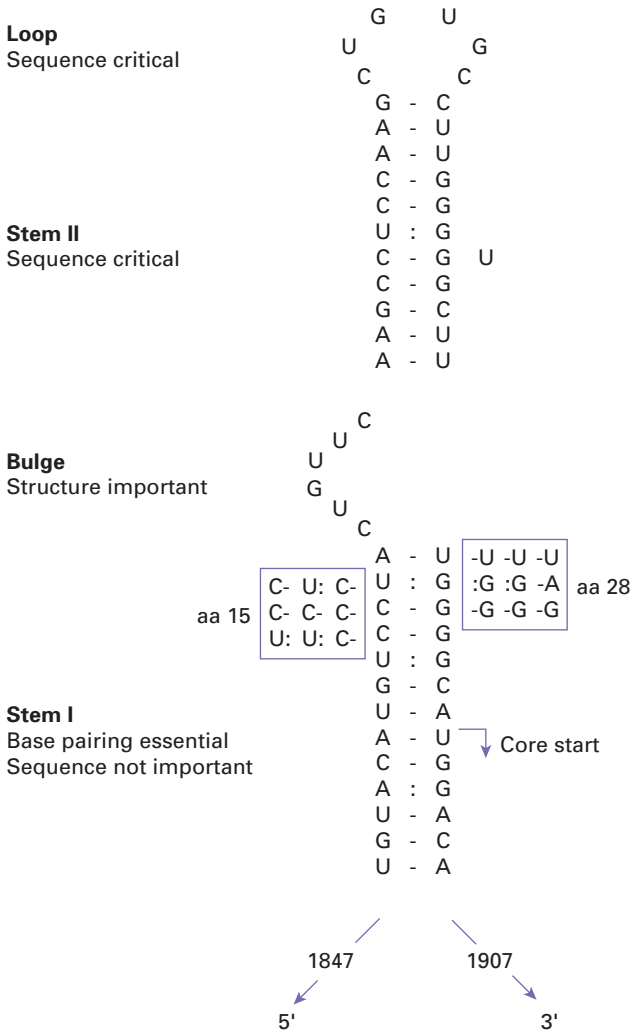

Figure 9.2 Secondary structure and some variants of the pregenomic RNA encapsidation signal (ε). The 5′ end of the pregenomic RNA, which starts just upstream of the precore AUG, folds into a stem loop structure as a result of base pairing of complementary nucleotide sequences. This secondary structure 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.

the stimulation of the BCP by ENII and CURS can be repressed by the effect of the upstream NRE.

Precore variants

A molecular explanation for the HBeAg-negative phase of hepatitis B viremia, first described in Mediterranean patients [8, 9], became apparent with the discovery of a variant of HBV with mutations in the precore region in 1989 [10]. This variant has a G-for-A substitution at position 1896 (denoted as G1896A or A1896) (Figure 9.1), which converts the codon for tryptophan (TGG, codon 28) to a translational stop codon (TAG), and is by far the commonest variant encountered in anti-HBe-positive patients. 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), mutation of the second codon to a stop codon, and frameshifts and deletions resulting in the synthesis of nonsense proteins. Precore amino acid substitutions have been detected in HBeAg-negative strains, 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. A1899 is primarily seen (with rare exceptions) together with A1896, implying that a second selection pressure is exerted on precore after emergence of A1896, and may relate to optimal function of the Kozak sequence for core protein translation.

A significant proportion of chronic hepatitis patients in Mediterranean countries [8] and in East Asia [11] are infected with HBeAg-negative variants. In fact, the HBeAg-negative chronic liver disease, as it is currently referred to, has become the commonest new presentation of chronic hepatitis in clinics in many countries of the world. In the Gambia and Brazil, as well as Northern Europe, there is a low prevalence of the precore stop codon mutation in anti-HBe-positive patients. These findings relate to the prevalent HBV genotypes that circulate in these populations. Precore variation, not all of which affects production of HBeAg, is distributed in particular genotypes. The A1896 stop codon mutation is not favored in genotype A, C, and F isolates, because these isolates possess C at position 1858. Genotypes B, C, D, and E with a T at this position are more amenable to the emergence of the A1896 mutation. C at position 1858 forms a canonical Watson–Crick pair with the G at position 1896 in wild-type strains, while T at position 1858 pairs with A at position 1896 in the precore stop codon variant. Inspection of the likely secondary structure of the encapsidation signal (Figure 9.2) 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 [5], and various groups have confirmed these findings on clinically observed variants. Viruses that have unstable stem loops do not encapsidate their pgRNA and do not compete successfully with other strains. Woodchucks may also exhibit precore variants of the woodchuck hepatitis virus (WHV), usually in the equivalent positions to HBV.

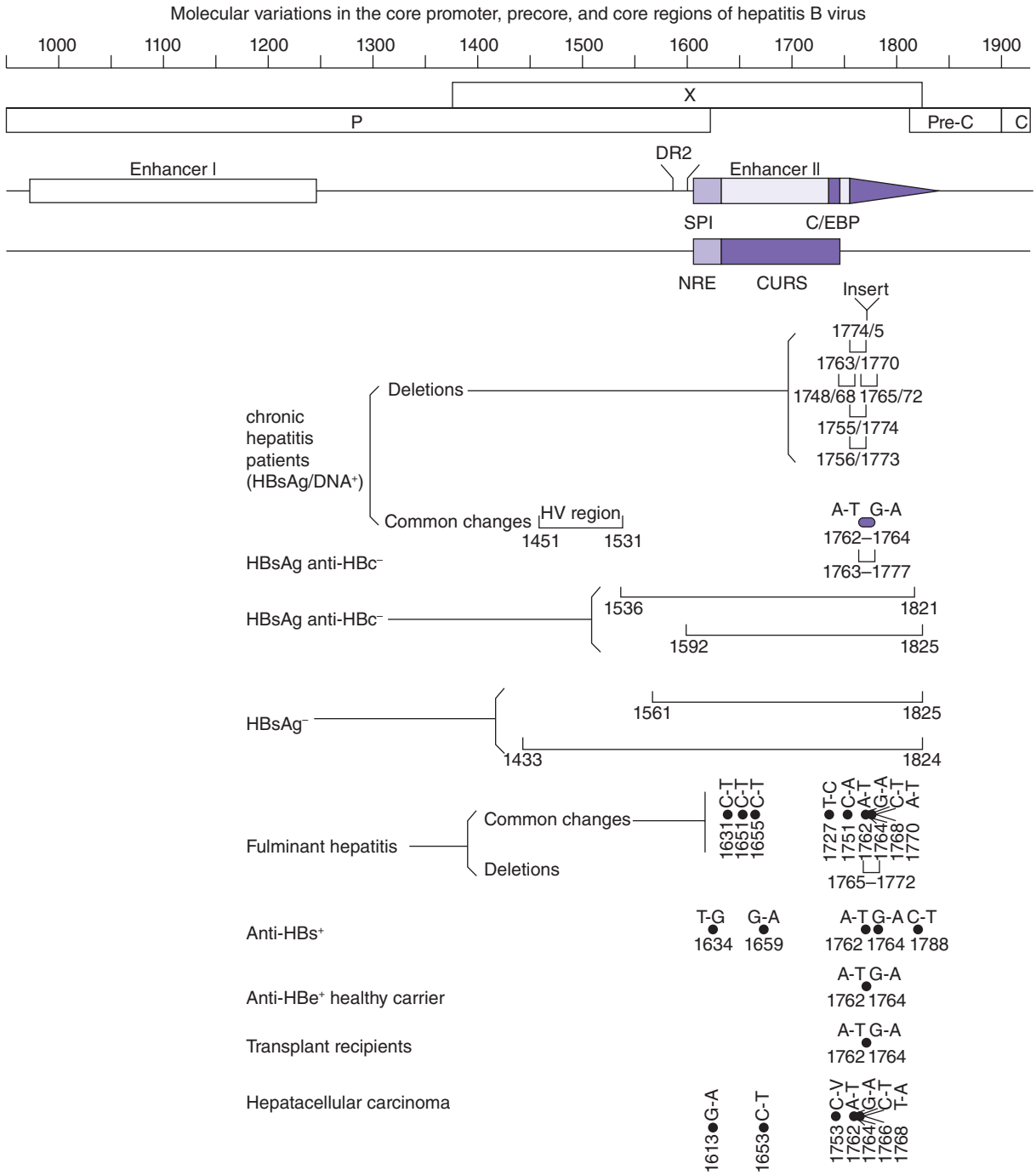

Figure 9.3 Transcriptional regulatory elements – the X gene and variants. The promoters and enhancers involved in HBV replication are indicated. Note that they are upstream of the precore/core gene and, in the case of the BCP, overlap the 5′ end. The X gene is embedded within these elements. Thus, any 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.

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). Viremia continues for many years, and initially there is very little inflammatory necrosis of infected hepatocytes (Figure 9.4) [11]. As the years go by, this immune-tolerant phase gives way to increased

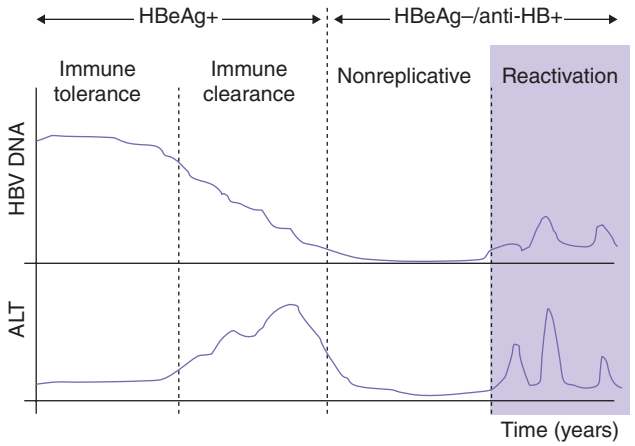

Figure 9.4 Diagram of the natural history of HBV infection showing the immune-tolerant, immune clearance, and nonreplicative phases of disease. In some patients, the latter phase may be followed by a reactivation phase characterized by increased viral replication and liver damage (shaded area). Precore and BCP variants are the main isolates seen in such patients during this latter phase.

hepatic activity, presumably reflecting immune activation, perhaps as a result of viral antigenic variation caused by transcriptional errors (as mentioned in this chapter). The role of $CD4^+$ lymphocytes in this process is supported by the observation of an increased response to the nucleocapsid proteins as HBeAg–anti-HBe seroconversion approaches [12]. These cells likely provide help to CTLs and B lymphocytes. Either because of CTL lysis of infected cells [13] or because of cytokine production by $CD4^+$ lymphocytes, which inhibit virion production [14], cells supporting productive HBV infection are cleared during the seroconversion hepatitis accounting for the hepatitis flares observed around the time of HBeAg seroconversion. About 5% of HBeAg-positive chronic infections may spontaneously seroconvert to anti-HBe per year. In the majority of these patients, HBs antigenemia persists predominantly because of the presence of small numbers of cells containing integrated HBV sequences as well as residual cells that support HBV DNA replication [10].

The A1896 variant is present in very small amounts during the immune clearance phase and increases in representation in the latter period of the HBeAg-positive phase of the disease and is selected at, or soon after, seroconversion to anti-HBe, while the HBeAg-producing strain is gradually being cleared. During the nonreplicative phase, both strains may be detectable at very low levels, if at all. In cases that reactivate after some years, the precore sequence tends to be similar to previous samples. It is unclear why in some patients the virus when reactivated replicates at high levels, causing further inflammatory liver disease [15], while other patients remain in remission. Mutations within epitopes recognized by T cells involved in control of the infection, or subsequent immunosuppression, may tip the balance in favor of the virus re-emerging. It is noteworthy that during this phase of the infection, amino acid substitution in the core protein is frequent (discussed in this chapter). In spite of early suggestions indicating that the precore stop-codon mutant was associated with severe chronic liver disease, emerging evidence suggests that this may not be the case, as the mutant seems to be present with equal frequency in patients with active liver disease and those asymptomatic [16]. Therefore, it seems likely that disease manifestation after seroconversion to anti-HBe may be dependent on additional mutations, particularly in the BCP and core encoding region in association with the A1896 substitution, as explained in this chapter.

Fulminant hepatitis

There is strong epidemiological evidence linking A1896 with fulminant hepatitis B (FHB) [17], suggesting that such a severe outcome may be due to viral factors rather than being solely dependent on an excessively vigorous immune response to the infecting viral strain. 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 [17]. This was confirmed recently in a study of 50 patients with fulminant hepatitis who were compared to an equal number of patients with acute self-limiting hepatitis B, and in whom multivariate analysis showed that G1896A, serum HBV DNA >5.23 log copies/ml, and total bilirubin >10.35 mg/ml were independently associated with FHB [18]. Other FHB strains have been shown to have a single nt insertion/deletion resulting in a frame shift of the precore protein, as observed in subgenotype B1/Bj strains. *In vitro* transfection studies indicated that such strains replicated at higher levels than did the wild-type virus [19].

In the case of the A1896 variants, as the source of the infection usually has minimal chronic hepatitis and is infected with a variant that has a similar or identical sequence to that found in the FHB patient [20, 21], 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 the result of infection with HBeAg-producing strains [17]. 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 the United Kingdom, Hong Kong, France, or the United States. 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 supports this hypothesis. However, the observation that transmission of A1896 strains to children seldom gives rise to chronic carriage supports the notion that HBeAg is a tolerogen and its absence decreases the risk of progression to chronic infection.

Variation within the *cis*-acting elements of the basal core promoter (BCP), which overlap with the HBx protein-encoding region (discussed further in this chapter), is also associated with FHB. The commonest variants contain a double mutation (T1762/A1764) in the BCP [22]. The current working hypothesis is that there are several routes to enhanced replication that, in a genetically predisposed host, may result in FHB. Although A1896 is often implicated, this does not necessarily mean that it is the variant *per se* that is causing the fulminant hepatitis. As the precore region of HBV is involved in inhibition of HBV replication [23, 24], it follows that BCP mutations in the presence of A1896 may result in higher levels of viral replication [25]. Detailed phylogenetic analysis of 20 epidemiologically unrelated FHB sequences [24], revealed six clusters of fulminant strains, each with distinct mutational patterns. Mutations were clustered in the *cis*-acting regulatory regions and HBx protein, but no unique variant was specifically associated with this disease outcome. There was statistical evidence of a heightened replication rate of the FHB genomes [26].

Summarizing, FHB is often the result of infection with variants carrying both the A1896 and the BCP double mutation, leading to an HBeAg-negative phenotype characterized by virus replication at high levels. In the absence of the tolerogenic effect of HBeAg, in a host genetic background that efficiently displays viral immune epitopes, the resulting effect is a strong immunological response causing severe liver disease and an unwelcome disease manifestation.

Fibrosing cholestatic hepatitis

Fibrosing cholestatic hepatitis (FCH) occurs after liver transplantation, occasionally after renal transplantation, and in HIV infection. The common factor is clearly immunosuppression. These patients rapidly progress to liver failure, 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. The issue has been raised whether cytopathic effect, rather than immune lysis, is primarily responsible for this form of hepatitis. It is also possible that some of these mutations result in truncated proteins that have lost secretory signal sequences or exhibit altered cellular localization causing cellular toxicity. S promoter variation with retention of S protein has also been described in patients with FCH [27]. Thus, retention of viral proteins within hepatocytes in association with high-replicating virus variants may be contributory factors in this disease manifestation.

Acute nonfulminant hepatitis

The incidence of the precore mutation during acute, nonfulminant hepatitis is presently unclear. Selection of A1896 strains does occur during acute infection, consistent with anti-HBe exerting an initial selection pressure followed by clearance of the HBeAg-negative virus by other immune mechanisms, possibly CTLs. However, others were unable to find evidence for this process during acute hepatitis. The description of a chronic carrier transmitting the A1896 variant in a pure form and resulting in typical acute hepatitis with seroconversion to anti-HBe without 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.

Basal 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 [22]. The double BCP mutation has been shown to be associated with HBeAg negativity in some studies, but not in others. These discrepant findings may be due to the fact that these are cross-sectional studies performed at different stages of chronic liver disease, and longitudinal studies showed that BCP mutations tend to emerge earlier than the precore mutation, often preceding HBeAg clearance by many years. The presence of the double mutation is clearly associated with downregulation of HBeAg production, as demonstrated by transfection studies [28, 29]. 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 coworkers demonstrated that this is indeed the case using a transcript-specific PCR approach and liver biopsy material from HBV carriers with the double mutation. In all cases, there was absence or a low level

of precore mRNA without a significant effect on total CP-directed transcription [30]. 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 [31]. Others, however, reported decreased precore mRNA levels, but wild-type levels of replication and gene expression [31]. 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 [29, 32]. 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). This effect has been investigated further in HepG2 cells and in the woodchuck animal model. Mutations in the HNF1 binding site decreased dramatically the synthesis of WHV 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.* [33], 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 the creation of the HNF1 site restored the pgRNA level. In addition, analysis of revertants with either one or the other of the BCP mutations showed that the T1762 change is critical for the mutant phenotype [29]. Transfection studies with cloned full-length HBV sequences derived from chronically infected patients have provided further information regarding the double A1762T/G1764A mutation in relation to genotype. The A1762T/G1764A BCP mutations in genotype C isolates correlated with increased replication capacity, while the A1752G/T mutation found in genotype B isolates correlated with low replication capacity. Interestingly, genotype C isolates with wild-type BCP sequences replicated less efficiently than genotype B isolates, due to less efficient transcription of the pgRNA, but more efficient virion release was observed with the former [34].

Chan and coworkers 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. Nevertheless, the double BCP and the precore stop codon mutations are far from being mutually exclusive [35]. A recent study from China comparing acute versus chronic hepatitis reported a lower prevalence of A1762T/G1764A, G1896A, and G1899A, but higher prevalence of T1758C mutations, in genotype C–infected patients. The T1758C and A1762T/G1764A mutations were mutually exclusive. Moreover, acutely infected patients with BCP/precore mutant viruses had higher viral load, whilst chronic patients with mutant viruses had lower load [36].

The BCP mutations, as mentioned here, 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. 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 carcinoma (HCC), and liver transplantation [21, 26, 37]. More recent studies have identified additional mutations in the BCP region that are prevalent in HCC patients. These include G1613A (NRE) and C1653T, both of which tended to occur simultaneously in HCC patients. Functional studies indicated that the G1613A mutant suppressed HBeAg production, enhanced viral DNA synthesis, and bound RFX1 with higher affinity. Since the 1762/1764 mutations overlap with the X ORF and lead to amino acid changes in the X protein, sequence analysis in patients from Korea indicated that this double substitution on its own or together with G1386A (V5M) was encountered at a significant rate in HCC patients [38]. Thus, to summarize, T1653, V1753, T1762, A1764, T1766, and A1768 have been found to occur more frequently in HCC patients [39]. From the REVEAL-HBV study, the risk of developing HCC over a 10-year period was 23% in patients infected with genotype B with the BCP double mutation. Moreover, the double BCP mutation was found to be associated with increased risk of HCC independent of genotype, while the precore A1896 mutation was associated with decreased risk.

Other variants

As mentioned in this chapter, CP mutations other than those at positions 1762/1764 could have a major impact on viral replication and HBeAg expression [40]. 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. Single mutations including T1753C/A/G (V), 1762/1764, 1896, and 1899, as well as triple mutations 1753/1762/1764 and 1762/1764/1766 or T1768A, were more frequently detected in patients with acute-on-chronic liver failure (ACLF) than in

patients with chronic hepatitis. This was mostly associated with genotype B– than C-infected patients [41]. Other mutations detected in ACLF patients include A1846T and A/G1913 [42].

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 [43], chronic hepatitis [42, 44], asymptomatic infection, serologically silent infection [45], HCC [46], renal dialysis, and liver and renal transplantation [47]; they are also found in patients who have survived hematological malignancies or solid tumors. Such deletion variants are often characterized by low viremia levels, 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. In contrast, three BCP deletion variants (either nt 1758–1777 or 1749–1768) from Singaporean chronic carriers had high viremia levels [48]. 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, those with HCC, and patients with fulminant hepatitis [43]. 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 [43]. 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. However, the detection of the variant on its own and in the absence of wild-type virus argues against this hypothesis. 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 [49], and then in Hong Kong Chinese patients with chronic hepatitis [50].

Chronic and fulminant hepatitis patients

The double mutation has been detected with increased frequency in patients with fulminant hepatitis, including children, those with HBeAg- and anti-HBe-positive chronic hepatitis, and HCC patients, but less so in asymptomatic chronic carriers. In addition, it has been detected in immunosuppressed, liver transplant, and seronegative patients. There are differences in the prevalence of such variants between studies in various settings, and this may relate to differences between prevalent genotypes, the importance of which is not always recognized and therefore not clearly reported.

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 East Asia (Japan) suggest that the BCP mutation is the one that appears first [51]. However, both can be present during the HBeAg-positive phase in mixtures with the wild-type virus [51]. A recent cross-sectional study has shown a gradual increase in the percentage of both precore and BCP mutants with age in HBeAg-positive carriers [52]. On follow-up, such mutant levels can remain stable for many years, and this may be followed by a steady increase from <10% to 50–100% within 3 years prior to seroconversion. 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. It seems, therefore, that reduced HBeAg expression (BCP mutation, and other mutations described in this chapter that result in reduced expression) or complete abrogation (precore stop codon) is a prerequisite for successful seroconversion to anti-HBe. The liver disease in many patients enters a quiescent phase with little, if any, virus replication; levels of viremia $<10^3$ copies/mL; and minimal changes in the liver. It is the cellular immune response to HBV that appears to hold the virus under control [53]. 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 in this chapter.

Table 9.1 Statistical analysis of correlation between mutation and B cell and Th epitopes in core protein[a].

		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

[a] 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 χ^2 test or Fisher's exact test. T/B: T or B cell epitope.

Core gene variants

Amino acid substitutions

The finding that precore mutants are present in 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 and geographical variation in incidence. There are well-defined epitopes in HBcAg that are recognized by antibodies, Th cells, and CTLs (Table 9.1). 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 East Asia with severe hepatitis, greater clustering of substitutions was noted between amino acids 84 and 101, compared with those with mild disease [54, 55] (Figure 9.5). 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 East Asian patients. Further analysis indicated clustering of mutations in B cell and Th epitopes. Chinese patients also select more variants during anti-HBe seroconversion than during the immune tolerant phase. In anti-HBe-positive Mediterranean patients with minimal hepatitis, very few changes were noted [56]. 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. A sequential study confirmed this finding for B cell epitopes but revealed a weaker association with Th epitopes [57, 58]. 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. 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. 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 [56]. 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 [56–59]. In some patients with acute exacerbations during the anti-HBe-positive phase, there is clearance of the preexisting strain and emergence of new viral variants with amino acid substitutions in epitopes suggestive of immune selection [57]. 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. Thus, the data suggest a more rapid selection of mutations in patients with active (immune pressure) than those with quiescent disease, and the site of mutation may differ depending on genotype and host genetics. These issues explain the varied findings from different geographical regions.

Finally, the sequence of HBcAg from FHB cases is quite variable compared with strains from patients with chronic hepatitis [20, 21]. This variation tends to cluster

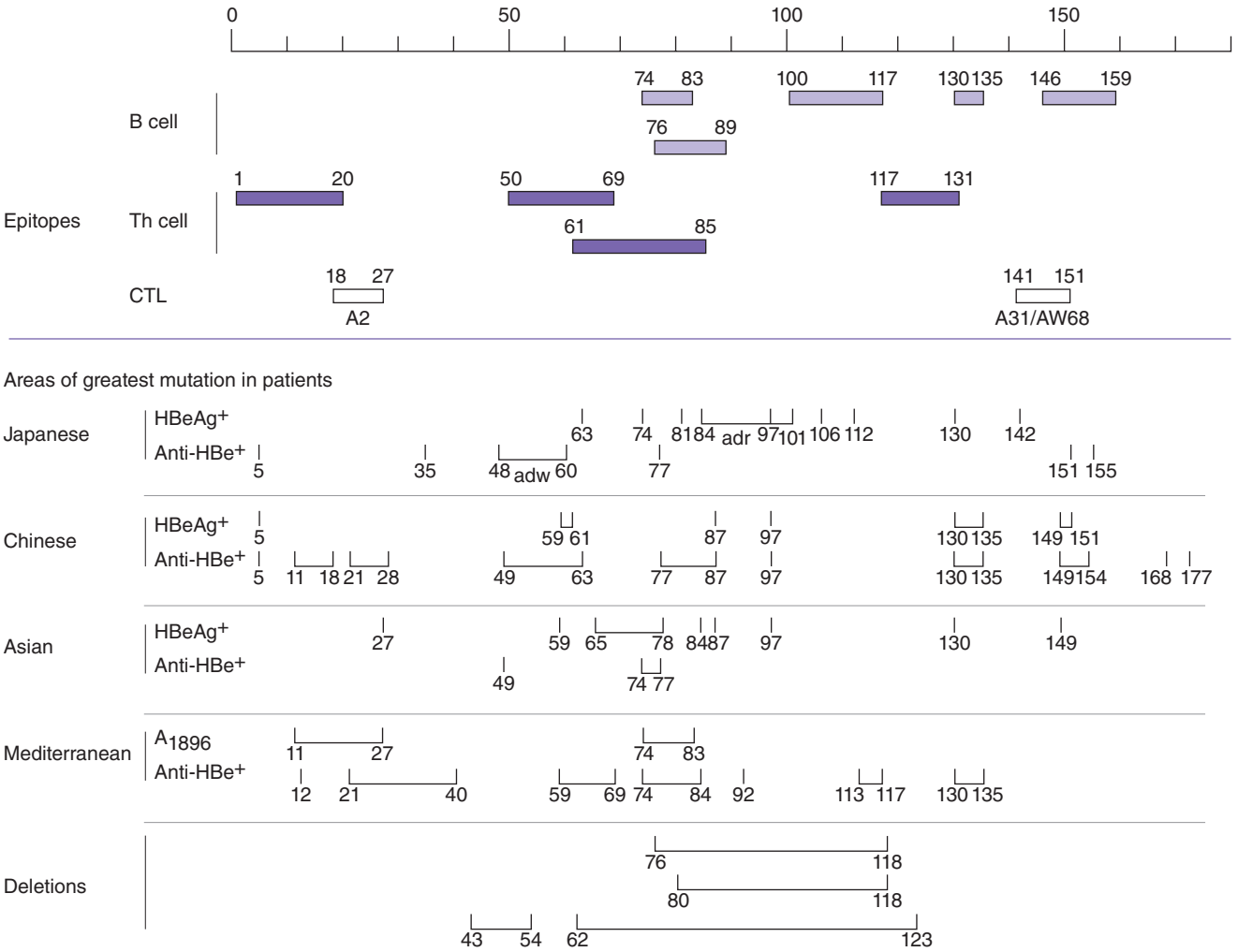

Figure 9.5 HBcAg variability correlated with immunologic epitopes. The Th, B cell, and CTL epitopes are shown above. Below are shown areas of greatest variability highlighted by the authors or, if not provided, our subjective interpretation. Note that there is little consistency in these results. This 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 human leukocyte antigen (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.

within epitopes and, as for chronic hepatitis, this is significantly associated with pre-selection of A1896.

Core deletion variants

As mentioned in this chapter, 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 [44, 59, 60, 61]. Such deletions can 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 coexist with the wild-type virus that provides the core protein for their encapsidation [62]. 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. Core deletion variants have been reported in both immunocompetent and immunosuppressed individuals [61]. In the former individuals, the detection of core internal deletion variants was associated with lower levels of viremia and early seroconversion to anti-HBe, with concurrent disappearance of the variants. 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.

Why this should be the case remains unanswered, but it seems likely that immunosuppression may increase HBV replication and therefore generation of such variants. 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.

Two further types of genetic variant exist. The first is a 36 bp insertion found around and including the core ATG associated with A1896 [63]. It was initially seen in a patient infected with HIV and a carrier treated with interferon (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 the core. It has also been observed that this genotype may coexist with genotype A, which explains the HBeAg positivity in such patients, in spite of the presence of the precore stop codon mutation. 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 [64]. 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 East Asia, 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 programs.

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 [14, 65]. A strong and multispecific 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 [66]. Such responses persist in recovered individuals, probably as a result of continued CTL activation in the presence of low levels of viremia, which they continue to keep under control. More recently, it has been shown that HBV replication within hepatocytes can be inhibited by antiviral cytokines without the requirement of hepatocyte lysis [67]. 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. Failure of antigen presentation to CD4+ helper T cells, failure of such cells to proliferate, immune modulation by secreted proteins, viral mutation, and integration of viral DNA into the cellular genome 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. This indicates that immune responses against HBV can recover under the appropriate conditions. The findings described here have in recent years been confirmed and extended using more sensitive assays than chromium release, such as ELISPOT and tetramer technology.

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 [11]. This latter phase, in view of what has been described in this chapter, can in a subgroup of patients be followed by a replicative or reactivation phase, characterized by the emergence of variant viruses [16]. 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. Moreover, HBeAg has been shown to elicit a Th-2-like immune response while downregulating Th-1 responses by inducing apoptosis of the effector cells. 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. It has been shown that some variants of the nuclear

localization region, whether found in natural isolates or generated *in vitro*, lead to redistribution to the cytoplasm after expression *in vitro*. Hepatocytes harboring 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 preexisting variants or variants arising during the nonreplicative phase escape immune recognition [65]. 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, and 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 [52, 57, 68, 69], while in others, mutations do not occur with each flare, at least in HBcAg [57]. Some may have transient variants that may attract immune attention and then be cleared [56]. Whether anti-HBe plays a role in antibody-dependent cell cytotoxicity during this period is not fully known, but is a possibility, as HBeAg can be expressed on the cell surface. These events would undoubtedly favor the elimination of HBeAg-expressing hepatocytes. During this period, mixtures of variants and wild-type virus are detectable at very low levels [10].

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. 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 have appeared to be coming to clinics more frequently in recent years. These reactivation episodes may occur at frequent intervals and may be accompanied by increases in IgM anti-HBc levels [57]. 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 [70]. 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 [57], or may affect areas that influence intracellular localization such as the carboxy terminus. Interestingly, patients who exhibit a more severe form of HBV infection such as FHB or who have had a liver transplant can have several mutations in the carboxy terminus of HBcAg. In mice transgenic for HBV replication, CTLs can clear active infection by a posttranscriptional mechanism mediated by IFNγ and tumor necrosis factor alpha (TNFα). 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, although this was not confirmed in two other studies, 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. Such differences may relate to the sensitivity of the method used for detection of variants. Others have found a low frequency of mutations in the BCP region in HBeAg-positive patients, which was of little help in predicting IFN response, 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. Anti-HBe-positive, HBV DNA–positive Mediterranean patients with chronic hepatitis often respond poorly to IFN therapy, and if they do respond, they tend to relapse [71]. Interestingly, T1856-containing strains may respond poorly to IFN, 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. A wide variety of stop codons, nonfunctional start codons, and amino acid substitutions arise in HBeAg-positive cases who respond to IFN therapy. One study also compared the frequency of selection of mutants after successful IFN therapy with natural seroconversion to anti-HBe. There was a trend toward less

frequent and slower emergence of mutants in those who seroconverted during IFN therapy. Sequence variability of HBcAg is greatest in anti-HBe-positive nonresponder patients. 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 nonresponders. However, no correlation was found by another group in Chinese patients.

Lamivudine

Lamivudine, a nucleoside analog, 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 while on treatment, with 65–96% showing normalization in ALT levels and loss of HBV DNA. However, on stopping therapy the majority relapse, and sustained response rates range between 10% and 15% [72]. 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 titers, and during prolonged treatment the variants reappear [73]. The reemergence of these variants appears to compensate for any replication capacity deficit that may be apparent as a result of the presence of lamivudine resistance mutations. In HBeAg-positive patients carrying genotype C, the presence of BCP and precore mutations was independently associated with HBeAg loss. Moreover, this occurred earlier in the presence of variant isolates than in the wild-type virus.

Other nucleos(t)ide analogs

In recent years, new nucleos(t)ide analogs such as adefovir, telbivudine, entecavir, and tenofovir have been introduced for the treatment of both HBeAg-positive and -negative patients. The latter two drugs, which have a very high genetic barrier that prevents the appearance of drug-resistant mutations in treatment-naïve patients, have permitted the long-term use of these drugs with effective suppression of virus replication. In view of this, there have been no studies investigating the role of the precore and BCP mutations in long-term treatment outcome, relapse after drug withdrawal (currently not indicated), or possible HBsAg loss.

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 and has been applied to clinical material to assess the relative importance of mutants to clinical severity. Alternatives are to use a common primer at one end of the DNA of interest and a second one that contains either a G or an A at the 3′ end, which has been applied to paraffin-fixed sections, a single-labeled nucleotide (G or A) addition to a primer that ends at either 1895 or 1897, and digestion of PCR products that have been generated with primers incorporating novel restriction sites. However, none of these are as comprehensive as sequencing or ultradeep pyrosequencing, which has been recently developed. A line probe assay developed by Inno-Lipa for the detection of the precore and BCP mutations, as well as HBV genotypes, has been in use for many years now. This has proved to be rapid, sensitive, and reliable.

Overall, in relation to antiviral treatment, testing for these variants is less accurate and more cumbersome in predicting response than ALT, HBV DNA, or HBV genotype and (more recently) HBsAg titer, so there is no clinical utility in testing for these variants

Concluding remarks

Undoubtedly, HBeAg-negative chronic liver disease is characterized by the presence of the variants described in this chapter, and will continue to constitute the main patient group presenting at clinics throughout the world for many years to come. Long-term carriage is associated with progressive disease and a high risk of developing cirrhosis and HCC. Effective antiviral regimens that suppress viral replication in the long term remain the only choice in successfully interrupting this process.

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Chapter 10
Natural history of chronic hepatitis B virus infection

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Summary

The natural course of perinatally acquired chronic hepatitis B virus (HBV) infection typically has three chronological phases: (1) the immune-tolerant phase, (2) the immune clearance phase, and (3) the inactive or residual phase. However, no obvious initial immune-tolerant phase is evident in adult-acquired chronic infection. Hepatitis B e antigen (HBeAg) seroconversion is a hallmark and usually signals the transition from active to inactive HBV infection with alanine aminotransferase (ALT) normalization and resolution of hepatitis activity. After long sustained remission, spontaneous hepatitis B surface antigen (HBsAg) seroclearance may occur and confers excellent prognosis. On the other hand, HBV may reactivate and trigger immune-mediated liver injuries at an incidence of 2–3% per year, usually in the first 10 years after HBeAg seroconversion. High HBV DNA levels and disease activity are the factors of adverse clinical outcomes such as hepatic decompensation, cirrhosis, hepatocellular carcinoma (HCC), and liver-related mortality. Sustained reduction of HBV replication before the onset of cirrhosis confers a favorable outcome.

Introduction

Chronic HBV infection is a global public health issue because of its worldwide distribution and its potential adverse sequelae, including cirrhosis, hepatic decompensation, and development of HCC. The prevalence of HBsAg is low (0.1% to 2.0%) in the United States and Northern and Western Europe; intermediate (2.0–8.0%) in the Mediterranean countries, Eastern Europe, Central Asia, the Middle East, South America, and Japan; and high (8.0–20.0%) in Southeast Asia, China, Taiwan, Alaska, and Sub-Saharan regions [1]. In high-prevalence areas, HBV infection usually occurs perinatally or during infancy and early childhood; in areas of intermediate endemicity, chronic HBV infection is generally caused by transmission during early childhood, mostly through household contacts; and in low-prevalence areas, hepatitis B is typically a disease of young adults, who acquire the infection through risky behavior, such as unprotected sexual contact or sharing of injection needles [2, 3].

Clinical presentations

Chronic HBV infection is a dynamic state involving interactions between the virus, host hepatocyte, and immune response. Accordingly, the clinical presentations of chronic HBV infection are complex and variable, and the natural course of chronic HBV infection consists of changing phases. In low- or intermediate-prevalence areas, approximately 30% to 50% of patients with chronic HBV infection have a history of acute hepatitis. In contrast, only a small percentage of patients with chronic HBV infection from high-prevalence areas have a history of clinical acute hepatitis. Most patients are incidentally

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identified to be HBsAg carriers during blood donation, during a health check-up or routine screening, or, in a few cases, due to nonspecific constitutional symptoms. Others present as HBsAg-positive patients with acute hepatitis who are seronegative for immunoglobulin class M (IgM) antibody against hepatitis B core antigen (anti-HBc); they are called "previously unrecognized HBsAg carriers with acute hepatitis flares or superimposed other forms of acute hepatitis" [3].

Upon initial evaluation, patients with chronic HBV infection may present with one of the following four biochemical and serological profiles: (1) HBeAg-positive with normal serum ALT levels; (2) HBeAg-positive with abnormal ALT levels; (3) HBeAg-negative, antibody to HBeAg (anti-HBe)-positive with normal ALT levels; and (4) HBeAg-negative, anti-HBe-positive with abnormal ALT levels. These four patterns of presentation actually represent different phases of chronic HBV infection.

Phases of chronic HBV infection

The natural course of chronic HBV infection consists of distinct phases, which are characterized by and diagnosed on the basis of serum HBsAg–HBeAg serology, HBV DNA level, ALT level, and liver histology. Typically, chronic HBV infection acquired perinatally or during infancy has three phases: the immune-tolerant phase, immune clearance phase, and inactive residual phase [2–4]. In a subset of inactive carriers, HBV may reactivate and trigger immune-mediated liver injuries. This reactive phase can be viewed as a variant of the immune clearance phase [3]. In adult-acquired chronic infection, there is usually no (or a very short) initial immune-tolerant phase. Otherwise, the clinical course is essentially the same as that seen in patients with perinatally acquired infection [2]. The clinical, serological, histological, and virological characteristics of these dynamic phases of chronic HBV infection are summarized in Figure 10.1.

Immune-tolerant phase

The initial phase of chronic HBV infection is characterized by the presence of serum HBeAg, very high levels of serum HBsAg (4.5–5.0 $\log_{10}$ IU/mL) and HBV DNA (8–10 $\log_{10}$ IU/mL), normal ALT levels, and normal or minimal histological changes [2–5], with intrahepatic hepatitis B core antigen (HBcAg) expressing diffusely and predominantly in the nuclei [6]. There is usually little or no disease progression as long as serum ALT levels remain normal and the immune tolerance is maintained [7].

The absence of liver disease, despite a high level of HBV replication during this phase, is believed to be a consequence of immune tolerance to HBV. Even though HBV does not cross the placenta, the HBeAg secreted by the virus does. Experiments in mice suggest that a transplacental transfer of maternal HBeAg may induce a specific unresponsiveness of helper T cells in neonates. Because HBeAg and HBcAg are highly cross-reactive at the T cell level, deletion of the helper T cell response to HBeAg results in an ineffective cytotoxic T lymphocyte (CTL) response to HBcAg, the major target of the immune response [8]. Once a chronic infection has been established, persistence of high viral load and continued secretion of HBeAg (the tolerogen) are necessary to maintain the tolerant state. The viral population identified during the immune-tolerant phase usually consists of exclusively wild-type HBeAg-positive HBV with little or no mutant-type HBeAg-negative HBV [9].

Immune clearance phase

The transition from the immune tolerance to immune clearance phase usually occurs between ages 20 and 40, but sometimes may start earlier and even occur in pediatric patients. Little is known about the mechanisms that regulate the loss of immune tolerance in chronic HBV infection. The finding that the immune clearance phase is accompanied by a change in the intrahepatic distribution of HBcAg from nuclear to cytoplasmic localization suggests that it may be triggered by a change in the presentation of viral antigens [6]. During this phase, the patients are still seropositive for HBeAg but ALT levels become abnormal. Liver biopsy in these patients demonstrates increased histological activity [4] and decreased nuclear HBcAg expression with a concomitant increase in cytoplasmic HBcAg expression [6]. These changes are associated with a decline in serum HBV DNA to 6–8 $\log_{10}$ IU/mL and in HBsAg to 3–4.5 $\log_{10}$ IU/mL [5], and also accumulation of mutant-type HBeAg-negative HBV with decreased secretion of HBeAg [9].

HBeAg-positive chronic hepatitis B

Most patients in the immune clearance phase are asymptomatic with mild to moderate elevation in serum ALT levels. They are said to have "HBeAg-positive chronic hepatitis B." Of note, the clinical course may be punctuated by spontaneous acute hepatitis flare with serum ALT elevation over five times the upper limit of normal (ULN). Patients may also present as previously unrecognized HBsAg carriers with clinical acute hepatitis, being HBeAg seropositive but seronegative for IgM anti-HBc [3]. These ALT elevations and acute hepatitis flares are considered to be the results of a human leukocyte antigen (HLA)–class I antigen-restricted, CTL-mediated immune response against HBV antigen(s) and its downstream apoptotic mechanisms [10]. The reasons for spontaneous acute hepatitis flares are not clear but

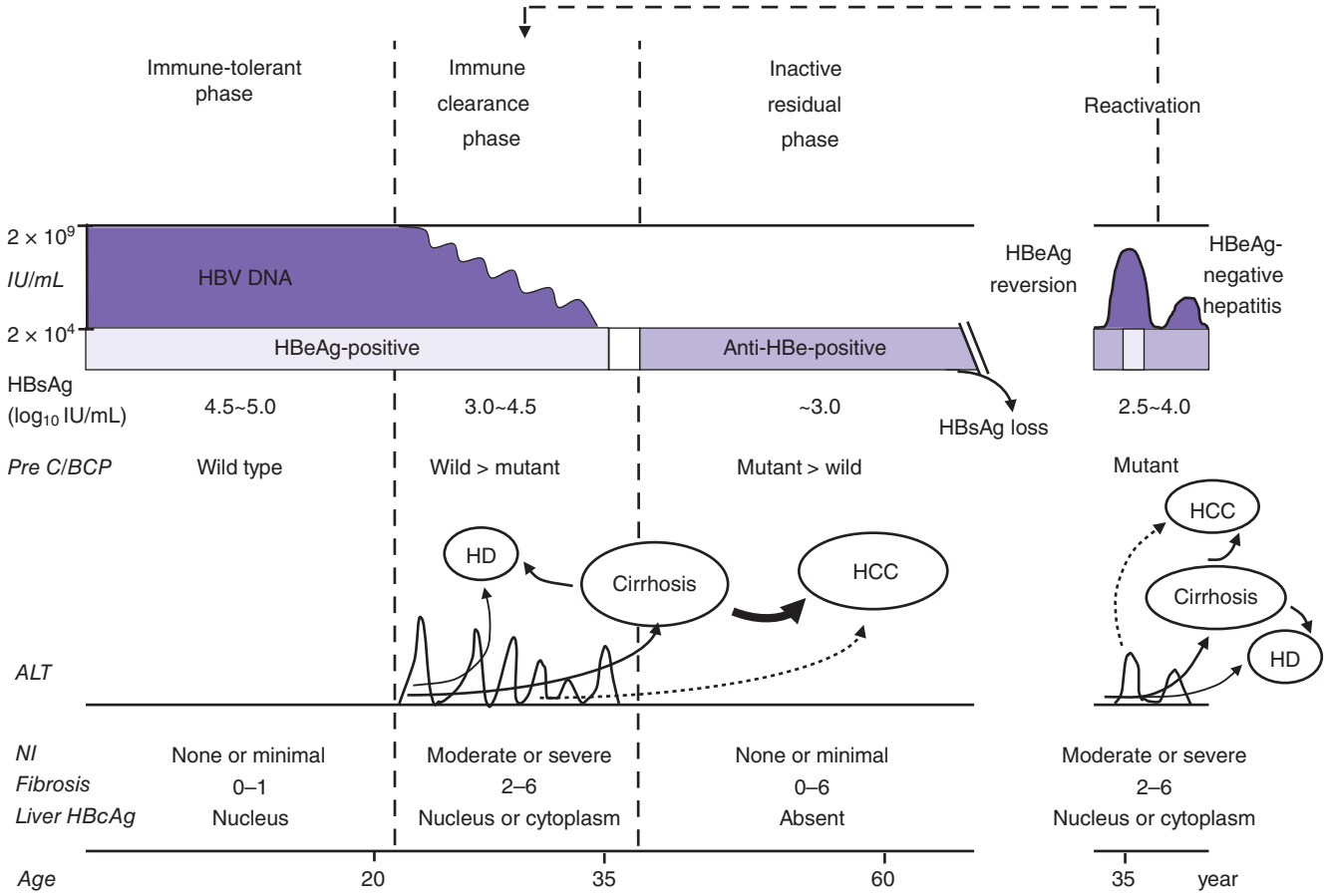

Figure 10.1 Natural course of chronic hepatitis B virus (HBV) infection acquired perinatally and during infancy. The reactivation phase is similar in every aspect to the immune clearance phase, except for HBeAg status. The events during the immune clearance and reactivation phases could lead to hepatic decompensation (HD), cirrhosis, and hepatocellular carcinoma (HCC). HBcAg: hepatitis B core antigen; HBeAg: hepatitis B e antigen; anti-HBe: antibody against HBeAg; HBsAg: hepatitis B surface antigen; pre-C: precore; BCP: basal core promoter; ALT: serum alanine aminotransferase; NI: necroinflammation; fibrosis: Ishak fibrosis score. (Source: Adapted from Liaw YF, Chu CM. Lancet 2009;373: 582–592 [3])

are likely explained by subtle changes in immunological controls of viral replication. Several studies have shown that acute hepatitis flares are often preceded by a sudden surge in serum HBV DNA levels [10] and enhanced T cell response to HBcAg and HBeAg [11]. This chronological sequence of HBV DNA and ALT changes in spontaneous acute flares is similar to that observed in acute hepatitis flares secondary to therapeutic intervention with corticosteroids or cancer chemotherapy [12]. Histological evidence of lobular hepatitis similar to acute hepatitis, even with bridging hepatic necrosis, is frequently observed during these flares [13]. IgM anti-HBc may also appear in some patients during acute hepatitis flare, but is generally at lower titers than in acute hepatitis B [14].

The reported incidence of acute hepatitis flare during the immune clearance phase varies among different patient cohorts. In hospital-based studies, the annual rate of acute ALT flare >5× the ULN was as high as 25% per year during the first 3 to 5 years of follow-up. Most acute ALT flares are asymptomatic, but around 20–40% of the episodes present with symptoms of acute hepatitis, and 3–5% of the acute flares may be complicated with hepatic decompensation [10, 14].

HBeAg to anti-HBe seroconversion

The immune clearance phase has a variable duration and often lasts many years until HBeAg seroconversion. HBeAg seroconversion is usually preceded by ALT elevation and decreasing HBV DNA level, followed by a marked reduction of serum HBV DNA to levels that are detectable only by polymerase chain reaction (PCR) assay ($<2 \times 10^{3-4}$ IU/mL), ALT normalization, and resolution of liver necroinflammation [15–18]. However, abnormal ALT levels and an HBV DNA level

Table 10.1 Common factors involved in spontaneous HBeAg seroconversion, HBV reactivation, and HBsAg seroclearance.

* Age
* Genotype (B vs. C; A vs. D)
* Immune status
* ALT level and hepatitis activity
* HBV DNA level

ALT: alanine aminotransferase; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen.

$>2 \times 10^4$ IU/mL persisted at the time of HBeAg seroconversion in about 5% of patients [15]. These patients progressed directly from HBeAg-positive chronic hepatitis to HBeAg-negative chronic hepatitis.

The average annual incidence of spontaneous HBeAg seroconversion is around 10% (2% to 15%), depending on factors such as geographical region, ethnicity, mode of transmission, age, ALT levels, histological activities, and HBV genotype [2, 8, 17–20]. A higher HBeAg seroconversion rate has been reported in non-Asian children with horizontal transmission than in Asian children with vertical transmission [2, 21]. Factors of HBeAg seroconversion are summarized in Table 10.1. The likelihood of HBeAg seroconversion correlates positively with baseline ALT levels: HBeAg seroconversion rates at 18 months of follow-up are 0%, 3–8%, 17%, and 59–70%, respectively, if baseline ALT levels are <1, 1–2.5, 2.5–5, and ≥5 times the ULN [10]. In patients with acute flare, 72% undergo HBeAg seroclearance within 3 months if the alpha-fetoprotein (AFP) level increases to >100 ng/ml during the flare, as do only 18% of those with AFP elevation <100 ng/mL [10]. The likelihood of HBeAg seroconversion also correlates with histological activities: the 5-year cumulative probabilities of HBeAg seroconversion are <25% and >65%, respectively, in patients with mild and more active hepatitis. HBeAg seroclearance may occur within 3 months in two-thirds of the patients with more extensive hepatocytolysis, such as bridging hepatic necrosis [10]. In a community cohort study including 439 HBe-positive subjects, being over age 30 years, being female, and having genotype B (vs. C), the presence of precore G1896A mutant, a higher baseline ALT, and a lower HBV DNA level were found to be independent factors associated with HBeAg seroclearance [18].

Studies from Taiwan have shown that HBeAg seroconversion occurs at a median (interquartile range) age of 32 (26–36) years, with the majority of cases (90%) occurring before 40 years of age [22]. In accordance with these data, the prevalence of serum HBeAg declines remarkably from 85% in carrier children (<15 years) to 5–10% in carrier adults over 40 years of age [22, 23]. These findings suggest that HBeAg seroconversion usually occurs between 15 and 40 years of age in perinatally acquired chronic HBV infection. Persistence of HBeAg over 40 years of age is rare and can be considered as "delayed" HBeAg seroconversion [22]. Studies have shown that patients infected with genotype B HBV, as compared with those with genotype C HBV, seroconvert earlier and more frequently [17–20]. Interestingly, HBeAg seroconversion is more frequently preceded by acute ALT flares in genotype C than in genotype B HBV infection, suggesting that a more vigorous immune-mediated hepatocytolysis may be needed to achieve HBeAg seroconversion in genotype C HBV infection [19]. In Alaska natives, spontaneous HBeAg seroconversion was similar between patients with genotype A, B, D, or F HBV infection but much slower in those with genotype C HBV infection. The median age of HBeAg seroclearance was <20 years in patients with genotypes A, B, D, and F but >40 years in patients with genotype C [20].

HBeAg persistence and its outcome

In some patients, the immune clearance phase may last for many years without HBeAg seroconversion during a long-term follow-up period. A recent cohort study from Taiwan demonstrated that the risk of progression to cirrhosis increased with increasing age of HBeAg seroconversion [24]. Notably, patients remaining HBeAg seropositive after 40 years of age are associated with a remarkably high risk of progression to cirrhosis compared with those with earlier HBeAg seroconversion [22]. A more recent study also showed significantly higher annual rates of cirrhosis and HCC (3.7% and 1.6%, respectively) in 147 patients with persistent HBeAg, as compared to 1.8% and 0.4%, respectively, in 86 patients who underwent spontaneous HBeAg seroconversion during a follow-up period of 7 years [25]. An earlier study from Germany in 53 untreated HBeAg-positive patients with chronic hepatitis showed that hepatic decompensation or liver-related death occurred significantly more often and that survival without clinical complications was significantly shorter in patients with persistent serum HBeAg than in those with spontaneous HBeAg seroconversion during a follow-up period of 38.5 months [26]. A study from Italy also showed high incidence of cirrhosis development and short survival in patients with persistent HBeAg seropositivity [27]. These findings indicate that a prolonged HBeAg-positive phase is associated with increased risk of disease progression.

Inactive or residual phase

The hallmark event of HBeAg seroconversion usually signals a transition from active hepatitis B to an "inactive HBsAg carrier state" [17]. Although HBV DNA is undetectable by hybridization assays, it is often detect-

able by PCR assays in these "inactive carriers." HBV DNA lower than 2000 IU/mL has been considered as a cutoff level to discriminate an inactive carrier state from HBeAg-negative chronic hepatitis. However, recent studies from Taiwan showed that 19–26% of inactive carriers with persistently normal ALT (PNALT) for more than 10 years and no disease progression had HBV DNA levels in the range between 2000 and 20 000 IU/mL but rarely >20 000 IU/mL [16, 28]. A large nested case–control study during a long-term follow-up period (median 13.4 years) also showed that maintenance of HBV DNA <4.39 $\log_{10}$ copies/mL (<4700 IU/mL) was associated with PNALT and decreased risk of HCC [29]. It therefore seems reasonable to define the "inactive HBsAg carrier state" as an HBeAg-negative noncirrhotic HBsAg carrier with PNALT and HBV DNA <20 000 IU/mL [30]. Studies have shown that serum HBV DNA and HBsAg levels decline gradually and slowly after spontaneous HBeAg seroconversion and to 1.5–3 $\log_{10}$ IU/mL in those with PNALT [5]. A study in genotype D HBV-infected HBeAg-negative HBsAg carriers has shown that combined serum HBV DNA <2000 IU/mL and HBsAg <1000 IU/mL at a single time point can reliably identify an inactive HBsAg carrier [31].

During this inactive phase, patients are usually asymptomatic and have PNALT. Liver biopsy shows no or mild necroinflammatory activity with variable degrees of fibrosis, including inactive cirrhosis, and intrahepatic HBcAg is absent [3]. Most HBsAg carriers in this phase show sustained remission of hepatitis and a lifelong inactive state, particularly if this phase is reached early in the disease course. Studies from Taiwan involving a large number of inactive HBsAg carriers have shown an estimated annual incidence of cirrhosis and HCC of 0.2% and 0.1% per year, respectively, and none of those who had PNALT over a follow-up period of 12.6±5.0 years died of liver disease [32]. For those who underwent HBeAg seroconversion before age 30, the incidences of cirrhosis and HCC were even lower [24]. The prognosis of inactive carriers from intermediate- or low-prevalence areas is even better, possibly due to the shorter duration of infection. In 317 asymptomatic HBV carriers from Montreal detected upon blood donation, the vast majority remained "healthy" and asymptomatic after 16 years of follow-up; only three died of HBV-related cirrhosis, and none of HCC [33]. In another study, from northern Italy, no difference in survival was observed between 296 healthy blood donors with positive HBsAg and 157 uninfected controls who were followed up over 30 years [34].

Spontaneous HBsAg seroclearance

During the inactive or residual phase, HBsAg level may decrease gradually and even disappear from the serum (HBsAg seroclearance) spontaneously. Short-term studies showed that the annual rate of spontaneous HBsAg seroclearance was 1% to 2% per year in Caucasian carriers in low-endemic areas, and even lower (~0.8%) in high-endemic areas where infection is usually acquired perinatally or in early childhood. However, a recent 11-year (mean) follow-up study in 1965 asymptomatic anti-HBe-positive subjects 16–76 years of age (median age 34) showed an annual HBsAg seroclearance rate of 1.2%; this was higher (1.8%) in those over age 50. The cumulative HBsAg seroclearance rate was 8% at 10 years, then it increased disproportionately to 25% at 20 years and 45% at 25 years of follow-up [35]. These findings suggest that the longer the duration of HBV infection, the higher the incidence of spontaneous HBsAg seroclearance.

In addition to PNALT and age or duration of HBV infection, factors significantly associated with spontaneous HBsAg seroclearance include low viral load, especially if HBV DNA is undetectable (<300 copies/ml); genotype A (> genotype D) or genotype B (> genotype C) HBV infection; and presence of cirrhosis or HCV superinfection (Table 10.1). Among these, older age was the most constant and important predictor for HBsAg seroclearance in most series. The annual incidence of HBsAg seroclearance varied among each series but correlated significantly with the mean or median age of patients at enrollment of each cohort [36]. Recent studies in predominantly genotype B or C HBV-infected HBeAg-negative patients with normal ALT have further shown that HBsAg levels <1000 IU/mL are predictive of spontaneous HBsAg seroclearance, the lower the higher, during a follow-up period of 7–12 years, and an HBsAg level ≤100 IU/mL is an appropriate cutoff for predicting HBsAg loss over time [37–40]. It was further demonstrated that HBsAg <200 IU/mL combined with a ≥1 $\log_{10}$ IU/mL decline of HBsAg in the preceding 2 years may predict HBsAg seroclearance in 3 years with high accuracy [40]. Interestingly, studies from Taiwan show that HBsAg carriers with spontaneous HBsAg seroclearance had significantly higher Body Mass Index (BMI) and higher degrees of fatty liver than those without [41], and fatty liver or BMI >30 kg/m^2 were independent factors significantly associated with HBsAg seroclearance [32, 42]. The underlying mechanism by which fatty liver enhanced HBsAg seroclearance remained unclear.

HBsAg seroclearance is eventually followed by loss of all serum markers of HBV replication, including HBV DNA becoming undetectable (<12 IU/mL) in >95% of the cases [43], and improved liver histology. However, intrahepatic HBV DNA, mainly in the form of covalently closed circular DNA, may persist after HBsAg seroclearance. Antibody to HBsAg (anti-HBs) may appear and increases over time to 59% at 51 months of follow-up after HBsAg seroclearance [36]. Patients with spontaneous HBsAg seroclearance had an excellent outcome because virtually none of the noncirrhotic

patients without concurrent HCV or HDV superinfection developed any untoward sequelae during a median follow-up period of 62 (12 to 179) months after HBsAg seroclearance [36, 39, 44]; HBsAg seroclearance has thus been considered as a state closest to a cure of chronic HBV infection. However, in patients who have preexisting cirrhosis or HCV superinfection, clinical outcomes of disease progression such as hepatic decompensation, HCC, or liver-related death may still occur [36].

Reactivation phase

Following HBeAg seroconversion, a subset of patients may encounter spontaneous reactivation of HBV replication, with reappearance of high levels of HBV DNA ($>2 \times 10^4$ IU/mL) and a rise in ALT levels. Only a small proportion of HBV reactivation is associated with the reappearance of serum HBeAg (HBeAg reversion), suggesting that reactivation of hepatitis B is mostly the result of HBV strain with precore or core promoter mutations that abolish or downregulate HBeAg production [15]. In addition, HBV replication can reactivate in inactive HBV carriers as a result of immunosuppression or chemotherapy [12].

HBeAg reversion

Following HBeAg seroconversion, HBeAg reversion may occur in a minority of patients. In a study from Alaska, 432 (80%) of 541 seroconverters remained HBeAg-negative and anti-HBe-positive throughout the study, whereas the remaining 109 (20%) encountered HBeAg reversion. Their HBeAg reversion episodes were frequently accompanied by a flare of hepatitis, and HBeAg tended to fluctuate between seroconversion and reversion, commonly having 2–3 reversions over the 6–7 years of the study [20]. However, HBeAg reversion was relatively uncommon in other studies. Studies from Taiwan have shown that HBeAg reversion occurred in <5% of the patients, usually within 3 years (median 1.1 years; range 0.3–3.2 years) after the onset of HBeAg seroconversion in several hundreds of patients [15, 24]. In a community cohort, HBeAg reversion was reported in 1.6% of 187 HBeAg seroconverters [18]. A study from Italy showed that only 1 (1.6%) of 61 seroconverters showed HBeAg reversion during follow-up [27]. Despite the low frequency, HBeAg reversion is significantly associated with increased risk of progression to cirrhosis [15], as well as development of HCC [20].

HBeAg-negative chronic hepatitis B

The majority of patients with HBV reactivation are HBeAg-negative, and these patients are said to have "HBeAg-negative chronic hepatitis B." HBeAg-negative chronic hepatitis B may present during follow-up in HBeAg-negative HBsAg carriers [15, 24] or present in previously unrecognized HBeAg-negative HBsAg carriers with clinical acute hepatitis [14]. Compared with HBeAg-positive patients with chronic hepatitis B, patients with HBeAg-negative chronic hepatitis B tend to be older and have lower serum HBsAg (2.5–4.0 $\log_{10}$ IU/mL) and HBV DNA levels (4.0–5.5 $\log_{10}$ IU/mL) [5], lower inflammatory activity, and fewer hepatitis flares but more advanced chronic liver disease.

The prevalence of HBeAg-negative chronic hepatitis B in chronic HBV infection varies widely between different geographical areas [2]. The relative proportions of HBeAg-negative cases among patients with chronic hepatitis B are 80% to 90% in the Mediterranean areas, 30% to 50% in Taiwan and Hong Kong, but less than 10% in the United States and Northern Europe [45]. However, the proportion of HBeAg-negative chronic hepatitis among anti-HBe positive carriers in different geographical areas remains largely unknown. This issue has been addressed in a few prospective studies that followed up the natural course following spontaneous HBeAg seroconversion. Studies from Taiwan have shown annual rates of HBeAg-negative chronic hepatitis of 2–3%, with a cumulative incidence of HBeAg-negative chronic hepatitis B of 25% after 16 years of follow-up following HBeAg seroconversion, but reactivation of hepatitis B typically occurred during the first 5 to 10 years following HBeAg seroconversion [15, 24]. In a study from Italy, 9 (14.8%) of 61 seroconverters developed HBeAg-negative chronic hepatitis during a mean follow-up of 22.8 years [27]. Notably, the incidence of HBeAg-negative hepatitis is related to the age of HBeAg seroconversion, being lower in those HBeAg-seroconverted before age 30 [24]. In pediatric patients, the rate is even lower: only 4 (6.3%) of 64 pediatric patients developed HBeAg-negative hepatitis during a mean period of 15 years following spontaneous HBeAg seroconversion [46].

The incidence of reactivation of hepatitis B among incidentally identified anti-HBe-positive carriers with normal ALT levels at entry also varies between Western and Eastern countries. In one study of 1241 anti-HBe-positive carriers from Taiwan, 211 (17.0%) developed HBeAg-negative chronic hepatitis during a mean period of 12.3 years, with the cumulative rate being 20.2% after 20 years of follow-up. Reactivation of hepatitis B occurred much more commonly during the first 5 to 10 years of follow-up and became extremely rare after 20 years [47]. A study from Italy showed that only 3 (4%) of 68 healthy blood donors seropositive for HBsAg developed HBV-related biochemical changes during a mean follow-up of 11 years [48]. A study from north Greece showed that only 4 (2.1%) of 195 "inactive" HBsAg carriers encountered HBV reactivation during a median follow-up of 5 years [49]. A more recent study

from Greece showed a cumulative incidence of HBeAg-negative hepatitis of 24% at the end of fourth-year follow-up of 85 "inactive carriers" [50]. This apparent difference among studies may be related to the different ethnic origin, different HBV genotype, mode of transmission, and age of the inactive carriers, and may therefore account for the different outcomes of inactive HBsAg carriers among these countries [2].

Factors predictive for HBV reactivation following HBeAg seroconversion have been identified (Table 10.1). Male gender, genotype C (> genotype B) [18, 51] or genotype D (> genotype A) HBV [52], and baseline HBV DNA levels $>10^4$ copies/ml or >2000 IU/mL [49] are associated with an increased risk of spontaneous HBV reactivation. It has been shown that serum HBV DNA <2000 IU/mL with HBsAg <1000 IU/mL is indicative of true inactive HBsAg carrier and has little chance of developing HBeAg-negative hepatitis over a median of 34.5 (6–110) months of follow-up [31]. HBeAg seroconversion before age 30 was associated with a particularly low incidence of HBV reactivation [24, 47]. In addition, ALT levels ≥5 times the ULN during the immune clearance phase and age of HBeAg seroconversion ≥40 years are also associated with increased risk of reactivation of hepatitis B [24, 51]. These findings suggest that hepatitis B is more likely to reactivate if more vigorous immune-mediated hepatocytolysis or a more prolonged immune clearance phase is needed to clear the HBeAg-producing HBV.

Studies have shown that patients with HBV reactivation have an increased risk of progression to cirrhosis, as compared to those with sustained remission. The annual rates of progression to cirrhosis and HCC were 2.3% and 0.5%, respectively, significantly higher than 0.1% and 0.2%, respectively, in those with sustained remission of hepatitis [15]. Notably, among patients with HBV reactivation, the incidence of cirrhosis correlated significantly with male gender and an age of reactivation >40 years [53].

Adverse sequelae and mortality

Liver cirrhosis

It is estimated that cirrhosis develops in approximately 20% of patients with chronic HBV infection. The annual incidence and cumulative probability of cirrhosis development in patients with chronic HBV infection varied considerably in different reported series, possibly due to inclusion of patients in different phases of infection as well as with variable severity of liver injury in each phase and also related to different geographical regions with different genotypes or mode of infection [2]. Studies have identified factors contributing to cirrhosis development. These include HBV genotype [19, 20]; HBeAg positivity or HBV DNA $>10^4$ copies/mL (2000 IU/mL) at recruitment [54, 55]; persistent HBeAg seropositivity [25–27]; HBeAg reversion [15, 20]; delayed HBeAg seroconversion over age 40 [22, 24]; reactivation of hepatitis B [15], especially if reactivation occurs at an age over 40 years [53]; persistence of serum HBV DNA during follow-up [56]; ALT flares with hepatic decompensation; and severe recurrent ALT flares with high AFP or bridging hepatic necrosis [57], as summarized in Table 10.2. In other words, many patients have developed cirrhosis during the HBeAg-positive phase, as shown in a recent study that 28 (30%) of 93 patients were HBeAg-positive at the onset of their cirrhosis [58]. These data implicate that the ultimate outcome of chronic HBV infection appears to depend on the duration of the immune clearance phase and reactivation phase following HBeAg seroconversion, as well as on the severity of liver damage during these phases. Other factors significantly correlate with progression to cirrhosis including older age, usually reflecting longer duration of infection; male gender; concurrent HCV, HDV, or HIV superinfection; alcoholism; and superimposed nonalcoholic fatty liver disease.

Table 10.2 Factors for the development of cirrhosis.

* Older age
* Genotype (C > B and D > A)
* Persistent HBeAg seropositivity and/or active HBV replication
* HBeAg seroreversion
* Persistent or intermitted ALT elevation
* Repeated hepatitis flares with bridging necrosis or hepatic decompensation
* Superinfection with hepatitis C or D virus or HIV
* Other factors: alcoholic or metabolic

ALT: alanine aminotransferase; HBeAg: hepatitis B e antigen; HBV: hepatitis B virus.

A large population-based long-term cohort study (the REVEAL-HBV study) from Taiwan revealed that the risk of cirrhosis increased proportionally with increasing HBV DNA levels at recruitment starting at a level $\geq 10^4$ copies/ml [55]. Patients included in the study were mostly HBeAg-negative (85%), and had normal ALT levels (94%) and a median age of 45 (30–65) years at recruitment. Consistent with the clinical finding that patients with serum HBV DNA $>10^4$ copies/ml have an increased risk of hepatitis B reactivation, it seems likely that the patients with high HBV DNA levels at baseline in this cohort study are prone to have hepatitis B reactivation and progress to HBeAg-negative chronic hepatitis prior to cirrhosis development [59].

Hepatic decompensation and mortality

HBV replication and necroinflammatory activity may have subsided at the onset of cirrhosis. However, at least

one-third to one-half of the patients with HBV cirrhosis still have a high level of HBV replication (HBeAg-positive or HBV DNA >2000 IU/mL) at the onset of cirrhosis [58, 60]. About 3–5% of patients with compensated HBV cirrhosis develop jaundice, ascites, hepatic encephalopathy, or esophageal variceal bleeding each year. The risk of hepatic decompensation is fourfold higher in HBeAg- or HBV DNA–positive (by hybridization assay) patients (4% per year) than in HBeAg- and HBV DNA–negative patients (1% per year) [60].

One major form of hepatic decompensation in HBV cirrhosis is secondary to hepatitis flares. The estimated annual incidence of hepatitis flare was 15–25% in HBeAg-positive patients and 5–10% in HBeAg-negative patients with cirrhosis [60]. Around 10–15% of acute flares were complicated with jaundice and 3–5% with ascites. Overall, the annual incidence of hepatic decompensation was 1.5% and the cumulative incidence was 30.8% during a mean duration of 102±60 (12–246) months [58].

The 5-year survival of compensated Child A HBV cirrhosis is approximately 80–85%, which correlates closely with the status of HBV replication. Survival probability is >95% in patients negative for HBeAg and HBV DNA (by hybridization assay) but only 60–72% in HBeAg- or HBV DNA–positive patients [60, 61]. Among the latter, HBeAg seroclearance is associated with a 2.2-fold decrease in mortality and ALT normalization is a better predictor of improved survival than HBeAg seroclearance [60].

Once hepatic decompensation has developed, survival probability decreases remarkably. The reported 5-year survival of decompensated Child B or Child C HBV cirrhosis varies considerably among different cohorts, ranging from 14% to 88% (average 30–50%) [60]. In one study from Hong Kong, the 5-year survival in patients seropositive for HBeAg at presentation was 57%, significantly lower than the 88% in patients seronegative for HBeAg at presentation [62]. In contrast, a study from Netherlands showed that the 5-year survival was extremely low (14%), independent of serum HBeAg at enrollment [63]. These data suggest that survival probability correlates significantly with the status of HBV replication in patients with less severe hepatic decompensation such as Child A and B cirrhosis, but not in patients with more severe hepatic decompensation such as Child C cirrhosis.

HCC

The incidence of HCC in chronic HBV infection correlates closely with the severity of underlying liver diseases. In East Asian countries, the summary annual rate of HCC ranged from 0.2% among inactive carriers to 0.8% in patients with chronic hepatitis B without cirrhosis and 3.7% in subjects with compensated cirrhosis; the corresponding 5-year cumulative incidence was 1%, 3%, and 17% respectively. In Western countries, the summary annual rate of HCC was 0.02% in inactive carriers, 0.3% in patients with chronic hepatitis B without cirrhosis, and 2.2% in patients with compensated cirrhosis; the corresponding 5-year cumulative incidence was 0.1%, 1%, and 10%, respectively [64]. These data confirm that liver cirrhosis is the most important risk factor of HCC development and also suggest that perinatally acquired chronic HBV infection is associated with a greater risk of HCC than infection acquired as an adult, possibly because of the longer duration of infection.

Since the risk of HCC correlates with underlying liver disease and most cases of HCC develop in patients with cirrhosis, HCC and cirrhosis share the same risk factors. Of the viral factors, HBV DNA levels, genotypes, and naturally occurring mutations such as HBV pre-S and basal core promoter A1762T/G1764A double mutations are associated with HCC development [65–68]. A multivariate analysis involving 2762 Taiwanese HBsAg carriers with 33 847 person-years of follow-up showed that A1762T/G1764A and genotype C were independent factors with an adjusted hazard ratio of 1.73 ($P = 0.013$) and 1.76 ($P = 0.005$), respectively [67]. It was also demonstrated that genotype C and high HBV DNA had a synergistic effect in promoting HCC development [65]. Investigators noted that the association between HBV mutations and HCC development was most profound in patients who had a pre-S deletion followed by basal core promoter mutations, and that combination of these mutations had a synergistic effect on the development of HCC [68]. Concurrent infection with HCV or HDV also increased the risk of cirrhosis and HCC [69]. Patients with a family history of HCC are at increased risk [70]. Other factors that contribute to HCC development include habitual alcohol consumption, cigarette smoking, and aflatoxin exposure. Studies in Africa and China showed a significant association between urinary aflatoxin levels and HCC, and a synergistic interaction with HBsAg carrier status [71]. The course and factors for the development of HCC are summarized in Figure 10.2.

Summary and conclusion

The natural history and events of perinatally acquired chronic HBV infection are summarized in Figure 10.1. The natural course of perinatally acquired chronic HBV infection typically has three chronological phases: an (1) immune-tolerant phase, (2) immune clearance phase, and (3) inactive or residual phase. In adult-acquired chronic infection, however, no obvious initial immune-tolerant phase is evident. HBeAg seroconversion is a

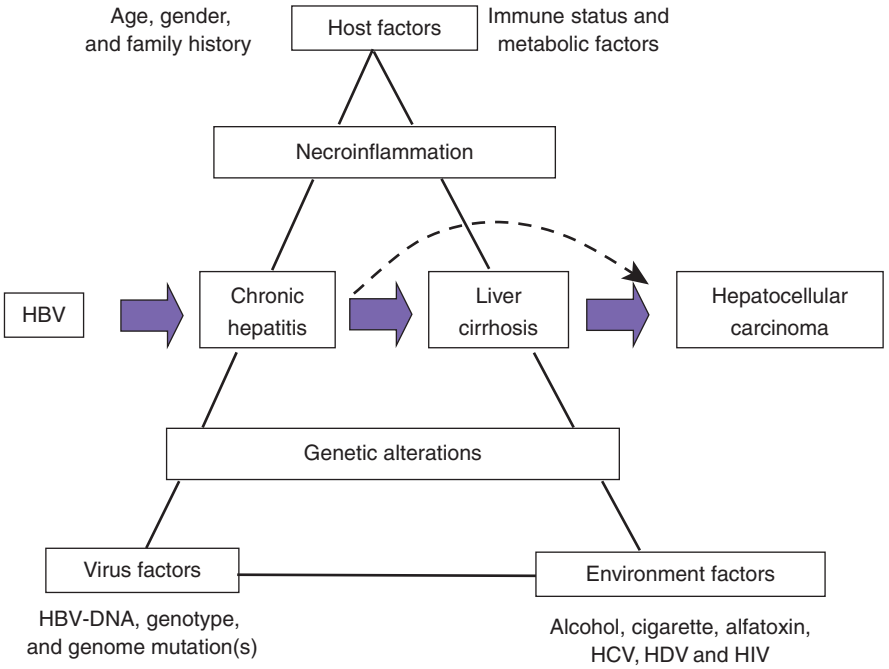

Figure 10.2 Hepatitis B virus (HBV) hepatocarcinogenesis is a multistage process involving multiple factors. Note the association of hepatic necroinflammation, liver disease progression, and genetic alterations during the course of chronic HBV infection. Host and environmental factors also contribute to the disease progression and hepatocarcinogenesis. HBV: hepatitis B virus; HCV: hepatitis C virus; HDV: hepatitis D virus; HIV: human immunodeficiency virus. ((Source: Adapted fro Liaw YF. Pros. Dig Liver Dis 2010; 42(Suppl 3):S293–S297 [71])

hallmark, and it usually signals transition from active to inactive HBV infection with ALT normalization and resolution of hepatitis activity. After long sustained remission, spontaneous HBsAg seroclearance may occur and confers excellent prognosis. On the other hand, HBV may reactivate and trigger immune-mediated liver injuries at an incidence of 2–3% per year, usually in the first 10 years after HBeAg seroconversion. This reactive phase can be viewed as a variant of the immune clearance phase.

HBV replication with subsequent interactions between HBV, hepatocytes, and immune cells during the immune clearance phase or reactive phase may lead to hepatitis activity and disease progression. High HBV DNA levels and disease activity are the best predictors of adverse clinical outcomes such as hepatic decompensation, cirrhosis, HCC, and liver-related mortality. Sustained reduction of HBV replication before the onset of cirrhosis confers a favorable outcome. Since active HBV replication is the key driving force of disease progression, HBV suppression is the primary target of therapy. Recent improvements in the understanding of the natural history of chronic hepatitis B and predictors of disease progression have helped in the management of patients with chronic HBV infection.

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Chapter 11
Extrahepatic manifestations of hepatitis B infection

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Summary

The hepatitis B (HBV) and hepatitis C (HCV) viruses are well-recognized causes of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Apart from liver disease, these viral infections are known to be associated with a wide spectrum of extrahepatic manifestations including polyarteritis nodosa (PAN) and vasculitis, glomerulonephritis, and cutaneous, neurological, and hematological complications. 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 are of paramount importance in facilitating early diagnosis and offering treatment. Such treatments may be highly successful, as for HBV-related PAN and glomerulonephritis, but in some cases treatment may be unsuccessful and the infection may continue with disabling extrahepatic manifestations.

Introduction

Hepatotropic HBV is 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. Treatment may be highly successful, as for HBV-related PAN, but it may also be less effective, and patients may continue with disabling extrahepatic manifestations.

Patients with chronic viral hepatitis commonly have immunological manifestations, including auto-antibody formation and concurrent immune diseases. These immunological findings may resemble those of autoimmune hepatitis, and they are not disease specific. High-titer auto-antibodies are uncommon in chronic viral hepatitis, as are multiple concurrent auto-antibodies. 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 (cryoglobulinemia, 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

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can have similar immune features, but patients with chronic hepatitis C more commonly have auto-antigen-driven processes.

Background

Although most patients infected with HBV remain symptom-free, a wide clinical and histopathological heterogeneity bears witness to complex interactions between HBV, the immune response, and other cofactors such as co-infection with HCV, hepatitis D virus, or HIV; consumption of cytotoxic, immunosuppressive, or glucocorticoid medicines; and alcohol abuse.

In acute HBV infection, in adults almost 75% of patients remain symptom-free and 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). 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 PAN, glomerulonephritis, polyradiculoneuritis, essential mixed cryoglobulinemia, 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 and central or peripheral nervous system manifestations, may also occur. Although extrahepatic manifestations do exist with all acute viral hepatitis, 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, which may be associated with fever and some urticarial maculopapular lesions. All these manifestations are usually transient and clear up as the hepatitis progresses. Rarely, extrahepatic manifestations of acute HBV infection may persist for months, usually reflecting the transition to chronic hepatitis. High viral replication levels and persistent infection may favor production of soluble immune complexes in antigen excess, which deposit on specific sites including skin, kidney, and small- and medium-caliber arteries. Different pathogenic mechanisms have been identified in the pathogenesis of vasculitis. Immune complexes are mainly thought to be responsible for vasculitis including PAN, glomerulonephritis, and mixed cryoglobulinemia. 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, and HBe antigens; immunoglobulins; and C3 have been documented together with low complement levels in the serum. Immunostaining and ultrastructure findings indicate that hepatitis B "e" antigen (HBeAg) is probably a responsible candidate antigen [2]. However, a PAN case was recently reported in association with an HBeAg-negative precore mutant infection [3].

Polyarteritis nodosa

Four decades ago, we recognized that HBV was responsible for a significant number of patients with PAN [4–6] (Table 11.1). It is a focal segmental vasculitis due to inflammation and necrosis of medium-sized arterial walls that lead to microaneurysm formation and organ infarction. It affects most commonly the vasculature of the kidneys, skin, muscles, nerves, and GI tract. There are two forms of PAN called idiopathic or primary and HBV-related or secondary form.

This viral infection is now considered as one criterion for the diagnosis of PAN vasculitis according to the American College of Rheumatology.

Epidemiology

PAN is a rare disease that affects all racial groups. Estimates of the annual incidence of PAN-type systemic vasculitis in the general population range from 4.6 per 1 000 000 in England [7] and 9.0 per 1 000 000 in Olmsted County, Minnesota, to 77 per 1 000 000 in a hepatitis B–hyperendemic Alaskan Eskimo population [8]. In a German study [9], the PAN incidence was extremely low (0.3–0.4/1 000 000, according to the year and part of the country). In France, PAN prevalence was 34 per 1 000 000 in Seine–Saint-Denis, a northern suburb of Paris [10]. Its prevalence was the highest among the vasculitides that were studied (microscopic polyangiitis [MPA], PAN, Churg–Strauss syndrome [CSS], and Wegener's granulomatosis [WG]), but its incidence has declined in parallel with that of HBV infection [11], especially wild-type HBeAg-positive ones.

In France, like in most developed countries, HBV infection transmitted by blood transfusion has now disappeared, with the last proven case occurring in 1987 [11], but intravenous drug abuse has rapidly become a major cause of HBV-related PAN [11], as are cases due to sexual transmission of HBV in individuals at risk who were not vaccinated. The development of vaccines against HBV and their administration to people at risk also explain the dramatic decrease of the number of new cases observed since 1989. Over the past few years, the frequency of HBV-related PAN has declined to 20% [11]. For the last 3 years, fewer than 10 patients a year have been diagnosed throughout France. Moreover, the frequency of PAN, whether due to HBV infection or not, has also decreased in parallel.

Table 11.1 Relevant clinical and biological symptoms in 115 patients with HBV-associated polyarteritis nodosa.

Clinical symptoms	Values[a]
Age, mean ± SD (years)	51.1 ± 17
Sex ratio	74 M and 41 F
General symptoms	111 (96.5)
Fever	79 (68.7)
Weight loss	100 (87)
Arthralgias	64 (55.7)
Myalgias	54 (47)
Mononeuritis multiplex	96 (83.5)
Superficial peroneal	82 (71.3)
Deep peroneal	46 (40)
Cubital	25 (21.7)
Radial	12 (10.4)
Bilateral	66/91 (72.5)
GI tract involvement	61 (53.0)
Abdominal pain	59 (51.3)
Bleeding	3 (2.6)
Appendicitis	2 (1.7)
Small intestine perforation	7 (6.1)
Cholecystitis	6 (5.2)
Pancreatitis	7 (6.1)
Renal and/or urogenital involvement	44 (38.3)
Proteinuria and/or hematuria	21 (18.3)
Glomerulonephritis[b]	1 (0.9)
Renal vasculitis	34 (29.6)
Creatininemia, mean ± SD (mg/dl)	1.52 ± 1.39
Anuria at entry	4 (3.5)
Orchitis	18/71 (25.3)
Microaneurysms[c]	46/67 (68.6)
Renal infarcts[b,c]	19/67 (28.3)
Skin involvement	36 (31.3)
Purpura	19 (16.5)
Infiltrated purpura	13 (11.3)
Livedo	12 (10.4)
Nodules	10 (8.7)
Edema (ankles)	18 (15.7)
Vascular manifestations	21 (18.3)
Hypertension	36 (31.3)
Malignant hypertension	6 (5.2)
Cardiac insufficiency	14 (12.2)
Raynaud's phenomenon	3 (2.6)
Pericarditis	6 (5.2)
Digital ischemia	4 (3.5)
Myocardial infarction	1 (0.9)
Central nervous system involvement	11 (9.6)
Retinal vasculitis	2 (1.7)
Erythrocyte sedimentation rate > 30 mm/1st h	90 (78.3)
Antineutrophil cytoplasm antibodies (ANCA)[d]	0

[a]Values are numbers (%), unless otherwise indicated.
[b]Not related to vasculitis.
[c]66 angiographies.
[d]66 searches.
(Source: Guillevin L, Mahr A, Callard P *et al.* Medicine (Baltimore). 2005 Sep;84(5):313–322[11])

Exposure to HBV before the onset of PAN is not easy to establish but, in a large series of patients, it was possible to determine the infection route in 43 out of 115 patients [11]. When identified, the interval between time for infection and first manifestation was 596 ± 628 days (range 30–1695 days). The immunological process responsible for PAN usually occurs within 12 months of the primary HBV infection. Hepatitis is rarely diagnosed as it remains mostly silent before PAN manifestations become clinically patent.

The pathogenic mechanism of HBV-related PAN has not been convincingly elucidated. Deposition of viral antigen–antibody complexes in vascular endothelium has been demonstrated. This deposition can lead to the activation of the complements cascade and the release of chemotactic factors attracting neutrophils to the site of inflammation.

One study also demonstrated the evidence of active HBV viral replication with detectable HBV RNA, replicative intermediates of HBV DNA, as well as HBsAg and HBcAg, localized to the vascular endothelium [12]. A combined role of immune complexes and local HBV replication within endothelium was, therefore, suggested as a pathogenic mechanism of HBV-related PAN [12].

Clinical manifestations

PAN typically presents with constitutional symptoms such as fever and weight loss associated with multiorgan dysfunction. All body organs could be affected except for the lungs, a feature that is helpful in distinguishing PAN from antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis.

Neurologic manifestation, which is usually presented by a mononeuritis multiplex or sensorimotor peripheral neuropathy, is the most common and is seen in up to 70% of patients [13]. Febrile polyneuritis is a signature of PAN.

This is followed by GI involvement that may occur in 40% to 60% of patients and can be manifested by mesenteric ischemia, GI hemorrhage, bowel perforation, and pancreatitis [14, 15]. Severe GI involvement is a major cause of death in patients with PAN.

Other common affections are renal involvement, skeletal-muscle and large-joint involvement, skin manifestations, and testicular artery ischemia, which presents by testicular pain and is a characteristic feature but occurs in only about 20% of patients.

A localized form of PAN involving a single organ (e.g., appendix) may occur and conversely is associated with good prognosis.

In the group of patients with HBV-related PAN, clinical data were roughly the same as those commonly observed for PAN, but with some differences. This type of PAN is more frequent in patients younger than 40 years, and malignant hypertension, renal infarction, and orchiepididymitis (25%) were more frequent [11, 16]. Regarding other symptoms, we frequently observed abdominal manifestations (53%) and especially surgical emergencies. In the study by Sergent *et al.* [17], two of

the three deaths (among nine patients) were attributed to colon vasculitis.

Digestive and renal manifestations resulted from ischemia, and angiography demonstrated the presence of microaneurysms and infarctions. It should be noted that subsequent angiograms showed that the aneurysms had disappeared when PAN had been effectively treated [18]. This disappearance could result from the thrombosis of aneurysms with evolution to fibrosis. HBV-related PAN is acute and initially severe, but the outcome can now become excellent in most cases if early adequate treatment is prescribed. Seroconversion usually leads to recovery. Sequelae are the consequence of vascular nephropathy, but, even in patients with renal insufficiency, it is possible to obtain recovery with little residual impairment of renal function.

Outcome

The outcome of HBV-related PAN is more serious unless the appropriate antiviral treatment is prescribed [11, 19–21]. Sequelae are the consequence of pathological damage including vascular nephropathy, as well as central and/or peripheral nervous system disorders. Hepatic manifestations associated with the course of PAN are clinically moderate, and transaminase elevation is 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 the pathogenesis of PAN, a few cases challenge this view, as they have been reported to be associated with a precore mutation that 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]. In a large cohort study based on 348 PAN patients who were followed up for a mean duration of 68.3 ± 63.5 months, 76 (21.8%) relapsed (63 [28%] non-HBV-related PAN versus 13 [10.6%] HBV-related PAN; $p < 0.001$) and 86 (24.7%) died (44 [19.6%] non-HBV-related PAN versus 42 [34.1%] HBV-related PAN; $p = 0.002$) [16].

Treatment

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 [22], 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. 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 [22]. Later, a first-generation nucleoside analog (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 [20]. The rationale of the therapeutic sequence was (1) to control the most severe life-threatening manifestations of PAN rapidly 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 favor HBeAg 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 HBsAg to anti-HBs seroconversion was obtained in about 20% of patients. Later studies enrolling small numbers of patients replaced vidarabin with interferon alpha (IFNα), allowing efficient recovery from PAN and HBeAg to anti-HBe seroconversion in about two-thirds of patients, HBsAg to anti-HBs seroconversion in about one-half of patients, and rare relapses over the long-term follow-up.

The dose of IFNα was comparable to that prescribed for hepatitis B, with the duration of administration not exceeding 6 months for the majority of patients. The triple therapy combining plasma exchanges, corticosteroids, and IFNα was again shown to be successful for treating a PAN case associated with a precore promoter mutant HBV infection [23]. Other similar antiviral approaches were also successful, including IFNα alone [24] or combined with famciclovir [25] or lamivudine [26–28]]|; [26–28]. Lamivudine has also been tested as a successful single first-line antiviral therapy for HBV-related PAN [27, 29]]|; [27, 29].

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 clear the immune complexes responsible for the disease rapidly. 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.

Renal manifestations

Renal manifestations related to HBV are renal vasculitis observed in PAN and glomerulonephritis. Hepatitis B virus is never responsible for rapid crescentic pauci-immune glomerulonephritis, which is the consequence of small-sized vessel vasculitis and the hallmark of ANCA-associated vasculitis. However, on rare occasions HBV can be responsible for other types of glomerulonephritis, which are detailed in this chapter.

Renal vasculitis

Renal artery involvement can be responsible for mild to severe and malignant arterial hypertension and/or vascular ischemic nephropathy with renal insufficiency [30], which has been identified as a poor prognosis factor [31, 32]. Angiography (when performed despite renal involvement) may show renal parenchymal infarcts and characteristic multiple stenoses and microaneurysms of branches of celiac-mesenteric and renal arteries. Microaneurysms can occasionally rupture, spontaneously or after renal biopsy, which is strongly contraindicated in the presence of microaneurysms. Renal hematoma can be extensive and requires embolization [33] or even nephrectomy [34].

Acute renal insufficiency usually occurs early during the disease course or during a flare. Plasma exchanges might be required initially, but renal function outcome remains unpredictable. Some patients may come off dialysis but end-stage renal failure, resulting from chronic renal ischemia, may develop later, sometimes years after the first manifestations of PAN.

Glomerulonephritis

Although most cases of glomerulopathy are idiopathic, they may be associated with a variety of chronic infections and immune complex diseases. HBV may cause membranous glomerulonephritis (MGN), rarely associated with concomitant PAN [35]; membranoproliferative glomerulonephritis (MPGN); mesangioproliferative glomerulonephritis (MSPGN); and, rarely, IgA nephropathy.

Chronic HBV infection is an etiologic factor for secondary glomerular diseases. HBV-related glomerular diseases are more common in children than adults and in men than women.

The first description of HBV-associated glomerulonephritis was reported by Combes and colleagues in 1971 [36]. The most common renal manifestation is MGN [37], and the other types of nephropathy such as MPGN, proliferative glomerulonephritis, and IgA nephropathy are less common. A possible link with focal segmental glomerulosclerosis has been suggested [38]. HBV-associated glomerulonephritis is a relatively rare manifestation of chronic HBV found in 3% of patients in a French study [30].

Pathophysiologically, immune complexes' depositions are thought to be the main pathogenic mechanism, especially in MGN. Whether these immune complexes are formed *in situ* or are deposits from circulating immune complexes trapped in the glomerulus remains controversial [39]. The immune complexes involved could result from various HBV antigens including HBsAg, HBeAg, and HBcAg. These viral antigens have been found in glomeruli as well as covalently closed circular DNA (cccDNA) in renal tissue [40]. Immunostaining and ultrastructural findings indicate that HBeAg is probably a responsible candidate antigen for the formation of subepithelial immune deposits [2]. Its low molecular weight makes it able to go through the glomerular basement membrane. Another evidence of involvement of HBeAg is the remission of proteinuria after its clearance. The deposited immune complexes likely activate complements, and glomerular injury occurs via the formation of a membrane attack complex and other downstream cascades of events [39].

The role of the HBV genotype has not been completely determined. A high prevalence of genotype A is suspected [39, 41]. The typical clinical presentation of HBV-related MGN is nephrotic range proteinuria associated with microscopic hematuria with otherwise normal renal function. In patients with MPGN, however, renal function impairment is common.

The clinical course of HBV-related membranous nephropathy is not the same for children and adults. In children, it usually has a benign course with a cumulative probability of spontaneous remission at 4 years of 64%, and the probability of progression to renal insufficiency is very rare [42]. Furthermore, the membrane deposits disappear after spontaneous remission of the nephrotic syndrome and HBe seroconversion frequently parallels remission.

In contrast to pediatric patients, adults patients are more likely to progress, as shown in an endemic country study with a low prevalence of spontaneous remission and, conversely, with progressive renal insufficiency in 29% and dialysis in 10% of patients after 60 months of follow-up [43].

Treatment of HBV-related nephropathy

Available data remain scarce and most come from studies in patients with MGN, the most common pres-

entation, and in pediatric patients, who generally have a more favorable course. The data on other types of nephropathy are anecdotal.

The treatment strategy is based on the use of antiviral agents. By contrast, the use of immunosuppressive drugs such as corticosteroid alone, which is usual in non-HBV-related nephropathy, carries additional risk of viral replication flares with liver disease exacerbation.

The reported results support the efficacy of both IFN [44] and nucleos(t)ide analog [45, 46] therapy in reducing proteinuria in patients , mostly children, with HBV-related MGN.

A randomized controlled trial by Lin [47] included 40 children with corticosteroid-resistant MGN in two study groups, the treatment group using IFNα and the control group using placebo. All 20 patients in the treatment group had regression of the proteinuria after 3 months of treatment and were free of proteinuria after 1 year of follow-up, and 80% of them showed HBe seroconversion. Conversely, no HBe seroconversion was observed in the placebo group and all patients continued to have proteinuria [47].

The results in adult patients are altogether disappointing [43, 48], as shown by Lai *et al.* [43], who treated five MGN patients by IFNα and obtained a complete response associated with HBe seroconversion in only one patient. However, a larger study confirmed the efficacy of IFNα in 15 HBV-related nephropathy patients, 10 of which were MGN and 5 MPGN [49]. In this study, sustained loss of HBeAg and HBV DNA occurred in eight (53%) patients, and most of these eight virological responders (seven of eight) also showed a gradual and marked improvement in proteinuria. While the nephropathy persisted in the seven virological nonresponders, the eight responder patients had MGN, whereas four of the seven nonresponder patients had MPGN.

Most of the data on nucleos(t)ide analog therapy in HBV-related nephropathy are anecdotal. This type of oral therapy with nucleos(t)ide analogs (lamivudine, telbivudine, adefovir, tenofovir, and entecavir) does not have the disadvantages of IFNα, including the subcutaneous injections and frequent side effects. However, it is a long-term treatment prone to selection of drug-resistant strains. Moreover in relation to the tubule-interstitial nephrotoxicity related to adefovir and, to a lesser extent, tenofovir, they should be used with caution in patients with renal impairment [39].

A lamivudine study in patients with HBV-related MGN showed a complete remission of proteinuria in 60% of patients after 12 months of treatment [46]. A recent meta-analysis [50] included 82 patients from six therapeutic trials with IFNα (72 patients) and lamivudine (10 patients) in HBV-related glomerulonephritis patients. It showed an overall remission of proteinuria in 65.2% and HBeAg seroconversion in 62%, with a close correlation between HBe seroconversion and the remission of proteinuria [50].

Although most published data so far are still based on lamivudine studies, the most logical choice in HBV MGN appears to be entecavir, which has been found superior in terms of efficacy without significant emergence of resistance in non-lamivudine-exposed patients and is not associated with potential renal toxicity like adefovir and tenofovir.

Cutaneous manifestations of HBV

Juvenile papular acrodermatitis and urticaria

These are the most common cutaneous manifestations of HBV. Juvenile papular acrodermatitis, also called Gianotti–Crosti syndrome, is a rapid-onset papular cutaneous rash that is often progressive and sometime pruriginous; it occurs in young children aged 1 to 6 years. Papular lesions are mostly found on the hands, legs, forearms, elbows, and knees. The face can also be affected. The rash spontaneously clears in 1 to 6 weeks. General symptoms such as splenomegaly, hepatomegaly, general weakness, and diarrhea may sometimes be associated. This juvenile papular acrodermatitis is mostly of viral origin, and HBV is one of the most common causes [51–53].

Acute urticaria

This is another cutaneous manifestation that can be associated with HBV. Although urticaria can be standalone, most of the time it happens together with violent headaches and arthralgia characteristic of Caroli's triad, which typically occurs at the acute pre-icteric phase of acute hepatitis B in 10% to 25% of cases. Remarkably, it will precede icterus by several weeks (1 to 7 weeks) and classically fades away when the patient turns yellow. As for PAN, the pathogenic mechanism of this acute serum sickness–like syndrome is believed to be due to the deposit of immune complexes involving viral antigens. Indeed, cutaneous biopsy did show vasculitis lesions associated with IgM and HBsAg deposits in the vessel walls [54, 55]. It should be emphasized that the CAROLI triad can be found with both hepatitis A and B infections and acute urticaria can be associated with many more viruses including EBV, enterovirus, and parvovirus. Urticaria is, in fact, one of the hallmarks of viral infection, especially in children.

Oral lichen planus

Oral lichen planus may questionably be associated with HBV [56, 57]. Other various cutaneous manifestations have been rarely reported, especially leucocytoclastic

vasculitis, which is mostly associated to purpura (Henoch–Schönlein type and erythema nodosum).

Hematologic manifestations

Although far less documented than with HCV, chronic HBV infection can be associated with B cell non-Hodgkin lymphoma (B-NHL). Indeed, it has been found more commonly in hospitalized patients than among general population controls. These preliminary data, which were initially based only on serological studies, have been confirmed in case–control studies [58, 59]. Furthermore, the incidence calculation of B-NHL has been carried out in HBV-positive and HBV-negative cohorts, which suggested an increased relative risk of 2.80 (confidence interval [CI] 95%: 1.16–6.75).

In HBV carriers, a newer study is being carried out in a big database of prospectively followed patients and is also confirming an increase for B-NHL in chronic HBV patients. Such recent data bring further support to active treatment and control of HBV carriers worldwide.

Neurologic manifestations

Acute polyradiculoneuritis has been reported in both acute and chronic HBV infection [60, 61]. Again, like for kidney, vascular, and other extrahepatic manifestations, evidence for immune complexes has been found in the serum and spinal fluid during the acute phase of neurological symptoms, suggesting the pathogenic role of such complexes.

Ocular manifestations

Uveitis has been suspected to be associated with HBV infection, and a Swiss study suggested that up to 13% of uveitis sufferers were positive for HBsAg [62]. Such results have, however, been challenged. One study in the United Kingdom detected HBsAg in only 2% of uveitis patients [63], and a similarly low prevalence of HBsAg was also found in French uveitis patients [64].

Conclusions

Extrahepatic manifestations of HBV infection are less common and have been partially overshadowed by those of hepatitis C. They are, however, well described and reported, and unfortunately could be invalidating or even life threatening. The two major entities are PAN or vasculitis and glomerulonephritis.

PAN is the most dramatic manifestation of primary HBV infection, while glomerulonephritis and nephrotic syndrome may develop during the course of chronic HBV infection. The pathogenesis of both vasculitis and glomerulonephritis is still believed to be mediated by circulating immune complexes, the formation of which is favored by high HBV replication. No evidence for an autoimmune pathogenic mechanism can be substantiated in PAN, contrary to other type 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 efficiently control HVB-related glomerulonephritis. Traditional immunosuppressive and corticosteroid therapy should no longer be used as a mainstay in those HBV-driven extrahepatic manifestations.

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Chapter 12
Hepatitis B and hepatocellular carcinoma

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Summary

Hepatocellular carcinoma (HCC) is among the most common cancers in the world and one of the few human cancers caused by viral infections. The hepatitis B virus (HBV) is a major risk factor of HCC development, but the underlying oncogenic mechanisms have not been fully elucidated. Malignant transformation occurs after a long period of chronic liver disease, which is frequently associated with cirrhosis, suggesting a nonspecific mechanism triggered by the host immune response. However, the virus might play a direct role as an insertional mutagen through viral DNA integration into the host genome. Viral proteins including the transcriptional activator HBx and the large and middle surface proteins can act as cofactors by interfering with regulatory processes. Because HCC carries a dismal prognosis, there is urgent need to develop early diagnostic markers of HCC and effective therapies against chronic hepatitis B. This chapter reviews various mechanisms linking chronic HBV infection to malignant transformation, with reference to recent advances provided by genomewide analysis of HBV-related HCC.

Introduction

Primary liver cancer, mostly HCC, ranks among the commonest cancers in many countries. A recent estimate indicates that HCC represents the second most common cancer in men and the sixth most common cancer in women, with a total of 748 300 new liver cancer cases and 695 900 liver cancer deaths each year, most of which occur in developing countries, and more than one-half in China alone [1]. Moreover, because of its very poor prognosis, HCC represents a leading cause of cancer death worldwide.

The causal relationship between chronic infection with HBV and the development of liver cancer has been firmly established by epidemiological criteria. For chronic HBV carriers, the risk of developing HCC is increased by 20- to 100-fold as compared with uninfected populations, placing HBV among human tumor viruses and at the first rank among known human carcinogens [2, 3]. Despite the availability of efficient and safe vaccines, HBV infection is still associated with 54% of HCC cases in the world [4]. However, whether HBV acts through any known oncogenic mechanism, either directly or indirectly, remains an 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 nonspecific mechanism triggered by the host immune response. Chronic inflammation of the liver, continuous cell death, and consequent cell proliferation increase the occurrence of genetic and epigenetic alterations ultimately leading to cancer. In a more specific pathway, long-term effects of viral

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proteins such as the X protein, a promiscuous transcriptional activator, or various forms of the large and middle surface proteins have been implicated in the tumoral process by enhancing immune-mediated liver disease. Alternatively, the virus might play a direct role through integration of viral DNA into the host genome. Viral integration events have multiple consequences by either inducing large-scale chromosomal changes or having *cis*-acting effects on the expression or function of nearby cellular genes. Indeed, viral integration nearby potential oncogenes has been described in an increasing number of HCC cases [5–7], and genetic instability resulting from viral integration has been documented. Integrated HBV sequences might also alter the host cell growth control in *trans*, through unregulated expression of mutated viral proteins. All these potential mechanisms might not be mutually exclusive [8, 9]. To date, although information on risk factors causally linked to HCC has accumulated, the role of viral agents and carcinogenic cofactors is only partially elucidated, and no unifying model accounting for the contribution of viral and cellular factors to liver oncogenesis has been established.

Epidemiological association between HBV and HCC

Epidemiological research has contributed largely to the understanding of the etiology of HCC. Different carcinogenic factors, such as chronic hepatitis B and C, exposure to dietary aflatoxins, excessive alcohol intake, and more recently diabetes and obesity, have been implicated as major risk factors in human hepatocarcinogenesis [10].

The first evidence linking chronic HBV infection with the development of HCC came from similar general geographical patterns 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 [4]. Case–control studies as well as prospective studies in many countries and in different ethnic or social groups have reported a marked increased risk of HCC among hepatitis B surface antigen (HBsAg) carriers compared with noncarriers (up to 100 risk factors have been reported using different methodologies of investigations). These studies have unveiled great variations in HCC incidence among different carrier populations, which may be attributed to differences in the intrinsic properties of HBV infection patterns and in host genetic and environmental determinants among chronic carriers. A gender discrepancy (men have a twofold to eightfold elevated risk of developing HCC compared with women) and familial tendency have been involved in the frequency of tumor development [4]. Chronic infections resulting from maternal-neonatal transmission present a greater risk of HCC than those acquired as adults. Among HBsAg carriers infected at an early age, an additional HCC risk has been associated with high levels of viral replication and with hepatitis B e antigen (HBeAg) carriage, suggesting that HBeAg positivity could be a predictive marker of the risk of cancer. It has also been shown that HBV genotypes might differ in their oncogenicity, as genotype C appears to be more oncogenic than genotype B [11]. Moreover, a meta-analysis of more than 40 studies, including a total of 11 582 HBV-infected individuals, has revealed that mutations accumulate at specific positions during the course of chronic hepatitis from healthy carrier state to HCC. In particular, mutations in the PreS domain combined with mutations C1653T, T1753V, and A1762T/G1764A in the basal core promoter (BCP) that overlaps with the 3′ end of the X gene have been associated with high probability of HCC development [12]. Interestingly, the mutation pattern of HBV DNA in plasma could represent a strong prognostic marker for HCC in chronic HBV carriers. Finally, the risk of cancer increases with the progression of liver damage and with the presence of cirrhosis. Liver cirrhosis is viewed as a preneoplastic condition, but with varying degrees of correlation. Macronodular cirrhosis precedes or accompanies a majority (from 60 to 80%) of HBV-associated HCCs in adults as well as children.

In addition, environmental cofactors have been shown to increase HCC incidence and mortality in HBV-endemic regions, where synergistic effects of aflatoxin B1 (AFB1) and HBV infection have been demonstrated. Excessive alcohol intake also increases the risk of HCC in HBV carriers, and diabetes mellitus (DM) further increases the risk of HCC in the presence of other risk factors such as hepatitis C, hepatitis B, and alcoholic cirrhosis [10].

In Western countries and Japan, only 15% to 20% of the tumors occur in HBsAg-positive patients, but epidemiological studies have shown a high prevalence of anti-HBs and anti-HBc antibodies in HBsAg-negative patients with HCC, indicating that past and resolved HBV infection might retain pro-oncogenic properties. A direct role of HBV in these liver cancers is suggested by the detection of HBV DNA in HCCs developing on noncirrhotic livers from HBsAg-negative patients. Thus, cirrhosis cannot solely account for the induction of the cancer in these cases. Another intriguing aspect is the repeated finding of increased risk of tumor development in HBsAg-negative patients that display characteristics of "occult" HBV infection, notably an absence of HBV serological markers but also low levels of HBV DNA detected by polymerase chain reaction (PCR) in the serum or in the liver [8, 13].

Finally, it is important to point out that the control of HBV transmission through systematic vaccination against hepatitis B has resulted in a recent decline of

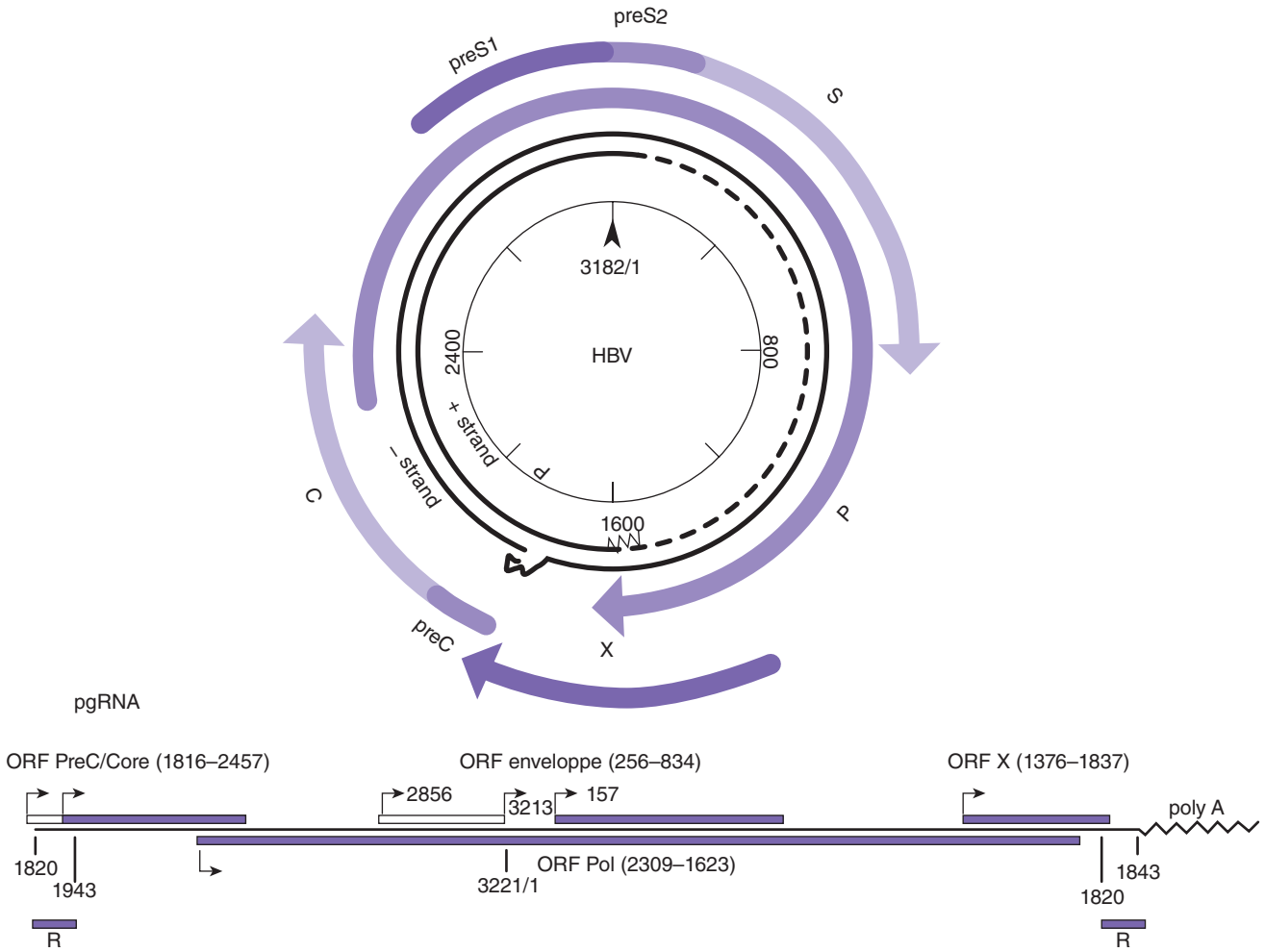

Figure 12.1 Genetic organization of HBV. The viral genome is made of two linear DNA strands (thick black lines) maintained in circular configuration by their cohesive ends. The minus strand is around 3.182 kb in length, while the plus strand has variable length with a fixed 5′ end position and variable 3′ end (dashed line). The protein bound to the 5′ end of the minus strand corresponds to the N-terminus of the HBV polymerase, also called primase. The broad arrows surrounding the viral genome represent four ORFs that encode seven different proteins. The lower panel depicts the organization of the pregenomic RNA (pgRNA). Nucleotide positions are given for HBV subtype *ayw* (Source: Galibert F, Mandart E, Fitoussi F *et al.* Nature 1979;281: 646–650 [15]). (Color plate 12.1)

pediatric HCC in endemic countries like Taiwan, with up to a 70% decrease of HCC-related mortality in children under 15 years of age [14].

The HBV genome

HBV belongs to the *Hepadnaviridae*, a family of small enveloped DNA viruses that infect a number of mammals and birds, including woodchucks, squirrels, and ducks. These viruses share a narrow host range and preferential tropism for hepatocytes. Sequencing of cloned HBV DNA from different genotypes has 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 [15] (Figure 12.1). 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 that contains primase and replicase activities; and the smallest, termed X, codes for a transcriptional transactivator. The entire viral genome is coding, with a large portion harboring two different reading frames due to overlapping of the P gene with the other viral genes. Moreover, because the genome is entirely coding, regulatory sequences (promoters, enhancers, and termination signal) are embedded within gene-encoding regions. The genomes of different HBV genotypes differ mainly by single-nucleotide substitutions or by the addition of

multiples of three nucleotide blocks, preserving the reading frames and leading primarily to conservative amino acid changes. 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. In addition, a number of mutations in the pre-S/S region, in the core gene or in the core gene promoter, and in the polymerase gene lead to the outcome of HBV variants with modified immunological and pathological properties [9].

The HBV genome shares with those of animal hepadnaviruses a basically identical genomic organization and extensive sequence homologies, mostly in the C and S genes and in the viral polymerase. 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, a variable region among hepadnaviral genomes that could account for the restrictive host range of *Hepadnaviridae*. The X internal domain is the less conserved region of the gene among mammalian Hepadnaviruses, but 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.

In infectious extracellular virions, the hepadnaviral genome is made of two complementary DNA strands of different length, maintained in a circular configuration by base pairing at their 5′ extremities (Figure 12.1). The viral replication cycle is virtually identical for all hepadnaviruses. It takes place in the nucleus and cytoplasm of infected cells, and it is entirely extrachromosomal [9]. Hepadnaviruses, like retroviruses and caulimoviruses, use a reverse-transcription step during replication, suggesting that they display a common evolutionary origin [16].

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 posttranscriptionally. Each individual HBV gene is controlled by an independent set of regulatory signals acting in cooperation with HBV enhancers that coordinate the relative level of viral gene expression. Two major HBV transcripts of 3.5 and 2.1 kb and three minor transcripts of 3.9, 2.4, and 0.8 kb direct the synthesis of seven viral proteins. Two RNA species of 3.5 kb encode the core protein, the e antigen, and the polymerase; the 2.1 kb RNA encodes the middle and major surface glycoproteins; the 2.4 kb RNA encodes the large surface protein; and the viral X transactivator activity is specified by the 3.9 and 0.8 kb transcripts. Moreover, a spliced HBV transcript can be reverse-transcribed and encapsidated in defective HBV particles or expressed as a new HBV protein, referred to as HBV spliced protein (HBSP) [8]. Viral transcription is controlled by four independent promoters and two enhancers: EN I, positioned about 450 upstream of the core promoter, and EN II, located in the X gene–coding region [17].

Transcription of viral RNAs including the 3.5 kb pregenomic RNA is a crucial step of the HBV life cycle. It occurs in the nucleus using as template the covalently closed circular DNA (cccDNA) (Figure 12.2). It has been shown that cccDNA is bound by histones and nonhistone proteins and organized into a chromatin-like structure that is subjected to epigenetic regulation such as recruitment of chromatin-modifying factors and posttranscriptional modifications of histone tails [18]. Because the persistence of cccDNA might be responsible for the failure of most antiviral treatments to eradicate the virus completely, it represents an interesting target for effective therapy of chronic hepatitis B.

Oncogenic properties of viral proteins

It is generally considered that HBV encodes no dominant oncogene. Transfection of cultured cells with HBV DNA is not 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 expression of different viral proteins, including the X gene product and the surface proteins in native or modified forms, might play a part as cofactors 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. However, the strong conservation of X sequences among HBV genotypes as well as in woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) genomes suggested that their products may be of importance for viral replication.

The HBx (or pX) protein encoded by the X gene is a small polypeptide of 154 amino acids (16.5 kDa). This protein is produced at low, barely detectable levels in acute and chronic infections, and it is able to induce humoral and cellular immune responses. The crucial role of the X gene product in viral infection was first demonstrated by the finding that X-deficient WHV genomes

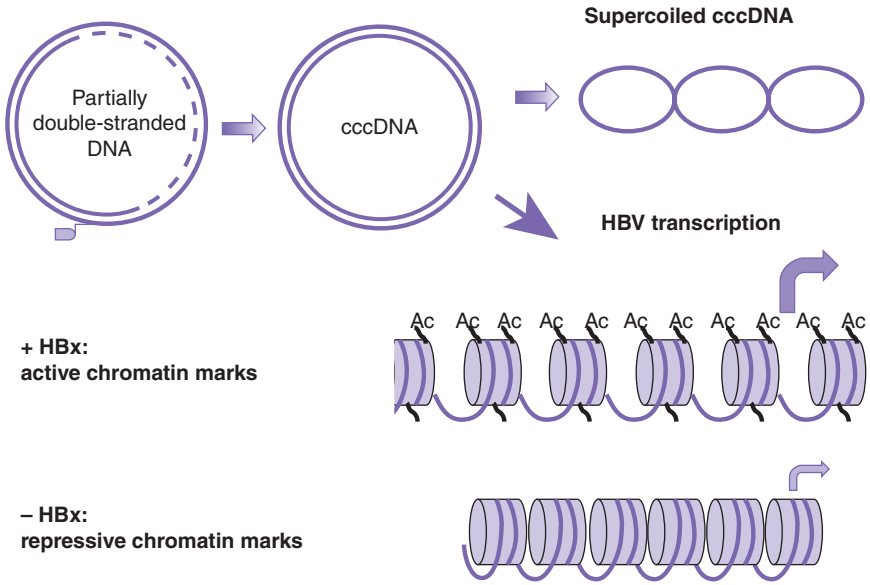

Figure 12.2 Epigenetic regulation of HBV transcription. After viral entry, the HBV genome is delivered into the nucleus. It is then converted into covalently closed circular DNA (cccDNA) by the cellular machinery. The cccDNA is organized into a mini-chromosome with bound histone and nonhistone proteins including HBx and the core protein. By recruiting different chromatin-modifying factors such as CBP/p300, HBx plays a pivotal role in the modulation of epigenetic marks such as histone tail acetylation and methylation that result in active HBV transcription. (Color plate 12.2)

inoculated into woodchuck livers were unable to initiate productive infections [19]. Moreover, HBx was shown to activate viral transcription and replication in cellular systems and a mouse model in which HBx expression was able to restore replication of an X-deficient HBV genome and viremia to wild-type levels [20]. Indeed, the first activity identified for this viral protein was the ability to activate transcription of viral and cellular genes. Thus, like other human tumor viruses, HBV encodes a small regulatory protein that can efficiently stimulate viral transcription and replication. It has been shown recently that HBx is recruited onto the HBV mini-chromosome, allowing transcription of the cccDNA through epigenetic regulation [21, 22] (Figure 12.2).

HBx has also been reported to activate transcription from cellular promoters including cytokines, growth factors, and oncogenes that play important roles in HCC pathogenesis. HBx has been localized mainly to the cytoplasm of *in vivo*–infected cells, near the plasma membrane and to the nuclear periphery, or to the mitochondrial membrane, as well as in the nucleus in transfected cells [23]. It may stimulate gene expression from different locations, either in the nucleus by interacting with transcription factors such as cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) and with elements of the basal transcription machinery, or in the cytoplasm by stimulating signaling pathways such as NFκB, NFAT, the Ras–Raf–MAPK cascade, or calcium-dependent Pyk2–Src signaling [24, 25] (Figure 12.3). A complex network of HBx interactions with cellular transcriptional co-activators and co-repressors has been evidenced recently, showing that HBx stimulates CREB-dependent transcription by stabilizing the CBP and p300 co-activators bound to phosphorylated CREB, and by inhibiting CREB dephosphorylation by the protein phosphatase 1 (PP1) [22, 26].

HBx is involved in protein–protein interactions with a growing number of cellular partners, providing more clues on the role of HBx in HBV pathogenesis (Figure 12.3). Different groups have shown that the binding of HBx to DDB1, a core subunit of the Cul4A-based ubiquitin E3 ligase complex, is essential for virus replication [27, 28]. DDB1 was first described as a subunit of the UV–DDB complex involved in DNA repair, suggesting that HBx might affect DNA repair through this interaction. HBx might also disturb the ubiquitin ligase activity of the Cul4A complex, thereby influencing the stability or other functions of cellular target proteins. The binding of HBx to DDB1 induces either apoptotic death or S phase progression; the latter is followed by formation of aberrant mitotic spindles and multinucleated cells, which in turn leads to genetic instability [29]. Data from other groups also converge to implicate HBx in deregulation of the mitotic checkpoint, notably in DNA

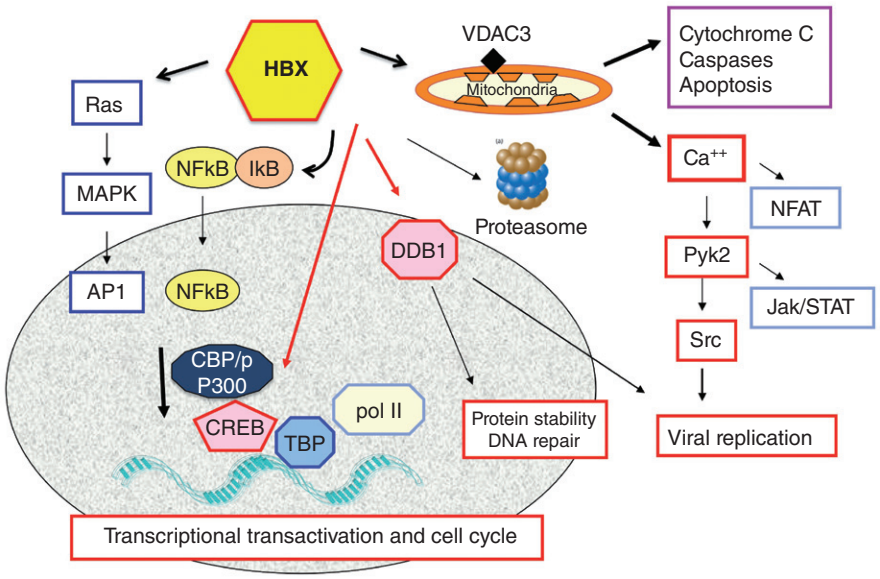

Figure 12.3 Multiple interactions of HBx with cellular partners from cytoplasmic and nuclear locations. Interactions of HBx with a large array of cellular partners may be relevant for HBx functions in cellular transformation, including stimulation of virus replication, cell cycle deregulation, activation of signaling pathways, induction or repression of apoptosis, and interference with DNA repair. Most of these functions are achieved through the transcriptional transactivation activity of HBx mediated by its interactions with cellular partners in the nucleus (transcription factors, co-activators, and/or basal machinery) or in the cytoplasm through the stimulation of the Src, Ras, MAPK, NFκB, and NFAT signaling pathways. Importantly, the binding of HBx to DDB1 is crucial for viral replication. (Color plate 12.3)

re-replication and polyploidy. It has been reported that HBx binds the tumor suppressor protein p53 and inactivates its functions, and this activity might be correlated with high chromosomal instability of HBV-related tumors. Moreover, inhibition of p53 together with activation of proliferation genes, including polo-like kinase 1 (Plk1), might account for the weak oncogenicity of HBx *in vitro* [30]. Other mechanisms have been proposed, such as targeting of the proteasome complex or modulating innate immune responses through down-regulation of interferon-beta. Thus, by enhancing liver cell injury mediated by the cellular immune response, HBx appears to be implicated through many different ways in the pathogenesis of chronic hepatitis B.

Although evidence for a direct oncogenic effect of HBx *in vivo* is scarce, it is now agreed that HBx participates as a cofactor in HBV-related carcinogenesis. While most HBx transgenic mouse lineages that express the protein in the liver develop no pathology, these mice are highly sensitive to liver tumorigenesis induced by chemical carcinogens or by c-Myc [31, 32]. Other studies have shown that expression of hypoxia-inducible factor 1 alpha (HIF1α) and vascular endothelial growth factor was increased in the liver of HBx-transgenic mice, suggesting that the cross-talk between HIF1α and HBx may activate HIF1α target genes that play a critical role in hepatocarcinogenesis. Different studies have demonstrated that HBx is able to interfere with liver regeneration, induce hepatocellular proliferation, and increase apoptosis in transgenic livers [33]. Collectively, these observations provide additional arguments for the contribution of HBx to viral-induced carcinogenesis.

The question of whether HBx is involved in cancer progression has been raised by the observation that HBx expression is usually retained in human HCCs, correlating with HBV methylome studies that showed global hypermethylation of the HBV genome in HCC, except for the X gene [34]. Analysis of integrated viral sequences in tumor DNA has highlighted one of the mechanisms leading to persistent expression of HBx in HCC. Upon HBV DNA integration into host cell DNA, a high percentage of viral–host junctions have been mapped near DR1 in the carboxy-terminal region of the X gene. The presence of viral–host transcripts containing a 3′ truncated version of the X-coding region fused with flanking cellular sequences has been described by different groups, and functional studies have outlined diverse properties compared to wild-type HBx. In some cases, these sequences retained transactivating capacity and the ability to bind p53 and block p53-mediated apoptosis, which may provide a growth advantage for neoplastic cells [35]. By contrast, in other cases, the truncated X sequences also harbored a number of missense mutations and were less effective in transcriptional transactivation, in apoptosis induction, and in supporting HBV replication [36]. Collectively, recent studies of the HBx

protein functions have contributed to better delineating the importance of the X gene product at different steps of the malignant process induced by persistent HBV infection.

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, including a large excess of the small (or major) envelope protein. While transgenic lineages that produce these proteins at normal physiological ratios develop no liver lesions or HCCs, overexpression of large S protein with respect to middle and major S leads to intracellular accumulation of nonsecretable filamentous envelope particles within the hepatocyte endoplasmic reticulum (ER). This results in histological features of "ground-glass" hepatocytes, which are considered to be typical of chronic hepatitis B in humans. In these transgenic mice, mild persistent hepatitis was followed by the development of regenerative nodules and eventually HCCs by 12 months of age [37]. Exogenous chemical cofactors were not required for tumor induction in this model, but adult transgenic mice displayed high sensitivity to hepatocarcinogens. Thus, inappropriate expression of the large S protein has the potential to be directly cytotoxic to the hepatocyte and may initiate a cascade of events including activation of oncogenes and growth-promoting signaling pathways that ultimately progress to malignant transformation. Additionally, naturally occurring mutants of the preS regions are frequently detected in HCC patients. Accumulation of these mutated proteins in the ER has been associated with the induction of stress signals, oxidative DNA damage, and genomic instability.

Studies of integrated HBV sequences in human liver tumors have suggested that rearranged viral S genes carrying truncated preS2/S sequences might play a role in HCC development. Deletion of the carboxy-terminal region of the S gene generates a novel transcriptional transactivation activity and activates c-Raf1–Erk2 signaling, resulting in an increased hepatocyte proliferation rate [38]. These findings support the hypothesis that accidental 3′ truncation of integrated preS2/S genes could be a causative factor in HBV-associated oncogenesis.

Viral integrations in HCC

Although hepadnavirus replication does not require a step of viral DNA integration in the host cell genome, the viral DNA can integrate into cellular chromosomes. Integrated HBV sequences have been observed in established hepatoma cell lines and in about 80% of human HCCs [39]. HBV DNA integration occurs at early stages in natural acute infections and in chronic hepatitis tissues, and they harbor a clonal pattern in HCCs that arise from outgrowth of one or a few transformed liver cells [8]. Single HBV insertions are common in childhood HCCs, but multiple insertions are seen more frequently in adult liver tumors, consisting of fragmented and rearranged sequences that cannot serve as a template for viral replication. Integration sites are dispersed over the viral genome, indicating that HBV integration does not occur through a unique mechanism, as for retro-elements and retroviruses. However, preferred integration sites in the HBV genome have been mapped within the "cohesive ends" region, which lies between two 11 bp direct repeats (DRs), DR1 and DR2. A narrow region encompassing DR1 has been shown to be particularly prone to recombination. 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 especially relaxed circular DNA might be preferential pre-integration substrates. Invasion of cellular DNA by single-stranded HBV DNA, using mainly free 3′ ends, might take place through a mechanism of illegitimate recombination involving Topoisomerase I. Such a mechanism is also supported by frequent patch homology between HBV and cellular sequences at the recombination breakpoints [40, 41].

As a consequence of the viral integration process, sequences of the S and X genes are almost systematically present in HBV inserts, whereas those of the C gene are less frequently represented. As detailed in this chapter, a significant percentage of viral junctions are localized in the carboxy-terminal part of the viral X gene, and evidence for transcriptional activity at integrated X sequences has been provided in tumors and chronically infected livers. Analysis of 194 paired HCCs and matched nontumor livers has led to detect carboxy-terminal truncated HBx in about 80% of tumors [42]. In other HCC cases, recombination breakpoints have been mapped in the S-coding region, leading to the expression of a truncated preS2/S product endowed with new, potentially oncogenic properties. Thus, viral DNA integration might play a part in *trans* in malignant progression of HBV-related HCCs.

Studies of the chromosomal sites of viral insertion in large series of human HCCs have revealed that integration can take place at multiple locations on virtually all chromosomes. 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 cellular genes in most cases. However, frequent insertions near or within fragile sites that are prone to instability have been noted. Furthermore, it has been proposed that simple repetitive elements are hotspots for HBV

insertion in the human genome. Indeed, Alu-type repeats, mini-satellite-like, satellite III, or VNTR (variable number of tandem repeat) sequences have frequently been identified near HBV insertion sites. This observation suggests 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 HBV, similar to that of retro-elements and retroviruses, might not be entirely random, and more recent studies have demonstrated frequent HBV insertions into actively transcribed chromosomal regions located in close vicinity of protein-encoding genes.

Evidence for a direct *cis*-acting promoter insertion mechanism was first provided in two independent HCC cases, consisting of early HCCs that developed in noncirrhotic livers and carried a single specific viral integration [5, 6]. In one case, the HBV insertion occurred in an exon of the retinoic acid receptor beta gene (*RARβ*) and fused 29 amino-terminal residues of the viral *preS1* gene to the DNA-binding and hormone-binding domains of *RARβ*. 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, *RARα*, is implicated in the chromosomal translocation t(15; 17) found in acute promyelocytic leukemias. In the human HCC, it seems most probable that inappropriate expression of a chimeric HBV–RARβ protein represented an important event in the tumorigenic process. In a second HCC, HBV DNA integration occurred in an intron of the human cyclin A2 gene, resulting in a strong expression of hybrid HBV–cyclin transcripts [6]. Cyclin A is an essential gene for cell cycle progression, and it plays a major role at both the G2/M and G1/S checkpoints. The hybrid RNAs code for a 430 amino acid chimeric protein, in which 152 amino-terminal amino acids of cyclin A are replaced by 150 amino acids from the preS2/S viral protein, while the carboxy-terminal two-thirds of cyclin A, including the cyclin box, remain intact. As the cyclin A degradation sequences located in the amino-terminal part of the protein are deleted, the hybrid HBV–cyclin A protein is not degradable. Furthermore, the transforming properties of the HBV–cyclin A protein have been demonstrated *in vitro*, suggesting a direct contribution in the tumoral process.

The notion that HBV might play a significant role as an insertional mutagen in liver cancer has been recently strengthened by large-scale analysis of viral insertion sites. It has been shown that HBV frequently targets cellular genes involved in cell signaling, and that some of them may represent preferential target sites of the viral integration. Analysis of 60 HCCs by using HBV Alu PCR has revealed HBV DNA integration sites in cellular genes in 90% of cases, including key regulators of cell proliferation and viability [7]. Notably, HBV DNA was found integrated into the following cellular genes: the sarco/endoplasmic reticulum calcium ATPase1 (*SercA1*) gene; the thyroid hormone receptor associated protein 150 alpha gene; the mini-chromosome maintenance protein (MCM)-related gene; *FR7*, a new gene expressed in human liver and cancer tissues; *p84*, a nuclear matrix protein gene; the neurotropic tyrosin receptor kinase 2 (*NTRK2*) gene; the IL1R-associated kinase 2 (*IRAK2*) gene; the p42 mitogen-activated protein kinase 1 (*p42MAPK1*) gene; the inositol 1,4,5-triphosphate receptor type 2 (*IP3R2*) gene; the inositol 1,4,5-triphosphate receptor (IP3R) type 1 (*IP3R1*) gene; the alpha 2,3 sialyltransferase (*ST3GAL VI* or *SITA*) the gene; thyroid hormone uncoupling protein (*TRUP*) gene; the EMX2-like gene; and the human telomerase reverse transcriptase (*hTERT*) gene. 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 [43, 44]. 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. All 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 viral integrations are nonrandom and that genes at the sites of viral integration may provide a growth advantage to a clonal cell population, leading to the accumulation of additional mutations and to cancer development [41].

The large variety of genes targeted by HBV integration in HCC contrasts sharply with the WHV–woodchuck animal model, in which WHV acts as a potent insertional mutagen by integrating into *myc* family genes (c-*myc* or N-*myc*) and activating these oncogenes in a large majority of woodchuck HCCs [45, 46]. This virus represents the first example of a DNA virus producing insertional events at such a frequency. No such preferential targeting has been observed in HBV-related human tumors and in GSHV-induced ground squirrel HCCs, in which the c-*myc* oncogene is frequently activated by chromosomal amplification [47]. Whether these striking differences are related to viral determinants or to species-specific factors remains to be determined.

HBV DNA integration may also enhance chromosomal instability: in many tumor cases, 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 [48]. In addition, HBV DNA may promote

homologous recombination at a distance from the insertion site. Although a precise role for most of these chromosomal abnormalities has not been assigned, high chromosomal instability is associated with aggressive and poorly differentiated tumors, all features that characterize a majority of HBV-related HCCs [49].

Genetic and epigenetic alterations in HBV-related HCC

Cancer development is associated with the accumulation of different genetic and epigenetic alterations that enable tumor growth and metastatic spread. Accordingly, genomic changes that cannot be clearly associated with a direct effect of viral infection have been described in human HCCs. These somatic changes include allele losses and DNA copy number changes on multiple chromosomal regions, mutation and activation of cellular genes showing oncogenic potential, deletion or mutation of tumor suppressor genes, as well as aberrant promoter methylation patterns and deregulated expression of microRNAs. The multi-step process of virally-induced hepatocarcinogenesis is schematically represented in Figure 12.4.

Loss of heterozygosity on chromosomes 1p, 4q, 6q, 8p, 9p, 13q, 16p, 16q, and 17q occurs frequently in human liver tumors, suggesting that these parts of the human genome contain some genes whose functional loss might be involved in hepatocellular carcinogenesis [50]. Essentially similar chromosomal regions were found to harbor changes in DNA copy number by comparative genomic hybridization, and these regions were further refined using array-comparative genomic hybridization (array-CGH) and single-nucleotide polymorphism (SNP) microarrays (reviewed in [51, 52]). Interestingly, HBV-related tumors harbor a higher rate of chromosomal abnormalities than tumors associated with other etiological factors [49]. A role of HBV DNA integration and of the HBx regulatory protein in promoting genetic instability has been evoked [29].

Allele loss of the short arm of chromosome 17, which includes the *p53* gene, has been observed in 30% to 60% of human HCCs and in hepatoma-derived cell lines. The wild-type *p53* gene negatively regulates cellular growth and, therefore, was designated a tumor suppressor gene or *anti-oncogene*. Genetic alterations of the gene generally consist of the deletion of one *p53* allele and mutation of the second allele, and represent the most common feature in human neoplasms. A hotspot 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 [53, 54]. This mutation, mainly a G-to-T transversion, appears to be specific for the mutagenic action of aflatoxin B1 [55]. This particular mutation was not observed in HCCs from patients who did not experience high exposure to the hepatocarcinogen. The *p53* codon 249 mutation was detected in apparently normal liver, and its frequency paralleled the level of aflatoxin B1 exposure, suggesting that it occurs early during HCC development. Structural aberrations of the *p53* gene (mutations at different codons and loss of one allele) have been observed more frequently in advanced, poorly differentiated HCCs with dismal prognosis [56], and they are often associated with abnormalities of

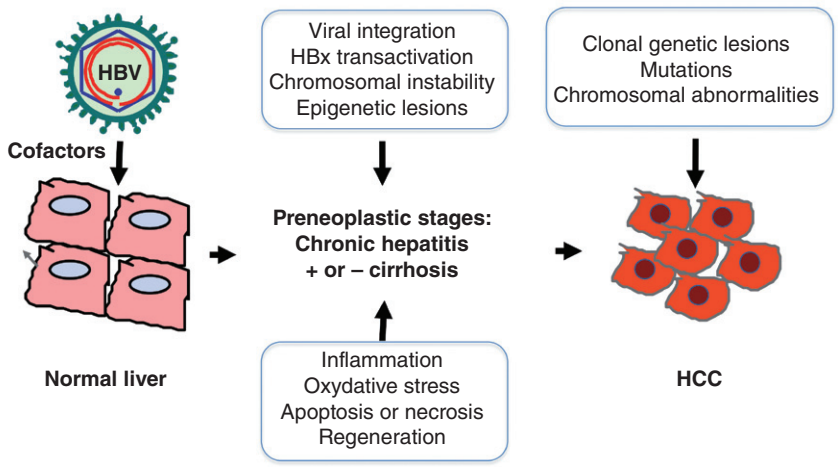

Figure 12.4 Multistep process of HBV-induced liver tumorigenesis. Chronic HBV infection, alone or in combination with cofactors such as aflatoxin B1 exposure or alcohol consumption, triggers a cascade of events ultimately leading to transformation. At pre-neoplastic stages, the immune response induces a necroinflammatory disease that creates a suitable environment for the occurrence of genetic and epigenetic lesions. Integration of HBV DNA sequences into the host genome as well as long-term production of the viral HBx and surface proteins may contribute to the tumoral process via multiple mechanisms. (Color plate 12.4)

another tumor suppressor gene, *RB1*. Interestingly, it has been shown that the rate of *p53* mutations is significantly higher in HBV-related tumors than in HCV-related and nonviral HCCs [49]. Although HBV DNA insertions in chromosome 17p have been described in some liver tumors, 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 RB1 locus on chromosome 13q have also been reported, but a low mutation rate was found in the remaining allele. Candidate tumor suppressor genes in other frequently deleted chromosomal regions include the mannose 6-phosphate/IGF-II receptor (*M6P/IGF2R*) gene on chromosome 6q, the *p16INK4A* and *p14ARF* genes on chromosome 9p, and the E-cadherin gene on chromosome 16q. In conclusion, recurring deletions spanning many human chromosomes might inactivate a number of tumor suppressors and thus play a key role in hepatocarcinogenesis.

Analysis of DNA copy number changes in HCC has shown frequent gains or amplifications at chromosomes 8q, 1q, 6p, and 17q, and amplification of the terminal region distal to 8q24 correlates with frequent overexpression of the c-*myc* oncogene [51]. A major breakthrough was the detection of oncogenic mutations in the β-catenin gene at significant rates in human and mouse liver tumors [57]. Activation of the Wnt–β-catenin pathway induces translocation of β-catenin to the nucleus and its association with the transcription factors of the TCF/LEF family, leading to transcriptional activation of target genes such as c-*myc* and cyclin D1. Activation of the Wnt signal 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. β-catenin mutations were found in around 30% of HCC cases, predominantly in HCV-related tumors and in tumors associated with alcoholic cirrhosis [49]. Additionally, loss-of-function mutations of the *Axin1* and *Axin2* genes have been detected in 5–10% of liver tumors, but mutations of the *APC* gene were rarely seen in HCC, contrasting with the predominant role of *APC* in colorectal cancer. Although activation of Wnt signaling through different mechanisms has been documented in HBV-related HCCs, these tumors harbor a lower β-catenin mutation rate (around 10%) than other etiologies. In addition, while the *p53* and β-catenin genes are the major targets for mutations in HCC, low rates (<10%) of mutations affect other genes, including oncogenes such as the Ras genes, *PIK3CA* and *MET*, and the tumor suppressor genes *RB1*, *CDKN2A*, *Axin1*, *Axin2*, *PTEN*, and *SMAD2/4*. Interestingly, recent exomic sequencing studies have revealed mutations in the chromatin-remodeling genes *ARID1A* and *ARID2* in HCCs of various etiologies, and *IRF2* was identified as a new tumor suppressor gene in HBV-related HCC [58, 59].

Besides genetic alterations, epigenetic changes like methylation-associated gene silencing play an important role in the deregulation of cell cycle control and proliferation. This epigenetic alteration occurs early during the tumoral process, and accumulates step by step during tumor progression. Frequent hypermethylation of CpG islands in the promoters of tumor suppressor genes such as *p16INK4A*, E-cadherin, and *APC* has been reported in HCC. It has been shown that the extent of genomewide hypomethylation and CpG hypermethylation correlates with biological features and clinical outcome of HCC patients [60]. As mentioned in this chapter, mutations in genes affecting epigenetic regulation of transcription have recently been unveiled, and this field will probably be subjected to further developments in the near future.

Genomewide studies of gene expression in HCC and in chronic hepatitis by microarray screening have undoubtedly provided insight into the complex mechanisms underlying liver tumorigenesis. This approach has been used to determine whether HBV-related HCCs harbor distinctive molecular mechanisms. Gene expression profiling has revealed several HCC subclasses, and the HCC subclasses associated with HBV infection display activation of the mitotic cell cycle, deregulated expression of developmental and imprinted genes such as *IGF2*, and activation of the AKT pathway [61]. A statistical meta-analysis integrating transcriptome data from eight independent cohorts has led to the identification of three major molecular subclasses of HCC associated with tumor differentiation and serum alpha-fetoprotein (AFP) levels [62]. In this study, chronic HBV infection was found to be significantly associated with moderate or poor differentiation of tumors. Moreover, molecular classification of HBV-related HCCs has been employed to predict early recurrence after curative resection. A high risk of recurrence was associated with a subgroup of tumors showing increased expression of genes involved in cell cycle progression, cell proliferation, migration, and motility, and in the Notch signaling pathway.

In recent years, attention has been focused on the role of microRNAs (miRNAs) in the pathogenesis of chronic hepatitis and HCC. miRNAs are highly conserved noncoding RNAs with an average length of 22 nt, which regulate gene expression via binding to the 5′-untranslated region or coding region of target messenger RNAs (mRNAs). So far, more than 1700 different miRNAs have been identified, and they serve as major factors within regulatory networks controlling gene expression. They intervene in virtually all cellular processes, and they play crucial roles in viral infections and in cancer. It has been reported that miRNAs, including the abundant, liver-specific miR122, can influence HBV expression and replication through direct binding of viral sequences or indirectly through regulation of host genes.

Conversely, the HBx protein might regulate host gene expression by targeting a number of miRNAs such as the tumor suppressor miRNAs *let7* and *miR16*. Deregulation of miRNA expression is observed from the early stages of hepatitis B, with important changes of miRNA profiles throughout the multistep tumorigenic process. Moreover, miRNAs can be detected and accurately measured in many body fluids including serum and urine, providing a noninvasive source of information. Differential expression of several miRNAs, including *miR16*, *miR122*, *miR21*, *miR223*, *miR25*, *miR375*, and *let7f*, has been described in the serum of patients with hepatitis B and HCC versus control individuals. Heterogeneous miRNA profiles have been detected in HCC tissues, reflecting multiple etiologies, genetic lesions, and tumor stage. Thus, miRNA profiles and especially those obtained from plasma or serum have a huge potential as diagnostic and prognostic markers for detecting early HCCs and for predicting metastatic spread and tumor recurrence [63, 64]. Finally, targeting the regulatory circuitries implicating miRNAs might be beneficial for adapted therapy of chronic hepatitis B and liver cancer.

Conclusions

Several lines of evidence contribute in linking chronic HBV infection and primary liver cancer. Firstly, a strong epidemiological association has been provided by extensive prospective and retrospective studies in many parts of the world. Secondly, 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 mutagenic action of viral integration into the host cell genome, the weak oncogenicity of the viral X transcriptional transactivator, and the long-term tumorigenic effect of surface glycoproteins, which may, directly or indirectly, contribute to deregulating 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 favor 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 HTLV1, EBV, human papilloma virus 16 (HPV16), and HPV18, 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 similar role has not been established for HBV, and it will be important to identify the cellular effectors connecting HBV infection and liver cell transformation.

The question of whether HBV could trigger distinct tumorigenic pathways compared to other risk factors can now be addressed via novel technologies for genomewide scans of genetic and epigenetic alterations, gene expression, and proteomics. Thus, despite high molecular heterogeneity, HBV-related tumors have been recently classified into subgroups that display high chromosomal instability, stemness features, increased frequency of *p53* mutation, and low rate of β-catenin mutation. It will now be important to apply these technologies to the analysis of gene expression and genetic and proteomic changes in chronic hepatitis B at different stages, in order to identify suitable prognostic markers and therapeutic targets. In addition, there is increasing evidence implicating epigenetic alterations such as methylation-associated gene silencing and abnormal expression of microRNAs in the deregulation of cellular functions leading to malignant transformation. Advances in this field will undoubtedly contribute to improve the management of chronic hepatitis B and liver cancer.

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Chapter 13
Murine models and human studies of pathogenesis of chronic hepatitis B

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Summary

This chapter describes immunologic findings from murine models of hepatitis B virus (HBV) infection and/or replication. Technologies, limitations, as well as major findings from three available HBV mouse models to date (i.e., transgenic mice, hydrodynamically transfected mice, and infected human liver chimeric mice) are discussed. Most importantly, the murine findings are compared to relevant findings in human studies in order to verify their relevance for elucidating immune clearance as well as the immunopathogenesis of HBV infection in humans.

Introduction

HBV infection is a major health problem worldwide with 240 million chronically infected individuals globally despite the availability of an effective prophylactic vaccine [1]. Complications of cirrhosis and hepatocellular carcinoma (HCC), following persistent HBV infection, contribute to 600 000 deaths per year [1].

The study of HBV infection has been greatly constrained by the limited range of appropriate animal models that recapitulate the human infection. Important insights on HBV replication and pathogenesis of the infection were obtained from studies of distinct animal species (ducks and woodchucks) that are naturally infected by other hepadnaviruses: woodchuck hepatitis B virus (WHBV) and duck hepatitis B virus (DHBV). Nevertheless, there are shortcomings of such models. WHBV and DHBV are similar but not identical to HBV and cause slightly different spectra of liver diseases. More importantly, the analysis of pathogenetic mechanisms related to viral control and liver damage is hampered in both ducks and woodchucks by the lack of appropriate reagents necessary to study the different components of adaptive and innate immunity.

Besides humans, HBV infects only chimpanzees and tree shrews, but strong ethical constraints, handling difficulties, as well as high costs restrict HBV studies in chimpanzees. Related studies in tree shrews are hindered by the low infectivity of the virus and the transient nature of HBV infection in addition to the lack of reagents to analyze the immune system [2]. Thus, an appropriate and well-defined, inbred, small-animal model is required for studying immune control and immunopathogenesis during HBV infection, and this

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necessity led to the production of different mouse models of HBV infection. In this chapter, we discuss immunologic findings derived from available mouse models to date and their relevance to HBV infection in humans.

HBV transgenic mouse models

Technology

HBV transgenic mice are generated through microinjection of HBV DNA (a partial or complete genome) into fertilized murine eggs (allowing chromosomal integration of viral DNA into the host genome) and subsequent implantation of the eggs into pseudo-pregnant female mice [3–5]. Of note, the usage of the HBV endogenous or liver-specific promoter allows hepatocytes to express viral proteins (reviewed in [6]), ensuring that HBV transgenic mice will express either a particular viral antigen or the complete HBV genome within the liver. This technology thus allows a well-defined, inbred, small-animal model system to be used to study many complex biological questions of HBV infection. For example, findings using this model support the concept that HBV is not directly cytopathic to the expressing hepatocytes [3–5]. Instead, this model provides direct evidence that hepatocellular injury in HBV infection is immune mediated [7], as will be discussed in detail in this chapter.

However, there are three major limitations with this model. Firstly, HBV does not infect murine hepatocytes, thus a true infection does not occur in the HBV transgenic mice even with the presence of circulating virus. Interpretations of data originating from such models would have to be performed carefully, bearing in mind that viral kinetics and mechanisms could differ when reinfection is occurring. Secondly, HBV transgenic mice are immunologically tolerant to viral antigens [8]. Hence, the original transgenic model does not display any sign of chronic liver disease. Nevertheless, this tolerance can be circumvented by adoptive transfer of syngeneic, unprimed splenocytes into HBV transgenic mice with severe combined immunodeficiency background [9] or by adoptive transfer of HBsAg-specific CD8+ T cells into thymectomized, irradiated, and bone marrow–reconstituted HBV transgenic mice [10], creating a model of chronic hepatitis. Importantly, the latter method results in the development of HCC, suggesting that viral hepatitis and subsequent HCC are immune mediated. Lastly, HBV covalently closed circular DNA (cccDNA), the primary viral transcriptional template during infection, is not found in the whole genome of HBV-replicating transgenic mice [5]. Hence, host attempts to clear HBV cccDNA cannot be studied.

Model of acute viral hepatitis

There are two main ways to generate immune responses against HBV in transgenic mice: (1) by injection of a specific agonist or activating cytokine, and (2) by adoptive transfer of antigen-specific immune cells or naïve splenocytes into transgenic mice. The latter method induces immune-mediated acute hepatitis in these mice. Important immunologic findings using this model are presented in this chapter.

Innate immune responses

Despite the immune-tolerant profile of HBV transgenic mice, proper activations of their innate immune systems were shown to be able to control viral replication. Toll-like receptors (TLRs), as pattern recognition receptors, are essential for the innate immunity to recognize invading pathogens. Indeed, activation of TLR3, TLR4, TLR5, TLR7, or TLR9 with its respective agonist suppressed HBV replication in an interferon alpha (IFNα) or beta (IFNβ) dependent manner [11]. Of note, TLRs are expressed by antigen-presenting cells (APCs), hence these results suggest that intrahepatic APCs can, if properly activated, play an important role for generating immune responses against HBV. Indeed, activation of intrahepatic murine APCs by anti-CD40 agonistic monoclonal antibody was sufficient to inhibit HBV replication noncytolytically [12].

Furthermore, activated intrahepatic APCs, through production of interleukin 12 (IL12) and IL18, can activate natural killer (NK) or natural killer T (NKT) cells to produce interferon gamma (IFNγ) that eventually inhibits HBV replication [12–14]. In line with these findings, an activation of invariant NKT cells with alpha-galactosylceramide (α-GalCer) induced production of IFNα, IFNβ, and IFNγ within the liver of HBV transgenic mice that ultimately inhibited HBV replication [15]. Collectively, these findings suggest that the cellular components of host innate immunity can potentially control HBV replication if properly activated. Furthermore, these findings also indicate the importance of type I and II IFNs to suppress HBV replication even though a recent work in HBV transgenic mice argued that the effects of type I IFN on HBV replication can be influenced by viral load, since in these mice IFNα and IFNβ suppressed HBV replication when viral load was high but enhanced HBV replication when viral load was low [16]. Whether such an effect can be translated to human infection, where HBV seems to be able to inhibit the antiviral effect of IFNα, has been discussed elsewhere [17].

Another interesting study that performed adoptive transfer of naïve splenocytes into HBV transgenic mice suggested a pathological role of nonclassical NKT cells

(CD1d restricted but nonreactive to α-GalCer) during acute viral hepatitis. Briefly, these transferred nonclassical NKT cells, but not NK or classical NKT cells, became activated in response to HBV-expressing hepatocytes through the NKG2D–RAE1 axis and subsequently caused acute hepatitis [18, 19]. Hence, this suggests that blockade of the NKG2D axis might be a potential treatment to attenuate the severity of liver injury during hepatitis B.

Adaptive immune responses

Similar to other viruses, HBV was widely assumed to be cleared by major histocompatibility complex (MHC) class I–restricted CD8+ T cells, mainly via destruction of infected hepatocytes. This hypothesis was partly supported by the adoptive transfer of HBsAg-specific CD8+ T cells into HBV envelope-expressing transgenic mice, where virus-specific CD8+ T cells recognized and lysed HBsAg-expressing hepatocytes in an MHC class I–restricted manner, subsequently causing immune-mediated acute hepatitis [7].

Interestingly, despite their ability to initiate the killing process, HBV-specific CD8+ T cells may have limited killing potential. Clinically, HBV clearance was usually associated with recovery without massive hepatocellular necrosis. It is also important to notice that in acutely infected chimpanzees, HBV DNA disappeared from the liver and the blood before the peak of liver injury [20]. This suggests the existence of noncytolytic mechanisms that may be physiologically important for HBV clearance without extensive hepatocyte destruction. Indeed, virus-specific CD8+ T cells have been shown to eliminate HBV gene expression and replication without killing the hepatocytes in HBV-replicating transgenic mice. Importantly, these noncytolytic mechanisms were mediated by the secretion of IFNγ and tumor necrosis factor alpha (TNFα), which eliminate HBV nucleocapsid particles and destabilize HBV RNA [21–23]. Excessive damage of the liver was instead mainly attributed to host-derived antigen-nonspecific lymphomononuclear and polymorphonuclear (neutrophil) cells [24, 25].

Even though IFNγ can mediate the antiviral activity of HBV-specific CD8+ T cells in a non-cytolytic-dependent manner, it is also involved in exacerbating liver injury [24]. Upon adoptive transfer of HBV-specific CD8+ T cells into HBV transgenic mice, the released IFNγ activated hepatocytes and other liver resident cells to secrete chemokine ligands 9 and 10 (CXCL9 and CXCL10) [26]. These chemokines in turn recruit host-derived antigen-nonspecific mononuclear cells to infiltrate the liver, hence enhancing the severity of liver injury. Importantly, this chemoattractant effect was independent of the antiviral property of IFNγ, suggesting that these chemokines could be neutralized as a novel therapeutic means to suppress viral replication without exacerbating the liver injury [26].

Subsequent adoptive transfer studies of HBV-specific CD8+ T cells into HBV transgenic mice revealed the involvement of neutrophils, platelets, and Kupffer cells in promoting or limiting liver destruction during acute viral hepatitis. Firstly, intrahepatic neutrophils were found to be responsible for the recruitment of antigen-nonspecific lymphomononuclear cells via production of metalloproteinase 8 and 9 (MMP8 and MMP9). Furthermore, depletion of neutrophils or inhibition of the MMP activity specifically inhibited the recruitment of antigen-nonspecific lymphomononuclear cells without affecting the migration or antiviral activity of HBV-specific CD8+ T cells [27, 28]. This suggests a pathologic role of MMP8 and MMP9 secreted by neutrophils in aggravating immune-mediated liver injury. Secondly, activated platelets were required to accumulate HBV-specific CD8+ T cells within the liver. This specific interaction may assist extravasation of HBV-specific CD8+ T cells from within the liver sinusoid and infiltrate the liver parenchyma, thus mediating antiviral activity as well as causing liver injury [29]. However, the exact mechanism is still elusive. Thirdly, Kupffer cells in this experimental setting were actually found to limit the severity of liver immunopathology by removing apoptotic hepatocytes. If the clearance by Kupffer cells did not ensue, the apoptotic hepatocytes became necrotic and released high-mobility group box 1 proteins. This in turn recruited other inflammatory cells, including neutrophils, and exacerbated the liver injury [30].

In contrast to CD8+ T cells, there is only limited information on CD4+ T and B cells from studies using HBV transgenic mice. As has been observed for HBV-specific CD8+ T cells, adoptively transferred HBV-specific CD4+ T cells could also recognize HBV-replicating hepatocytes and subsequently secrete IFNγ [31]. However, these CD4+ T cells only partially suppressed HBV replication, suggesting that they are not the principal effector responsible for HBV clearance. Nevertheless, transferred CD4+ T cells caused liver injury in some cases. Further studies suggest that IL22, secreted by activated CD4+ T cells, was responsible for the recruitment of antigen-nonspecific inflammatory cells into the liver, causing acute hepatitis in HBV transgenic mice [32]. Next, B cells in HBV transgenic mice were reversibly tolerant to viral proteins due to the permanent immune-tolerant profile of T cells within transgenic mice [8, 33]. Indeed, exogenous antigenic stimulation along with appropriate T cell help reversed this unresponsiveness, as noticed by the production of anti-HBc and anti-HBs antibodies. In sum, little progress has been achieved by

using this mouse model to elucidate the roles of CD4+ T and B cells.

Model of immune tolerance

As mentioned in this chapter, HBV transgenic mice are immune tolerant at the T cell level. Hence, a good method is required to distinguish T cell immune tolerance due to the transgenic system from tolerance due to the actual tolerogenic property of HBV antigens. One way is by analyzing T cell responses in the nontransgenic offspring of HBV transgenic mice. An interesting study of offspring from HBe-expressing transgenic mice (exposed to HBeAg/HBV e antigen *in utero*) demonstrated that the T cell proliferation response of nontransgenic offspring was significantly weaker compared to immunized nontransgenic control mice, but not completely tolerant as compared to the transgenic offspring [33]. This indicates that exposure to HBeAg *in utero* can induce T cell immune tolerance.

Another important observation of natural HBV infection is that viral persistence occurs more frequently when infection is acquired during infancy than in adulthood. In order to delineate the involved mechanism, adoptive transfer of adult splenocytes into adult as well as young HBV transgenic mice was performed [34]. Interestingly, following the adoptive transfer, young transgenic mice never developed alanine aminotransferase (ALT) elevation, only exhibited minimal histological inflammatory activity, and only generated anti-HBc but not anti-HBs antibodies, resulting in persistence of HBsAg expression. In contrast, the adult transgenic mice, upon adoptive transfer, developed typical acute hepatitis and cleared HBsAg from circulation. In addition, ineffective hepatic T follicular helper cell priming and/or trafficking was found in young HBV transgenic mice. This phenomenon led to reduced IL21 production that may be responsible for the nonoptimal generation of virus-specific CD8+ T and B cell immune responses [34].

Hydrodynamically transfected HBV mouse models

Technology

In an effort to develop mouse models of HBV infection without some of the constraints described in this chapter, researchers have established several HBV-transfected mouse models that are not immunologically tolerant and are easier to produce than classical transgenic mice. Particularly, recent emphasis has been placed on the technique of hydrodynamic transfection. This involves the injection of approximately 8–12% of the body weight in volume of HBV genome-containing plasmid into the tail vein within 5–8 seconds [35]. Unlike transgenic mice in which the hepatic expression of the HBV genome is controlled primarily by either endogenous (HBV) or liver-specific promoters, the systemic administration of the HBV plasmid under hydrodynamic conditions preferentially, but not exclusively, delivers the HBV transgene into the hepatocytes [35]. Integration of the HBV genome is then mediated through the action of the inverted terminal repeats of adeno-associated virus [36] or by the Sleeping Beauty Tranposase system [37], while expression is controlled by the endogenous or liver-specific promoters. Hence, this technique provides a simple and convenient method to generate mice with HBV-expressing hepatocytes for the study of viral dynamics and immune response to HBV.

However, as with all animal models of HBV, there are some limitations of hydrodynamically transfected mice. In addition to the fact that mice are not permissive for HBV infection, the technique is only partially specific for the liver, often resulting in off-target transfections of other organs. How these unintended transfections could alter the various systems in the mice is difficult to predict and could potentially interfere with subsequent analysis. Furthermore, hydrodynamic transfection typically reaches peak transgene expression after approximately 8 hours post injection of plasmid DNA, and expression levels decrease thereafter [35]. This transient transfection property of the technique precludes the generation of persistently HBV-transfected mice without additional manipulations and is not clearly mimicking the kinetics of viral replication of the natural infection. In Figure 13.1, we depict the differences in the kinetics of viral replication and antiviral immunity in HBV-transgenic mice, hydrodynamically transfected mice, and adult humans (and chimpanzees) acutely infected by HBV. See Table 13.1.

Model of acute viral hepatitis

The first HBV mouse model established by hydrodynamic transfection of a replication-competent HBV genome was a model of acute infection [37]. In this model, viral antigens and replicative intermediates were synthesized in the liver and HBV was secreted into the blood [37]. More importantly, the appearance of antiviral antibodies and HBV-specific CD8+ T cells within 15 days post transfection coincided with the clearance of HBV antigens, transcripts, and replicative intermediates from both the peripheral blood and the liver [37]. In contrast, transfection of immunodeficient NOD/SCID mice lacking T, B, and NK cells resulted in HBV that persisted for at least 81 days [37]. Hence, the outcome

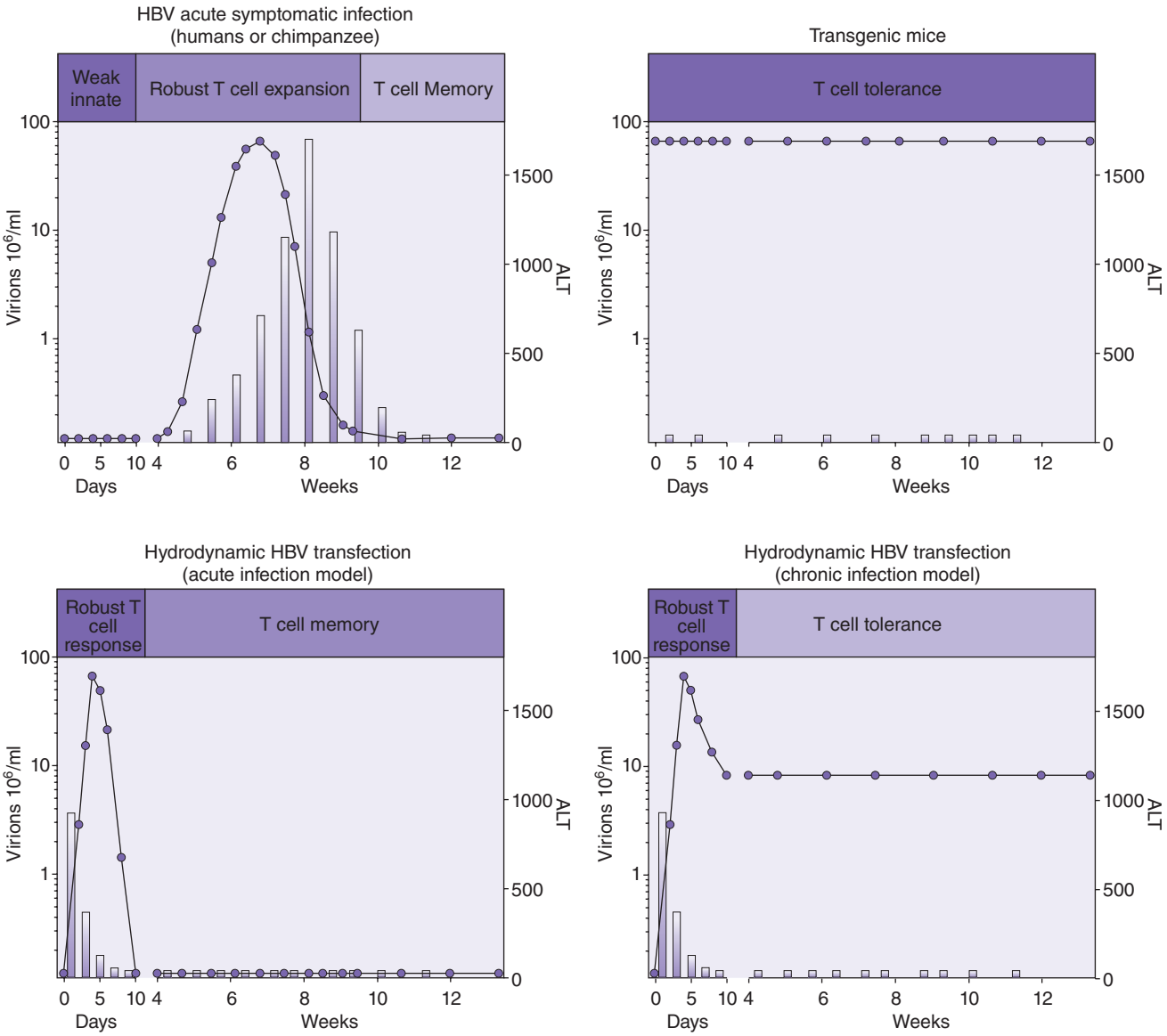

Figure 13.1 Kinetics of HBV replication (serum HBV DNA; lines), liver damage (ALT; bars) and T cell response in acutely infected humans/chimpanzees or in common murine transfection models.

of hydrodynamic transfection of HBV is determined by the host immune response.

Further insights into the complexity of the systems involved in HBV control have also been delineated using a similar model. Hydrodynamic transfection of mice genetically deficient in a variety of cellular (CD8+ T, CD4+ T, NK, and B cells) and molecular (IFNγ, IFNα and IFNβ receptor-1, TNFα receptor-1, perforin, and TNF receptor superfamily, member 6 [FAS]) effectors revealed that, with the exception of the B cell and perforin knockout mice, each of the other lineages resulted in persistent HBV presence for at least 60 days [38]. Interestingly, HBV replication was ultimately terminated in all the lineages except the NOD/SCID mice [38]. These results identified the key mediators of HBV control and suggest the existence of redundant mechanisms that inhibit HBV replication.

Model of chronic viral hepatitis

More recently, an immunocompetent mouse model for chronic HBV infection was established through hydrodynamic transfection of replication-competent HBV DNA. Instead of the rapid clearance of HBV observed in the model described here, the use of both the specific transfection vector (pAAV/HBV1.2) and mouse strain (C56BL/6) resulted in persistent HBV presence (HBsAg in circulation and hepatic detection of replication

Table 13.1 Summary of the technology, suitable applications, limitations, and major findings of the various HBV mouse models.

Models	Technology	Suitable applications	Limitations	Major findings	Reference
Transgenic mouse	• Microinjection of HBV DNA (full genome or specific HBV genes) into fertilized murine eggs allows the hepatic expression of HBV antigens or virions. • Adoptive transfer of HBV-specific immune cells or naïve splenocytes induces acute hepatitis.	• Innate and adaptive immune response — Antiviral mechanisms • Immunopathogenesis — Mediators of hepatic damage • Immunotolerance mechanisms • Testing of immunomodulatory therapies	1. Murine hepatocytes cannot be infected by HBV. 2. Mice are immunologically tolerant to HBV antigens. 3. cccDNA formation does not occur.	• Nonclassical NKT cells recognize HBV-replicating hepatocyte. • Virus-specific T cells can clear HBV via noncytolytic means. • IFNγ can exacerbate liver injury through the induction of CXCL9 and CXCL10. • Activated platelets contribute to HBV-associated liver damage. • *In utero* exposure to HBeAg tolerizes HBV-specific T cells.	[17, 21, 25, 28, 33]
Hydrodynamically transfected mouse	• Tail vein injection of HBV plasmid under hydrodynamic conditions preferentially delivers the HBV transgene into hepatocytes and induces acute hepatitis. • Persistent HBV antigenemia can be generated through transfection of C56BL/6 mice using only a specific adeno-associated viral vector.	• Innate and adaptive immune responses during acute HBV exposure — Antiviral mechanisms	1. Murine hepatocytes cannot be infected by HBV. 2. Effects of off-target transfections are hard to predict. 3. The inherent transient property of the technique precludes the generation of persistently transfected mice without additional manipulations	• CD8 T cells, CD4 T cells, NK cells, IFNγ, IFNα/β receptor-1, TNFα receptor-1, and FAS all contribute to the control of HBV. • HBcAg seems to be important in determining HBV persistence. However, the strict requirement for the generation of persistent HBV antigenemia is a major caveat of this finding.	[37, 38]
Human hepatocyte chimeric mouse	• Xeno-transplantation of human hepatocytes into uPA-SCID mice or $Fah^{-/-}$ $Rag2^{-/-}$ $IL2R\gamma^{-/-}$ knockout mice. • Transgene-induced, hepatocyte-specific damage allows the human hepatocytes to progressively repopulate and replace the murine liver. • Chimeric mice are permissive for HBV infection.	• Host innate immune response — IFNα response — TLRs • HBV infection and virology — Infection and replication kinetics • Antiviral testing	1. The immunodeficient nature of the model restricts the study of HBV immunopathogenesis and subsequent development of hepatocellular carcinoma. 2. It is difficult to standardize the degree of human hepatocyte engraftment. 3. There is a limited availability of human hepatocytes.	• HBV is likely to be detected by the host innate immune system, but it possesses the ability to inhibit the IFNα response actively.	[47]

intermediates, transcripts, and proteins) for over 6 months in 40% of the transfected mice [36]. These mice also developed a tolerance toward HBV surface antigen, similar to that observed in humans. Utilizing this model together with the introduction of premature termination codons into the various HBV genes, it was observed that mice receiving the HBV core–deficient mutants had a higher incidence of HBsAg (HBV surface antigen) persistence [39]. The analysis of serial HBcAg (HBV core antigen) deletion mutants revealed a 10 amino acid region at the carboxy terminus that was essential for the generation of a vigorous T cell response (IFNγ production) and rapid HBV clearance [39]. Data in this model therefore suggest that the immune response triggered by HBcAg is important in determining HBV persistence.

Chimeric mouse models

Technology

The latest advancement in HBV mouse models was made possible by the generation of chimeric mice where xeno-transplanted human hepatocytes progressively repopulate the mouse liver, with the eventual goal of replacing the entire mouse organ with functional human hepatocytes. Two major models were established: (1) the urokinase-type plasminogen activator (uPA) transgenic mice [40], and (2) the fumarylacetoacetate hydrolase (Fah) knockout mice [41]. In both models, transplantation rejection was prevented by the use of immune-deficient mice, while transgene-induced hepatocyte-specific damage provides the necessary growth stimuli and space for the xeno-transplanted human hepatocytes to proliferate and repopulate the mouse liver. Though both models operate through a similar concept, the Fah knockout mice comprise an inducible system where hepatotoxicity is induced by withdrawal of oral 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC) administration. This is different from the uPA transgenic mice in which hepatocyte damage is uncontrollable and progressive, often leading to excessive neonatal mortality, low breeding efficiency, and technically more demanding colony management [42].

These chimeric mice, being different from the transfection mouse models where HBV virions were produced artificially without infection, were fully permissive for HBV infection [43, 44], essentially creating a reproducible small-animal model of true HBV infection and replication. This combination of *bona fide* HBV infection in a mouse model provides, at the moment, the most physiologically relevant platform for the analysis of human HBV infection, virology, and antiviral testing. However, due to the immunodeficient nature of the model, the analysis of HBV immunopathogenesis and the subsequent development of HCC would be restricted. The technical difficulties in standardizing the number of grafted human hepatocytes and the limited availability of human hepatocytes could also prove to be significant shortcomings of this model.

Findings

Despite the immunodeficient state of chimeric mice, this model was used to provide some insights into the host response of HBV. Experiments in chimpanzees have shown that the type I IFN pathways were not activated upon HBV infection [45]. This led to the idea that HBV was able to establish itself without alerting the innate immune system [46]. However, recent work *in vivo* suggests that the innate immune system is capable of sensing HBV infection [47, 48]. Whether HBV has evolved an ability to suppress the innate immune system of the host actively or to evade detection completely has yet to be determined. This issue was investigated by administering IFNα to HBV-infected and noninfected chimeric mice and monitoring the response through the expression of interferon-stimulated genes (ISGs) [49]. Introduction of IFNα into HBV-infected chimeric mice failed to stimulate the expression of ISGs and STAT1 compared to the uninfected control mice [49]. Furthermore, there was a slight increase of basal ISG expression in HBV-infected chimeric mice in comparison to the control. These results suggest that HBV is likely to be detected by the innate immune system, but it has the capacity to inhibit innate immunity actively and promote viral persistence or limit the effectiveness of IFNα therapy in patients. It is unlikely that HBV deploys similar strategies as HCV does to counteract the antiviral response (e.g., cleaving mitochondrial antiviral signaling protein [MAVS] and Toll–IL1 receptor [TIR]-domain-containing adapter-inducing interferon-β [TRIF] proteins) (reviewed in [50]), hence it is still an open question how HBV can deceive the innate immunity.

Murine models for assessing HBV treatments

In addition to addressing numerous questions related to HBV immunopathogenesis, the murine models are also used to assess efficacy as well as mechanisms of potential or established HBV treatments. Chapter 14 will cover HBV treatments in detail; hence we concisely discuss the suitability as well as limitations of each mouse model for assessing HBV treatments.

The immunotolerant nature of HBV transgenic mice makes it attractive for studies using immunomodulatory therapies. For example, immunizing HBV transgenic mice with dendritic cells loaded or transduced with HBV antigens (HBcAg or HBsAg) can break the

typical T cell tolerance observed in these mice [51–53]. These studies might support the use of dendritic cell vaccination as a therapy, since introducing HBV antigens to dendritic cells obtained from chronic HBV-infected patients has been shown in some studies to induce *in vitro* proliferation of autologous T cells [54, 55]. HBV hydrodynamically transfected mice might also be useful to test similar or other immunomodulatory treatments. However, the transient expression of the HBV transgene and the diversity of the tolerant state present in different transfected mice make the studies more difficult to perform.

Due to their immunodeficiency state, HBV-infected chimeric mice cannot be used for characterization of immunomodulatory therapies. Nevertheless, differing from HBV transgenic and hydrodynamically transfected mice, the chimeric mice are well suited for testing the direct antiviral effects of different drugs or cytokines since such studies require a system where *bona fide* HBV infection occurs. This explains why these mice have been used to test the ability of small peptides to block HBV infection of human hepatocytes [56] or why a recent study has used human liver chimeric mice to demonstrate that IFNα inhibits HBV transcription and replication by targeting the epigenetic regulation of the nuclear cccDNA mini-chromosome [57], demonstrating how a new HBV animal model can be exploited to decipher the fine mechanism of the direct antiviral effect of a cytokine currently used in patients.

Relevance to HBV infection in humans

HBV mouse models provided many important findings, particularly on the immune pathogenesis during HBV infection. The next question, logically, is whether the obtained data on the innate and adaptive immune responses, as well as immune tolerance, can be extrapolated to elucidate the pathogenesis of HBV infection in humans. Thus, a comparison with relevant findings in HBV-infected humans is extremely crucial.

Observations from human studies support the concept that HBV is not directly cytopathic to infected hepatocytes. In some acute HBV-infected patients, high levels of HBV DNA with completely normal ALT levels were observed [58]. In addition, massive reduction of HBV DNA levels occurred before peak ALT levels in patients with acute hepatitis [58]. These observations are in line with the general concept found in mice where liver injury was primarily mediated by the host immune system.

Innate immune responses

In contrast to the murine innate immune system, the role of the human innate immune system in controlling HBV infection and causing liver injury is elusive due to the difficulties in obtaining patient material at the very early stage of HBV infection. However, some studies do provide insights into the early stages of the host innate immune response against HBV, particularly the role of NK and NKT cells, as well as the antiviral effect of IFNs.

Despite the abundance of NK cells in the normal human liver (30–40% of intrahepatic lymphocytes) and the increased frequency of circulating NK cells at the time of first detection of HBV DNA and HBsAg [48, 58], their activation and effector function were reduced during peak viremia, probably due to induction of the immunosuppressive cytokine IL10 [47]. Also, NK activation in these patients peaked only when viremia was resolving. Thus, in contrast to the antiviral role of NK cells in hydrodynamically HBV-transfected mice [37, 38], it is unclear whether an increased frequency of activated NK cells reduces HBV replication in patients.

With regard to α-GalCer, this agonist of invariant NKT cells has been tested in a randomized placebo-controlled phase I/II clinical trial as a novel treatment for chronic HBV-infected patients. However, α-GalCer caused a strong decrease of NKT cells instead and did not clearly reduce HBV DNA levels in these patients [59]. Furthermore, unlike in mice, the frequency of CD1d-restricted invariant NKT cells is actually low in humans (reviewed in [60]), further questioning the antiviral role of these invariant NKT cells in HBV-infected patients.

The antiviral role of type I IFN was strongly supported by the fact that IFNα2a and IFNα2b are currently used for hepatitis B treatment (reviewed in [61]). However, it is important to note that many patients with high viral loads do not respond effectively to the IFNα treatment. Thus, in accordance with the findings in chimeric mice, HBV not only can be a virus able to evade efficient recognition by the innate immune system, but also can possess mechanisms that are able to counteract the innate IFNα response actively [49]. The antiviral role of type II IFN was also supported by an *in vitro* study, demonstrating that addition of IFNγ reduced HBV DNA levels in naturally HBV-infected human hepatocytes [62]. However, the usage of IFNγ as hepatitis B treatment is not recommended yet due to its lower efficacy in suppressing HBV replication and its side effects in certain populations [63, 64].

Adaptive immune responses

Unlike the elusive role of innate immunity, the host adaptive immune response against HBV is better characterized. Studies using hydrodynamically transfected mice models clearly illustrate the importance of T cells in HBV control, in line with numerous analyses of the HBV-specific T cell response in humans where it was

quantitatively detectable in patients with a self-limited infection but not in patients with viral persistence [65–68]. It was also well established that HBV-specific CD4+ and CD8+ T cell–mediated immune responses in acute infected patients are typically multispecific, T_H1 oriented, and polyfunctional compared to those observed in the chronic counterparts (reviewed in [17]). Importantly, depletion studies of CD4+ or CD8+ T cells in HBV-infected chimpanzees also confirmed the crucial roles of T cells to control HBV infection. Briefly, immunodepletion of CD4+ T cells at the early stages, but not at the peak, of HBV infection resulted in persistent HBV infection [69, 70], suggesting that early priming of the CD4+ T cells is required to induce the CD8+ T cell response in order to clear the infection. Indeed, immunodepletion of CD8+ T cells at the peak of viremia delayed the viral clearance and onset of viral hepatitis, indicating that HBV-specific CD8+ T cells are the key effector to clear the virus (primarily via IFNγ) and also to cause liver injury [69].

Similar with findings in HBV transgenic mice [24, 25], the liver injury in hepatitis B patients was not primarily caused by virus-specific CD8+ T cells. Among different chronic HBV-infected patients, irrespective of the level of HBV replication or liver inflammation, the absolute numbers of intrahepatic HBV-specific CD8+ T cells remain similar [71]. Instead, the liver injury observed in chronic HBV-infected patients with high levels of HBV DNA and ALT was more associated with massive liver infiltration by antigen-nonspecific CD8+ T cells [71]. Nevertheless, it is important to notice that this massive recruitment may also be mediated by HBV-specific CD8+ T cells, due to the fact that chronic HBV-infected patients with hepatic flares displayed high levels of IFNγ-induced chemokines CXCL9 and CXCL10 [72], similar to what has been reported in transgenic mice [26]. Whether HBV-associated liver injury in humans can also be influenced by other immune components like the Kupffer cells, neutrophils, and platelets has not been elucidated in humans, even though it was well described using HBV transgenic mice [27, 29, 30].

In contrast to the findings in HBV transgenic mice [32], the pathogenic role of IL22 in hepatitis B patients is questionable. Serum levels of IL22 in chronic HBV-infected patients with liver injury were similar to the levels observed in healthy controls and chronic patients without ALT elevation, indicating that IL22 might not mediate liver damage in humans [73].

Interestingly, high levels of activated but not exhausted B cells (based on high expression of the CD69, CD71, and CD86 activation markers and low expression of the FcRL4 exhaustion marker) were observed in chronic HBV-infected patients [74]. This suggests that B cells are not severely impaired during persistent viral infection, which was in agreement with the findings in HBV transgenic mice [8, 33] Furthermore, it is widely acknowledged that anti-HBs antibodies appear only late during HBV infection. Hence, these antibodies are unlikely to contribute to the early phase of viral clearance. Instead, they most likely serve as neutralizing antibodies that prevent viral spread from infecting remaining cells during the resolution of HBV infection (reviewed in [75]). Thus, this observation can partially explain why the hydrodynamic transfection of HBV in B cell knockout mice did not result in HBV persistence [38].

Immune tolerance

It is known that vertical transmission accounts for most chronic HBV-infected cases. The majority of untreated infants born to HBeAg-positive mothers, but not from HBeAg-seroconverted mothers, become infected, and more than 90% of them became persistently infected [76, 77], suggesting an immunotolerogenic property of HBeAg *in utero*. Indeed, HBeAg can traverse the placenta as it has been detected in cord blood of infants born to HBeAg-positive mothers [78, 79]. Importantly, peripheral as well as cord blood mononuclear cells from infants born to HBeAg-positive mothers were unresponsive to HBcAg stimulation *in vitro* [80], as has been reported in HBeAg-expressing transgenic mice [33].

In contrast to the findings in mice [34], it is unclear as yet whether low levels of IL21 may contribute to HBV persistence in humans. Nevertheless, high serum levels of IL21 after 12 weeks of telbivudine treatment can predict HBeAg seroconversion in chronic HBV-infected patients [81], suggesting a protective role of IL21 during HBV infection.

Conclusions

For almost three decades, mice have been used to study the immunopathogenesis of HBV. The hurdle of a lack of an HBV tropism to infect mice was partially circumvented by creating transgenic mice, injecting HBV plasmid hydrodynamically, or infecting a chimera of human hepatocytes within mouse liver. Many interesting findings were obtained by using this inbred, small-animal model. Furthermore, several of these findings were in agreement with observations in HBV-infected humans, such as the crucial role of HBV-specific CD8+ T cells to control HBV replication. However, it is important to bear in mind that HBV infection in humans, particularly during chronic infection, is a complex process. This complexity cannot be entirely recapitulated using the available mouse models, due to differences in host–virus interactions. In sum, findings from mouse models that can be verified by observations in HBV-infected humans have significantly contributed to the understanding of immune control and pathogenesis

during HBV infection and are likely to help in the quest of new efficient treatment for this important chronic viral disease still affecting millions worldwide.

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Chapter 14
Treatment of hepatitis B

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Summary

Treatment of hepatitis B has evolved with seven approved drugs, including five nucleos(t)ide analogs and two formulations of interferon. Antiviral therapy not only suppresses hepatitis B virus (HBV) replication, but also decreases inflammation and fibrosis in the liver with resultant improvement in clinical outcome. With multiple treatment options that are efficacious and safe, the key question is which patients need immediate treatment and which patients can be monitored. In general, indications for treatment are based on activity or stage of liver disease, but viral factors such as HBV DNA levels play an increasingly important role in determining when treatment should be initiated. In this chapter, we will consider which patients will benefit from hepatitis B therapy, review the safety and efficacy of currently available treatments, and discuss management of patients with antiviral drug resistance.

Introduction

Over the past decade, significant advances have been made in understanding the natural history of chronic HBV infection and in the development of new treatments for chronic hepatitis B.

Prospective cohort studies have provided reliable estimates of the rate of progression of HBV-related liver disease and identified predictors of disease progression. In addition to age, gender, alanine aminotransferase (ALT) levels, and presence of cirrhosis, these studies demonstrated that viral factors including serum HBV DNA level, HBV genotype, and HBV precore/core promoter variants predict disease progression [1]. The most notable finding from these studies is that high serum HBV DNA level increases the risk of cirrhosis and hepatocellular carcinoma (HCC) [2, 3]. This was unexpected because HBV had been considered a noncytopathic virus and liver damage had been attributed to immune response to HBV. The finding that high serum HBV DNA level is a strong predictor of cirrhosis and HCC has led to treatment recommendations incorporating serum HBV DNA level as one of the criteria for initiating treatment [4–6].

The past decade also witnessed studies questioning the definition and meaning of normal ALT level. Studies of blood donors and persons being evaluated for living liver donation found that healthy persons who test negative for hepatitis B and C and who denied regular alcohol consumption and use of potentially hepatotoxic medications have ALT levels well below the upper limit of normal (ULN) determined by clinical diagnostic laboratories [7, 8]. These findings have led to recommendations that the ULN for ALT should be lowered to 30 for men and 19 for women. Adoption of this recommendation will markedly increase the number of patients with chronic hepatitis B who should receive treatment. Support for lowering the ULN for ALT has also been garnered from studies showing that patients with ALT levels within the normal range defined by diagnostic laboratories can have inflammation and fibrosis on liver biopsy, and studies demonstrating that patients with

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ALT levels 0.5–1.0 times the ULN have a higher incidence of liver decompensation and HCC compared to those with ALT levels <0.5 times the ULN [9, 10].

During the last 5 years, there has been intense interest in the role of quantitative hepatitis B surface antigen (HBsAg) as a surrogate for immune control of HBV and as an indirect measure of covalently closed circular HBV DNA (cccDNA). Quantitative HBsAg has been proposed to be a marker for differentiating inactive carriers from patients with hepatitis B e antigen (HBeAg)–negative hepatitis and for predicting the risk of HCC among inactive carriers [11, 12].

Interferon alpha (IFNα) and lamivudine were approved for the treatment of chronic hepatitis B in the 1990s. During the last decade, four additional nucleos(t)ide analogs (NUCs) and two formulations of pegylated IFN (PEG-IFN) have been approved for treatment of chronic hepatitis B. The newer NUCs have a higher barrier to resistance and are more potent than lamivudine. PEG-IFN has replaced conventional IFN, allowing weekly dosing instead of thrice-weekly injections with improved tolerance and increased rates of response. Quantifying serum HBV DNA levels, a key measure of the success of antiviral therapy for chronic hepatitis B, has also been revolutionized. A decade ago, non-PCR (polymerase chain reaction)–based assays with a lower limit of detection (LLOD) >100 000 copies/mL (~20 000 IU/mL) were still used in many countries, and PCR assays available at that time had an LLOD around 1000 copies/mL (~200 IU/mL) and a range of linearity up to 6–8 $\log_{10}$ copies/mL (~5–7 $\log_{10}$ IU/mL). Real-time PCR assays with an LLOD of 10–30 IU/mL and a range of linearity up to 8–9 $\log_{10}$ IU/mL are now widely used for monitoring virologic response to antiviral therapy.

These advances have expanded treatment options for patients and improved response rates, but many questions remain. Should antiviral treatment be recommended for all patients with chronic HBV infection? If not, for whom should treatment be recommended and when? Which is the best treatment? Is there a role for *de novo* combination therapy? Can treatment be tailored to the individual patient? Is it possible to stop treatment? In this chapter, we will summarize the efficacy of approved treatments and discuss indications for treatment, selection of first-line therapy, and management of treatment failure.

Treatment goals

The goal of treatment of chronic hepatitis B is to prevent cirrhosis, HCC, and liver failure. Conducting studies with these clinical outcomes as endpoints is not feasible because of the long interval between infection and the occurrence of these outcomes, necessitating the enrollment of hundreds of patients followed for many years. Therefore, treatment trials use surrogate endpoints to determine efficacy, and in clinical practice these same surrogate endpoints are used to monitor response. Surrogate endpoints that have been used include normalization of serum ALT levels, decrease in inflammation scores by ≥2 points with no worsening in fibrosis on liver biopsies, suppression of serum HBV DNA to undetectable levels, loss of hepatitis B e antigen (HBeAg) and seroconversion to HBe antibody (anti-HBe), and loss of HBsAg and seroconversion to HBs antibody (anti-HBs).

Indications for HBV treatment

Every person with chronic HBV infection is at risk of development of cirrhosis, liver failure, and HCC. With the availability of safe and potent antiviral agents, the question is not "Whom should be treated?" but rather "Should treatment be initiated now?" The patient who is not a treatment candidate at the time of presentation may become one in the future because of the dynamic nature of chronic HBV infection. Treatment should be initiated in patients who have active or advanced liver disease at presentation or who are predicted to have a high risk of cirrhosis or HCC in the foreseeable future (Table 14.1). Treatment may be deferred in patients who have quiescent, early-stage liver disease, and who are predicted to have a low risk of cirrhosis and HCC. The latter patients should continue to be monitored, with treatment initiated if and when the indications arise.

Decisions regarding hepatitis B treatment are usually made based on clinical features, levels of serum ALT and HBV DNA, and, when available, liver histology. It is important to emphasize that for most patients, assessment on a single occasion does not capture the overall state of HBV replication or liver damage, and monitoring of HBV DNA and ALT levels on 2–3 occasions over a 3–12-month period is necessary to determine whether treatment should be initiated. A short duration of monitoring prior to initiation of treatment is warranted in patients with compensated liver disease given that most patients will require many years and, in some instances, lifelong treatment.

Life-threatening liver disease

The decision to initiate antiviral therapy is clear in patients who present with life-threatening liver disease: acute liver failure, decompensated cirrhosis, and severe exacerbations of chronic hepatitis B (defined as ALT flares accompanied by jaundice and/or coagulopathy). Although most studies of antiviral therapy in these settings were uncontrolled or utilized historical controls and included small numbers of patients, these studies demonstrated that NUCs are effective not only

Table 14.1 Treatment indications for hepatitis B.

Treatment is indicated	HBV DNA (IU/mL)	ALT	Age	Histology	Clinical presentation
Acute liver failure or decompensated cirrhosis	Detectable	Any	Any	Not required	
Compensated cirrhosis	≥2000	Any	Any	Not required	
Severe exacerbation of chronic HBV	Detectable	Elevated	Any	Not required	Jaundice and/or coagulatopathy
HBeAg-positive chronic HBV	≥20 000	≥2 × ULN	Any	Not required	
		1–2 × ULN	Any	Moderate to severe inflammation or significant fibrosis	
		1 × ULN	>40	Moderate to severe inflammation or significant fibrosis	
HBeAg-negative chronic HBV	≥20 000	≥ 2 × ULN	Any	Not required	
	2000–20 000	1–2 × ULN	Any	Moderate to severe inflammation or significant fibrosis	
Treatment is not indicated					
HBeAg-negative inactive carrier	<2000	Persistently normal	Any		
HBeAg-positive immune tolerance phase	Generally >20 000	Persistently normal	<40		

in suppressing HBV replication but also in reversing liver failure (with decreases in the Child–Turcotte–Pugh score and Model for End Stage Liver Disease score) and improving survival [13–15]. These studies also showed that patients with advanced liver failure did not derive a clinical benefit and that most died or needed a liver transplant, prompting recommendations that treatment should be initiated as soon as possible in patients with liver failure. Additionally, starting antiviral treatment early in advanced liver failure will prevent recurrence of HBV infection in patients who ultimately need a liver transplant.

Compensated cirrhosis

The decision to initiate antiviral therapy is also obvious in patients with compensated cirrhosis, although the recommended HBV DNA cutoff levels for initiating treatment across professional society guidelines differ. Whereas all the guidelines recommend antiviral therapy regardless of ALT level, the American Association for Study of Liver Diseases (AASLD) and Asian Pacific Association for the Study of the Liver (APASL) guidelines recommend antiviral therapy if HBV DNA >2000 IU/mL, while the European Association for the Study of the Liver (EASL) guidelines recommend antiviral therapy in all patients with detectable HBV DNA [4–6]. The landmark double-blind, randomized, placebo-controlled trial of lamivudine in patients with advanced fibrosis or cirrhosis enrolled patients who were HBeAg-positive or had serum HBV DNA levels >700 000 Eq/mL (roughly 140 000 IU/mL) [16]. After a median duration of 32 months, 7.8% of treated patients and 17.7% of controls (P = .001) met predefined criteria for disease progression. Lower HBV DNA cutoffs are used in professional society guidelines based on the findings of the Risk Evaluation of Viral Load Elevation and Associated Liver Disease (REVEAL) study showing that a serum HBV DNA level >10 000 copies/mL (~2000 IU/mL) is associated with significantly higher risk of cirrhosis, HCC, and liver-related mortality [2, 3].

Compensated noncirrhotic liver disease

In patients who have not progressed to cirrhosis, the decision regarding when to start treatment is based on levels of ALT, HBV DNA, and liver histology (Table 14.1). All professional guidelines recommend antiviral treatment when ALT is persistently >2 times ULN. EASL uses 40 IU/L as the ULN, while lower values are recommended by AASLD and APASL. When ALT is 1–2 times the ULN, all guidelines recommend considering a liver biopsy and treatment if there is moderate to severe necroinflammation or fibrosis [4–6].

A cutoff HBV DNA level of >20 000 IU/mL (~100 000 copies/mL) was arbitrarily chosen as an indication for initiating treatment at a National Institutes of Health conference in 2000 [17]. At that time, the LLOD for most HBV DNA assays was 10^5–10^6 copies/mL. The REVEAL study found that risks of cirrhosis and HCC increase

with levels of HBV DNA >10 000 copies/mL (~2000 IU/mL) [2]. This has led to suggestions that the cutoff HBV DNA level for initiating treatment should be lower. It is rare for HBeAg-positive patients to have HBV DNA levels <2000 IU/mL except during ALT flares or during the process of HBeAg clearance. HBeAg-negative patients have lower levels of HBV DNA, and they are generally older and have more advanced fibrosis; therefore, it might be appropriate to lower the HBV DNA cutoff for initiation of treatment. However, HBeAg-negative patients with HBV DNA 2000–20 000 IU/mL and persistently normal ALT rarely have histologically significant liver disease [18].

Liver histology is a prerequisite in clinical trials and a criterion for initiating treatment in the EASL guidelines, but liver biopsies are not always performed in clinical practice. Liver biopsy is invasive, and interpretation of liver biopsy is limited by the size of the biopsy and sampling error. In many countries, noninvasive tests such as measurement of liver stiffness and panels of routine laboratory tests or serum markers of fibrosis have replaced liver biopsies for assessment of stage of liver disease in patients with chronic hepatitis C. The application of these noninvasive tests to hepatitis B is more complicated. Inflammation is usually mild and stable during the course of chronic hepatitis C virus (HCV) infection, while fluctuation in inflammatory activity is a hallmark of chronic HBV infection. Thus, measurement of liver stiffness in a patient with chronic hepatitis B reflects not only fibrosis but also inflammation and may be falsely high during ALT flares [19]. Similarly, serum markers of fibrosis such as the Aspartate Aminotransferase to Platelet Ratio Index (APRI) and Fibrotest that include markers of inflammation may overestimate the stage of fibrosis in patients with moderate to severe inflammation. Furthermore, activity is a better predictor of response to hepatitis B treatment than fibrosis, and development of HBV-related HCC can occur in patients who have not progressed to cirrhosis.

This discussion highlights the dilemma in deciding when antiviral therapy should be initiated in patients who do not have cirrhosis. Despite variations in HBV DNA and ALT cutoff levels, AASLD, EASL, and APASL guidelines all recommend that patients with HBeAg-positive or HBeAg-negative chronic hepatitis should be treated. HBeAg-positive patients with elevated ALT and normal hepatic synthetic function may be observed for 3–6 months to determine if they will undergo spontaneous HBeAg seroconversion prior to starting treatment. HBeAg-negative patients with elevated ALT are unlikely to achieve spontaneous sustained remission and may initiate treatment as soon as it is clear that the liver disease can be attributed to HBV (i.e., after exclusion of HCV and hepatitis D virus [HDV] co-infection and other causes of chronic liver disease, including fatty liver).

Antiviral treatment may be deferred in young HBeAg-positive patients who are in the immune tolerance phase and in HBeAg-negative patients who are in the inactive carrier phase. HBeAg-positive patients in the immune tolerance phase typically are young Asians who acquired HBV infection during infancy or early childhood, and have high levels of HBV DNA and persistently normal ALT. Follow-up of these patients for up to 10 years revealed a very low rate of progression to cirrhosis or HCC, and up to 85% underwent spontaneous HBeAg seroconversion [20]. Patients who underwent HBeAg seroconversion before the age of 30 years had an excellent prognosis with a low rate of progression to HBeAg-negative hepatitis, cirrhosis, and HCC compared to patients who did so after age 40 [21]. These data indicate that presence of HBeAg or a high level of HBV DNA at a single time point is not indicative of poor prognosis; rather, the risk of cirrhosis and HCC is increased in patients with a persistent presence of HBeAg or persistently high level of HBV DNA. This is supported by data from the REVEAL study showing that patients who had high levels of HBV DNA at presentation that declined during follow-up had significantly lower risks of HCC compared to those who maintained high levels of HBV DNA throughout the study [22]. Thus, while antiviral treatment is in general not recommended for patients in the immune tolerance phase, it should be considered in patients who remain in this phase after age 40. An additional reason for not recommending treatment to patients in the immune tolerance phase is the lower rates of response to both IFN and NUC therapy [23, 24].

HBeAg-negative patients who are deemed to be in the inactive carrier phase after 12 months of monitoring with three or more ALT and HBV DNA tests do not require antiviral treatment [25]. Long-term follow-up studies showed that the prognosis of inactive carriers is excellent [26]. These patients need to be monitored every 6–12 months so treatment can be initiated if and when they progress to HBeAg-negative hepatitis.

Some patients have HBV DNA and ALT levels that fall in the gray zone. A liver biopsy is helpful if, after 3–12 months of monitoring, it remains unclear if a patient meets criteria for initiating treatment. The benefit of a liver biopsy over noninvasive assessment of fibrosis is that it provides information regarding not only fibrosis but also inflammation and steatosis.

Special situations

Besides HBV DNA, ALT, and liver histology, the decision regarding when to start treatment should take into consideration the age of the patient, plans to start a family, family history of HCC, occupational considerations, co-infection with HIV, other medical conditions requiring immunosuppressive therapy or cancer

chemotherapy, and willingness to commit to years of antiviral therapy.

Patients who will be receiving chemotherapy or immunosuppressive therapy

Studies have shown that reactivation of HBV replication occurs at a rate of 20% to 50% in HBsAg-positive patients receiving immunosuppressive therapy [27, 28]. Most cases of HBV reactivation are asymptomatic, but severe hepatitis and death due to hepatic failure can occur. Reactivation of HBV has also been reported in HBsAg-negative, hepatitis B core antibody (anti-HBc)–positive patients; the incidence is lower and occurs mainly in the setting of potent immunosuppression such as regimens for hematologic malignancies and regimens that include rituximab [27, 29]. A systematic review of 14 studies of HBsAg-positive patients receiving chemotherapy, including 275 who received prophylactic lamivudine and 475 who did not, showed that with preventive lamivudine, the relative risk of both HBV reactivation and HBV-related hepatitis decreased by 80–100% [30]. Randomized trials showed that administration of antiviral therapy after the onset of HBV reactivation is less effective than prophylactic administration prior to or at the start of immunosuppressive therapy [31].

AASLD, EASL, and APASL guidelines recommend that patients be tested for HBsAg and anti-HBc prior to starting immunosuppressive therapy or cancer chemotherapy [4–6]. Patients who are found to be HBsAg-positive should receive prophylactic antiviral therapy. The role of prophylactic antiviral therapy is less clear in patients who are HBsAg-negative and anti-HBc positive; however, most experts would recommend prophylactic antiviral therapy for patients who will be receiving very potent immunosuppressive therapy such as treatment for hematologic malignancy or treatment regimens that include rituximab. Ideally, antiviral therapy should be started before or as soon as possible after the initiation of immunosuppressive therapy and continued for at least 6 months after the discontinuation of immunosuppressive therapy. Prophylactic antiviral therapy should be continued for at least 12 months after the discontinuation of rituximab because of the long delay in recovery of immune function. Treatment should be continued until the endpoint of chronic hepatitis B treatment is accomplished in patients with a baseline HBV DNA level >2000 IU/mL. Lamivudine was used in most studies of prophylactic antiviral therapy in patients receiving immunosuppressive therapy, but it is associated with a high risk of drug resistance. While lamivudine may be used in patients with undetectable HBV DNA prior to immunosuppressive therapy, entecavir or tenofovir disoproxil fumarate, which are more potent and have a higher genetic barrier to resistance, should be used in patients with detectable HBV DNA and those who are anticipated to require >12 months of antiviral therapy.

Women of reproductive age and pregnant women

Women who are of reproductive age, who have not completed their family, and who otherwise meet criteria for starting antiviral therapy should start treatment if they have life-threatening liver disease or cirrhosis. For patients with precirrhotic liver disease, the decision whether they should start treatment immediately or defer until after they complete their family needs to be carefully discussed. One option would be to use PEG-IFN, which is administered for a finite duration and can be completed prior to becoming pregnant. IFN is a US Food and Drug Administration (FDA) teratogenicity risk category C drug; therefore, emphasis must be made on the importance of contraception during treatment, and treatment must be stopped if the patient becomes pregnant. Another option is to use a NUC such as tenofovir disoproxil fumarate or telbivudine, which are FDA teratogenicity risk category B drugs. Because most patients will require a long duration of therapy, tenofovir disoproxil fumarate, which has a higher barrier to resistance, is preferred. If the patient becomes pregnant on tenofovir disoproxil fumarate, treatment can be continued. A third option is to monitor the patient and initiate antiviral therapy after she has completed her family.

HBsAg-positive pregnant women with high serum HBV DNA levels may transmit HBV infection to their infants despite administration of hepatitis B vaccine and hepatitis B immunoglobulin (HBIG) within 12 hours of birth and completion of the three-dose vaccine. A prospective study of 313 HBsAg-positive pregnant women found that perinatal transmission occurred in 8.5% of mothers with HBV DNA >8 $\log_{10}$ copies/mL compared to 0% in mothers with HBV DNA <8 $\log_{10}$ copies/mL [32]. Several studies have demonstrated that administration of lamivudine or telbivudine to mothers with high viremia in the late second or early third trimester can reduce the risk of HBV transmission. A randomized controlled trial of lamivudine versus placebo in 115 pregnant women who had HBV DNA >1000 MEq/mL (~8 $\log_{10}$ IU/mL) found that infants born to mothers in the treated group had a significantly lower rate of HBsAg seropositivity at 1 year of age, 18% versus 39% ($p = 0.014$) by intention-to-treat analysis and 7% versus 18% (p = NS) in adjusted analysis accounting for missing data, compared to infants born to mothers in the placebo group [33]. In another study, pregnant women with HBV DNA >6 $\log_{10}$ copies/mL chose to receive telbivudine 600 mg daily ($n = 135$) from weeks 20–32 of gestation or no treatment ($n = 94$). The incidence of perinatal transmission was 0% versus 8% ($p = 0.001$) in the telbivudine versus control group [34]. These data have prompted some experts to recommend antiviral therapy

to highly viremic mothers to prevent perinatal transmission; however, the cutoff HBV DNA levels to initiate treatment and the optimal time to start antiviral therapy have not been determined. Although lamivudine is a teratogenicity risk category C drug, it has been widely used in mothers with HIV infection, and data from the antiretroviral pregnancy registry showed that there was no increased risk of birth defects compared to non-exposed infants among 4185 and 1612 infants exposed to lamivudine and tenofovir disoproxil fumarate *in utero*, respectively [35]. Thus, the choices of antiviral drugs in pregnant women include lamivudine, telbivudine, and tenofovir disoproxil fumarate. Tenofovir disoproxil fumarate is preferred because of the low risk of drug resistance if treatment is intended to continue after delivery. Treatment can be stopped after delivery of the child if the patient did not have indications for treatment prior to the pregnancy. Patients should be monitored for hepatitis flares after treatment is stopped. NUCs can be secreted in breast milk, but the concentration in breast milk is much lower than in serum, and in the case of tenofovir disoproxil fumarate the active drug is poorly absorbed.

Healthcare providers infected with HBV

Transmission of HBV infection from healthcare providers to patients is rare but can occur if the provider is engaged in exposure-prone procedures. The Society for Healthcare Epidemiology of America (SHEA) recommend that providers with HBV DNA $>10^4$ GE/mL or positive HBeAg routinely double-glove for all invasive procedures and for all aspects of patient care where gloving is recommended [36]. These providers should not perform exposure-prone procedures until they have been initiated on antiviral therapy with serum HBV DNA suppressed to $<10^4$ GE/mL and have consented to regular monitoring to ensure that HBV DNA remains suppressed. In July 2012, the US Centers for Disease Control and Prevention recommended that individuals with chronic HBV infection should not be excluded from practice or study to be a healthcare provider. Healthcare workers with HBV DNA levels <1000 IU/mL should be permitted to perform exposure-prone procedures provided they are guided by an expert review panel and agree to monitoring at 6-month intervals [37]. Similar policies are in place in many European countries, although the cutoff HBV DNA and frequency for HBV monitoring vary [38].

While these policies provide a framework for healthcare providers with chronic HBV infection to continue working, they have compelled healthcare providers with high-level viremia who need to be engaged in exposure-prone procedures to initiate antiviral therapy for occupational reasons even when there is no clinical indication. This situation is most common in young providers in the immune tolerance phase. While antiviral therapy is effective in suppressing serum HBV DNA levels, it may take several months for HBV DNA to decrease to acceptable levels and these providers will likely need to remain on antiviral therapy throughout their entire career.

Family history of HCC

A family history of HCC has been shown to be an independent risk factor for HCC [39]. Some experts recommend treatment of HBV based on a family history of HCC regardless of whether the patient meets criteria for treatment. It is questionable whether this approach is beneficial to patients who otherwise have very low risk of HCC. For these patients, close monitoring and initiation of treatment when they meet treatment criteria may be more appropriate.

Co-infection with hepatitis C, hepatitis D, or HIV

Patients with HCV co-infection should be evaluated to determine which virus is dominant. In most cases, HCV is dominant and treatment of hepatitis C should be initiated as indicated. Studies using standard IFN or PEG-IFN with ribavirin have shown that the sustained virologic response rate is comparable in patients co-infected with HBV and HCV and those with HCV mono-infection [40]. These patients should be carefully monitored for HBV DNA rebound, which may occur due to removal of the interfering effects of HCV. Treatment of hepatitis D is discussed in Chapter 29.

For patients co-infected with HIV and HBV, an important decision is whether treatment of HIV, HBV, or both is needed. If both HIV and HBV should be treated, a combination of two drugs (tenofovir disoproxil fumarate plus lamivudine or emtricitabine) that has antiviral activity against both viruses should be used. Most experts recommend a lower threshold for initiating HBV treatment in patients with HIV co-infection and caution when changing antiretroviral regimens with anti-HBV activity to prevent hepatitis flares [41]. For the minority of patients who do not require HIV treatment, PEG-IFN or adefovir dipivoxil, which has no anti-HIV activity, can be considered.

Safety and efficacy of approved HBV drugs

Currently seven drugs have been approved for the treatment of hepatitis B: conventional and pegylated IFNα and five NUCs. Although IFN has weak antiviral activity, it enhances immune clearance. The advantages of IFN therapy are a finite duration of therapy, more durable HBeAg seroconversion, and a higher rate of HBsAg loss, especially in patients with genotype A

infection. NUCs have potent inhibitory effects on HBV DNA replication. The advantages of NUCs are convenience (once-daily oral administration) and tolerability. Unfortunately, viral relapse is common when NUCs are discontinued, thus a long-duration and often lifelong therapy is required with resultant risk of drug resistance and high cumulative costs. Currently monotherapy with PEG-IFN or a NUC is recommended when treatment for HBV is indicated. IFN should not be used in patients with decompensated cirrhosis, acute liver failure, immunosuppression, pregnancy, or medical or psychiatric contraindications. Patients with cirrhosis and no evidence of portal hypertension may be treated with PEG-IFN.

IFNα

Response rates

Responses to IFN in clinical trials are summarized in Tables 14.2 and 14.3 [42–48]. For HBeAg-positive patients, HBV DNA decreases to undetectable levels in

Table 14.2 Response rates to approved therapies for HBeAg-positive chronic hepatitis B.

	Lamivudine	Adefovir dipivoxil	Entecavir	Telbivudine	Tenofovir disoproxil fumarate	Pegylated interferon*	Pegylated interferon and lamivudine*
At week 48 or 52							
Histology Improved	49–56	53	72	65	74	38	41
Undetectable HBV DNA	36–44	13–21	67	60	76	25	69
HBeAg seroconversion	16–21	12–18	21	22	21	27	24
HBsAg loss	<1	0	2	<1	3	3	3–7
During extended treatment (years)**							
Undetectable HBV DNA	39	39	94	79	97	19***	26***
	(2)	(5)	(5)	(4)	(5)	(3.5)	(3)
HBeAg seroconversion	47	48	41	42	40	37%***	25***
	(3)	(5)	(5)	(4)	(5)	(3.5)	(3)
HBsAg loss	0–3	2	5	1.3	10	11***	15***
	(2–3)	(5)	(5)	(2)	(5)	(3.5)	(3)
Genotypic resistance	65	42	1.2	21	0	0	NA
	(5)	(5)	(6)	(2)	(5)		

Response expressed in %. "Histologic improvement" defined as a >2-point decrease in necroinflammatory score and no worsening of fibrosis score. NA = not available.
*Liver biopsy performed 24 weeks after stopping treatment.
**Timepoint in which response was assessed in years in brackets.
***Assessment performed off treatment.
(Source: Adapted from Scaglione SJ, Lok AS. Gastroenterology 2012;142(6):1360–1368 e1[48])

Table 14.3 Response rates to approved therapies for HBeAg-negative chronic hepatitis B.

	Lamivudine	Adefovir dipivoxil	Entecavir	Telbivudine	Tenofovir disoproxil fumarate	Peginterferon*	Peginterferon + lamivudine*
At week 48 or 52							
Histology Improved	60–66	64–69	70	67	72	48	38
Undetectable HBV DNA	60–73	51	90	88	93	63	87
HBsAg loss	<1	0	<1	<1	0	4	3
During extended treatment (years)**							
Undetectable HBV DNA	6	67	NA	84	99	18***	13***
	(4)	(5)		(4)	(5)	(3)	(3)
HBsAg loss	<1	5	NA	<1	0.3	8***	8***
	(4)	(5)		(2)	(5)	(3)	(3)

Response expressed in %. "Histologic improvement" defined as a >2-point decrease in necroinflammatory score and no worsening of fibrosis score. NA = not available.
*Liver biopsy performed 24 weeks after stopping treatment.
**Timepoint in which response was assessed in years in brackets.
***Assessment performed off treatment.
(Source: Adapted from Scaglione SJ, Lok AS. Gastoenterology 2012;142(6):1360–1368 e 1[48])

25% of patients at the end of 1 year of PEG-IFN treatment and remains undetectable in 19% at year 4. HBeAg seroconversion is observed in 21% of patients at year 1 and increases to 37% at year 4. HBsAg loss is seen in 3% at year 1 and increases to 11% at year 4. For HBeAg-negative patients, HBV DNA decreases to undetectable levels in 63% at the end of a 1-year course of PEG-IFN therapy and remains undetectable in 18% at year 4. HBsAg loss is seen in 4% at year 1 and increases to 8% at year 4.

Long-term follow-up showed that IFN-treated patients had a reduced incidence of cirrhosis, decompensation, liver-related death, and HCC compared with untreated patients, but the benefit was observed mainly in responders [49, 50].

Predictors of response

For HBeAg-positive patients, pretreatment predictors of response include high ALT, high histological activity index, low serum HBV DNA levels, and HBV genotype A or B. A retrospective analysis of two large trials of PEG-IFN defined sustained response as HBV DNA <2000 IU/mL and HBeAg loss 6 months after treatment. Patients with genotype A infection who had HBV DNA <9 $\log_{10}$ copies/mL and ALT >2 times the ULN had 54% predicted sustained response compared to 7% in patients with genotype D infection, HBV DNA >9 $\log_{10}$ copies/mL, and ALT <2 times the ULN [23]. Recent studies suggest that HBsAg decline on treatment predicts off-treatment response and may be used as a guide to determine whether treatment should be continued [51]. These data suggest that inadequate or lack of HBsAg decline after 12 weeks of PEG-IFN may be used as a stop rule, as the likelihood of a sustained response with continued treatment would be very low; however, these findings need to be validated prospectively before response-guided therapy can be recommended.

Treatment regimen

PEG-IFN-α2a should be administered in doses of 180 μg subcutaneously weekly. PEG-IFN-α2b is approved for hepatitis B in many countries but not in the United States. Clinical trials have used various doses from 0.5 to 1.5 μg/kg body weight weekly. The recommended duration of treatment for both HBeAg-positive and HBeAg-negative patients is 48–52 weeks. A recent study in HBeAg-positive patients with predominantly genotype B or C infection showed that 180 μg was superior to 90 μg and 48 weeks was superior to 24 weeks of treatment [52]. Another study in HBeAg-negative patients with genotype D infection found that virologic response defined as HBV DNA <2000 IU/mL was 59% versus 67% at the end of treatment, and 12% versus 29% ($P = 0.03$) 1 year post treatment, in the two groups that received 48 versus 96 weeks of PEG-IFN, respectively [53]. These data suggest that a longer duration of therapy may increase the rate of sustained response in HBeAg-negative patients.

Adverse events

IFN therapy produces a wide range of side effects including fatigue, flulike symptoms, depression, bone marrow suppression, and unmasking or exacerbation of autoimmune disorders. Flares in ALT can occur in as many as 40% of patients during IFN therapy, and although these flares are often associated with response, they can precipitate liver failure in patients with cirrhosis.

Nucleos(t)ide analogs (NUCs)

The five NUCs approved by the FDA, the European Medicines Agency, and many countries worldwide are categorized into L-nucleoside (lamivudine and telbivudine), acyclic nucleoside phosphonates (adefovir dipivoxil and tenofovir disoproxil fumarate), and deoxyguanosine analogs (entecavir). The main action of these drugs is to inhibit reverse transcription of the pregenomic HBV RNA to the first-strand HBV DNA. These drugs do not have any direct effect on covalently closed circular DNA (cccDNA), the template for transcription of pregenomic RNA and translation of viral proteins; therefore, viral relapse is common when treatment is discontinued. The lack of effect of NUCs on cccDNA explains why there is a discrepancy between potent HBV DNA suppression and a low rate of HBeAg or HBsAg loss. Because of the high rates of drug resistance, most professional organizations' guidelines and many health authorities do not recommend use of lamivudine or telbivudine, and adefovir dipivoxil is not recommended because of its weak antiviral activity.

Response rates

Responses to NUCs in clinical trials are summarized in Tables 14.2 and 14.3 [48, 54–64]. In HBeAg-positive patients, HBV DNA decreases to undetectable levels in 13–76% of patients at the end of 1 year of treatment and in 39–94% of patients at the end of 5 years of treatment. HBeAg seroconversion is seen in 12–22% of patients at year 1 and increases to 40–48% at year 5. HBsAg loss is seen in 0–3% of patients at year 1 and remains low at 0–10% at year 5. For HBeAg-negative patients, HBV DNA becomes undetectable in 51–93% of patients at the end of 1 year of treatment and remains undetectable in 67–99% at the end of 5 years of treatment. HBsAg loss is seen in 0–1% at year 1 and remains low at 0–5% at year 5. Viral suppression is accompanied by decrease in

necroinflammation as well as reversal of fibrosis. Long-term follow-up of patients receiving entecavir showed that after a median of 6 years on treatment, 96% of patients had decreased necroinflammatory scores and 88% had decreased fibrosis scores, including 10 patients who had advanced fibrosis or cirrhosis at baseline [65]. Similar findings were observed in patients receiving tenofovir disoproxil fumarate [64]. These data indicate that long-term viral suppression not only prevents further liver damage but also reverses previous damage. Indeed, NUCs have been shown to prevent progression of liver disease and to reduce the incidence of HCC [16, 66].

Predictors of response

As with IFN, high ALT and low HBV DNA pretreatment are associated with a higher rate of response; however, there is no correlation between HBV genotype and response to NUC. A study that combined data from four studies with a total of 406 patients treated with lamivudine for approximately a year showed that HBeAg loss was 2%, 9%, 21%, and 47% for patients with pretreatment ALT levels that were, respectively, normal or 1–2, 2–5, or >5 times the ULN [67]. Similarly, in the phase III study of entecavir, HBeAg-positive patients with pretreatment ALT >2 times the ULN had higher rates of undetectable HBV DNA (73% vs. 48%), ALT normalization (73% vs. 55%), histologic improvement (75% vs. 62%), and HBeAg seroconversion (26% vs. 8%) at the end of year 1, compared to those with ALT 1.3–2.0 times the ULN [24]. Unlike IFN, NUC therapy has minimal effects in reducing HBsAg levels, and the accuracy of HBsAg levels at week 12 or 24 in predicting a long-term response to NUC is low [51].

Adverse events

In general, the NUCs are tolerated well with minimal side effects. All NUCs can cause lactic acidosis, but there had been very few reports of lactic acidosis associated with NUCs approved for HBV treatment. Entecavir was reported to cause lactic acidosis in one case series of patients with decompensated liver disease [68]; however, two large prospective studies of patients with decompensated hepatitis B receiving entecavir, tenofovir disoproxil fumarate, a combination of entecavir and tenofovir disoproxil fumarate, or a combination of emtricitabine and tenofovir disoproxil fumarate did not observe any cases of lactic acidosis, although lactate levels were not systematically monitored [13, 14].

Adefovir dipivoxil and tenofovir disoproxil fumarate can cause nephrotoxicity and renal tubular abnormalities. Nephrotoxicity was observed in 3% of patients with compensated liver disease after 4–5 years of continued adefovir dipivoxil [63]. The incidence of nephrotoxicity is higher in patients with decompensated liver disease and in those who had a liver transplant. Nephrotoxicity was observed in 1% of patients with compensated liver disease after 5 years of tenofovir disoproxil fumarate treatment [64]. Among patients with decompensated liver disease, the incidence of nephrotoxicity after 1–2 years of tenofovir disoproxil fumarate or entecavir treatment was similar [14]. Decrease in bone mineral density had also been reported in patients with HIV who received tenofovir disoproxil fumarate as part of their antiretroviral therapy [69, 70]. To date, there is no evidence that tenofovir disoproxil fumarate causes bone abnormalities in patients with HBV mono-infection.

In animal studies in which entecavir was given in doses that are 3 to 40 times that in humans, there was an increased incidence of lung adenomas, brain gliomas, and HCC [71]. To date, there is no evidence that entecavir increases the incidence of neoplasms in humans.

Telbivudine can cause myopathy and peripheral neuropathy [57, 72, 73]. Peripheral neuropathy is more common when telbivudine is combined with PEG-IFN, leading to termination of the trial on this combination regimen.

Treatment regimen

All five NUCs are administered orally once daily, and doses must be adjusted in patients with renal insufficiency. Treatment should be continued indefinitely in patients with cirrhosis. For patients without cirrhosis, treatment may be discontinued in HBeAg-positive patients who have completed 12 months of consolidation therapy after achieving HBeAg seroconversion. Although the durability of NUC-induced HBeAg seroconversion is lower than that of IFN-induced HBeAg seroconversion, one study of 178 patients showed that HBeAg seroconversion was durable in 91% of patients who completed 12 months of consolidation lamivudine therapy [74]. The endpoint for treatment of HBeAg-negative patients is unclear. Guidelines recommend lifelong treatment or until HBsAg clearance. A recent study of 33 noncirrhotic HBeAg-negative patients who discontinued treatment after 4–5 years of adefovir dipivoxil therapy found that although all patients experienced viral relapse, 18 (55%) had sustained remission and 13 (39%) lost HBsAg a median of 69 months after treatment discontinuation [75], suggesting that NUC treatment may be discontinued in some HBeAg-negative patients.

De novo combination therapy in treatment-naïve patients

Studies comparing a combination of IFN and lamivudine with lamivudine alone and/or IFN alone showed

that combination therapy resulted in greater on-treatment viral suppression, but there was no difference in sustained off-treatment response compared with IFN alone [42, 43].

Studies comparing a combination of two NUCs with NUC monotherapy in general found no benefit of combination therapy. In one study, 115 HBeAg-positive patients were randomized to a combination of lamivudine and adefovir dipivoxil versus lamivudine monotherapy. At week 104, 26% and 14% had undetectable HBV DNA, 13% and 20% had HBeAg seroconversion, and 19% and 44% had virologic breakthrough in the groups that received combination therapy and monotherapy, respectively [76].

More recently, a combination of entecavir and tenofovir disoproxil fumarate was compared to entecavir monotherapy in 379 NUC-naïve patients [77]. At week 96, comparable proportions of patients in the two treatment arms achieved HBV DNA <50 IU/mL (83% vs. 76%, P = .088). Among HBeAg-positive patients, a greater proportion given combination therapy achieved HBV DNA <50 IU/mL than those given entecavir alone (80% vs. 70%, P = .046); however, this difference was observed only in patients with baseline HBV DNA $\geq 10^8$ IU/mL. Rates of HBeAg loss and HBeAg seroconversion were similar in the two treatment groups. No variants associated with entecavir or tenofovir disoproxil fumarate resistance were detected. These data indicate that *de novo* combination therapy with entecavir and tenofovir disoproxil fumarate does not offer any benefit compared to monotherapy in the majority of NUC-naïve patients; however, combination therapy may be beneficial in patients with high baseline HBV DNA in whom rapid viral suppression is desirable.

Antiviral drug resistance

HBV DNA replicates via reverse transcription of the pregenomic RNA. Because of the high rate of HBV replication and the lack of proofreading of reverse transcriptase, the spontaneous mutation rate of HBV DNA is high, and it is estimated that all possible mutations may be present in the viral pool of most patients. However, due to the overlapping of open reading frames, not all variants would be viable. Exposure of HBV to NUCs with anti-HBV activity selects for mutations in the polymerase (P) gene that confer resistance or decreased susceptibility to the drugs. The rapidity with which drug resistance variants are selected depends on the potency of the antiviral drug and its genetic barrier to resistance, replication rate of HBV in the host, replication fitness of the variant, and replication space (pool of uninfected hepatocytes that will accommodate the resistance variants) [78]. Antiviral drug resistance mutations in the HBV polymerase gene may result in amino acid changes in the overlapping HBV surface gene, including alteration in a major epitope of the surface protein (e.g., M204I in the P gene can result in W196*/S/L in the surface gene) or decreased secretion of surface protein (e.g., A181T in the P gene can result in W172*). Such changes can give rise to false negative results in serology assays for HBsAg, and may potentially abrogate the protective effects of HBIG or HBV vaccine with serious public health consequences. The rtA181T/sW172* mutation had also been reported to be associated with increased risk of HCC in *in vitro* studies [79]; whether this is true in humans remains to be confirmed.

Location of primary antiviral resistance mutations

Figure 14.1 depicts the conserved domains of the HBV reverse transcriptase and the location of the primary antiviral drug resistance mutations [78, 80]. Resistance to lamivudine is primarily due to substitution of methionine for valine or isoleucine in the YMDD motif (M204V/I) and in some instances to substitution of

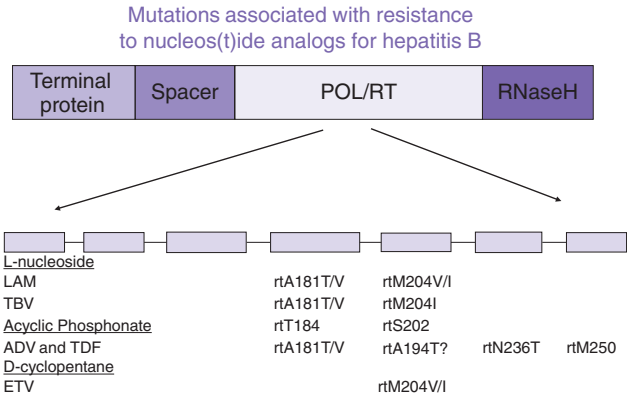

Figure 14.1 Mutations in the reverse transcriptase region of HBV polymerase that are associated with resistance to nucleos(t)ide analog therapy. The HBV polymerase consists of four regions: terminal protein, spacer, POL/RT, and RNaseH. The POL/RT region itself has seven domains (A, B, C, D, E, F, and G), and amino acid substitutions in this region confer resistance to nucleos(t)ide analogs. Multiple amino acid substitutions at positions 184, 202, and 250 have been reported to be associated with resistance to entecavir. Abbreviations: A181T/V: alanine to threonine or valine; A194T: alanine to threonine; M204V/I: methionine to valine or isoleucine; N236T: asparagine to threonine; rtT184A/L/F/M/C/G/S/I: threonine to alanine, leucine, phenylalanine, methionine, cysteine, glycine, serine, or isoleucine; rtS202G/C: serine to glycine or cysteine; rtM250V/L/I: methionine to valine, leucine, or isoleucine; LAM: lamivudine; TBV: telbivudine; ADV: adefovir dipivoxil; TDF: tenofovir disoproxil fumarate; ETV: entecavir.

Table 14.4 Incidence of genotypic resistance to approved HBV nucleos(t)ide analogs.

	Incidence of genotypic resistance at year (%)				
	1	2	3	4	5
Nucleos(t)ide-naïve patients					
Lamivudine	~20	45	55	71	65
Telbivudine	5–10	10–20	NA	NA	NA
Adefovir dipivoxil	0	3	11	18	29
Entecavir*	0	~1	1–2	1–2	1–2
Tenofovir disoproxil fumarate**	0	0	0	0	0
Patients with lamivudine resistance					
Switch to adefovir dipivoxil	~5	~20	NA	NA	62–66 [88]
Add adefovir dipivoxil	10 [89]				
Entecavir	6	15	36	46	51

*Entecavir 1.0 mg daily used after year 2.
**Most patients with detectable HBV DNA at week 72 received additional treatment with emtricitabine.

alanine for valine or threonine at position 181 (A181V/T). These mutations, particularly M204V, decrease replication fitness of HBV and may be accompanied by compensatory mutations such as substitution of valine for leucine at position 173 (V173L) and substitution of leucine for methionine at position 180 (L180M). Resistance to telbivudine is primarily due to M204I but not M204V and in some instances A181V/T. The A181V/T mutation also confers resistance to adefovir dipivoxil. Another mutation associated with adefovir dipivoxil resistance involves substitution of asparagine for threonine at position 236 (N236T), which may be present on its own or together with A181V/T mutation. Resistance to entecavir occurs through a two-hit mechanism with initial selection of M204V/I mutation followed by amino acid substitutions at T184, S202, or M250. *In vitro* studies showed that the mutations at positions 184, 202, or 250 on their own have minimal effect on susceptibility to entecavir, but susceptibility to entecavir is decreased by 10–250-fold when one of these mutations is present with M204V/I, and by >500-fold when two or more entecavir resistance mutations are present with M204V/I [81]. This explains the high incidence of entecavir resistance in patients with lamivudine resistance HBV. Resistance to tenofovir disoproxil fumarate is rare and remains to be characterized. Substitution of alanine for threonine at position 194 (A194T) had been reported to be associated with resistance to tenofovir disoproxil fumarate [82], but this remains to be confirmed. *In vitro* studies showed that mutations associated with adefovir dipivoxil resistance, A181V/T and N236T, particularly when present together, decrease susceptibility to tenofovir disoproxil fumarate, and clinical studies confirmed that the presence of these mutations impairs virologic response to tenofovir disoproxil fumarate [83].

Factors associated with antiviral drug resistance

Clinically, antiviral drug resistance is related to viral, host, and treatment factors. Viral factors include high pretreatment HBV DNA and preexistence of drug resistance variants. Host factors include prior exposure to HBV NUCs, particularly those with cross-resistance; the immune status of the patient; pharmacodynamics; and medication adherence. Treatment factors include potency of the drug, genetic barrier to resistance, and rapidity of virologic response.

Incidence of genotypic resistance

The incidence of genotypic resistance varies from 0% to 20% after 1 year of NUC therapy, and it increases to 0–70% after 5 years of treatment (Table 14.4). Entecavir and tenofovir disoproxil fumarate are the preferred NUCs in NUC-naïve patients because of the low rate of drug resistance. Patients with slow or suboptimal virologic response are at greater risk of antiviral drug resistance. This has led to the proposal of the road map approach in which patients with inadequate HBV DNA suppression after the first 24 weeks of treatment should have a second NUC added [84]; however, this approach was based on data from clinical trials of lamivudine and telbivudine – drugs that have very low barriers to resistance. Clinical trials as well as data from real-life practice showed that patients with detectable serum HBV DNA after 48 weeks of entecavir or tenofovir disoproxil fumarate experienced further viral decline during continued treatment with monotherapy of these drugs with very low rates of drug resistance.

Antiviral resistance is initially manifested as a virologic breakthrough defined as a >1 log increase in serum

Table 14.5 Salvage therapies for antiviral drug-resistant HBV.

Types of resistance	Preferred treatment	Alternative treatment
Lamivudine or telbivudine	Switch to or add tenofovir disoproxil fumarate.	Add adefovir dipivoxil. Stop lamivudine or telbivudine, and switch to Truvada.
Adefovir dipivoxil	Switch to or add entecavir.	Stop adefovir dipivoxil and switch to Truvada.
Entecavir*	Switch to or add tenofovir disoproxil fumarate.	Stop entecavir and switch to Truvada.
Tenofovir disoproxil fumarate*	Switch to or add entecavir.	Stop tenofovir disoproxil fumarate and switch to Truvada.
Multidrug resistance	Tenofovir disoproxil fumarate and entecavir.	

Truvada: a combination pill with emtricitabine and tenofovir disoproxil fumarate; not approved for HBV.
*Limited clinical data.

HBV DNA from nadir while the patient is still on treatment. Virologic breakthrough may occur simultaneous with but more often precedes biochemical breakthrough (defined as elevated ALT after normalization). In some instances, this is followed by hepatitis flares, hepatic decompensation, and death. Not all cases of virologic breakthrough are due to antiviral drug resistance. One study found that up to 38% of virologic breakthrough observed in clinical practice could not be confirmed on retesting, and 67% had no evidence of genotypic resistance [85]. These data highlight the importance of counseling patients on the importance of medication adherence and the need to confirm the presence of genotypic resistance before initiating rescue therapy.

Prevention of antiviral drug resistance

Once resistance develops, it will limit the available treatment options; thus, preventing the development of resistance is important. The first step is to determine whether treatment is indicated and whether the patient is committed to adhering to the treatment regimen. Once treatment is decided upon, a drug with a high genetic barrier to resistance and potent antiviral activity – namely, entecavir or tenofovir disoproxil fumarate – should be used as the first-line treatment and the patient should be educated on the importance of medication adherence. Virologic response should be monitored by checking serum HBV DNA every 3–6 months so that inadequate response or virologic breakthrough can be detected early.

Management of antiviral drug resistance

Detection of virologic breakthrough should prompt further evaluation. If there is no evidence of biochemical breakthrough, counseling on medication adherence and retesting for HBV DNA after 1–3 months or testing for genotypic resistance should be performed before initiating rescue therapy. Table 14.5 summarizes the options for rescue therapy. In general, addition of a second drug that has no cross-resistance is preferred to a switch; however, recent studies suggest that tenofovir disoproxil fumarate monotherapy is as effective as tenofovir disoproxil fumarate plus emtricitabine in patients with lamivudine resistance [86]. Patients who had sequential monotherapy may have multidrug resistance HBV [87]. Characterization of the resistance mutations is important in guiding the selection of rescue therapy. In most patients, a combination of entecavir and tenofovir disoproxil fumarate would be necessary.

Conclusions and future perspectives

Substantial progress has been made in the treatment of hepatitis B in the past decade, however when treatment should be initiated in noncirrhotic patients remains a topic of debate. While currently available treatment is effective in suppressing HBV replication, it fails to eradicate the virus, necessitating long-lasting and often lifelong treatment. The future of hepatitis B treatment will involve more personalized decisions regarding when to initiate treatment based on prognostic models that include genetic markers that predict cirrhosis and HCC as well as choice of treatment based on pharmacogenetics and predicted response. Most important is the need for novel therapies – antiviral agents with new targets in the HBV replication cycle combined with immunotherapies that can restore the host immune response to HBV.

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Chapter 15
Liver transplantation for chronic hepatitis B and C

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Summary

Decompensated cirrhosis and hepatocellular carcinoma (HCC) secondary to chronic hepatitis C are the most common indications for liver transplantation in Europe, North America, and Australasia, and the incidence of both is projected to double over the next decade. In contrast, although chronic hepatitis B virus (HBV) is the most common indication in the Asia–Pacific region, the demand will decrease secondary to the widespread availability of effective, well-tolerated oral antivirals against HBV that can successfully rescue most cases of decompensated cirrhosis. Hepatitis B virus (HBV)–related HCC will eventually decrease because of the effects of universal neonatal vaccination.

Without antiviral prophylaxis, the allograft is re-infected at the time of transplantation for chronic hepatitis B or C. If untreated, recurrent HBV and hepatitis C virus (HCV) infection will result in severe graft injury with accelerated disease progression to cirrhosis, graft loss, and death. Although now uncommon, recurrent HBV Infection can be successfully treated by oral antivirals that must be maintained for life. Unfortunately, the current standard of care for HCV is poorly tolerated following liver transplantation, and the efficacy is much lower than in nontransplant patients – only 20–25% of those suitable for treatment will have a sustained virologic response (SVR) to pegylated IFN plus ribavirin (PEG-IFN/RBV). The addition of protease inhibitors should increase the SVR rate, but triple therapy is complicated because of significant toxicities and drug interactions.

The best strategy in transplantation for chronic viral hepatitis is prevention of recurrent infection through peritransplant prophylaxis. Recurrent HBV infection has become rare since the introduction of safe and effective antiviral prophylaxis with a combination of nucleos(t)ide analogs plus hepatitis B immune globulin (HBIG). However, the high cost and inconvenience of lifelong HBIG have driven the development of HBIG minimization strategies. The use of potent nucleos(t)ides from the time of listing should allow a completely HBIG-free regimen for the majority of patients. Unfortunately, recurrent HCV infection is universal as PEG-IFN/RBV is poorly tolerated either before or immediately following liver transplantation.

Outcomes following liver transplantation for chronic hepatitis B are excellent because of the availability of safe and effective antiviral prophylaxis. Prevention and treatment of recurrent hepatitis C remain huge unmet medical needs. Pre- or peritransplant trials of combination direct-acting antivirals in patients listed for end-stage chronic hepatitis C should be considered high priorities.

Liver transplantation for chronic hepatitis C

Epidemiology

Almost 180 million people are chronically infected with HCV, with the highest prevalence rates reported in the Middle East, Eastern Europe, and Southeast Asia [1].

The incidence of HCV infection has decreased by more than 50% since 2000, thanks to the successful implementation of public health prevention strategies including routine screening of all blood and organ donors and reduction of intravenous (IV) drug use [2,

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3]. Although the cohort of individuals with chronic HCV infection is now stable in size, this represents an aging, untreated population with slowly progressive fibrosis [4]. Over the next two decades, the proportion with established cirrhosis is projected to double, and the incidence of HCC and liver failure will triple [5–9]. Chronic hepatitis C is already the leading indication for listing for elective liver transplantation, accounting for 30% of listed adults in Australia and New Zealand and 40% in Europe and North America [10–12]. The projected increase in demand for liver transplantation will soon outstrip the total deceased donor supply, which will further increase HCV-related mortality. This huge health burden can be averted only through successful eradication of HCV infection in patients with chronic hepatitis C. Unfortunately, currently less than 10% of infected individuals have received antiviral therapy, with less than one-half of this figure cured. The current treatment numbers would need to increase more than threefold in order to prevent this projected health burden [1, 8].

Table 15.1 Predictors of rapid fibrosis progression.

Pretransplant	Posttransplant
Viral factors	Immunosuppression
High viral load	Adjuvant therapy for rejection
HIV co-infection	Rapid weaning of prednisone
Host factors	Pattern of recurrence
↓ CD4+ and CD8+ responses	Cholestatic hepatitis
↓ Innate NK and NKT responses	Severe inflammation at 1 year
Cryoglobulinemia	Persistently elevated alanine aminotransferase (ALT)
IL28B genotype	Early high HCV RNA levels
	Posttransplant diabetes
Donor	Herpesvirus reactivation
Older age	Cytomegalovirus
Steatosis	HHV6
Diabetes	
Ischemic time	
IL28B genotype	

Outcome and predictors of outcome

In all patients who have active HCV infection at the time of transplant, the donor allograft becomes infected immediately following reperfusion [13]. Although recurrent HCV infection is usually asymptomatic, most patients will develop biochemical graft dysfunction within the first 4–24 postoperative weeks secondary to acute hepatitis C [14, 15]. Rarely, acute hepatitis C may develop within the first 2 weeks post transplant, confounding the diagnosis of acute allograft rejection [16]. The steep rise in HCV viral load that accompanies acute hepatitis C may help distinguish this from uncomplicated acute cellular rejection [15–19]. Spontaneous resolution of acute hepatitis C is accompanied by falling viremia levels, which subsequently plateau at 1–2 logs higher than pretransplant levels, reflecting the indirect effects of immunosuppression [15, 20–25]. The natural history of recurrent hepatitis C is accelerated, with 20–40% progressing to cirrhosis within 5 years. However, the rate of fibrosis progression is not uniform and may change over time. Recurrent hepatitis C is associated with reduced graft and patient survival. The increasing demand for transplantation for HCV-related HCC and decompensated cirrhosis, the increasing problem of HCV-related graft loss, and the inability to prevent or treat recurrent HCV infection have meant that recurrent hepatitis C has become the biggest challenge facing adult liver transplant programs worldwide.

Multiple host, donor, and viral pretransplant factors are associated with increased risk of severe recurrence and HCV-related graft failure (Table 15.1). Both high pretransplant and high early posttransplant viral load are associated with more severe recurrent hepatitis C [26–28]. HIV co-infection is also associated with increased fibrosis progression and reduced pre- and posttransplant survival [29, 30]. Posttransplant diabetes mellitus (PTDM), which develops in almost 40% of patients transplanted for HCV–cirrhosis, is also associated with increased fibrosis progression and reduced posttransplant survival [31–34].

Recent registry reports demonstrate a steady improvement in patient and graft survival following transplantation for non-HCV diseases but not following transplantation for HCV–cirrhosis. Potential reasons include deterioration in donor quality and changes in immunosuppression strategies.

The widening gap between donor supply and demand has resulted in increased use of donor organs previously considered as marginal. Between 1995 and 2005, the mean deceased donor age has almost doubled [35, 36]. Increased donor age is associated with accelerated fibrosis progression in patients with recurrent HCV infection. This aging effect reflects altered interaction between HCV and the senescent hepatocyte, resulting from cumulative mitochondrial mutations and telomere shortening, both of which alter the hepatocyte's response to viral infection [37–40].

Early reports of inferior outcomes following live-donor liver transplantation for HCV–cirrhosis are now attributed to increased biliary complications in the early learning period of live-donor liver transplantation. Later studies that incorporated protocol allograft biopsies have demonstrated no difference in the rate of progression of recurrent hepatitis C in live-donor and deceased-donor liver transplant recipients [41, 42].

The relationship between immunosuppression and HCV progression is complex. An episode of treated rejection is associated with reduced survival in recipients with HCV infection, suggesting an adverse effect of corticosteroids on the natural history of recurrent hepatitis C [26, 43]. High-dose IV steroid therapy for acute rejection leads to an early and massive increase in hepatitis C viremia following liver transplantation and is associated with earlier onset and more rapid progression of recurrent hepatitis C [26, 43–47]. This association between bolus steroids and rapid fibrosis progression encouraged steroid-sparing immunosuppressive protocols for patients with recurrent hepatitis C [48]. However, studies of steroid-sparing protocols have failed to demonstrate any benefit over traditional steroid-containing protocols [49, 50]. Compared to rapid-weaning protocols, maintaining recipients on low-dose prednisone and/or azathioprine may protect against rapid fibrosis progression [51–55]. The popular practices of early discontinuation of steroids and avoidance of maintenance azathioprine have been implicated in the observed worsening outcomes for HCV during the past decade [56]. The choice of calcineurin inhibitor following transplantation for HCV remains controversial. Cyclosporine, but not tacrolimus, possesses an intrinsic antiviral effect against HCV. Cyclosporine binds to cyclophyllin B, inhibiting interaction with the HCV NS5A protein, which is essential for activation of the HCV polymerase complex. Cyclosporine is also associated with lower risk of PTDM than tacrolimus following transplantation for HCV [57, 58]. Despite these intrinsic antiviral and metabolic effects of cyclosporine, no long-term benefit of cyclosporine over tacrolimus-based immunosuppression following transplantation for HCV has been demonstrated in either single-center series or large meta-analyses [59, 60]. A recent retrospective analysis of almost 10 000 liver transplants for HCV conducted by the United Network for Organ Sharing (UNOS) and the Organ Procurement and Transplantation Network (OPTN) demonstrated an increased risk of primary graft failure and biopsy-proven acute rejection in those patients who received cyclosporine rather than tacrolimus-based immunosuppression [61].

Finally, there is no demonstrable effect of mycophenylate, basiliximab, or sirolimus on the natural history of recurrent HCV.

The best predictor of rapid fibrosis progression is the grade of necroinflammation at one year post transplant, and a protocol liver biopsy at 12 months is recommended in all patients with recurrent HCV infection [62–64]. Thereafter, annual assessment by transient elastography has been advocated as a non-invasive means of monitoring fibrosis progression in liver transplant recipients with recurrent hepatitis C [65–68].

Treatment of recurrent hepatitis C

The only factor associated with improved graft and patient survival following transplantation for hepatitis C is HCV eradication through successful antiviral therapy [69, 70]. The past decade has seen huge improvements in the efficacy of antiviral therapy for chronic hepatitis C in nontransplant patients, where SVR rates in HCV genotype 1 have increased from <10% with standard interferon monotherapy to 65–75% with a combination of telaprevir or boceprevir plus PEG-IFN/RBV (the current standard of care). However, liver transplant recipients with recurrent HCV infection represent a "born-to-lose" population, with a high prevalence of baseline negative predictors for response to interferon-based therapy, including HCV genotype 1, high baseline HCV RNA level (a direct effect of immunosuppression), interleukin 28B (IL28B) non-CC genotype, and previous nonresponse to PEG-IFN/RBV. Immunosuppression may also inhibit responses to PEG-IFN/RBV through increased baseline viremia, reduced IFN sensitivity, and blunted innate and HCV-specific T cell responses [71–73]. However, the most important negative predictive factor of response to current antiviral therapy in liver transplant recipients with recurrent HCV is poor adherence, due to poor tolerability of both interferon and ribavirin. As many as 50% of patients require dose reduction, and almost 30% have to stop therapy because of adverse effects.

Most side effects of IFN–, including flulike symptoms, weight loss, and dose-related myelosuppression, are increased in liver transplant recipients and necessitate dose reduction in 30–60% and withdrawal in 20%. Depression may also be more common, leading to treatment cessation in almost 20% of liver transplant recipients [74–76]. Interferon is an immunomodulator that upregulates HLA class I antigen expression, which could potentially increase the rate of immune-mediated graft injury [77]. Certainly, interferon therapy in renal transplant recipients is associated with increased rejection and graft loss [78–81]. Although an early study in liver transplant recipients reported increased rate of chronic rejection, subsequent single center studies did not confirm this [82–87]. However, a recent case-controlled multicenter study demonstrated a significant association between PEG use and onset of severe immune-mediated liver allograft dysfunction, characterized by plasma cell hepatitis or rejection [88].

The major adverse effect of ribavirin is dose-related hemolysis. Unfortunately, this is more severe in transplant recipients because of reduced renal clearance secondary to calcineurin inhibitors [89]. The ribavirin dose needs to be decreased in 50–70% in liver transplant recipients, compared to only 10% of nontransplant patients [90]. The essential role of ribavirin, however,

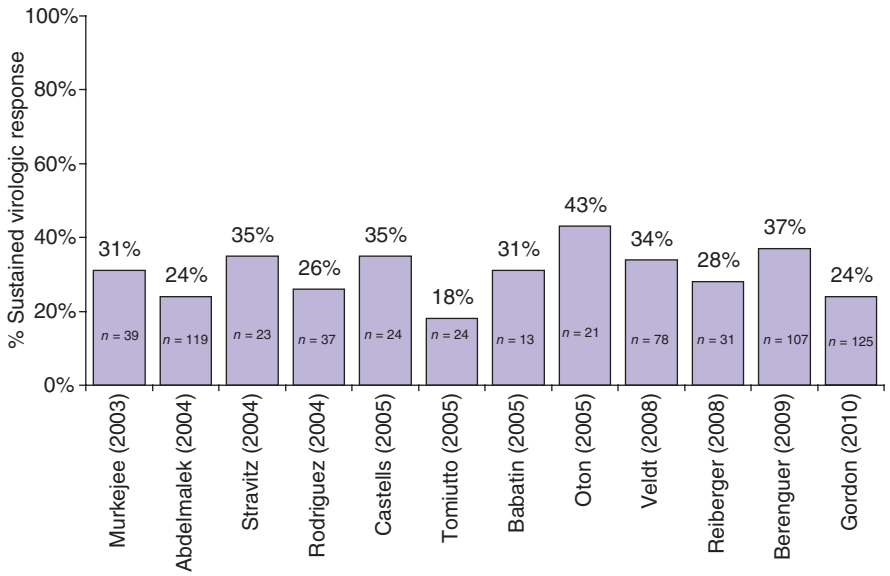

Figure 15.1 Treatment of recurrent HCV infection with pegylated interferon plus ribavirin.

was confirmed in a recent study where transplant recipients were randomized to receive either PEG-IFN/RBV or PEG monotherapy. Although the incidence of serious adverse effects, dose reduction, and treatment cessation was significantly lower in the RBV-free arm, the removal of ribavirin was associated with a significant decrease in SVR rate (44% vs. 14%, OR = 5.02; CI 1.32–19.08) [91]. Ribavirin-induced hemolysis can be avoided through close drug monitoring, targeting a trough level of 10–15 mmol/L, but testing for ribavirin level is not readily available [92]. Substitution of ribavirin with the liver-targeting taribavirin has been delayed because of inferior response rates and gastrointestinal side effects [93, 94]. An inherited single nucleotide polymorphism (SNP) on chromosome 20 at rs1127354, which determines the activity of inosine triphosphatase, reliably predicts protection from ribavirin-induced hemolysis [95]. Baseline testing for this SNP may improve the safety of ribavirin use following liver transplantation, but its utility in clinical practice is uncertain.

Overall SVR rates with PEG-IFN/RBV in liver transplant recipients with recurrent hepatitis C genotype (GT) 1 range from 15% to 35% (Figure 15.1) [96–99]. A number of baseline and on-treatment predictors of SVR have been identified, including HCV genotype, host IL28B genotype, stage of liver fibrosis, timing of treatment, and choice of maintenance immunosuppression during treatment (Table 15.2).

Both before and after liver transplantation, the HCV genotype is the most important predictor of response to interferon-based treatment. In a large US multicenter, open-label study of 48 weeks of PEG-IFN/RBV in 125 liver transplant recipients with recurrent hepatitis C (the PROTECT study), the SVR rate was 24% in patients infected with HCV GT1 and 55% in those infected with GT2/3 [100]. Other studies have also reported higher SVR rates in patients with recurrent HCV GT2/3 infection [95–98].

Table 15.2 Predictors of response to pegylated interferon (PEG-IFN) plus ribavirin.

Viral factors
Low pretransplant viremia level
Low posttransplant viremia level
HCV genotype 2/3
Host factors
Lack of severe fibrosis
Donor IL28B genotype CC
Recipient IL28B genotype CC
Younger donor age
Treatment-related factors
Reduced time since transplant
Maintenance cyclosporine
Adherence to ribavirin
Adherence to PEG-IFN

Independent genomewide association studies have identified an SNP on chromosome 19, upstream from the IL28B gene, which codes for IFN lambda (IFNλ) and may determine innate immunity and IFN sensitivity in chronic HCV infection. The rs12979860 locus has two alleles termed T (unfavorable) and C (favorable). In non-transplant HCV infection, the IL28B CC genotype is

associated with spontaneous clearance and slower fibrosis progression [101]. In retrospective studies of liver transplant recipients with recurrent hepatitis C, IL28B genotype influences the natural history of recurrent hepatitis C in the allograft. In a large multicenter German study, recipient IL28B genotype influenced the grade of necroinflammation and the mean levels of both serum aminotransferases and HCV RNA [102]. A recent study by the US multicenter study group demonstrated that whilst recipient CC genotype was associated with earlier hepatitis and more benign disease, donor CC genotype was associated with later recurrence but more rapid fibrosis progression [103]. Most recently, the recipient IL28B genotype also predicted a risk of developing PTDM [104]. IL28B genotype also predicts response to IFN-based antiviral therapy. In nontransplant HCV infection, SVR in the homozygous CC individual approaches 80%, while it is less than 40% for both T/T and T/C [105–108]. Recent retrospective studies in liver transplant recipients with recurrent hepatitis C have demonstrated that both recipient and donor IL28B genotype determine response to PEG-IFN/RBV, reflecting that both infected donor hepatocytes and recipient immune effector cells express IL28B. The lowest SVR rates were achieved when both donor and recipient were non-CC genotype (16%), intermediate SVR rates when either was IL28B CC (50% and 42%, respectively), and highest rates when both were CC (86%) [101, 109, 110]. In a recent case report from Japan, a patient with decompensated HCV–cirrhosis was transplanted with dual left-lobe grafts, one from a donor with a favorable IL28B genotype and the other from a donor with an unfavorable IL28B genotype [111]. The patient responded to PEG-IFN/RBV at 2 years post transplant but relapsed shortly thereafter. At 4 years post transplant, both grafts were biopsied. The graft that carried the favorable genotype had no histological hepatitis and no evidence of HCV infection, while the other graft had significant fibrosis and active infection. Although selection of CC donors for all candidates with HCV GT1 infection is not practical, genotyping of both donor and recipient may help predict chance of response to posttransplant PEG-IFN/RBV and help guide decisions on therapy.

Response-guided therapy, using both rapid virologic response (RVR) (undetectable at 4 weeks) and early virologic response (EVR) (>2 log reduction at 12 weeks), improves both the tolerability and cost-effectiveness of PEG-IFN/RBV in nontransplant HCV treatment and is an accepted part of routine clinical practice for GT1 infection. Both early on-treatment responses are also reliable predictors of SVR during treatment of recurrent HCV infection. The strongest positive predictor for SVR is RVR (PPV 90–100%), whilst the strongest negative predictor for SVR is EVR (NPV 89–100%) [112–116]. These studies would support the adoption of a 12-week stopping rule for patients who fail to achieve EVR, but there are no studies yet that support shortened duration of therapy for those who achieve RVR.

Earlier treatment prior to the development of severe fibrosis also is associated with improved SVR to PEG-IFN/RBV [117]. As in nontransplant HCV, rates of SVR to PEG-IFN/RBV are lowest in patients with established cirrhosis. However, the timing of antiviral therapy is determined largely by tolerability. Treatment is currently recommended for patients with established recurrent hepatitis C in the allograft, usually later than 6 months post transplant, when the risks of cytopenias and rejection are reduced. Institution of antiviral therapy in the immediate posttransplant period before the development of graft injury ("preemptive" therapy) is attractive as the low serum HCV RNA levels, lack of quasi-species diversity, and lack of fibrosis should all favor eradication and prevent disease progression. However, current treatment is tolerated poorly in the early posttransplant period because of renal impairment as well as risk of cytopenias from concomitant medications, viral infections, and persisting hypersplenism. In the largest study to date of preemptive PEG-IFN/RBV conducted at the University of California, San Francisco (UCSF), two-thirds of screened subjects were excluded because of renal or hematologic abnormalities, recent rejection, or sepsis [118]. Of those who started treatment, almost 90% required dose reduction and 40% stopped treatment. Only 9% achieved SVR. In a recent large, randomized, US multicenter study of early preemptive therapy (the PHOENIX study), 115 patients, who were HCV RNA–positive prior to transplant, were randomized to receive either PEG-IFN/RBV or no treatment between 10 and 26 weeks post transplant, with the control group permitted to start open-labeled treatment if they developed significant histological recurrence [119]. Rates of histological recurrence, SVR, graft loss, and death were similar in the two arms.

Several recent reports, including a large Spanish multicenter study of more than 400 patients, have suggested that patients receiving cyclosporine-based immunosuppression have higher response rates to antiviral therapy than those receiving tacrolimus because of lower risk of relapse [120–122]. However, other reports have not shown such an association, and no recommendations can be made at this time regarding preferred maintenance immunosuppression [123, 124].

At this time, antiviral therapy should be treatment of established recurrence rather than preemptive therapy. Given the limitations of current therapy, treatment should target those recipients with the greatest chance of response (including HCV GT2/3 or GT1/4 plus the D/R IL28B CC genotype) and those at highest risk for recurrent cirrhosis and graft loss. The most reliable predictor of rapid fibrosis progression is moderate

histological necroinflammation or fibrosis in the one-year protocol allograft biopsy [125–128].

Prevention of recurrent hepatitis C

Pretransplant treatment of HCV infection in order to prevent recurrent hepatitis C infection is difficult. Both PEG-IFN and RBV are poorly tolerated in patients with end-stage liver disease because of increased risks of sepsis and bleeding and also increased risks of acute decompensation from interferon-induced flares [129]. In a pilot study of standard IFN/RBV in 32 patients listed with decompensated cirrhosis (mean Child–Pugh score [CPS] = 12 ± 1), most were unsuitable for IFN/RBV, and of those who started treatment, 90% withdrew because of sepsis, encephalopathy, or bleeding. No patients achieved SVR [130]. In a second larger study of 124 patients with less advanced disease (mean CPS = 7 ± 2), patients were started on a half-dose of both PEG-IFN/RBV with incremental increases as tolerated over the next 4 weeks. This was better tolerated, with 15 (12%) developing serious adverse events of whom four died [131]. Thirty (24%) patients achieved SVR, one-half of whom were delisted because of clinical improvement. None of those with documented SVR, who subsequently underwent transplantation, developed recurrent HCV infection. The most recent consensus guidelines recommend that patients with decompensated hepatitis C should be considered for treatment only in an experienced liver unit, preferably a transplant center [132]. To avoid precipitating life-threatening encephalopathy, spontaneous bacterial peritonitis, and hepatorenal syndrome, treatment should be limited to those with a Child–Pugh score ≤7, with a Model for End-stage Liver Disease (MELD) score ≤18, without clinical ascites, and with a baseline platelet count >60 000. A low-ascending dose regimen (LADR) should be used.

Treatment of HCV before and after liver transplantation is a huge unmet medical need because of the poor tolerability and efficacy of PEG-IFN and RBV. It is hoped that the arrival of direct-acting antiviral agents (DAAs) in clinical practice will help fill this need. Already, more than 60 DAAs have entered clinical trials.

Two protease inhibitors (boceprevir and telaprevir) have been recently approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for use in combination with PEG-IFN/RBV. The addition of telaprevir to PEG-IFN/RBV increases SVR rates in treatment-naïve patients from 44% to 73% and in treatment-experienced patients from 17% to 64% [133–136]. The greatest increments in SVR rates were seen in the most difficult-to-treat patients, including cirrhotics, African Americans, patients with the non-CC IL28B genotype, and nonresponders. Unfortunately, this improved efficacy is offset by reduced tolerability from toxicities of these first-generation protease inhibitors, in particular anemia and skin rash. The anemia is likely to be particularly problematic before and after liver transplantation, where reduced renal function results in increased ribavirin-induced hemolysis, secondary to reduced ribavirin clearance. An additional problem with boceprevir and telaprevir following liver transplantation will be the direct drug interactions. Both protease inhibitors are potent inhibitors of the CYP-3A4 enzymes and therefore should decrease metabolism of both tacrolimus and cyclosporine, although the relative effects will differ given the different metabolic pathways of the two calcineurin inhibitors. In a phase I drug–drug interaction study in healthy volunteers, boceprevir increased the mean drug exposure (AUC) of tacrolimus 17-fold and that of cyclosporine 2.7-fold [137]. In a separate study, telaprevir increased the mean drug exposure (AUC) of tacrolimus 70-fold and that of cyclosporine 4.6-fold. As a result, the half-life of tacrolimus increased to 196 hours [138]. A large phase 3b study is currently studying telaprevir-based triple therapy in liver transplant recipients with recurrent hepatitis C [139]. A dramatic reduction in dosage and an increase in dosing interval will be required along with frequent, careful monitoring in order to minimize the risk of severe calcineurin inhibitor toxicity (e.g., renal failure or central nervous system toxicity) and inadvertent under-immunosuppression, resulting in allograft rejection. Interruption of either PEG-IFN or RBV dosing must be avoided to reduce the risk of emergence of drug resistance to the protease inhibitor. Finally, the validity of the current response-guided approaches (extended rapid virologic response) for both telaprevir and boceprevir and the initial lead-in PEG-IFN/RBV phase for boceprevir will need to be validated in this population.

Unfortunately, these first-generation protease inhibitors will not be effective in recipients re-infected with HCV genotypes 2 or 3 or in those patients unable to tolerate PEG-IFN or RBV. The recent clinical development of DAAs targeting other steps of HCV replication (NS5A, NS5B RNA-dependent RNA polymerase, and cyclophyllin B) has allowed the first trial of combinations of two or more agents without cross-resistance in an all-oral, IFN-free antiviral regimen that may provide pre- and posttransplant suppression of recurrent hepatitis. In contrast to the first-generation protease inhibitors, many new DAAs in development such as the nucleotide inhibitor sofosbuvir are well tolerated, lack significant direct drug interactions with immunosuppressants, and demonstrate excellent antiviral efficacy across all HCV genotype and subtypes. Phase II results in nontransplant HCV infection suggest that the combination of sofosbuvir plus ribavirin or a second DAA for 12–24 weeks may provide a pangenotypic, all-oral, IFN-free regimen, further improving the safety and tolerabil-

ity in liver transplant recipients. The short duration and lack of significant hepatic metabolism have expedited a study in patients awaiting liver transplantation, where it is hoped that this approach will allow successful eradication without delaying transplantation, thereby preventing posttransplant recurrence, although the efficacy of this strategy in patients with genotype 1 infection is unclear. Future studies are planned in patients with decompensated cirrhosis, and it is hoped that the rapid biochemical response observed in the phase II studies may lead to improved liver synthetic function and rescue from the waiting list.

IFN-free combination DAA regimens are about to be studied in patients awaiting transplantation. The advantage of these is that the likely duration of therapy will be short (<=24 weeks), thereby allowing eradication prior to transplantation. The phase II studies demonstrated rapid virologic response (to PCR nondetectability) and biochemical response (normalization of ALT) within 3 weeks of starting treatment. Such a rapid response is likely to be associated with rapid improvement in liver synthetic function. Direct antiviral agents may rescue some patients listed for decompensated cirrhosis, as seen previously in patients listed with decompensated chronic hepatitis B.

Summary and recommendations

There is an urgent need to develop more effective and better tolerated antiviral regimens for this area of unmet need. In patients with advanced liver disease, HCV eradication may rescue some patients who are on the waiting list and, for those who require transplantation, may prevent recurrent infection of the allograft.

Liver transplantation for hepatitis B

Epidemiology

Although the global prevalence of chronic HBV infection is falling steadily because of successful neonatal vaccination programs, it is still the leading cause of liver-related mortality in Asia and the Pacific, where 600 000 to 1 million people die annually from HBV-related HCC and liver failure. The improved long-term outcomes of liver transplantation, the successful development of living-related liver donation, and rapid economic growth have all contributed to the rapid expansion of liver transplantation within the Asia–Pacific region, where more than 10 000 transplants are now performed each year. Chronic hepatitis B now accounts for almost 80% of emergency and elective liver transplants in this region. Potent oral nucleos(t)ide analogs, tenofovir (TDF) and entecavir (ETV), are able to rescue many patients with decompensated chronic hepatitis B or acute-on-chronic liver failure related to hepatitis B [140, 141]. As a result, HCC has replaced liver failure as the chief indication for listing in patients with chronic hepatitis B [142].

Outcomes and predictors of outcome

Without perioperative antiviral prophylaxis, HBV infection will recur in the allograft in >80% of patients who are HBV DNA positive by hybridization assay (>4–5 $\log_{10}$IU/mL) prior to transplantation [143]. If untreated, recurrent HBV infection will rapidly progress to graft loss and death.

Treatment of recurrent hepatitis B

Following diagnosis of recurrent hepatitis B (reappearance of both HBsAg and HBV DNA in serum), HBIG should be stopped for futility and to avoid the risk of immune-complex disease. Recurrence is secondary to either noncompliance with prophylaxis or emergence of drug resistance. Appropriate rescue therapy should be started promptly by adding an oral nucleos(t)ide analog, without cross-resistance, such as adding adefovir (ADV) or tenofovir (TDF) to patients receiving lamivudine (LAM), telbivudine (TBV), or entecavir (ETV); adding ETV to patients receiving ADV or TDF; or switching to emtricitabine/TDF (truvada™). Most patients will be successfully rescued, but treatment should be continued indefinitely.

Prevention of recurrent hepatitis B

The best approach is, of course, prevention of recurrent infection through effective antiviral prophylaxis. Passive immunoprophylaxis with hepatitis B immunoglobulin (HBIG), commenced intra-operatively and continued in the long term post transplant, protects the allograft from HBV infection by complexing circulating virions and also by blocking intrahepatic spread. In the landmark Eurohep study, high-dose IV HBIG reduced the overall recurrence rate by 60% (Figure 15.2) [138]. The best results were achieved in those patients with fulminant hepatic failure and hepatitis D co-infection, who had low HBV DNA levels at the time of transplant [144]. In contrast, the rate of HBV recurrence remained high in those patients with high pretransplant HBV DNA levels. Early recurrence reflected insufficient neutralizing antibody during the immediate postoperative period, whereas late recurrence resulted from selection of surface escape mutants in the "a" determinant of the HBV pre-S/S genome.

Increasing the total dose of IV HBIG improves the efficacy of passive immunoprophylaxis in patients with

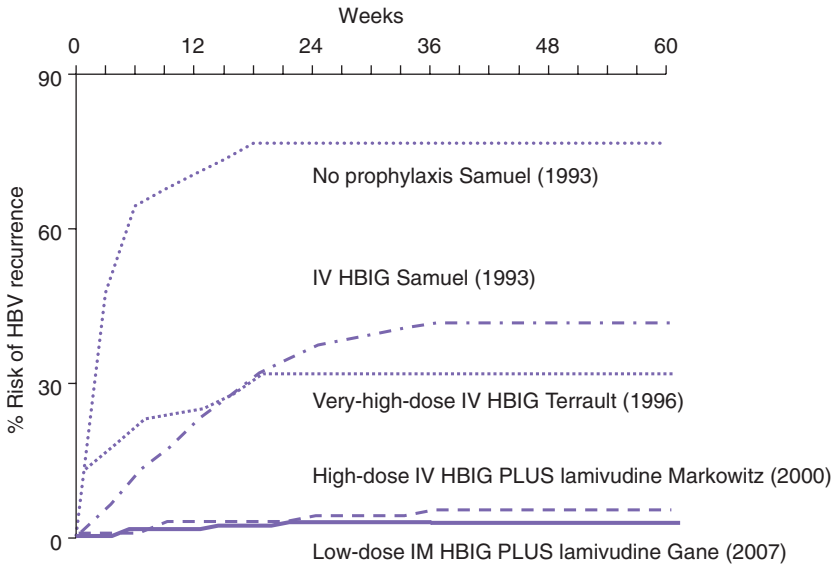

Figure 15.2 Prevention of recurrent HBV infection with antiviral prophylaxis.

high levels of circulating HBV DNA. High-dose HBIG regimens include both fixed high doses (10 000 IU intra-operative, daily for 1 week, weekly, then monthly over the long term) [145] and on-demand high doses (targeting "protective" trough [anti-HBs] titers above 500 IU/mL) [146]. These aggressive dosing protocols require 300 000–400 000 IU for the first year and 120 000–200 000 IU each subsequent year, with an associated annual cost of around US$75 000–120 000 for the first year and US$50 000–100 000 thereafter.

HBIG monotherapy must be maintained over the long term to prevent late recurrence. Persistent, low-level HBV replication can be detected in allografts up to 10 years post transplant, indicating a high risk of recurrence following HBIG withdrawal at any time post transplant [147, 148].

The high cost and inconvenience of monthly high-dose IV HBIG have prevented the adoption of this approach in the Asia–Pacific region and have inspired the development of alternative, less expensive strategies, including HBIG dose reduction, intramuscular (IM) administration, late HBIG withdrawal, and total HBIG avoidance.

Reducing the dose of IV HBIG to 2000 IU/month, and targeting a lower trough anti-HBs level, has similar efficacy to the standard high-dose regimen (10 000 IU/month) but reduces the cost by more than 50% [149].

The efficacy of high-dose IV HBIG is improved by the addition of a nucleos(t)ide analog. This is because pre-transplant nucleos(t)ide analog therapy will suppress the circulating viral load prior to transplantation, thereby preventing early recurrence and reducing the required perioperative neutralizing dose of anti-HBs. Continuing the oral nucleos(t)ide analog post transplant in combination with HBIG should protect the allograft from late HBV recurrence through prevention of the emergence of surface escape mutants. The addition of LAM to high-dose IV HBIG has reduced the rate of recurrent HBV below 10% [150].

Conversion from the intravenous to the intramuscular route of HBIG administration in the late maintenance phase reduces the total dose necessary to maintain protective (anti-HBs) titers (>100 IU/mL), which should prevent late recurrence [151, 152]. This approach improves both cost-effectiveness and quality of life compared to long-term high-dose IV HBIG [147, 153].

Although an intra-operative intramuscular injection of HBIG cannot produce the high neutralizing (anti-HBs) titers achieved following an IV infusion of HBIG [154], low-dose IM HBIG can be administered from the time of transplant when combined with a nucleos(t)ide analog [155]. In a large multicenter study of 150 patients, who received LAM from the time of listing and low-dose IM HBIG from the time of transplant, the actuarial risk of HBV recurrence was only 4% after 5 years and at a cost of only 10% that of the standard high-dose IV HBIG regimen. In this study, median duration of LAM prior to transplant was 92 days, and one-half of the patients had undetectable HBV DNA (<300 copies/mL) at transplant. These factors may explain the low rate of HBV recurrence and the low rate of LAM resistance. Late recurrence reflects a selection of variants conferring resistance to both HBIG and LAM, because of overlapping reading frames of major catalytic regions of the HBV polymerase gene and neutralization domains of the surface gene [156, 157].

Late HBIG withdrawal has been evaluated in some "low-risk" patients in order to remove the cost of lifelong HBIG. Patients who had undetectable or low ($<10^5$ copies per ml) pretransplant HBV DNA levels maintained on combination HBIG plus LAM prophylaxis for at least 12 months can be safely converted to LAM monotherapy [158–161].

Attempts to replace HBIG through *de novo* production of anti-HBs with standard recombinant HBV vaccination have been disappointing [162, 163]. The use of newer, more immunogenic vaccines such as the GSK experimental vaccine HBsAg-AS02 and the Sci-B-Vac™ Pre-S vaccine have achieved protective neutralizing antibody titers in some patients, although these waned overtime [164, 165]. At this time, posttransplant vaccination cannot be recommended as a strategy to prevent late HBV recurrence.

Replacing maintenance HBIG with a second oral nucleos(t)ide analog without cross-resistance should prevent late HBV recurrence even in "high-risk" patients with high pretransplant HBV DNA levels. In a randomized controlled trial, in patients maintained on LAM plus HBIG for at least 1 year post transplant, switching to LAM plus adefovir provided equivalent protection against HBV recurrence, but with better tolerability and reduced cost [166]. In a recent study, the replacement of LAM plus HBIG by TDF plus FTC was safe and well tolerated, with no recurrence after 24 months [167].

Complete HBIG avoidance remains the ultimate goal given the costs and lack of availability of reliable HBIG in many countries. Posttransplant LAM prophylaxis without any HBIG is associated with a high rate of late HBV recurrence from emergence of LAM resistance [168]. The reported rate of HBV recurrence during LAM monotherapy is higher in European compared to Asian series (45% vs. 20%), which could reflect differences in the virus (genotype) or possibly in the host (adoptive immunity from live-related HBV-immune donors in Asia) [169, 170]. TDF and ETV may be better candidates than LAM for HBIG avoidance strategies because of the higher barrier to antiviral resistance. In countries where TDF is not available, ADV may be used instead.

In a recent study in Hong Kong, ETV monotherapy was used as antiviral prophylaxis in 75 patients transplanted for HBV–cirrhosis [171]. The cumulative rate of HBsAg loss was 86% at 12 months and 91% at 24 months following liver transplantation, with 98.8% achieving undetectable HBV DNA levels. In comparison, in a recent Australasian prospective open-labeled study, 65 HBsAg-positive patients received combination LAM plus ADV from the time of listing for transplantation. This combination prevented emergence of LAM resistance and suppressed HBV DNA to undetectable levels in most patients prior to transplant [172]. No perioperative HBIG was administered to the 18 patients who suppressed pretransplant HBV DNA levels below 1000 IU/mL, while the remaining patients, including those with unknown HBV DNA levels, received daily 800 IU IM HBIG for one week. No patient had HBV recurrence after a median follow-up of almost 3 years. In this study, the median time to HBsAg loss was only 2 weeks compared to 8 weeks in the Hong Kong study. This difference in posttransplant HBsAg clearance reflects greater pretransplant viral suppression in the Australasian study, where all patients received pretransplant antiviral compared to less than one-half the patients in the Hong Kong study. A recent report from India confirmed the safety and efficacy of HBIG-free prophylaxis in 47 patients, who suppressed HBV DNA below 2000 IU/mL prior to transplantation through single or combined nucleos(t)ide analog therapy [173].

These preliminary data suggest that either a single potent nucleos(t)ide analog with a high genetic barrier (ETV or TDF) or a combination of nucleos(t)ide analogs without cross-resistance (LAM/ADV, TDF/FTC) will provide similar safety and efficacy to current LAM plus HBIG prophylaxis but without the cost and inconvenience of long-term monthly HBIG administration. The future use of HBIG immunoprophylaxis may become limited to perioperative use only in patients with high viral load at the time of transplant.

Despite the low barrier to resistance, LAM remains in widespread use in the developing world because of its low cost. As a result, an increasing proportion of patients with end-stage chronic hepatitis B have established LAM resistance. In this "high-risk" population, antiviral prophylaxis with combination LAM and ADV therapy is effective for preventing re-infection of the graft, with or without concomitant HBIG [174, 175]. Either TDF monotherapy or TDF plus LAM/emtricitabine is also likely to be effective in this population.

Renal dysfunction is common before and after liver transplantation. Most patients with decompensated cirrhosis have functional renal insufficiency related to hepatorenal syndrome. This may be exacerbated by prerenal (e.g., overdiuresis or sepsis) and renal injury (e.g., HBV-related glomerulonephritis and nephrotoxicity from concomitant medications, such as aminoglycosides). Following transplantation, the nephrotoxic effects of calcineurin inhibitors further reduce renal function. The oral nucleos(t)ide analogs for chronic hepatitis B should be used with caution in patients with renal dysfunction. All are excreted by the kidneys and so should be dosed according to renal function. In addition, the acyclic phosphonates ADV and TDF, which are excreted through active proximal tubular transport, may cause direct renal tubular injury and, rarely, Fanconi's syndrome [140]. In studies of ADV and TDF for decompensated chronic hepatitis B, renal impairment defined as an increase of serum creatinine by ≥0.5 mg/dl

over baseline or hypophosphatemia was reported in 5–10% of patients, but was usually resolved following dose reduction [140, 141, 174]. Entecavir treatment was recently associated with lactic acidosis in patients with decompensated chronic hepatitis B. All cases occurred in patients with very advanced liver disease (MELD scores >20), suggesting that renal dysfunction in these cases may result in mitochondrial toxicity from increased drug exposure [176]. Another study suggested that ETV treatment increased short-term liver-related mortality in patients with severe acute exacerbation of chronic hepatitis [177]. In a large randomized multicenter study on decompensated chronic hepatitis B, ETV was associated with a single case of lactic acidosis, and mortality was similar to that with ADV [141].

Posttransplant HBV recurrence is defined as reappearance of HBsAg in serum. Following reappearance of HBsAg, the patient should be tested for HBV DNA to confirm recurrent HBV infection, and if it is detectable, then testing for antiviral resistance should be performed. In some patients, particularly those who receive prophylaxis with nucleos(t)ide monotherapy, reappearance of HBV DNA in serum may be the first sign of HBV recurrence.

Almost 75% of all donors in the Asia–Pacific region have prior exposure to HBV (anti-HBc-positive). A liver from an anti-HBc-positive donor carries a significant risk of *de novo* HBV infection if it is transplanted into an HBV-naïve recipient. This risk becomes negligible if the recipient is seronegative for HBsAg but positive for anti-HBs either through natural immunity from previous infection or through vaccination [178]. In the HBV-naïve recipient, prophylaxis with either LAM or HBIG will prevent *de novo* infection, although long-term LAM is preferred because of convenience and cost.

Summary and recommendations

Recurrent HBV infection is rare following liver transplantation for HBV because of safe and effective antiviral prophylaxis with a combination of nucleos(t)ide analogs plus HBIG. The cost and inconvenience of lifelong HBIG have driven the development of HBIG minimization and avoidance strategies. The use of potent nucleos(t)ides from the time of listing should provide a completely HBIG-free oral prophylaxis regimen and should further improve the outcomes, tolerability, and cost-effectiveness of transplantation for chronic hepatitis B.

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Section IV
Hepatitis C Virus

Chapter 16
Structure and molecular virology

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Summary

There have been major advances recently in several areas of hepatitis C virus (HCV). The array of host receptors important for HCV entry now includes HSGP, LDLR, CD81, SRB1, CLDN1, occludin, and NPC1L1. The interaction of host and nonstructural viral proteins is essential for genome replication. Long-range interactions between *cis*-acting elements in the 5′ and 3′ UTRs and the NS5B coding region may control the switching between genome replication and genome packaging into virions, as well as modulate translation. Capsid assembly also involves NS5A, NS2, NS3/4A, and viral RNA as well as core protein. HCV assembly is intimately connected to host lipid metabolism and the synthesis of very-low-density lipoproteins (VLDLs) resulting in a lipoviroparticle. Core, NS5A, NS2, NS3, and NS4B can regulate host metabolic processes. HCV proteins have been shown to compromise host defenses at many levels, such as NS3 and NS5A inhibition of interferon alpha and NS5A and NS5B inhibition of apoptosis.

Introduction

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus of the genus *Hepacivirus* in the *Flaviviridae* family. HCV causes chronic infection in around 80% of cases [1, 2], which then predisposes patients to the sequential development of cirrhosis and primary hepatocellular carcinoma (HCC) [3]. The HCV genome is composed of a positive-stranded RNA molecule of about 9500 nucleotides (nt) [4] 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 [5, 6] bounded by 5′ and 3′ untranslated regions (UTRs) of approximately 341 and 230 nucleotides (Figure 16.1). The 5′ UTR of the HCV genome has substantial primary sequence identity with the corresponding region of pestivirus genomes [5, 7], and the nucleotide triphosphatase (NTPase) region of the NS3 helicase has significant sequence identity with those of the pestiviruses and, to a lesser extent, the flaviviruses [5, 8, 9]. Protease and RNA-dependent RNA polymerase (RdRp) 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 collinear among the three types of viral polyproteins [5]. 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 [5]. Nucleotide and protein sequence analyses also show that HCV is more closely related to GBV-B virus than to the other members of the *Flaviviridae* family, and together they comprise the *Hepacivirus* genus. It has recently been proposed that the other GBV viruses, GBV-A, GBV-C (HGV), and GBV-D, should be classified as a separate genus, the *Pegiviruses* [10]. There is substantial sequence variation in the HCV genome resulting from the lack of proofreading of the NS5B RdRp and

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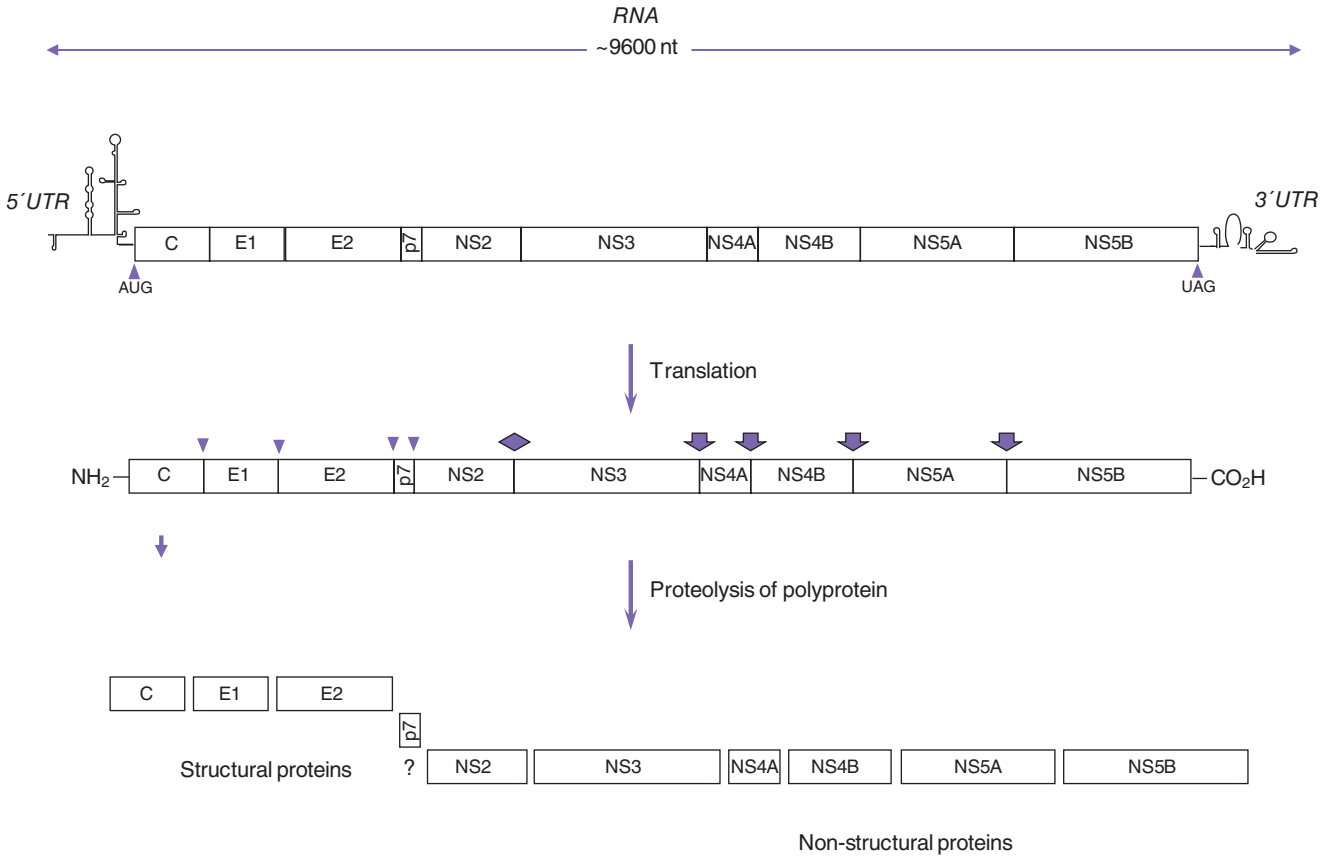

Figure 16.1 Schematic organization of the HCV genome and the encoded proteins. The viral RNA consists of a single open reading frame (ORF), encoding about 3011 amino acids, bounded by 5′ and 3′ untranslated regions (UTRs). The polyprotein is cleaved posttranslationally with signal peptidases (▼) and the virus-encoded NS2–3 (◆) and NS3–4 (⬥) proteases to generate the mature structural and nonstructural proteins.

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 [11]. 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. To standardize the various systems that had developed in different laboratories, a uniform system for the nomenclature of HCV genotypes was derived from sequence comparisons in 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 [12, 13]. Originally six genotypes or clades (1, 2, 3, 4, 5, and 6) with a variable number of subtypes (designated a, b, etc.) for each of these genotypes were identified, and this was used as the standard system for the nomenclature of HCV genotypes [11]. However, more recently a single isolate of a seventh genotype has been identified [14]. In addition, there have been a number of reports of possible recombinant genotypes [15]. Most recently a non-primate hepacivirus with strong homology to HCV has been described [16].

Significant advances in the study of HCV have come from the development of subgenomic replicating HCV RNAs (RNA replicons) as cell-based models for HCV RNA replication. These were developed from full-length HCV genomes in which the sequence coding from core to p7 or from core to NS2 was replaced by a gene cassette that consisted of neomycin phosphotransferase gene (i.e., a "neo"-selectable marker) and a downstream encephalomyocarditis virus (EMCV) internal ribosome entry site (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. Human hepatoma 7 (Huh7) cells transfected with HCV replicon RNA are grown in culture medium that contains G418, which only allows the growth of only cell lines that contain the replicating

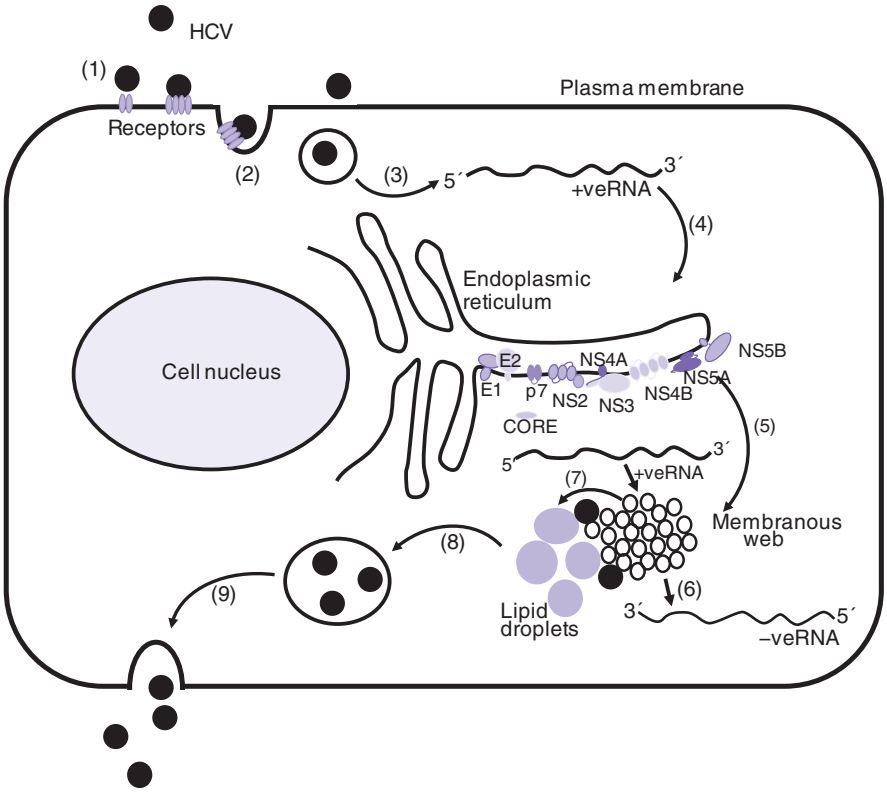

Figure 16.2 Replication cycle of HCV. (1) Binding of virus to receptors. (2) Endocytosis. (3) Uncoating. (4) Translation of genomic RNA and proteolytic processing of the polyprotein associated with the endoplasmic reticulum. (5) RNA replication complex formation in the membranous web. (6) Genome replication. (7) Encapsidation of genomic RNA and assembly of virus particles through interactions between the ER and lipid droplets. (8) Exocytosis of virus. (9) Release of virus particles from the cell.

HCV RNA replicons [17, 18]. More recently, the full replication cycle of HCV (Figure 16.2) in cell culture (HCVcc) has been achieved by cloning a HCV genome from a genotype 2a HCV-infected patient in Japan who had fulminant hepatitis (i.e., Japanese fulminant hepatitis-1 [JFH-1]). Full-length RNA was then synthesized from the cloned JFH-1 cDNA, and after transfection into Huh7 cells or the more permissive Huh7.5 derivative cell line, infectious virus particles were produced [19, 20]. These virus particles were then used to infect naïve cells. The JFH-1 HCV replication system and a small number of chimeric viruses derived from JFH-1 and some additional genotypes have become a standard model for HCV replication in laboratories throughout the world [21, 22].

The HCV virion

Particles with a diameter of 45–65 nm have been observed by electron microscopy (EM) in human plasma [23] and in chimpanzee and human liver chronically infected with HCV [24, 25]. Non-enveloped nucleocapsids have also been detected in the plasma of infected chimpanzees [26]. Analysis of the structure of HCV virions from cell culture, using negative-staining electromicroscopy, has shown that they are spherical and pleomophic with a diameter of 40–75 nm. However, using cryoelectron microscopy, it was found that there are two main populations of particles of 60 and 45 nm diameter [27]. The 60 nm particles possess an internal structure that appears to correspond to the capsid and a separate membrane bilayer, and they have a high RNA-to-infectivity ratio and appear to correspond to complete virions. The 45 nm particles lack the membrane bilayer, were less infectious, and may correspond to non-enveloped viral capsids. Analysis of the lipid composition of purified E2-FLAG tagged virus particles by mass spectrometry showed that HCV particles possess a unique lipid composition that is very distinct from all other viruses, with cholesteryl esters accounting for almost one-half of the total HCV lipid content [28]. Electron microscopy showed that the particles were heterogeneous, mostly spherical structures, with an average diameter of about 73 nm, and immuno-EM showed that most E2-containing particles also contained apolipoprotein E (apoE) on their surface [28]. There is

evidence that HCV particles are present in the circulation as immune complexes [29] or in association with serum lipoproteins [30–32]. Analysis of virus particles from HCV-infected patients shows that lower density particles have a much higher infectivity than higher density particles [33–35]. Particles of around 100 nm, which are rich in triacylglycerol and probably contain apoB, apoE, and apoC1-C3, have been described [33]. HCV also appears to be associated with apoE to form lipoviroparticles (LVPs), which contain components of very-low-density lipoproteins (VLDLs) [36].

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 [5, 37, 38]. As in the case for pestiviruses 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 nonstructural (NS) proteins (Figure 16.1). Structural proteins are processed from the N-terminal region of the HCV polyprotein precursor, beginning with the 23 kDa basic capsid or core RNA-binding protein (C) followed by two envelope glycoproteins – E1 (33–35 kDa) and E2 (68–72 kDa) (Figure 16.2).

Core protein and virus assembly

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 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) [29, 39, 40]. HCV core protein has been identified in the sera of HCV-infected humans as a 21 kDa protein. 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 anticore antibodies in the presence of core protein, showing that core interacts with E1. In contrast, the E2 protein is not co-precipitated with anticore 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 [41, 42]. 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, and deletion studies indicate that the C-terminal regions of both proteins are important for their interaction [43]. Both membrane-bound and membrane-free core exist in dimeric and multimeric forms. 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 [44]. After cleavage of the signal sequence between core and E1, the intramembrane cleaving protease, signal peptide peptidase (SPP), cleaves the signal sequence, releasing core that can then associate with lipid droplets (LDs) [45].

The association of core with LDs causes them to increase rapidly in size [46], and this may be part of a mechanism for virus assembly. LDs are a storage organelle for triglycerides and cholesterol esters that are surrounded by a monolayer of phospholipids. Specific cellular proteins (e.g., adipose differentiation-related protein [ARDP]) are also associated with LDs [47]. Core interacts with LDs via its central domain (residues 125–144). Core proteins lacking part of the central domain are degraded by the proteosome [48]. Similar sequences in the core protein of GBV-B and in plant oleosin protein were shown to facilitate the association of these proteins with LDs. The HCV lipid-binding domain can substitute for the equivalent domain in oleosin, allowing the hybrid protein to bind to LDs [49]. Core inhibits the microsomal triglyceride transfer protein (MTP), which is needed for VLDL synthesis, and this may lead to the increase in LDs and subsequently contribute to steatosis [50]. Core expression induces the clustering of LDs around the nucleus, and a reduction in the level of LD-associated ADRP by core appears to trigger this [51]. Three-dimensional EM analysis showed that an increase in the synthesis of LD was associated with LD emergence from ER and that LD clustering was induced by core coating of LDs [52]. Other evidence indicates that this clustering may also involve microtubules [51]. The interaction of the triacylglycide-synthesizing enzyme diacylglycerol transferase-1 (DGAT1) with core is necessary to deliver core to LDs, and siRNA ablation of DGAT1 greatly reduces the production of virus particles [53]. The association between core and DGAT1 also interferes with triglyceride synthesis. This helps to reduce the turnover of LDs, and may also contribute to steatosis, since DGAT(−/−) mice expressing core protein are protected from steatosis [54]. Growing evidence shows that core can alter lipid metabolism by a variety of mechanisms (e.g., by activation of delta-9 desaturase [55] and by the accumulation of lipids by core domain 3 from genotype 3 [56]). SKI/SIP has a critical role in the proteolytic activation of SREBPs, key enzymes that control cholesterol and fatty acid metabolism. Use of a specific protein inhibitor of SKI/SIP inhibited HCV assembly, which may be due to a reduction in neutral lipids, LDs, and ARDP [57]. In addition, siRNA knockdown of ACSL3, which incorporates fatty acids into

phosphatidyl choline (PC), inhibited secretion of the virus [58].

The amino-terminal hydrophilic portion of the core protein contains a homotypic domain that allows the p21 core protein to interact with itself [59]. In contrast, core that was expressed as p23 did not interact with itself, indicating that the C-terminal domain may inhibit multimerization [59]. AA C128 is highly conserved between HCV isolates, and mutational analysis of core amino acids showed that it is necessary for the formation of disulfide-bonded core dimers that are essential for nucleocapsid formation but not RNA binding or genome replication [60]. Mutational analysis of JFH-1 HCV core protein domain 1 (D1),showed that it has an essential role in the formation of infectious virus particles [61]. In one study, mutation of basic amino acids R50, K51, R59, and R62 prevented infectious virus particle formation [62]. In a different study, mutation of amino acids 64–66 also blocked virus production. However, passaging these mutant core constructs in cell culture resulted in a compensatory mutation in the NS3 helicase domain that rescued their ability to produce infectious virus particles. This indicates that the interaction of core D1 and NS3 helicase is essential for the late stage of virus particle formation [63]. Transcomplementation studies have shown that replicon RNA that lacks the core–NS2 region can be efficiently packaged into particles that are able to penetrate naïve cells. This indicates that HCV RNA packaging sequences lie outside the core–NS2 region [64]. Analysis of core interaction with the 3′ UTR RNA showed that the amino terminal domain D1 can act as a chaperone and alter the structure of the RNA, and may therefore promote switching between different RNA conformations at different stages in the HCV replication cycle [65]. The DEAD box family RNA helicase DDX3 was shown to bind to core [66], and siRNA knockdown of DDX3 reduced HCV genome replication [67]. Mutation Y35A disrupted the interaction of core with DDX3; however, this did not affect the production of infectious viruses or RNA replication, indicating that the direct interaction of core with DDX3 is not required for the function of DDX3 in HCV replication [68].

Core is recruited to form large cap areas on LDs next to the putative capsid assembly sites. This appears to involve interactions between core, NS5A, NS2, NS3/NS4A, and possibly viral RNA to form structures that initiate genome encapsidation [69]. Models of this process indicate that this may occur during the interactions of LDs and ER. Subsequent events include the transfer of E1E2 from ER to the surface of nascent virions and the association of these virions with newly formed LDs in the lumen of the ER. These would then appear to fuse to form LVPs that contain apoE. This is consistent with the evidence from HCV cell culture (HCVcc) where inhibition of apoE with antibodies or siRNAs resulted in a large reduction in HCV infectivity, indicating that apoE-enriched lipoprotein particles are required for HCV assembly [70, 71]. However it is likely that these LVPs then fuse with VLDLs, also synthesized in the lumen of the ER, to form the low-density LVPs found in HCV-infected patients. Following assembly next to LDs, nascent virus particles were shown to be targeted to early and then late endosomes, and this involved microtubules. This could facilitate the egress of virus particles to the sites where the virus is then released (e.g., the cell plasma membrane or cell junctions) [72]. The inhibition of HCV virion release from infected cells, by dominant negative Vsp4 or other ESCRT-III components or a reduction in Hrs (a component of the ESCRT-O), indicates that the ESCRT pathway is essential for HCV budding through the exosomal secretion pathway and HCV virion release [73, 74]. Other evidence has shown that core interacts with alpha and beta tubulin *in vitro* to promote their polymerization. This could also facilitate HCV capsid transport by microtubules at the postfusion stage of HCV infection [75].

Serine residues S99 and S116 of core are the major phosphorylation sites for PKA and PKC, respectively, and the phosphorylation of S116 by both PKA and PKC may enhance the nuclear localization of core [76]. 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 [77]. The proteosome activator PA28γ was shown to bind to core, and deletion of the PA28γ binding region from core led to its export from the nucleus to the cytoplasm [78]. HCV core can regulate the activity of cellular genes, including c-*myc* and c-*fos* [79]. Core activation of transcription from the interleukin-2 (IL2) promoters via nuclear factor of activated T cells (NFAT) suggests that core expression may have consequences for IL2-mediated T cell development [80].

Different reports have presented evidence that core can stimulate or inhibit apoptosis. Core can bind to the lymphotoxin beta receptor (LTβR) and activate NFκB activity by increasing the degradation of I kappa B kinase (IKKβ) [81, 82]. Consequently, both Fas- and tumor necrosis factor alpha (TNFα)-mediated apoptosis are inhibited. Expression of core in HepG2 and HeLa cells activated the NFκB signaling pathway in a dose-dependent manner. The use of dominant negative forms of TNF receptor-associated factor 2 (TRAF2) showed significant blockage of the activation of this pathway by core. This suggests that core protein mimics pro-inflammatory cytokine activation of NFκB through

TRAF2 [83, 84]. 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 TNFα signaling pathways [85] primarily by facilitating Fas-associated death domain (FADD) recruitment to TNFR1 [86]. 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 [87]. Aggregation of Fas receptors by cytoplasmic core was shown to facilitate apoptosis in Jurkat cells [88]. By contrast, core expression has also been reported to inhibit TNFα-induced apoptosis [89] and increase the level of bcl-x1 mRNA, which may inhibit apoptosis. Core expression caused mitochondria to be more sensitive to oxidative stress–induced cell death [90] and also caspase-independent apoptosis [91]. Core has also been implicated in cell transformation. It has been shown to bind to *p53* and enhance the expression of *p21* (waf1/Cip1), indicating that this binding may lead to *p53* activation [92, 93]. Core inactivates a bZIP nuclear transcription protein, LZIP, by sequestering it in the cytoplasm and subsequently potentiating cellular transformation in NIH 3T3 cells [94]. Core expression is also able to immortalize primary human hepatocytes [95]. 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 may be responsible for the regulation of the immortalized phenotype [96]. Core may also have the ability to alter the methylation status of E-cadhedrin promoter, which may be important for the development of HCC [97].

Envelope proteins and cell entry

HCV has two envelope glycoproteins, E1 and E2, of molecular weights 33–35 kDa and 70–72 kDa, respectively. 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 ER, where they are translocated into the lumen, and after signal sequence cleavage [98–100], they remain anchored inside the lumen [98, 101]. 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 their mass [98–100]. The C-terminal region of E1 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 flavivirus envelope proteins [41, 42]. 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 [99, 100]. 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 as to the E1E2 complex. These TMDs are sufficient to localize E1 and E2 to the ER, and to serve as membrane anchors, and they are involved in the heterodimerization of E1 and E2 [102, 103]. The anchor domains of E1 and E2 are similar in structure to those of other members of the *Flaviviridae* family. They comprise two short stretches of hydrophobic residues separated by a short region that 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 [104]. 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. Analysis of the regions of E2 responsible for interaction with E1 shows that the TMD is critical for native complex formation [105]. 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 re-orientating their C-terminals toward 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) [106]. However, while intracellular E1 and E2 were shown to form noncovalent heterodimers, virion-associated E1 and E2 formed large covalent complexes stabilized by disulfide bridges [107]. E2 contains 18 highly conserved cysteine residues that form disulfide bonds required for the formation of the E2 tertiary structure. Alanine scanning has shown that all of these are required for HCV entry [108]. Furthermore, alkylation of the eight E1 and 18 E2 cysteine residues abolished infectivity, indicating that this is dependent on the presence of free thiol groups on these cysteines [109]. Mutational analysis of E2 histidine 445 indicates that histidine protonation in endosomes may alter the structure of E2 and be a key regulator of HCV fusion. Essential alterations to the structure and functions of class II fusion proteins from other viruses occur in endosomes due to histidine protonation [110]. E2 amino acids 705–715 in the protein stem region may be involved in the reorganization of E1–E2 complexes during fusion [111].

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 [112]. 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. 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 E1 or E2 [113], indicating that HCV buds from the ER 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 [114], mostly 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 chaperones. One chaperone, Calnexin, appears to play a role in the productive folding of the HCV glycoproteins [115, 116], and may therefore help to retain the E1E2 glycoproteins in the ER and thus prevent their migration to the plasma membrane and facilitate their interaction with the nucleocapsid [112, 117].

HCV binds to at least six cellular receptors on the surface of hepatocytes: heparin sulfate proteoglycans (HSPGs), low-density-lipoprotein receptor (LDLR), CD81, scavenger receptor class B type 1 (SR-B1), claudin-1 (CLDN-1), and occludin. Binding of E2 to HSPG may be the first step in the interaction between HCV and the cell surface, resulting in subsequent specific receptor-mediated entry and initiation of infection [118]. E2 glycoprotein was shown to bind to cell surface HSPGs, possible through HVR-1 [119], and liver-derived highly sulfated heparin sulfate specifically inhibited cellular binding and entry of E2 pseudotyped VSV in a dose-dependent manner [120]. Lipoprotein lipase (LPL) has been shown to retain HCV at the cell surface, thereby inhibiting its uptake. This inhibition appears to be mainly mediated by the LDLR, which acts as a bridge between virus-associated lipoproteins and surface heparin sulfate [121]. Silencing of the LDLR in Huh7 cells reduced HCV infection [122], and anti-apoE antibodies were also able to block HCV entry. Analysis of HCV from cell culture by density gradients showed that apoE is present in fractions with the highest density of HCV to form lipoviroparticles (LVPs). This indicates that apoE in LVPs helps HCV to infect cells by binding to the LDLR [123]. However, apoE plays an additional role in HCV infectivity by also binding to SR-B1 [124]. E2 binds to CD81, a tetraspanin that is present on the surface of B lymphocytes and hepatocytes. The binding region of E2 was mapped to the large 89 aa extracellular loop on CD81. Recombinant CD81 molecules that contain this loop were able to bind HCV and anti-HCV antibodies that inhibited virus binding to CD81 [125]. A model of the tertiary structure of E2, derived from analysis of a soluble protein that corresponds to the E2 ectodomain, shows that the CD81 receptor binding site mapped to the interface between E2 domains I and III [126]. Deletion analysis has shown that E2 HVR1 occludes CD81 binding and neutralizing epitopes, and this may help in virus replication, in immune evasion, and to establish and maintain a chronic virus infection [127]. CD81 binding to E1E2 primes HCV for pH-induced conformational changes that promote fusion of the virus envelope with endosomes, necessary for infection [128]. In the absence of CD81, HCV still has been shown to be transmitted directly from cell to cell. However, the significance of this mode of virus transmission is unclear [129]. Inhibition of SR-B1 with antibodies or siRNA blocks HCV entry [130–133]. However, it is unclear if SR-B1 binds to HCV directly or through the associated LDL as SR-B1 has different binding regions for E2 and LDL [134–136]. PDZK1 is a scaffold protein that interacts with SR-B1 thereby enhancing its activity. Knockdown of PDZK1 expression reduced HCV entry through interaction with SR-B1, indicating that PDZK1 plays a role in HCV infection [137]. The tight junction protein CLDN-1 was shown to be an essential factor for HCV by expression cloning [138]. Other evidence indicates that CLDN-6 and CLDN-9 may mediate HCV entry [139]. CD81 interacts with CLDN-1 to form a receptor complex, and inhibition of protein kinase A (PKA) inhibited CD81–CLDN-1 interaction and HCV entry [140]. RNA kinase screening also demonstrated that epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EpHA2) are host factors needed for HCV entry. They may act by regulating CD81-claudin-1 association and E1E2-dependent fusion [141]. HCV infection after virus internalization is inhibited by anti-CD81 antibodies. HCV virions stimulate CD81 and CLDN-1 endocytosis, suggesting that HCV facilitates its internalization by increased receptor trafficking [142]. Live cell imaging of HCV-infected cells shows endocytosis of both CD81 and CLDN-1 and fusion with endosomes that express Rab5. This indicates that this receptor complex has a role in HCV internalization. Occludin is another tight junction protein and has an essential role in infection by HCV. Knockdown of occludin by siRNA inhibits HCV entry [143]; however, this did not affect the attachment

of HCV to cells via the interaction with CD-81 and claudin-1. This indicates that occludin has a role in the later events of entry [144]. During HCVcc infection, the expression of occludin was downregulated, indicating that this could prevent HCV superinfection [145]. Another cell factor, Niemman–Pick C1-like 1 (NPC1L1) cholesterol uptake receptor, has also been shown to be necessary for HCV cell entry. Inhibition of NPC1L1 by silencing of its expression reduces HCV infection [146]. Although hepatocytes are the main host for HCV infection, evidence suggests that other cells (e.g., in neural tissue) may also be capable of being infected. Neuroepithelial cell lines that expressed CD-81 and SR-B1, claudin-1, and occludin were shown to be capable of being infected by HCV [147]. E2 binds to dendritic cell–specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and liver endothelial L-SIGN via high-mannose glycans [148, 149]; however, the importance of this for HCV infectivity is still not clear.

E2 contains a sequence identical to the phosphorylation sites of IFN-induced protein kinase R (PKR) and eIF2α, a target for PKR. E2 expression blocked PKR kinase activity and its subsequent inhibitory effects on protein synthesis and cell growth [150]. 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 [151].

p7

Located between E2 and NS2, p7 is a 63 aa integral membrane polypeptide composed of two transmembrane domains connected by a cytoplasmic loop and intraluminal amino- and carboxyl-terminal tails [152, 153]. It is mainly located in intracellular membranes, which may be associated with the ER [154] or the mitochondria. Studies *in vitro* have shown that p7 acts as a cation ion channel [155, 156] and can be inhibited by amantidine. NMR analysis indicates that the p7 monomers are mainly α-helical [157], and a 3D structure model of the p7 ion channel from single-particle EM analysis indicated that it had a hexameric flower-shaped structure with petals oriented toward the ER lumen [158]. Other *in vitro* evidence indicated that it may form heptameric complexes [159]. p7 appears to be essential for infection as transcomplementation analysis shows that it is necessary for the production of infectious HCV [160], and deletion or substitution mutations in the p7 region of an infectious HCV cDNA clone were not viable in chimpanzee infection studies [161]. In addition, p7 expression reduces the pH of intracellular compartments. This loss of acidification may protect nascent virus particles and is needed for HCV replication [162]. P7, together with NS2, is also a key requirement for the localization of core to the ER, which is required for efficient virus assembly [163].

Nonstructural proteins and virus replication

The organization of the nonstructural (NS) protein-coding region of HCV (Figure 16.1) resembles those of flaviviruses and pestiviruses. Small amino acid sequence motifs conserved among proteases, helicases, and replicases are collinear in all three types of viral-encoded polyproteins [5, 9]; 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 [5].

NS3/NS4A protease

The NS3 protease domain contains a serine protease catalytic triad, H57, D81, and S139 [164, 165], 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, NS4B, NS5A, and NS5B [164], and showed that small uncharged residues (e.g., S or A) appear to be preferred at P1′ and that polar residues C or T are found at position P1 with a G or D found at position P5 [165, 166].

NS4A was also shown to be important for NS3-mediated cleavage, particularly at the NS4B–NS5A site [24, 167–169]. However, cleavage at the other nonstructural protein junctions was variable in efficiency, with cleavage at the NS5A–NS5B junction being most efficient [170, 171]. The N-terminal 181 residues of NS3 form the protease domain and can cleave all of the nonstructural protein sites in *trans* except the NS3–NS4A site. NS4A stabilizes NS3 and helps to localize it in the ER membrane [172]. ER anchoring is critical for NS3–NS4A cleavage of substrates encoding for the NS4B–NS5A and NS5A–NS5B junctions, but not for the NS3–NS4A and NS4A–NS4B junction substrates. NS4A binding to NS3 rearranges the secondary structure of both the N-terminus and catalytic site of the NS3 protease, which results in a rigid and tight structure of the catalytic site needed for the binding and hydrolysis of substrates [173]. NS3–NS4A protease is associated with intracellular membranes, including the mitochondrion-associated ER membrane (MAM), through targeting domains in NS4A and in the amphipathic helix a(0) of NS3 [174]. Hydrophobic amino acids in the helix are required for the cleavage of membrane-anchored parts of the HCV polyprotein and the MAM-associated mitochondrial antiviral signaling (MAVS) protein, which

is essential for HCV RNA replication in cells with intact RIG-I signaling [174]. A point mutation M21P destroyed NS3 helix a(0) formation, disrupted the membrane association of NS3, and abolished HCV replication. However, protease and NTPase–helicase activities were not affected, but the stability of NS3 was reduced [175]. Analysis of NS3 protease activity in full-length and truncated NS3 complexes showed that protease activity is enhanced by the presence of the NS3 helicase domain [176, 177].

Kinetic cleavage analysis of P6-P4′ peptides corresponding to all the intermolecular cleavage sites of the nonstructural region of the HCV polyprotein had the following order of cleavage: NS5A–NS5B > NS4A–NS4B >> NS4B–NS5B. This indicates that the primary structure of the cleavage site is an important determinant for the efficiency with which each site is cleaved. 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 [178]. NMR 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 [179], and they appear to induce stabilization of the enzyme by causing tightening of the interdomain interaction in NS3 [180] by an induced fit mechanism [181].

Amino acids C97, C99, C145, and H149 are likely to represent the zinc-binding motif in the NS3 protease. Changes in the NMR signals of H149 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 [182]. No similar zinc-binding domain was found in NS3 of other members of the *Flaviviridae* family [183]. 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 [183, 184].

X-ray crystallography at 2.2–2.7 Å resolution of NS3 protease domains from the HCV BK [185, 186] and H [187] strains showed that 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, nonpolar, and 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 [166]. 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 neighboring molecules. The interaction of NS3 with NS4A [185] 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 that are important for the production of a catalytically favorable conformation of the active site [186, 187]. A comparison of NS3 protease and an 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 [188].

NS3 NTPase and helicase

The C-terminal 442 aa of the HCV NS3 protein contains a DECH amino acid sequence motif [5, 189], which is characteristic of the DExH subfamily, of DEAD-box superfamily-2 NTPase–RNA helicases [190, 191]. HCV NS3 NTPase activity can be stimulated by polynucleotides in a similar fashion to those of pestiviruses and flaviviruses. 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 that contain 5′ and 3′ single-stranded regions (5′/3′) or substrates containing only 3′ single-stranded regions (3′/3′) but not substrates that contain 5′ single-stranded regions only (5′/5′) or on substrates lacking a single-stranded region [192]. This indicates that the HCV helicase, like most RNA helicases, is unidirectional in a 3′ to 5′ direction [193]. HCV NS3 helicase was also shown to unwind DNA–DNA and DNA–RNA substrates efficiently. 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 a role in viral pathogenesis or persistence [194]. The concentration of RNA 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 [195]. A comparison of ATP hydrolysis and helicase translocation with RNA and DNA substrates showed that hydrolysis of each ATP molecule by the full-length NS3 resulted in a translocation step nearly every time, whereas the helicase domain on its own resulted in translocation in only 20% of hydrolysis events. This indicates that stabilization of the helicase domain by the protease domain may lead to better cross-talk between the ATP and nucleic acid–binding sites [196], and the interaction between the protease and helicase domains is important during virus replication. Mutational analysis that removed the tether between the helicase and protease domains reduced helicase activity, indicating that an open extended form of NS3 is necessary for RNA helicase activity [177].

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 [197]. 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 [198, 199]. 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 [200, 201], indicating that these two activities are coupled. Mutants in both the GxGK and DExH motifs that had lost NTPase activity could still bind RNA efficiently. However, NS3 with a mutation in the QRxGRxGR motif was defective in NTPase and could no longer bind double-stranded RNA (dsRNA). This indicates that the conserved motifs cooperatively constitute a larger functional domain rather than act as several independent domains [202]. NS3 RNA helicase undergoes conformational changes, on binding of NTPs or single-stranded RNA (ssRNA) and after NTP hydrolysis, which drive helicase translocation along a nucleic acid. Binding of an NTP reduces the affinity of NS3 for single-stranded (ss) nucleic acid but increases its affinity for double-stranded (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 promote tight binding to the ss nucleic acid [203–205].

Kinetic analysis suggests that NS3 helicase appears to function as an oligomer [206], and one study indicates that NS3 complexed with NS4A has a higher RNA helicase activity than NS3 alone [194]. Protein interaction studies and RNA unwinding experiments have shown that NS4A can enhance NS3 binding to RNA by binding via the C-terminal acidic domain [207]. Amino acids in the C-terminal of NS4A are required for this interaction, as shown by mutational analysis where a large reduction in NS3 unwinding activity was observed [208]. The aa motif EFDEMEE of NS4A may interfere with the ATP-binding site of NS3 helicase, thereby regulating the ATPase activity of NS3 helicase as well as activating the NS3 protease [209]. The NS3 helicase was also shown to facilitate the conversion between different possible RNA structures, indicating that it may have a role in structural rearrangement of RNA *in vivo* [210]. Y-box-binding protein 1 (YB1) was shown to interact with NS3–N4A and HCV RNA. Knockdown of YB1 expression by siRNA increased the production of infectious HCV virus particles but inhibited HCV RNA replication. This indicates that YB1 NS3–N4A–HCV RNA complexes may regulate the balance between RNA replication and virus particle formation [211]. Creatine kinase B (CRB) was shown to associate with NS4A and form a ternary complex with NS3 in replication centers. siRNA knockdown of CRB reduced RNA replication, suggesting that it may play a role in ensuring that local ATP levels are maintained in RCs [212]. The interaction of the NS3 helicase and the core protein is essential for capsid formation [63].

NS3 interactions with the cell

The N-terminus of NS3 was shown to interact with the C-terminus of p53 and form a complex [213, 214]. 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 [215]. Staining of hepatocytes from HCV-infected patients with anti-NS3 antisera showed that while NS3 is mainly present in the cytoplasm, in a minority of infected cells NS3 was also present in the nucleus [216]. 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 of NS3 in the nucleus [217]. The NS3–NS4A serine protease was shown to block IFN production in response to dsRNA signaling mediated by Toll-like receptor 3 (TLR3) by proteolysis of the adaptor protein, Toll-IL-1 receptor domain containing adaptor-inducing interferon-β (TRIF). The NS3–NS4A cleavage site in TRIF is very similar to that of the NS4B–NS5A junction [218]. In a similar way, NS3–NSA4 blocks IFN production in response to 5′ triphosphates present on viral RNAs being detected by the retinoic acid-inducible gene 1 (RIG1) protein. NS3–NS4A cleaves the N-terminal fragment of RIG1 adaptor protein and mitochondrial antiviral signaling (MAVS) protein from the mitochondria, thus blocking signal transmission to the nucleus [219]. During HCV replication, the core protein alters the localization of sMaf proteins from the nucleus to the cytoplasmic HCV RNA replication complex, where they bind to NS3. Extranuclear sMaf proteins prevent NF-E2-related factor 2 (Nrf2) entry into the nucleus and thus inhibit the expression of cytoprotective genes that are normally induced by Nrf2 binding to antioxidant-response elements (AREs) in their promoters. This indicates that HCV infection can cause increased levels of reactive oxygen species (ROS) and subsequent cellular damage during its replication cycle [220]. HCV infection leads to PKR and eIf2a phosphorylation at 12–15 hours post infection. Inhibition of this phosphorylation restores interferon expression and leads to decreased yields of HCV until 18 hours post infection, when NS3–NS4A

cleavage of MAVS inhibits interferon production [221]. NS3–NS4A also cleaves T cell protein tyrosine phosphatase (TC-PTP), which is expressed ubiquitously, *in vitro*, thereby inactivating it. Evidence for this cleavage was also found in hepatocytes from HCV-infected humans and in a HCV transgenic mouse model. TC-PTP downregulation leads to enhanced epithelial growth factor (EGF)-induced signal transduction and increases the basal activity of Akt, which has been shown to be necessary for HCV replication [222].

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) [5, 8], and NS5B has been shown to have RdRp activity [223]. X-ray crystallography shows that most of the NS5B protein (the first 530 aa) forms a catalytic domain with "finger" and "thumb" subdomains encircling the enzyme active site, in the "palm" subdomain, typical of single-chain polymerases [224]. X-ray crystallography also indicates that the fingers domain has a long binding groove that guides template RNA to the catalytic site [225] and an NTP-binding region close to the active site [226]. 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-loop I [227] and that *de novo* synthesis favors initiation from the +1 position to maintain the integrity of the template [228]. NS5B has two successive crucial steps in *de novo* initiation of RNA synthesis: dinucleotide formation, which requires a closed conformation of NS5B, and the transition to elongation, which requires the opening of NS5B. Structural and functional analyses demonstrated that residue 405 of NS5B acts as a molecular switch between the closed and the open forms [229]. GTP has also been shown to stimulate the transition between these two forms [230]. Two nonnucleoside analog 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 [231]. 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 [232]. Two residues (E18 and H502) that lie outside the catalytic site are critical for RdRp and are also necessary for the oligomerization of NS5B [233], which occurs in the presence of template RNA [226, 234]. Oligomerization of NS5B may also stimulate *de novo* initiation [235]. The C-terminal 21 amino acids of NS5B form an alpha-helical transmembrane domain that anchors it to the cytosolic face of the ER [236]. 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 [237].

HCV infection induces the formation of autophagosomes, and both NS5A and NS5B were shown to be localized to autophagosomes. Silencing of the expression of LC3 or Atg7, two protein factors critical for the formation of autophagosomes, suppresses the replication of HCV RNA. Inhibition of Class III PI3K activity had no effect on the autophagosomes induced by HCV. This indicates that HCV induces autophagosomes via a Class III PI3K-independent pathway and that it uses autophagosomal membranes as sites for its RNA replication [238]. NS5B has also been shown to interact with autophagy factor Atg5, and they are co-localized in perinuclear regions during early infection with HCV. Atg5 silencing inhibits autophagy and blocks HCV replication [239].

NS5A

NS5A occurs as a basally phosphorylated 56 kDa protein and as a 58 kDa hyperphosphorylated form of the 56 kDa protein [240]. The production of p58 is enhanced by the presence of NS4A [240] or NS4B [241, 242], which may modulate the activity of a cellular protein kinase. The N-terminal 30 aa form an amphipathic α-helix that functions as a membrane anchor [243].

NS5A contains three domains, D1–D3, separated by linker sequences, and distributed among them are three clusters of serine residues that are the target for phosphorylation (PI, PII, and PIII), with PI being hyperphosphorylated. Domain I and the linker that separates D1 and D2 bind RNA. When U-rich RNA is present, the equilibrium between NS5A monomers and dimer shifts in favor of dimers. This suggests that RNA interaction with NS5A may regulate HCV and cellular gene expression [244]. Another study that showed that D1 and D2 interact specifically with the 3′ UTR RNA suggests that NS5A interaction with RNA may play a role in the control or enhancement of HCV genome replication [245].

Cyclophilin A (CypA), a peptidyl-prolyl isomerase, binds to D2 and appears to stimulate D2 binding to RNA [246, 247]. CypA has also been shown to interact with D3 [248]. This indicates that CypA may play an important role in HCV RNA replication and CypA also locates

to the HCV RNA replication centers (RCs). However, depletion of CypA from RCs does not appear to influence the association of NS5A and NS5B with the RC [249]. NS5A D1 interacts with PI4KIIIα and stimulates its kinase activity, resulting in increased levels of phosphatidylinositol-4-phosphate (PI4P). Knockdown of PI4KIIIα activity by siRNAs induced a dramatic change in the ultrastructural morphology of the membranous HCV RC and a reduction in HCV RNA replication and virus release [250, 251]. The interaction between NS5A and NS5B requires the binding of c-Src, and the inhibition of c-Src by siRNA knockout or chemical inhibitors strongly reduced HCV RNA replication [252]. NS5A interacts with lipid droplets (LDs) and also recruits the Rab GTPase activation protein TBC1D20 and its cognate GTPase, Rab1 to LDs. Dominant-negative Rab1 proteins inhibit the interaction of NS5A at viral replication sites, indicating that the interaction of NS5A with TBC1D20 and Rab1 affects the lipid droplet metabolism required to promote the viral replication cycle [253]. NS5A plays a central role in the early stages of HCV virion assembly through the interaction of its D3 with core [254, 255]. One serine residue in D3 that is phosphorylated by casein kinase II appears to be crucial for NS5A to bind core in the early stages of assembly [256]. Annexin A2 (ANXA2) co-localized with viral nonstructural proteins in HCV replication complexes. Deletion analysis showed that NS5A D3 was responsible for ANXA2 recruitment. Knockdown of ANXA2 expression had no effect on viral RNA replication but reduced extra- and intracellular virus titers, indicating that it has a role in HCV assembly but not in genome replication or virion release [257]. ApoE is necessary for HCV assembly and interacts via its C-terminal domain with NS5A [258].

NS5A has been implicated as having a role in inhibiting host cell responses to IFN [259]. 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 IFNα) dimerization domain. This has the effect of disrupting kinase dimerization, thereby inhibiting its ability to phosphorylate eIF2α and to shut off translation [260]. However, the role of NS5A in the inhibition of IFN-induced PKR remains unclear.

NS5A inhibits the oxidative stress–induced p38 MAPK phosphorylation of Kv2.1, preventing oxidative stress–induced apoptosis in HCV-infected cells and cells containing subgenomic replicons [261]. HCV NS5A also downregulates TLR4 signaling and LPS-mediated apoptotic pathways, and this may contribute to HCV pathogenesis [262]. NS5A may also inhibit apoptosis by binding the cellular protein, FK506-binding protein 38 (FKBP38), thereby impairing the interaction between mTOR and FKBP38 [263]. NS5A upregulates the host cap-dependent translation machinery by stimulating PI3K–AKT–mTOR, activating eIF4E, and increasing eIF4F complex assembly, thereby increasing the efficiency of cap-dependent translation [264].

NS5A blocked TNFα-mediated apoptosis [265] but not Fas-induced apoptosis. The binding of NS5A to TNFα receptor–associated death domain protein (TRADD) suggests that the protection against TNFα may be by the inhibition of TRADD-mediated nuclear factor kappa-light-chain-enhancer of activated B (NFκB) activation [266]. NS5A can stimulate monocytes through TLR4 and induce IL10 production, triggering the secretion of TGFβ, which downmodulates the NKG2D-activating receptor expression on natural killer (NK) cells. This NS5A-induced decrease in NKG2D may allow HCV to evade NK-cell-mediated responses [267]. NS5A expression inhibits the transcription of complement 3 (C3), thereby reducing levels in the circulation during HCV infection [268].

Huh7.5 cells expressing HCV-3a NS5A showed increased nuclear accumulation of Sterol Regulatory Element Binding Protein-1c (SREBP-1c), a key transcription factor that activates the transcription of lipogenic genes. Furthermore, inhibition of transcriptional factor Sp1 abrogated SREBP-1c promoter activation by HCV-3a, indicating that NS5A is a contributing factor to steatosis caused by HCV-3a infection, and this is mediated by Sp1 activation of SREBP-1c transcription [269]. HCV infection also induced JNK activation due to increased mitochondrial ROS production, caused by NS5A, resulting in decreased FoxO1 phosphorylation and FoxO1 nuclear accumulation, and, eventually, increased glucose production. This indicates that HCV promotes hepatic gluconeogenesis through an NS5A-mediated, FoxO1-dependent pathway [270].

Binding studies and confocal microscopy indicate that NS5A can bind to p53 [271] and suppress p53-mediated transcriptional transactivation and apoptosis [272]. NS5A inhibits growth factor receptor bound protein 2 (Grb2) by binding NS5A polyproline motifs to Grb2 Scr homology 3 (SH3) domains [273] and inhibiting Grb2-mediated mitogenic signaling [274]. NS5A inhibited the mitogenic and stress-activated transcription factor activating protein-1 (AP1) by binding via polyproline motifs, thereby perturbing mitogenic signaling pathways in HCV-infected hepatocytes [275]. 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) [276]. NS5A activates beta-catenin signaling by binding to and stabilizing beta-catenin, and this may contribute to cell transformation and the development of HCC [277, 278].

NS2

NS2 is a 21–23 kDa protein [279, 280] forming part of the NS2-3 cysteine protease that is specific for the NS2–NS3

cleavage site [280]. However, NS2 has basal protease activity in the absence of NS3. This can be greatly enhanced by the addition of the NS3 Zn^{2+}-binding domain that modulates the NS2 protease activity [281]. 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 that altered the conformation of the region P5 to P3′ (e.g., proline at P1 or P1′) had a major inhibitory effect on cleavage [282]. NS2 is a transmembrane protein with two internal signal sequences that can target it to the ER posttranslationally, unlike most mammalian membrane or secretory proteins [283]. It appears to have multiple transmembrane domains, and it may have both the amino- and carboxyl-termini located in the lumen of the ER. This association with the ER membrane may be required to assist in the proper folding of the nascent precursor for processing at the NS2–NS3 junction [283]. NS2 is essential for virus assembly and is found in ER-derived structures in association with p7 and E2, which then interact with NS3 and NS5A during virus assembly [284]. Protein–protein interaction studies showed that NS2 also bound to E2, p7, NS3, and NS2 itself [285]. NS2 therefore appears to mediate virus assembly by coordinating interactions between NS3, N5A, and the envelope proteins in replication complexes near LDs [286]. The NS2 protease domain, though not the protease activity, is required for virus particle assembly [287]. 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 [288]. In addition, NS2 expression also upregulated the transcription of SERBP-1c and FAS and may therefore contribute to steatosis [289].

NS4B

NS4B is a cytoplasmic transmembrane protein. The carboxyl-terminus is located in the cytoplasm, though the location of the amino terminus is not clear. NS4B has two N-terminal amphipathic helical transmembrane domains (TMD1 and TMD2), a hydrophobic central domain (TMD3), and a highly conserved C-terminal domain (TMD4) that may have two alpha helices. Both TMD1 and TMD4 act to induce the formation of a membranous web in the ER, which is required for the formation of HCV RNA RCs and with which all of the HCV proteins [290] and genomic RNA are associated [291]. Mutational analysis of amino acids in the highly conserved C-terminal domain TMD4 indicates that it has an important role in NS4B self-interaction and in the formation of HCV replication complexes [292]. However, another study reported that NS4B forms homodimers mediated by a bZIP motif in the TMD1 N-terminal region [293]. NS4B is present as punctate foci in the ER at HCV RNA synthesis sites [294]. Mutations in TMDs 1–3 led to disruption of these focal structures in the ER, indicating that the TMDs play a role in HCV RC formation [295]. Formation of NS4B foci formation may also require the interaction of Rab5 with TMD4 [296]. NS4B has been shown to induce autophagy that may also facilitate HCV replication. Inhibition of either Rab5 expression or Vsp34 function prevented NS4B-induced autophagy, indicating that NS4B-induced autophagy involves the formation of a complex with Rab5 and Vsp34 [297]. By stimulating the production of ROS initiated by perturbing intracellular calcium, NS4B expression leads to the activation of unfolded protein response (UPR), resulting in ER stress, and this in turn may contribute to the pathological effects of chronic HCV infection [298]. NS4B expression may also lead to apoptosis through the activation of caspases 3, 7, and 9 and poly(ADP-ribose) polymerase (PARP) and a reduction in mitochondrial membrane potential and the release of cytochrome C from the mitochondria [299]. NS4B may play an important role in hepatic steatosis by increasing the transcriptional activities of SREBPs, enhancing the protein expression levels of SREBPs and FAS, and inducing lipid accumulation in hepatoma cells [300]. There is also evidence that NS4B inhibits the traffic of proteins between the ER and the Golgi in infected cells [301], inhibits protein synthesis [302, 303], and modulates the hyperphosphorylation of NS5A.

The 5′ terminal UTR

The 5′ untranslated region (5′ UTR) of 341 nucleotides in length precedes the initiator methionine codon of the large ORF encoding the polyprotein precursor [5, 6, 37, 38]. 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 [5, 7] and up to 60% identity with the GBV-B 5′ UTR [304]. The 5′ UTR contains four domains (I–IV) that have extensive stem-loop structures [305, 306]. Domain IV extends beyond the 3′ UTR and contains the start codon of the polyprotein with a 3′ boundary between nucleotides +12 and +30 [307–309], and together with domains II and III forms the HCV internal ribosome entry site (IRES), which controls HCV protein translation. 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 shown to bind to domain III [310].

The maintenance 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 [306]. NMR studies show that the free form of domain II is similar in shape to the 40S bound form [311]. Mutants that stabilize stem-loop IV reduce the translational efficiency of the IRES. Long-range interactions between nucleotides 24–38 and 428–442 in stem-loop IV also reduce IRES-mediated translational efficiency [312]. Stem-loop IV may therefore regulate the translational efficiency of HCV. This would require stem-loop IV to melt before 40S ribosomal subunits could bind to the AUG and begin the process of translation [313]. In binding studies, it was shown that core protein preferentially binds to oligonucleotides corresponding to the stem loop IIId domain of the 5′ UTR [314], suggesting a possible site of interaction between the nucleocapsid and the genome. However, other evidence indicates that core interaction with this region of the genome is not required for RNA packaging [64]. 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 [315, 316].

Chemical and enzymatic analysis of the structure of the IRES under physiological conditions shows that it folds into a unique 3D structure in the absence of the translational apparatus and additional co-factors [317]. 40S ribosomal subunits form a stable pre-initiation complex with HCV IRES RNA, and 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 [318]. The binding of IRES to eIF3 HCV drives translation by recruiting 40S ribosomal subunits [319]. Hsp90 binds indirectly to eIF3 subunits through interaction with the IRES. This prevents eIF3 degradation and enhances translation [320]. IRES 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 sequence of the HCV genomic RNA [321]. After the 40S subunit binds, the stem loop containing the initiation codon unwinds and forms a stable configuration in the 40S subunit mRNA binding cleft. However, this also requires interaction with domain IIb, indicating that distant interactions within the IRES are required for translational initiation.

Stem-loops I and II of the 5′ UTR are essential for RNA replication; however, stem-loop I is not required for translation. Knockdown of poly(C)-binding protein 2 (PCBP2) reduced IRES-dependent translation and decreased the levels of HCV RNA and nonstructural proteins. PCBP2 also interacts with HCV 5′ and 3′ UTRs to form an RNA–protein complex and induces the circularization of HCV RNA, which may be important in the regulation of translation and is commonly found in positive-strand RNA viruses [322]. Some evidence indicates that HCV IRES RNA inhibits PKR, while other evidence suggests that it activates PKR. Analysis of the IRES domain II structure suggests it may mimic dsRNA. One model suggests that at low NS5A concentrations, PKR is activated by HCV RNA shutting off host but not viral protein synthesis. As NS5A levels increase, it inhibits PKR, allowing host protein translation to provide factors necessary for virus replication [323].

The liver-specific microRNA-122 (miR-122) binds to two sites (S1 and S2) in the 5′ UTR, and this switches the IRES to an open conformation that may enhance HCV RNA translation [324]. Selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) mapping of the 5′ UTR RNA secondary structure during miR-122 interaction shows that both the tail region of miR-122 and the seed region bind to the 5′ UTR S2 site, and this enhances HCV replication [325]. The 3′ terminal nucleotides in miR-122 were absolutely required for maintaining HCV RNA abundance. When it binds to S1, the 3′ terminal nucleotides of miR-122 form an overhang and mask the 5′ terminal sequences of the HCV genome sequences, and this may protect them from nucleolytic degradation or from inducing innate immune responses to the RNA terminus [326]. In addition to miR-122, the 5′ UTR also binds Ago2 to form a RISC-like complex that also protects the HCV RNA genome from 5′ exonuclease activity of the host mRNA decay machinery, thereby stimulating translation and promoting genome replication [327]. Regions upstream of the S1 and S2 miR-122 binding sites have also been shown to be important in the recruitment of Argo2 to stabilize HCV RNA [328]. RNA translation and replication also depend on a number of host factors (e.g., Rck/p54, LSm1, and PatL1), which regulate the fate of cellular mRNAs from translation to degradation in the 5′-3′-deadenylation-dependent mRNA decay pathway. In addition LSm1–7 heptameric ring complexes specifically interacted with *cis*-acting HCV RNA elements located in the 5′ and 3′ UTRs [329]. HepG2 cells are deficient in miR-122 and do not support HCV RNA replication. However, exogenous miR-122 allows HCV RNA replication in HepG2 cells [330]. A comparison of HCV translation at different stages in the cell cycle in Huh7 cells shows that this is higher in the G_0, G_1, and G2–M phases and lowest in the S phase. The levels of miR-122 show that they are lowest in the S phase and are highest in the G_0–G_1 state found in hepatocytes in the liver, which may therefore facilitate high levels of HCV expression partly due to the greater abundance of miR-122 [331]. Autophagy proteins including Beclin-1, Atg4B, and Atg12 have been implicated in the translation of incoming HCV RNA; however, once RNA replication has been established, they are not required for subsequent translation [332]. HCV infection has been shown to disrupt P-body and stress granule formation

by hijacking some of their components for HCV RNA replication [333].

The 3′ terminal UTR

The 3′ terminal UTR is located downstream of the stop codon terminating the large ORF encoding the viral polyprotein, and it plays an important role in minus- and plus-strand RNA synthesis, in the regulation of protein translation, and in the packaging of viral RNAs. The 3′ UTR comprises three structurally distinct RNA domains. From 5′ to 3′, these are an upstream variable region (VR) of about 40 nucleotides that varies considerably between different genotypes; a long poly(U)-poly(U/UC) tract; and a 98 nucleotide (3′X) sequence, which forms three stem-loop structures (SL1, SL2, and SL3), terminates in a U residue, and is highly conserved between all HCV genotypes [334].

An RNA element within the NS5B-coding region, previously known as 5BSL3.2 but now known as SL9266, forms a functional kissing-loop tertiary structure with the 3′ UTR, SL2, which is required for HCV replication [335]. These *cis*-acting replication elements (CREs) also form long-range interactions between the 3′ UTR SL2 and another RNA sequence located 3′ to SL9033 in the NS5B-coding sequence. Alternative long-range interactions between 3′ UTR SL2 and the different elements in NS5B may function as a molecular switch controlling a critical aspect of HCV genome replication, and may also direct plus-strand genomic RNA from RNA synthesis to packaging, and eventually to cellular export [336]. In addition, subdomain IIId of the IRES also interacts with the CRE centered on 3′ UTR and NS5B elements. This can modulate IRES-dependent HCV translation [337] as mutations in SL9266, which prevent it from participating in the CRE and can increase translation by up to eightfold [336]. The first 55 nt of the 98 nt 3′ UTR X region (X55 RNA) are 100% conserved among all HCV strains. *In vitro* experiments have shown that X55 contains a dimer linkage sequence (DLS) region that causes dimerization of HCV genomic RNA in the presence of the viral core protein, and mutations in this region can reduce RNA replication. The involvement of the 3′ UTR in dimer formation would preclude 3′ UTR from engaging in the long-range kissing interaction with the NS5B-coding region that is known to be essential for RNA replication. It has therefore been proposed that the DLS region might have a role in the formation of alternative conformations of the 3′ X RNA [338].

The highly conserved 3′ X sequence interacts with viral and cellular proteins and/or other 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 [339] and possibly to 3′ X stem-loops 2 and 3 [340]. A 35 kDa protein corresponding to heterogeneous nuclear ribonucleoprotein C (hnRNPC) was also shown to bind to this region [339]. In one study, the presence of a 3′ X region that is capable of binding PTB has been shown to enhance the efficiency of translation from the HCV IRES three-to-five fold [341]. Infectivity studies in chimpanzees show that the 3′ X sequence [342, 343] 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 [342]. Deletion of the variable region still allowed replication but at a much lower level. However, deletion of either the poly(U/UC) or the 3′ X was not viable [344, 345]. 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 [199].

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Chapter 17
Epidemiology and prevention

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Summary

Hepatitis C virus (HCV) is distributed globally, affecting all countries. The majority of new HCV infections in developed countries are currently due to needle sharing by intravenous drug use (IDU) and nosocomial patient-to-patient transmissions in healthcare settings. In developing countries, blood transfusion remains a major cause, followed by transmission by medical procedures (specifically those using contaminated injection equipment) and IDU. The large reservoir of asymptomatic chronically infected individuals, the fluxes of immigration from endemic areas to less prevalent regions, and the uncontrolled epidemic of IDUs continue to spread HCV throughout the world. The goal of this chapter is to review the substantial changes in HCV transmission routes of groups at risk of HCV infection, genotype distribution, and prevalence in the general population. The data describe a worldwide HCV prevalence of 2.27% or 160 million persons infected with this virus. HCV prevalence and transmission routes are the basis for the development of measures to prevent new infections, to provide HCV screening and early treatment, to reduce complications associated with HCV infection, and to attenuate the huge socioeconomic impact associated with HCV infection.

Introduction

HCV infection is one of the leading causes of chronic hepatitis, cirrhosis, and liver cancer and accounts for more than 50% of adult liver transplantations in the Western world. With an estimated worldwide prevalence of 2.27% of the general population (Table 17.1) or 160 million people [1], chronic HCV infection may cause 350 000 deaths each year [2].

Hepatitis C virus is a bloodborne infection, and transfusion of blood and blood products was the most frequent mode of acquisition until the late 1980s; studies in this setting were determinant to the cloning of the agent and subsequent development of antibody tests for donor screening and of disease diagnosis. Implementation of universal screening of blood donors drastically decreased transfusion-associated hepatitis C, while other parenteral routes such as IDU and inapparent nosocomial exposure emerged as the predominant modes of HCV transmission in the Western world. Injections and invasive procedures with nondisposable sharps, syringes, and needles in the medical setting have traditionally fueled HCV spread throughout the world to a much larger extent than blood transfusion, and they continue to comprise the most frequent mode of transmission in developing countries with overstretched public health systems. An epidemic explosion of IDU shortly followed the iatrogenic spread. The timing and the extent to which health-care-related transmission or IDU fueled the epidemic in different coun-

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Table 17.1 Estimated prevalence and number infected according to world population (2011) by continents*.

Continent	Population 2011 (census or official estimates), by continent.	Anti-HCV (%)	Estimate HCV-infected individuals. (2011)
Oceania	36992477	1.36	501665
Europe	740020696	1.87	13828253
Americas	944124136	1.48	13996958
Middle East	553644000	5.08	28115565
Africa	963742000	3.13	30178423
Asia	3735892247	1.92	71767793
	6974415556	2.27	158388657
	Global prevalence	2,27%	

*Total population (July 1 interpolated). Medium variant based on WUP Revision 2011. From [34]. Corrected by official census when possible.

tries explain the different epidemiological profiles between Northwestern and Southeastern European countries and the United States.

The large reservoir of asymptomatic chronically infected individuals, the often unnecessary use of blood transfusion in countries with poorly regulated blood bank systems, the use of contaminated injection equipment and the administration of unnecessary injections in developing countries, the global expansion of the human population with increasing fluxes of immigration from endemic areas to less prevalent regions, and the uncontrolled epidemic of IDU continue to spread HCV throughout the world. As a consequence, substantial changes in epidemiological factors such as prevalence, incidence, disease transmission patterns, and genotype distribution have been observed in Europe during the last 15 years, in which HCV continues to spread despite the near-zero risk of transmission by blood transfusion, due to immigration from endemic areas, the ongoing IDU epidemic, frequently unrecognized patient-to-patient transmission during healthcare-related procedures, and the absence of a preventive vaccine [3].

Transmission mechanisms and groups at risk for HCV infection

Hepatitis C is a strictly bloodborne infection, and humans are the only natural reservoir for the virus. Given the high rate of viral persistence after HCV exposure and the asymptomatic and smoldering course of chronic infection, percutaneous exposure to infected blood is the main transmission mechanism in the vast majority of cases. Large and/or repeated direct percutaneous exposure to blood (such as via blood transfusion or IDU) is the most efficient mode of HCV transmission. HCV is less efficiently transmitted by single small-dose percutaneous exposure (such as accidental needlesticks). Mucosal exposure to infected blood uncommonly transmits infection unless there is obvious disruption of mucosal integrity or concurrent exposure to large amounts of blood (such as during birth by an infected mother or high-risk sexual exposure). HCV is not spread by breastfeeding, sneezing, hugging, coughing, food or water, sharing eating utensils, or casual contact.

The worldwide spread of HCV occurred through massive unsafe injection campaigns that were started during the first decades of the twentieth century, when glass syringes and other medical devices were produced on a large scale, and during World War II, when blood transfusion became a common clinical practice. In fact, some molecular epidemiologic studies have shown that major HCV subtypes were widely spread in different countries coinciding with massive campaigns of parenteral treatments such as antischistosomal therapy with antimony, which spread HCV subtypes 4a and 1b in Egypt and Japan during the 1930s and 1920s, respectively.

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, unsafe parenteral treatments, and IDU were the predominant mode of transmission in the world. As a result, most chronic transfusion recipients and virtually all patients receiving clotting factor concentrates were exposed to HCV. During the 1990s, the incidence of new infections in developed countries decreased rapidly due to improvements in infection control standards, the implementation of effective virus-inactivation procedures for blood derivatives in the late 1980s (developed to prevent transmission of HIV), and the introduction of second-generation anti-HCV tests for blood donors in 1992. As a consequence, the majority of new HCV infections in these countries are currently

Table 17.2 Estimated 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 C virus (HBV), human immunodeficiency virus (HIV), and human T lymphotropic virus (HTLV).

Viral agent	Incidence (per 100 000 person years)	Window period (days)	Risk per unit				
			United States	Spain	France	Italy	Canada
HBV	1.267	45	1:640 000	1:74 000	1:470 000		1:7 Md
HCV (Aby)	1.889	70	1:276 000	1:149 000	1:860 000	1:127 000	
HCV (+NAT)	1.889	10	1:1 935 900	1:425 714*	1:8 300 000*	1:747 058*	1:6.7 Md
HIV (Aby+Ag)	1.554	16	1:1 468 000	1:513 000	1:1 370 000	1:435 000	
HIV (+NAT)	1.554	11	1:2 135 000	1:1 026 000*	1:2 700 000*	1:870 000*	1:8 Md
HTLV	0.239	51	1:2 993 000		Nil		

* Predicted residual risk after implementation of NAT in 2001. Aby: antibody test only; +NAT: antibody plus nucleic acid technology: TMA (transcription mediated amplification) or PCR (polymerase chain reaction); Aby+Ag: antibody plus p24 antigen; Md: million donations.

due to needle sharing by intravenous drug abusers with occasional outbreaks of nosocomial patient-to-patient transmissions in healthcare settings [4].

In sharp contrast, in developing countries, including several of the most populous nations in the world, which have a high prevalence of HCV infection in all age groups, blood transfusions remain a major cause of HCV spread (due to inadequate blood bank policies and nonexistent or suboptimal donor screening), and the risk of acquiring HCV infection through medical procedures is not limited to occasional outbreaks but occurs on a daily basis through the use of contaminated injection equipment, accounting for more than 2.3 million HCV new infections every year.

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. 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 lower incidence of infection.

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 to 1.1% in Australia, 3–4% in the United States, 7.7% in Japan, 11% in Spain, 12.5% in Taiwan, and 13% in Greece. As expected, patients requiring multiple transfusions, such as thalassemics or multitransfused patients in long-term remission from leukemia, have a high prevalence of HCV infection.

In developed countries, improvements in blood donor selection and deferral criteria, and especially the introduction of second-generation anti-HCV screening tests for blood donors, drastically reduced the risk of HCV transmission to very low levels, with the residual risk essentially limited to units collected during the serological window period. The implementation of nucleic acid technologies (NATs) to detect HCV RNA in pools or individual blood donations further improved (albeit marginally) the safety of the blood supply by reducing the window period. Consequently, transfusion of screened blood should no longer be considered a primary risk factor for HCV infection (Table 17.2). The residual risk of a blood recipient becoming infected with HCV is so low that it cannot directly be measured in prospective surveys.

In developing and transitional countries, in contrast, blood transfusions still remain a major cause of HCV infection. A combination of factors, including a low proportion of volunteer unpaid blood donors, a higher HCV infection prevalence among blood donors, and nonexistent or unreliable screening systems (because of staff shortages, lack of laboratory services, and a poor-quality or irregular supply of test kits) underlie the unsafeness of the blood supply. Despite significant improvements during the last decade, the most recent update of the Global Database on Blood Safety (GDBS) [5] showed that in 2008 in 40 countries, less than 25% of the blood supply was collected from volunteer unpaid donors and still depended mostly on family–replacement and paid donors, with at least 800 000 donations having been collected from paid donors in 26 countries. Furthermore, 41 developing countries screened less than 100% of blood units or did not screen at all for HCV, and in most cases screening was not done following basic-

quality standard procedures. Unfortunately, the prevalence of HCV infection among blood donors in these countries is high, and most blood transfusions are given to children with severe childhood anemia and to young females with pregnancy-related complications.

Plasma product recipients

The prevalence of HCV among hemophiliacs correlates with the amount and type of product transfused. Virtually all hemophiliacs exposed to untreated commercial clotting factor concentrates before anti-HCV screening have evidence of HCV, whereas among those treated exclusively with cryoprecipitates, the anti-HCV rate was 66%. In contrast, hemophiliacs who have exclusively received appropriately inactivated coagulation components or single-donor cryoprecipitate are generally anti-HCV-negative. Screening of plasma pools used to manufacture concentrates combined with viral inactivation steps virtually eliminated the risk of infection. Because of the widespread use of recombinant clotting factors in most patients, young hemophiliacs are no longer considered a risk group for HCV infection.

Although intravenous immune globulin (IVIG) has excellent safety records, two outbreaks of HCV infection were described in either intramuscular- or intravenous-contaminated anti-D immunoglobulins in the late 1970s, and two major and three minor outbreaks of hepatitis C in agammaglobulinemic patients treated with different products manufactured by the Cohn fractionation method from unscreened donor plasma have been described. Surprisingly, in 1993 a major outbreak of hepatitis C in agammaglobulinemic patients was caused by a commercial immune globulin (Gammagard) prepared from enzyme immunoassay (EIA)-2-screened plasma.

Hemodialysis patients

The prevalence of HCV infection in patients on hemodialysis varies widely between geographic areas and between centers in the same country, ranging from less than 5% in Northern Europe to 30% to 50% in Japan, and reaching 70.4% in Egypt, with rates of 30.25% in Saudi Arabia, 37.6% in Taiwan, 39% in Brazil 41.5% in Poland, and 43.51% in Bosnia-Herzegovina. Prevalence between 5% and 30% had been reported in the United States (23% in New York), 5% in Kenya, 8.2% in Denmark, 8.4% in Germany, 16.6% in Hong Kong, 20% in Thailand, 21.1% to 23% in Italy, 23.6% in France, and 24.5% in India. The high prevalence of HCV infection in hemodialysis patients has been consistently associated with the number of blood transfusions and the length of time on dialysis, suggesting that HCV may be transmitted among patients in the dialysis unit. In fact, although blood donor screening and use of erythropoietin to reduce transfusion requirements were associated with a progressive decrease of new infections, they did not eliminate incident infections. A prospective study conducted between 1997 and 2001 in seven countries involving more than 8000 patients found a median prevalence of HCV of 14% (range: 2.6% to 22.9%) and median seroconversion rates of 2.5 per 100 person years (range 1.2 to 3.9). Outbreaks of HCV infection in dialysis units have been attributed to poor infection control practices. HCV in plasma remains viable after drying and environmental exposure to room temperature for at least 16 hours. Therefore, blood-contaminated surfaces and objects can serve as sources for HCV transmission [6].

Since exposure to blood and contaminated items is common in dialysis facilities, precautions to prevent HCV transmission in this setting include wearing gloves for patient care and equipment handling; restricting the use of common supplies, instruments, and medications for multiple patients; and not using storage or distribution carts within treatment areas. Adherence to these safe practices requires adequate staffing and appropriate timing between shifts and continuing education of all staff members. That strict application of standard precautions is sufficient to avoid patient-to-patient transmission in dialysis centers was proven in a prospective, longitudinal study in Belgian patients in which a consistent reduction in annual incidence of HCV infection from 1.4% to 0.56% and then to 0% was achieved simply by reinforcing standard hygienic measures.

Organ transplantation

Organ transplant recipients are at high risk of acquiring HCV infection. Infection in this setting almost invariably occurs in HCV-infected, liver transplant recipients as a result of recurrence of HCV infection in the liver graft, but may also occur in HCV-negative transplant recipients through accidental nosocomial transmission when not strictly applying safe practices.

The risk of transmission from an anti-HCV-positive organ donor to a seronegative recipient is very high. In some studies, 90% to 100% of recipients of kidneys, liver, or heart from HCV-infected donors acquired HCV infection after transplantation. In other studies, infection rates have been reported to be much lower (approximately 50% to 60%). It is unknown whether differences in procurement or organ preservation techniques might account for these different transmission rates.

Other healthcare-associated nosocomial transmissions

Previous hospitalization has historically been shown to be an epidemiological risk factor in patients with HCV infection. Currently, despite the widespread

use of disposable injection items, improvement of disinfection techniques for nondisposable equipment, and adherence to standard infection control measures, healthcare-related infections (mostly patient-to-patient transmissions) continue to occur outside the dialysis setting (either sporadically or as small outbreaks) in countries with high sanitary standards [7].

Because the prevalence of HCV infection among hospitalized patients is rather high (between 2% and 20%, depending on the patient setting), nosocomial transmission is likely if disinfection procedures are inadequate and contaminated equipment is shared between patients. Studies of seroconverting blood donors and of patients with acute hepatitis C suggest that nosocomial transmission is not uncommon and may account for a substantial proportion of HCV infection among patients lacking a history of obvious parenteral exposure. Medical and surgical invasive procedures were the main risk factors associated with seroconversion and acute hepatitis in Italian blood donors and among patients enrolled in the Italian National Hepatitis Surveillance System. Similarly, among 214 consecutive patients diagnosed with acute hepatitis C at 12 Italian medical centers between 1999 and 2004, an invasive procedure was involved in transmission in 32% [8]. The fact that high HCV prevalence among hospitalized patients increases the risk of patient-to-patient transmission is clearly exemplified by a prospective study conducted between 2000 and 2002 at a tertiary-care liver unit in Barcelona, in which six of 1540 hospitalized patients seroconverted (incidence: 0.27 per 100 hospital admissions per year), despite the fact that the staff was fully aware of the ongoing study, and, hence, adherence to standard safety precautions should have been optimal due to the "Hawthorne effect" [4]. In a report of 103 patients with acute hepatitis C seen at different Spanish hospitals between 2000 and 2005, 70% had been infected during hospitalization [9]. In a recent French case–control study, a substantial proportion of patients with community acquired chronic hepatitis C had inhospital or outpatient treatments as the most likely risk factor for infection [10]. Hence, it seems that reference tertiary hospitals with a higher HCV prevalence are likely to concentrate a significant proportion of HCV carriers, increasing the risk of nosocomial transmission when adherence to standard safety measures is not optimal.

However, outbreaks with the highest impact have been reported in outpatient settings, where risk perception may be lower and attention to infection control practices more frequently overlooked. Such outbreaks of HCV transmission have been associated with several medical procedures, including the use of contaminated multidose vials, spring-loaded finger sticks, gastrointestinal endoscopy, and other outpatient medical procedures [7, 11].

In most of these outbreaks, investigated HCV or HBV transmission has been attributed to syringe reuse and other breaks in standard precautions, resulting in contamination of injectable medications or flush solutions, and in many cases delivery of anesthetics has been a common factor. It is likely that the misperception underlying improper handling of syringes is the erroneous belief that needles but not syringes become contaminated during injection, that intervening length of tubing or heparin locks or valves prevent contamination during IV administration, or that reusing a syringe to draw medication from a vial for a single patient and then discarding it carry no risk to subsequent patients. It must be remembered that syringe contamination invariably occurs from the negative pressure created when the contaminated needle is removed and that reuse of that syringe to draw additional medication from a vial can contaminate the remaining product within the vial. On the other hand, the fact that anesthetics are so frequently involved in viral hepatitis outbreaks may be related to the volume of the vials and characteristics of the medication. In a recent study, it was shown that HCV remained viable in propofol solution for up to 35 days at room temperature as compared to 28 days in standard cell culture medium, suggesting that the high lipid content of the propofol emulsion (soybean oil, glycerol, and egg lecithin) provides an ideal environment for HCV infectivity [12].

Knowledge of the basic concepts of injection safety and infection control, and strict adherence to standard precautions, should prevent the vast majority of nosocomial HCV infections [7]. A few examples of healthcare worker–to-patient transmission have been reported, but fortunately they appear to be very rare [13].

In developing and transitional economy countries, the nosocomial transmission of new HCV infections is a major problem because of the reuse of contaminated or inadequately sterilized syringes and needles used in medical, paramedical, and dental procedures [7], with an estimated 2.3–4.7 million new infections occurring each year. In Egypt, the treatment of endemic schistosomiasis in mass programs (discontinued in the 1980s) that frequently used unsterilized needles and syringes has led to a national HCV prevalence of more than 14%, with rates of 20–30% in young male adults. The highest reported rates of needle reuse are found in the Middle East, Southeast Asia, and the Western Pacific, and the most frequently injected medications include antibiotics, vitamins, and analgesics that could be taken orally most of the time, and that are usually administered for nonspecific symptoms. Special attention should focus on the fact that injection solutions and equipment are frequently purchased outside of the formal healthcare system, and injections are dispensed by unqualified personnel in pharmacies or marketplaces [1].

Healthcare workers (HCWs)

Occupational HCV transmission has been documented among HCWs who have sustained contaminated needlestick injuries. In prospective studies, the average risk of infection after a needlestick injury involving HCV-positive blood has been estimated at 1.8% with a range between 0.013% and 10% in different studies. Most cases are associated with hollow-bore needles and deep injuries. In one prospective study, no seroconversions were detected after 105 accidental injuries caused by suture needles or sharp objects, as compared with 1.2% after 331 needlestick injuries involving hollow-bore needles [14]. Exceptionally, transmission has been reported after mucous membrane or non-intact skin exposure to infected blood, and no cases have been associated after intact skin exposure.

Reported prevalence among general healthcare workers have ranged between 0.3% and 0.7% in the United Kingdom and are 0.8% in Germany, 1.8% in Spain, 1.97–2.2% in Italy, and 2.7% in Hungary. Hence, the prevalence of HCV infection among HCWs is not greater than that of the general population or the specific high-risk group they serve. Guidelines on the management of HCV-infected HCWs often are controversial, because despite HCWs being at low risk for HCV infection and despite transmission from HCV-infected HCWs to patients being rare, HCWs who directly perform invasive procedures, such as surgeons, are at high risk of HCV transmission. To reduce the risk of HCV transmission and to safeguard both patients' and diseased workers' health, it has been proposed that improvement of compliance with existing infection control measures should be accentuated in such high-risk situations, and those responsible for the medical surveillance of workers should judge on a case-by-case basis [15].

Intravenous drug use

IDU is one of the most efficient routes for HCV transmission, and HCV is acquired more rapidly after initiation of IDU than other viral infections during the first year. In certain European countries (the United Kingdom, Sweden, and Norway), the United States, and Australia, IDU has been the dominant mode of HCV transmission for the past 50 years, accounting for 60% to 90% of prevalent infections; but, with the virtual eradication of transfusion-associated infections and the explosive increase of IDU in Eastern Europe, IDU has also become the main transmission mechanism of HCV in Europe [3].

The efficiency of IDU in HCV transmission may be explained by the prolonged survival of infectious particles in contaminated syringes. In a recent study using a cell culture–adapted HCV clone, viable virus was found in 71% of high-void volume syringes left at room temperature for 7 days [16]. In addition to syringes, the sharing of drug injection paraphernalia other than syringes, such as "cookers" or cotton filters, has long been implicated as a cause of HCV transmission between IDUs. In a recent meta-analysis of 21 studies, syringe sharing was associated with HCV seroconversion (RR = 1.97), as was sharing drug preparation containers (RR = 2.42), filters (RR = 2.61), and rinse water (RR = 1.98), as well as "backloading," a syringe-mediated form of sharing prepared drugs (RR = 1.86). Metaregression results showed that the risk of HCV infection through shared syringes is similar to that of sharing drug preparation equipment, and both are dependent upon HCV seroprevalence in the population.

The prevalence of HCV infection among IDUs is very high in most countries. In EU countries, reported prevalence ranged from 30% to 98%, with incidence rates of 6.2 to 39.2 per 100 person years. In the United States, a sentinel surveillance reporting system showed that between 1982 and 2006, intravenous drug abuse was responsible for 32–46% of cases of acute hepatitis C acquired in the community, and global studies found prevalence of anti-HCV between 70% and 90% of intravenous drug users in the early 1990s. In a recent systematic review of anti-HCV prevalence in IDUs in 77 of the 152 countries where IDU has been reported, midpoint values ranged from 9.8% to 97.4%, with prevalence of 60–80% in 25 and >80% in 12 countries. Countries with the largest estimated population of anti-HCV-positive IDUs were China (1.6 million), Russia (1.3 million), and the United States (1.5 million). After extrapolating to all countries, it was estimated that, in 2010, 10 million IDUs were HCV infected, of whom 2.3 million lived in Eastern Europe and 2.6 million in East and Southeast Asia [17]. These data support the WHO resolution to consider IDUs a key group requiring targeted actions for viral hepatitis prevention and treatment, both of which have been found to be cost-effective [18].

Non-apparent parenteral transmission

Tattooing and acupuncture

Tattoos have been involved in HCV transmission. In a Taiwanese study, 12.6% of 87 tattooed healthy young men without other risk factors were found anti-HCV-positive as compared to 2.4% of 126 matched control subjects. Tattooing has been epidemiologically linked to HCV infection in Australia. Not exceptionally, tattoos are surrogate indicators of unconfessed intravenous drug abuse [19]. Acupuncture can be a potential risk factor when improperly sterilized needles are used together by an inexperienced acupuncturist. Acupuncture has been associated with an increased risk of HCV infection among Korean adults with chronic liver disease.

Perinatal transmission

Perinatal mother-to-child transmission of HCV is uncommon. The prevalence of HCV-infected pregnant women is 0.1% to 2.4%, which is no different than in the general age-matched population. Transmission of HCV occurs only when HCV RNA is detectable in serum at the time of delivery and may be associated with higher viral loads (above 10^6 copies per mL), although most studies were conducted when quantitative tests for HCV RNA had a narrow dynamic range. The overall rate of vertical transmission is 4% to 7% per pregnancy. Co-infection with HIV increases the rate of transmission four- to fivefold, and active IDU has also been associated with higher transmission rates. [Other factors that have been found to increase transmission rates include premature membrane rupture and invasive fetal monitoring. Although the precise timing of mother-to-infant transmission remains unknown, the facts that most infected babies take several weeks to reach detectable HCV RNA and that cord blood is usually negative suggest perinatal transmission during delivery rather than intrauterine infection. Cesarean section, however, has no effect on transmission and is not recommended. Breastfeeding carries no further risk of transmission.

Sexual and household transmission

Sexual transmission of HCV is still a controversial issue. Although cross-sectional studies conducted since the early 1990s reported prevalence rates up to 28% among spouses of chronic HCV carriers that increased with duration of partnership, these initial reports were not confirmed when molecular typing became available and prospective cohort studies of heterosexual persons in long-term monogamous relationship with an HCV-infected partner provided little evidence for sexual transmission of HCV. Occasional cases of acute hepatitis C through sexual transmission in heterosexual couples (virtually all male-to-female transmissions), usually associated with obvious mucosal damage and bleeding, have been reported. The finding of HCV-specific T cell responses in female spouses of chronic carriers suggests that unapparent transmission without seroconversion may occur more frequently than serological tests would suggest and in turn might explain resistance to overt infection over time. Hence, available evidence suggests that HCV is rarely transmitted by monogamous heterosexual intercourse and that this mode of transmission has no impact on HCV-related disease burden.

Since 2000, however, outbreaks of acute hepatitis C have been reported among men who have sex with men (MSM) who are infected with HIV and denied IDU; these outbreaks have been reported from Europe, the United States, and Australia. In the United Kingdom, presence of HCV in primary HIV infection increased from 0% in 1999 to 4% in 2006, and prevalence in the Netherlands increased from 1–4% before 2000 to 15% in 2007 and 21% in 2008. Phylogenetic analysis in Europe revealed sexual HCV transmission networks among MSM with monophyletic transmission clusters in England, France, the Netherlands, and Germany and a large European MSM-specific network. Molecular clock analysis was consistent with the expansion of the MSM-specific 1a and 4d strains around 1996, when HAART was introduced, and coinciding with an increase in high-risk sexual behavior and sexually transmitted infections. Factors associated with HCV transmission exclusively among MSM infected with HIV, which increased 10-fold since 1996, include permucosal traumatic sexual activities often practiced in a group and with the use of non-injecting recreational drugs.

There is no evidence that familial household transmission of HCV does occur. Although the pooled prevalence of anti-HCV among siblings and household contacts of patients with chronic liver disease has been reported to be higher than that of relatives of uninfected individuals (4% compared to 0% among contacts of anti-HCV-negative controls) and that of offspring of Japanese HCV-infected chronic liver disease patients was 17% compared to 10.4% of controls, these findings may reflect only familial clustering of percutaneous risk factors rather than intrafamilial nonpercutaneous transmission.

Global prevalence of HCV infection among the general population

Global prevalence of HCV should be based on community-based studies; however, in most countries, studies have been performed in specific groups not representative of the general population, such as blood donors, drug users, pregnant women, or groups with specific sexual behaviors. Indeed, vulnerable groups such as IDUs, migrants, homeless persons, and prisoners tend to be underrepresented in general population studies, especially in low-prevalence countries. Therefore, the global estimates for HCV prevalence are still associated with some uncertainty, and the true prevalence may be underestimated [20, 2]. However, there is a need to perform accurate estimations using representative population samples to evaluate future education, prevention, and control activities.

A recompilation of the global prevalence of HCV infection in the general population using community-based studies and/or extrapolations to neighboring countries (when information was not reported) is shown in Table 17.1, and it is expanded from country to country (234 countries and territories) in Table 17.3 and repre-

Table 17.3 Estimated HCV prevalence among the general population by countries.

	Flag	Country	Population, 2011 **(census or official estimates)**	Anti-HCV (%)	Number of infected estimates, January 2012	Reference
AS		Afghanistan	32 358 000	0.5	161 790	[1]
EU		Albania	3 216 000	1.5	48 240	[1]
AF		Algeria	36 300 000	0.2	72 600	[1]
EU		Andorra	68 900	2.5	1 723	As Spain
AF		Angola	19 618 000	5	980 900	[1]
AM		Anguilla (UK)	15 236	0.75	114	As Caribbean
AM		Antigua and Barbuda	89 138	0.75	669	[1]
AM		Argentina	40 765 000	2	815 300	[27]
AS		Armenia	3 266 300	4	130 652	[1]
AM		Aruba (the Netherlands)	108 000	0.75	810	As Caribbean
O		Australia	22 606 000	1.3	293 878	[21]
EU		Austria	8 413 000	1	84 130	[1]
AS		Azerbaijan	9 306 000	4	372 240	[1]
AM		Bahamas	347 000	0.75	2 603	[1]
ME		Bahrain	1 324 000	1.8	23 832	[1]
AS		Bangladesh	150 494 000	0.6	902 964	[1]
AM		Barbados	274 000	0.75	2 055	[1]
EU		Belarus	9 559 000	2.2	210 298	[1]
EU		Belgium	10 754 000	0.9	96 786	[1]
AM		Belize	318 000	0.75	2 385	[1]
AF		Benin	9 100 000	1.6	145 600	[1, 29]
AM		Bermuda (UK)	65 000	0.75	488	As Caribbean
AS		Bhutan	738 000	1.3	9 594	[1]
AM		Bolivia	10 088 000	4.7	474 136	[1]
EU		Bosnia and Herzegovina	3 752 000	1.5	56 280	[1]
AF		Botswana	2 031 000	1.6	32 496	[1]
AM		Brazil	196 655 000	1.5	2 949 825	[27]
AM		British Virgin Islands (UK)	23 000	0.75	173	As Caribbean
AS		Brunei	406 000	2.9	11 774	[1]
EU		Bulgaria	7 446 000	1.8	134 028	[1]
AF		Burkina Faso	16 968 000	5.2	882 336	[1]
AF		Burundi	8 575 000	11.3	968 975	[1, 29]
AS		Cambodia	14 305 000	4.1	586 505	[1]
AF		Cameroon	20 030 000	13.8	2 764 140	[1, 29]
AM		Canada	34 350 000	0.82	281 670	[23]
AF		Cape Verde	501 000	3	15 030	[1]
AM		Cayman Islands (UK)	57 000	0.75	428	As Caribbean
AF		Central Africa Republic	4 487 000	2.4	107 688	[1, 29]
AF		Chad	11 525 000	4.8	553 200	[35, 29]
AF		Chile	17 227 000	0.85	146 430	[1]
AS		China	1 347 565 000	1.9	25 603 735	[21]
AM		Colombia	46 927 000	0.97	455 192	[1]

(*Continued*)

Table 17.3 (*Continued*)

	Flag	Country	Population, 2011 **(census or official estimates)**	Anti-HCV (%)	Number of infected estimates, January 2012	Reference
AF		Comoros	754 000	1	7 540	[1]
AF		Republic of the Congo (Congo-Brazzaville)	4 140 000	6.4	264 960	[35]
O		Cook Islands	20 000	1.5	300	[1]
AM		Costa Rica	4 727 000	0.75	35 453	[1]
AF		Côte d'Ivoire	20 153 000	3.3	665 049	[1, 29]
EU		Croatia	4 396 000	1.5	65 940	[1]
AM		Cuba	11 241 161	1.8	202 341	[1]
AM		Curaçao (the Netherlands)	142 180	0.75	1 066	As Caribbean and Venezuela
ME		Cyprus	1 117 000	0.5	5 585	[1]
EU		Czech Republic	10 534 000	1.5	158 010	[20]
AF		Democratic Republic of Congo (Kinshasa ex Zaire)	67 758 000	5.5	3 726 690	[29]
EU		Denmark	5 573 000	0.5	27 865	[1]
AF		Djibouti	906 000	0.3	2 718	[1, 29]
AM		Dominica	68 000	0.75	510	[1]
AM		Dominican Republic	10 056 000	0.75	75 420	[1]
AM		Ecuador	14 666 000	1.4	205 324	[1]
ME		Egypt	82 537 000	14.9	12 298 013	[21, 32]
AM		El Salvador	6 227 000	2.5	155 675	[1]
AF		Equatorial Guinea	720 000	1.7	12 240	[1, 29]
AF		Eritrea	5 415 000	1.9	102 885	[1, 29]
EU		Estonia	1 341 000	5	67 050	[1]
AF		Ethiopia	84 734 000	1.9	1 609 946	[1, 29]
AM		Falkland Islands (UK)	3 000	0.75	23	Close to Argentina; estimate intermediate United Kingdom
EU		Faroe Islands (Denmark)	49 000	0.75	368	North of Scotland, estimate UK
O		Federal States of Micronesia	112 000	2	2 240	[1]
O		Fiji	868 000	2	17 360	[1]
EU		Finland	5 385 000	0.5	26 925	[1]
EU		France	63 126 000	0.84	530 258	[20]
AM		French Guiana	237 000	0.75	1 778	As Caribbean/Brazil
O		French Polynesia	274 000	2	5 480	As Fiji and Micronesia
AF		Gabon	1 534 000	9.2	141 128	[1, 29]
AF		Gambia	1 776 000	2.4	42 624	[1, 29]
AS		Georgia	4 329 000	6.7	290 043	[1]
EU		Germany	82 163 000	0.4	328 652	[20]
AF		Ghana	24 966 000	1.7	424 422	[1, 29]
EU		Gibraltar (UK)	29 441	2.5	736	As Spain
EU		Greece	11 390 000	1.5	170 850	[1].
AM		Greenland (Denmark)	56 890	0.5	284	[1]
AM		Grenada	105 000	5	5 250	[1]
AM		Guadalupe (France)	463 000	0.75	3 473	As Caribbean

Table 17.3 (*Continued*)

	Flag	Country	Population, 2011 **(census or official estimates)**	Anti-HCV (%)	Number of infected estimates, January 2012	Reference
AS		Guam (USA)	182 000	2	3 640	As Philippines
AM		Guatemala	14 757 000	0.8	118 056	[1]
EU		Guernsey (UK)	62 431	0.75	468	Between UK and France., estimate UK
AF		Guinea	10 222 000	5.5	562 210	[1, 29]
AF		Guinea-Bissau	1 547 000	4.7	72 709	[1]
AM		Guyana	756 000	0.75	5 670	[1]
AM		Haiti	10 124 000	4.4	445 456	[1]
AM		Honduras	7 755 000	0.5	38 775	[1]
AS		Hong Kong (China)	7 122 000	1	71 220	As mean of China
EU		Hungary	9 966 000	0.6	59 796	[20]
EU		Iceland	324 000	0.2	648	[36]
AS		India	1 241 492 000	1.5	18 622 380	[1, 21]
AS		Indonesia	242 326 000	3.9	9 450 714	[1]
ME		Iran	74 799 000	0.9	673 191	[30, 1]
ME		Iraq	32 105 000	3.21	1 030 571	[1]
EU		Ireland	4 526 000	1.1	49 786	[1]
EU		Ille of Man (UK)	83 000	0.75	623	In between UK and Ireland
ME		Israel	7 562 000	1.5	113 430	[1]
EU		Italy	60 789 000	3.2	3 161 028	[20]
AM		Jamaica	2 751 000	0.75	20 633	[1]
AS		Japan	126 497 000	2.4	3 035 928	[1]
EU		Jersey (UK)	92 500	0.75	694	In between UK/Ireland
ME		Jordan	6 330 000	2.1	132 930	[1]
AS		Kazakhstan	16 207 000	3.2	518 624	[1]
AF		Kenya	41 610 000	0.9	374 490	[1, 29]
O		Kiribati	101 000	2	2 020	[1]
ME		Kuwait	2 818 000	3.1	87 358	[1]
AS		Kyrgyzstan	5 393 000	4	215 720	[1]
AS		Laos	6 288 000	1.1	69 168	[1]
EU		Latvia	2 243 000	2.2	49 346	[1]
ME		Lebanon	4 259 000	0.7	29 813	[1]
AF		Lesotho	2 194 000	1	21 940	[1]
AF		Liberia	4 129 000	3	123 870	[1]
AF		Libya	6 423 000	7.9	507 417	[35, 37]
EU		Liechteinstein	36 157	0.75	271	Low European mean
EU		Lithuania	3 307 000	2.2	72 754	[1]
EU		Luxembourg	516 000	1	5 160	[1]
AS		Macau (China)	556 000	1	5 560	As Hong Kong
EU		Macedonia	2 064 000	2	41 280	[1]
AF		Madagascar	21 315 000	2.1	447 615	[35, 29]
AF		Malawi	15 381 000	6.8	1 045 908	[35, 1]
AS		Malaysia	28 859 000	1.5	432 885	[1]

(*Continued*)

Table 17.3 (*Continued*)

	Flag	Country	Population, 2011 **(census or official estimates)**	Anti-HCV (%)	Number of infected estimates, January 2012	Reference
AS		Maldives	320 000	1	3 200	[1]
AF		Mali	15 840 000	3.3	522 720	[1]
EU		Malta	418 000	1	4 180	[1]
O		Marshall Islands	55 000	1.5	825	[1]
AM		Martinique (France)	407 000	0.75	3 053	As Caribbean (0.75%)
AF		Mauritania	3 542 000	1.1	38 962	[1, 29, 35]
AF		Mauritius	1 307 000	2.1	27 447	[1]
AF		Mayotte (France)	211 000	1	2 110	As Mauritania
AM		Mexico	114 793 000	1.4	1 607 102	[27]
EU		Moldova	3 545 000	2.3	81 535	[1]
EU		Monaco	35 881	1	359	As France but close to Italy
AS		Mongolia	2 800 000	10.7	299 600	[1]
EU		Montenegro	632 000	1.5	9 480	[1]
AM		Montserrat (UK)	6 000	0.75	45	As Caribbean
AF		Morocco	32 273 000	1.93	622 869	[1]
AF		Mozambique	23 930 000	3.2	765 760	[1, 35]
AS		Myanmar	48 337 000	1	483 370	[1]
AF		Namibia	2 324 000	0.9	20 916	[1, 35]
O		Nauru	10 000	2	200	[1]
AS		Nepal	30 486 000	0.64	195 110	[1]
O		New Caledonia (France)	255 000	1.3	3 315	As Australia
O		New Zealand	4 415 000	0.3	13 245	[1, 22]
AM		Nicaragua	5 870 000	0.35	20 545	[1]
AF		Niger	16 069 000	3.2	514 208	[1]
AF		Nigeria	162 471 000	2.1	3 411 891	[1, 29]
O		Niue (NZ)	1 000	2	20	[1]
AS		North Korea	24 451 000	1	244 510	[1]
O		Northern Mariana Islands (USA)	61 000	1.3	793	as Australia
EU		Norway	4 925 000	0.7	34 475	[20]
ME		Oman	2 846 000	1.2	34 152	[1]
ME		Pakistan	176 745 000	5.9	10 427 955	[1, 21]
O		Palau	21 000	2	420	[1]
ME		Palestinian territories (West Bank+Gaza strip)	4 152 000	2.2	91 344	[30]
AM		Panama	3 571 000	0.75	26 783	[1]
O		Papua New Guinea	7 014 000	2	140 280	[1]
AM		Paraguay	6 568 000	1.23	80 786	[1]
AM		Peru	29 400 000	1	294 000	[27]
AS		Philippines	94 852 000	2.2	2 086 744	[1]
EU		Poland	38 299 000	1.9	727 681	[20]
EU		Portugal	10 690 000	1.5	160 350	[20]
AM		Puerto Rico	3 746 000	2.3	86 158	[27]
ME		Qatar	1 870 000	6.3	117 810	[30]

Table 17.3 (*Continued*)

	Flag	Country	Population, 2011 **(census or official estimates)**	Anti-HCV (%)	Number of infected estimates, January 2012	Reference
AF		Reunion (France)	856 000	0.14	1 198	[1]
AF		Republic of the Sudan	36 370 000	2.2	800 140	[30]
EU		Romania	21 436 000	3.5	750 260	[20]
EU		Russia	142 836 000	2.5	3 570 900	[20]
AF		Rwanda	10 943 000	4.9	536 207	[1]
AM		Saint Barthelemy (France)	8823	0.75	66	As Caribbean (0.75%)
AM		Saint Helena, Ascension and Tristan da Cunha (UK)	4255	0.75	32	Estimate UK
AM		St Martin (France)	37 163	0.75	279	As Caribbean
AM		St Pierre and Miquelon (France)	6290	0.75	47	Near Canada
EU		San Marino	32 093	2.6	834	North Italy
O		Samoa	184 032	0.75	1 380	[1]
AF		Sao Tome and Principe	169 000	10	16 900	[1]
ME		Saudi Arabia	28 083 000	1.8	505 494	[1, 21]
AF		Senegal	12 768 000	3	383 040	[1]
EU		Serbia	9 854 000	1.5	147 810	[1]
AF		Seychelles	87 000	0.34	296	[1]
AF		Sierra Leone	5 997 000	2	119 940	[1, 35]
AS		Singapore	5 188 000	1	51 880	[1]
EU		Slovakia	5 472 000	1	54 720	[1]
EU		Slovenia	2 035 000	1	20 350	[1]
O		Solomon Islands	550 000	2	11 000	[1]
AF		Somalia	9 557 000	1	95 570	[1]
AF		South Africa	50 460 000	1.7	857 820	[1]
AS		South Korea	48 219 000	1.29	622 025	[21]
AF		South Sudan	8 300 000	2.2	182 600	As Republic of Sudan
EU		Spain	47 190 493	2.5	1 179 762	[3, 38]
AS		Sri Lanka	21 045 000	1	210 450	[1]
AM		St Kitts and Nevis	53 000	2.2	1 166	[1]
AM		St Lucia	176 000	0.75	1 320	[1]
AM		St Vincent and the Grenadines	109 000	1	1 090	[1]
AM		Suriname	529 000	0.75	3 968	[1]
AF		Swaziland	1 203 000	1.5	18 045	[1, 29, 35]
EU		Sweden	9 441 000	0.59	55 702	[20]
EU		Switzerland	7 702 000	1.75	134 785	[20]
ME		Syria	20 766 000	1.9	394 554	[30]
AS		Taiwan	23 197 947	4.4	1 020 710	[21]
AS		Tajikistan	6 977 000	4	279 080	[1]
AF		Tanzania	46 218 000	3.2	1 478 976	[1, 29, 35]
AS		Thailand	69 519 000	2.8	1 946 532	[22]

(*Continued*)

Table 17.3 (*Continued*)

	Flag	Country	Population, 2011 **(census or official estimates)**	Anti-HCV (%)	Number of infected estimates, January 2012	Reference
EU		The Netherlands	16 665 000	1	166 650	[1]
AS		Timor-Leste	1 154 000	3.9	45 006	As Indonesia
AF		Togo	6 155 000	3.3	203 115	[1, 35]
O		Tokelau (NZ)	1 000	2	20	As Tonga
O		Tonga	105 000	2	2 100	[1]
AM		Trinidad and Tobago	1 346 000	3.9	52 494	[1]
AF		Tunisia	10 594 000	1.2	127 128	[1]
ME		Turkey	73 640 000	2.1	1 546 440	[30]
AS		Turkmenistan	5 105 000	4	204 200	[1]
AM		Turks and Caicos Islands (UK)	39 000	0.75	293	As Caribbean
O		Tuvalu	10 000	2	200	[1]
AF		Uganda	34 509 000	6.6	2 277 594	[1, 29]
EU		Ukraine	45 190 000	4	1 807 600	[1]
ME		United Arab Emirates	7 891 000	2.3	181 493	[1]
EU		United Kingdom	62 417 000	1.1	686 587	[1]
AM		United States of America	313 085 000	1.68	5 259 828	[25, 39]
AM		United States Virgin Islands	109 000	0.75	818	As Caribbean
AM		Uruguay	3 380 000	1	33 800	[1]
AS		Uzbekistan	27 760 000	6.5	1 804 400	[1]
O		Vanuatu	246 000	2	4 920	[1]
EU		Vatican city	800	2.6	21	As Northern Italy
AM		Venezuela	29 437 000	0.95	279 652	[27]
AS		Vietnam	88 792 000	2	1 775 840	[21]
O		Wallis and Futuna (France)	13 445	2	269	As Tonga
AF		Western Sahara	548 000	3	16 440	[1]
O		Western Samoa	70 000	2	1 400	[1]
ME		Yemen	24 800 000	1.7	421 600	[1, 30]
AF		Zambia	13 475 000	1.5	202 125	[1]
AF		Zimbabwe	12 754 000	2	255 080	[1, 29]
			6 974 415 556	**2.27**	**158 388 657**	

World population according to medium variant based on WUP Revision 2011. AF: Africa; AM: Americas; AS: Asia; EU: Europe; ME: Middle East; O: Oceania. From [34]. Corrected by oficial census when possible.

sented in Figure 17.1. Prevalence data differences and dynamics within and between countries may sometimes be explained by local and regional variances in transmission routes or different public health measures.

Epidemiological trends

Prevalence estimates are 500 000 chronically infected subjects in Oceania, 14 million in the Americas and in Europe, 28 million in the Middle East, 30 million in Africa, and 71 million in Asia (Table 17.1).

Oceania

Oceania has the lowest mean HCV prevalence of 1.36%, or 501 665 infected individuals. In Australia, because of the elimination of transmission via blood supply, IDU and immigration from endemic countries have been

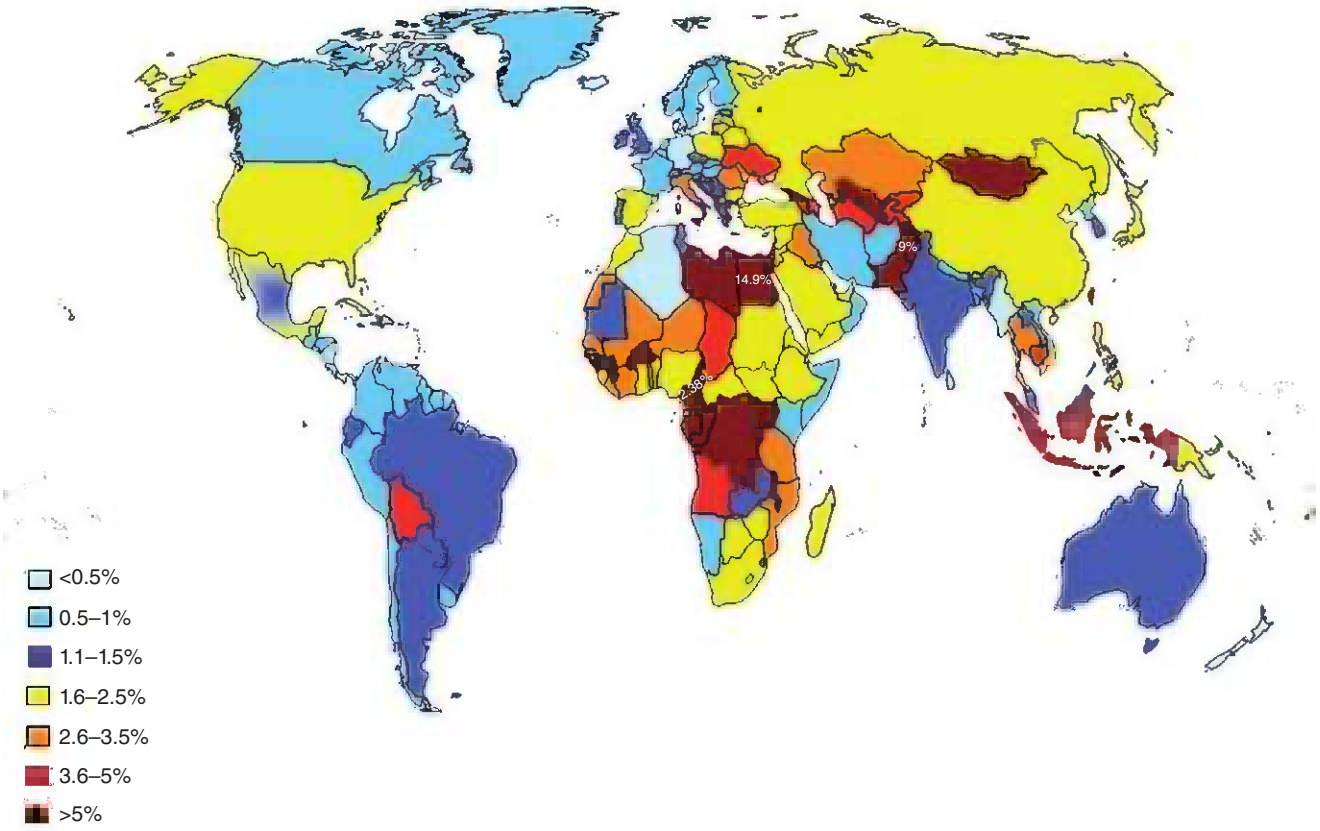

Figure 17.1 Hepatitis C virus (HCV) prevalence (%) in the general population. (Color plate 17.1)

identified as the main risk factors of HCV transmission. The peak of prevalence was observed in 1999 at 2.3%, but the latest study from 2005 reported a drop to 1.3%, consistent with a reduction in IDU. The sequela of this peak of transmission was that the highest prevalence was observed in individuals in their 20s and 40s, and genotype 3 was the most prevalent in this cohort in concordance with the evidence that HCV genotype 3a is currently the most prevalent genotype in IDU patients, while 1b is identified more frequently among patients with transfusion-acquired HCV. Prevalence in New Zealand was 0.3%, with genotype 3 being the most prevalent. The genotype distribution in Australia and New Zealand was 52% of genotype 1 (14% G1, 15% G1a, and 23% G1b), 9% of G2, 32% of G3, 5% of G4, 2% of G6, and 1% mixed.

At present, IDU continues to be the main driver of HCV infection in Oceania, which had a relatively high proportion of G3-infected individuals compared with other developed countries [21, 22].

Americas

Globally, the Americas have a mean HCV prevalence of 1.48%, or 13 996 957 infected individuals. High variation is observed between countries, regions, and populations. Indeed, Aboriginal North American natives are disproportionately affected by chronic liver disease (CLD) with high disparities on HCV seroprevalence. US Native Americans (South Dakota residents, Navajos, and those in urban and veterans populations) have a seroprevalence from 3% to 32%, while US Alaska Natives have a prevalence of 0.8%. In the case of Canadian Aborigines (Manitoba, British Columbia, and Inuit), Inuit have a prevalence of 1.1% while non-Inuit have 2–20% and Native Greenlanders (Inuit) have lower than 1% [23].

In the United States, the rate of new HCV infections is decreasing, yet the number of infected people with complications of the disease is increasing, especially in the cohort of people born between 1945 and 1964 (baby boomers) [24]. The total HCV-infected population in the United States was estimated to decline 24%, from 3.15 million in 2005 to 2.47 million in 2021, while disease burden will increase as the remaining infected population ages and the mortality rate is forecast to increase from 2.1% to 3.1% [25]. Besides, ethnic groups have disparate behavior to HCV infection, and it has been reported that African Americans not only have lower response to anti-HCV therapy but also are less likely to

clear HCV naturally, potentially contributing to higher prevalence of HCV. Genotype distribution in the United States shows a major prevalence of G1 (73.7%) followed by G2 (14.9%), G3 (7.4%), and others (4%) [26].

Latin American countries have low or moderate HCV prevalence. In contrast to Australia, the United States, or Europe, IDU is not a large problem except for in northern Mexico. This suggest that other risk factors play major roles in new infections, such as nosocomial infections, non-IDU forms of injection such as vitamin shots, inadequate needle disposal, dental procedures, tattooing, cosmetic procedures, or other situations involving contact with infected blood.

In Central America, prevalence ranges from 0.35% in Nicaragua to 2.5% in El Salvador. In Mexico, the reported mean prevalence is 1.4%, but with a heterogeneous prevalence of 2.0% in the north section of the country, 1.5% in the south, and 1.1% in central Mexico, being mainly infected from contaminated blood (64–72%) and IDU (2.5%). The most prevalent genotype is G1 (70.36%), followed by G2 (21.77%) and G3 (7.18%), with subtype G1b being the most prevalent (32–41%).

In South America, prevalence ranges from 1% to 2%, except for Bolivia with a reported prevalence of 4.7%. Genotype distribution is similar in all countries, with G1 being the most prevalent. In Argentina and Brazil, G1a and G1b have similar percentages. In Venezuela the split between G1a and G1b was dependent on the population, with G1a being more common in blood donors and hemophiliacs, while G1b was more common in hemodialyzed and chronic hepatitis C patients. In Peru, G1a was the highest prevalent genotype (74%). Interestingly, G2 was the second most prevalent in Venezuela and Argentina, but minor in Brazil and Peru, probably as a consequence of the different origin and route of HCV transmission in Latin American [27].

Europe

The epidemic of hepatitis C virus (HCV) infection in Europe is continuously evolving, and epidemiological parameters (prevalence, incidence, disease transmission patterns, and genotype distribution) have changed substantially during the last 15 years [3]. The main risk for HCV transmission in countries with well-established HCV-screening programs, improved healthcare conditions, and lower HCV prevalence was IDU, which was associated with younger age at the time of infection and a higher infection rate among males. In other regions, contaminated glass syringes and nosocomial infections continue to play important roles in new infections. Immigration from endemic areas was another factor impacting the total number of infections and the genotype distribution and diversification. Hence, prevalence data from studies conducted a decade ago may not be useful to estimate the current and future burden of HCV infection, and additional epidemiological studies should be conducted, as well as new preventive strategies implemented to control the silent epidemic. Despite the eradication of transmission by blood products, HCV infection continues to be one of the leading bloodborne infections in Europe [20].

The mean prevalence of HCV in Europe is 1.87% affecting 13 828 253 individuals; it varies from 0.2% in Iceland to over 2–3% in Greece, Bulgaria, Moldova, and central and southern Italy and reaches the top in Ukraine, some areas of Italy, Romania, and Estonia (3.2–5%) [2, 3, 20].

Despite the limited number of population-based studies on the age-specific prevalence of infection, three patterns of HCV transmission have been recognized in Europe. In Northern Europe, the epidemic was mainly transmitted by IDU (accounting for more than half of HCV infected patients, i.e., Norway 67%, Sweden 65%, and the United Kingdom 90%), with an overall prevalence between 0.2% and 1%, and with the highest prevalence among adults (30–50 years old). In Central Europe, HCV prevalence is intermediate, ranging from 0.4% in Germany to 1.75% in Switzerland. In Southern Europe, the overall prevalence ranges between 1% in Malta and 3.2% in Italy. In these southern countries, an initial epidemic of iatrogenic nature, occurring >50 years ago, led to a high infection prevalence in older people, followed, some 30 years later, by a still-ongoing IDU-related epidemic that spread the infection among younger people. The result is that in isolated areas in Italy and Greece, prevalence reaches 7% to 20% of the general adult population. Using epidemiological data and molecular evolutionary methods, some research groups showed that the spread of genotype 1b in Spain and France, and that of genotype 3a in the former Soviet Union, coincided with local outbreaks of unsafe parenteral treatments.

Most Eastern European countries have prevalence between 1% and 2.5%, but Romania and Estonia report 3.5%, and 5% respectively. In former communist countries such as Poland, Czech Republic, and Russia, IDU was less frequent, and nosocomial transmission (inhospital diagnostic or treatment procedures) appeared to play a major role in HCV infection (40% to 70% of prevalent cases). At present, HCV incidence is still increasing in Eastern European countries, mainly because of an increase in IDU; for instance, in a 2006 study from the Czech Republic, HCV G1a and G3 prevalence increased, respectively, from 23% and 3% to 40.5% and 23.5%. In Russia, there has been an increase in HCV incidence over the last 10 years, especially in adults aged 20–40, and some studies report a high prevalence of HCV G3 infection. Without effective prevention measures, HCV transmission occurs rapidly within the IDU population. Thus, considering the increasing numbers of those

engaging in IDU, there is an unmet need for effective prevention measures within the IDU population, especially in Eastern Europe. In fact, awareness of HCV infection is one of the most important prevention measures that caused a decline in newly acquired HCV infections in Western countries such as France; in France, where effective needle share programs were instituted between 1994 and 2004, IDU as a risk factor for newly diagnosed HCV patients declined from 51% in 1997 to 31% in 2003. The consequence of access to treatment and reduction of transmission has been a decline of prevalence in France from 1.05% to 0.85%. In Germany, the use of Methadone substitute programs for IDUs and increasing numbers of effectively treated patients have caused a yearly decline of approximately 10% per year [20].

Italy has a particularly south-to-north prevalence gradient, with very high prevalence in south (7.3%) and central (6.1%) Italy, while the north has a prevalence of 2.6% [28]. The very high prevalence in the south is explained mainly because of intravenous therapies with contaminated glass multi-use syringes.

In Spain (mean prevalence of 2.5%), hospital admission was among the most frequently identified transmission routes in multiple studies. The pattern of prevalence, increasing with age and no gender difference, indicates a historical basis for the transmission of HCV, as observed in Spain and many other countries. However, a high rate of G3 detected in Spain and some urban areas in Italy implicates IDU as the primary risk factor of the future. In those areas in which nosocomial infections are frequent, it is essential to implement high-quality sanitation protocols concerning equipment sterilization, washing, and other hygiene measures to prevent further HCV transmission. However, even in countries such as Germany with lower HCV prevalence, occupational HCV transmission occurs [3].

Countries such as Greece, Turkey, and Romania with intermediate-to-high HCV prevalence may face different problems, especially in rural areas of the country in which IDU is not yet the main cause for HCV transmission, but limited medical care is. Contaminated syringes account for the majority of new HCV infections, resulting in high HCV prevalence rates (i.e., up to 10.9% reported for Crete and 7% for rural areas in Romania). The main HCV genotype in those patients is G1b. It is interesting to note that HCV G1a is more frequent among Turks living abroad (60%), with IDU being the main risk factor, while patients living in Turkey have a higher prevalence of HCV G1b (90%).

Another important issue in Europe related to prevalence is immigration from high-endemic countries that will lead to an increase in the transmission of infectious disease. Immigration is a major issue in Israel, as 70% of current HCV-infected individuals were born in the former Soviet Union. In Switzerland, about one-third of the infected population was not born in Switzerland. Similarly, a large study from Germany reported that 37% of HCV patients were not from Germany; most of the patients were German repatriates returning from the former Soviet Union. In the United Kingdom, immigration in particular from countries with historical links and high HCV endemicity (India and Pakistan) has a major impact on HCV prevalence. Immigration will also lead to a change in HCV genotype distribution. An example is a high prevalence of HCV G4 in Greece because of immigration from Africa.

In the early 1990s, HCV genotypes 1, 2, and 3 accounted for most infections in blood donors and patients in Europe. After G1, the next most prevalent was G3a, except for southern Italy in which G2c accounted for 25% to 30% of infections among older adults. G4 infections were found at low frequencies (4–6%) in Southern Europe. It was already known that genotype distribution was associated with the mode of transmission, with subtypes 1a, 3a, and 4 being mostly IDU-related and genotypes 1b and 2 being associated with blood transfusion and unsafe medical procedures. Consistent with this suggestion, HCV genotypes 1a and 3 are more frequent in countries where IDU is the main reason for HCV infection Recent studies have reported that HCV genotype 5a, once believed to be restricted to South Africa, had been endemic for a long time in isolated areas of central France and west Flanders [3].

Africa

The overall HCV prevalence in Africa is 3.13%, ranging from 0.2% in Algeria to 13.8% in Cameroon, and infecting a total of 30 178 423 individuals. Africa can be divided into four parts: the Maghreb (Northwest); West Sub-Saharan Africa, including 15 countries from the Atlantic Ocean to the borders of Cameroon and Chad; the Central African region, which includes 13 countries (Libya, Cameroon, Chad, Republic of Sudan, South Sudan, Republic of the Congo, Equatorial Guinea, Democratic Republic of the Congo [formerly Zaire], the Central African Republic, Uganda, Rwanda, Burundi, and Angola); and the Southern and East Africa region, including 16 countries bordering the Indian Ocean and the Atlantic South.

The Maghreb (Northwest) and Southern and East Africa have the lowest prevalence values, being in most cases below 2%. In the Maghreb, prevalence ranges between 0.2% and 1.93%, except for West Sahara with 3%. In Southern and East Africa and the Indian Ocean Islands, prevalence is mostly close to or below 2% except for Tanzania (3.2%), Mozambique (3.7%), and Malawi (6.8%). A particularity of South Africa is that

HCV genotype 5 has been isolated almost exclusively in this country.

West Sub-Saharan Africa has moderate to high prevalence. Most of the countries have prevalence higher than 2%, and the highest is Guinea with 5.5%.

The highest HCV prevalence rates are found in Central Africa, concurrent with HIV epidemic. Except for Equatorial Guinea (a previous Spanish colony) with 1.7% and the Central African Republic with 2.4%, very high HCV prevalence is reported in these countries, in which Cameroon, the best studied in this area, appears to have the highest prevalence (13.8%), followed by Burundi 11.3%, Sao Tome and Principe 10%, Gabon 9.2%, and Libya 7.9%. The rest of the countries had very high prevalence between 4.8% and 6.6%.

The substantial differences in the prevalence of HCV in Africa, from 0.2% to 13.8%, and the history of mass injecting campaigns, such as the treatment for trypanosomiasis between 1920 and 1960 in Central Africa, have focused attention on unsterile injecting and contaminated medical procedures and equipment as a potential factor in the epidemiology of HCV in the general population of Africa. Other causes are the absence of consistent screening of blood donors in many areas except South Africa and Zimbabwe. IDU is not a major problem, but there are early indications of its appearance in the largest cities of Nigeria, Kenya, and South Africa [29].

Middle East

The Middle East is a geographical region of Africa–Eurasia with no clear definition; it includes Bahrain, Cyprus, Egypt, Iran, Iraq, Israel, Jordan, Kuwait, Lebanon, Oman, the western part of Pakistan, the Palestinian territories (West Bank and the Gaza Strip), Qatar, Saudi Arabia, Syria, Turkey, the United Arab Emirates, and Yemen [30].

Estimations indicate that there are at least 28 million HCV carriers in the Middle Eastern countries, which is higher than the number of carriers estimated in the Americas and Europe combined. HCV prevalence ranges between 0.7% in Lebanon to the highest reported in the world, 14.9% in Egypt. Most of the other Middle Eastern countries have low to moderate (close to or below 2%) prevalence, except for Iraq (3.21%), Kuwait (3.1%), Pakistan (5.9%), and Qatar (6.3%). High prevalence disparities have been observed when comparing subpopulations in the same country: for instance, the prevalence in Kuwait first-blood donors is 0.8% for Kuwaiti nationals and 5.4% for non-Kuwaiti Arabs; in Jordan, the differences are between 0.65% and 6.5% depending on the subpopulation studied; and differences are noted when comparing geographic areas like the Nile River, which has become an endemic area for HCV in Egypt. Extreme variances existed in Pakistan with countrywide prevalence estimated between 2.4% and 6.5%, and with reported rates up to 30% in the region of Punjab, associated with reuse of syringes for antibiotics, vitamins, and drugs and mainly acquired in hospitals, suggesting nosocomial infections as the primary source of transmission [21].

As noted, Egypt has the highest HCV prevalence in the world (nearly 15% of the population). In Egypt, schistosomiasis was traditionally the most important public health problem, and infection with *Schistosoma mansoni* was the major cause of liver disease. To control the schistosomiasis epidemic, the Egyptian Ministry of Health undertook large control campaigns from the 1950s until the 1980s using intravenous tartar emetic, and indirectly caused an HCV spread through needle reuse, establishing a very large reservoir of HCV in the country. By the mid-1980s, the effective oral drug praziquantel replaced tartar emetic as treatment for schistosomiasis in the entire country. This both reduced schistosomal transmission and disease and interrupted the HCV epidemic. Consequently, the older generations have a higher HCV prevalence than younger ones Geographically, the lowest rates are in Cairo and Alexandria (<8%), the highest are in rural areas of the Nile Delta (Lower Egypt >15%), and rates are intermediate (8–16%) in rural areas along the Nile south of Cairo (Middle and Upper Egypt). Recent modeling data have also revealed a continuing trend of high incidence rates despite better blood-screening measures and better sanitization practices within hospitals. This is in part because of the large reservoir of infected individuals, which increases the potential for continued transmission [21, 31, 32].

A particularity of the Middle East is the high presence of G4. The distribution of HCV genotypes in the Middle East follows two main patterns. One is peculiar to the Arab countries, in which G4 predominates in Egypt, the Palestinian territories, Kuwait, Lebanon, Saudi Arabia, and Syria. The exception is Jordan, with G1a being the most prevalent (40%), but it also has a high proportion of G4 (26.6%). The other pattern, of G1a and G1b predominating, is characteristic of the non-Arab countries (Iran, Israel, Turkey, and Pakistan). It is noteworthy that G4 is almost exclusive (93%) in Egypt [21]. The highest genotype in Pakistan was G3, and this prevalence has been associated with reusing needles and glass syringes given for a variety of treatments in Pakistan and probably also IDU. In the northwest region of Iran, the highest genotype was G3 (83%), probably associated with IDU. South Iran shows a different epidemiological pattern with a dominance of G1a (70%). Immigration of Indian workers to Qatar or of Turkish soldiers to Cyprus may explain the patterns of infection in these countries. Unique factors in some countries related to cultural or educational situations may explain high prevalence, such as the case of Pakistan, where public shaving of

beards and other body hair is common and has been identified as a route of acquiring HCV and HBV infection [31].

Asia

The overall HCV prevalence in Asia is not the highest (1.92%), but in absolute numbers it represents almost one-half of the world's HCV-infected population (71 765 518 individuals). Mongolia has the highest prevalence (10.7%), followed by all the former Russian republics (from 3.2% of Kazakhstan to 6.7% of Georgia), Cambodia (4.1%), and Taiwan (4.4%). The lowest prevalence is found in Afghanistan (0.5%), Bangladesh (0.6%), and Nepal (0.64%). Nosocomial infection, underregulated medical procedures, blood transfusion (before screening), and IDU were identified as common risk factors in the region. Tattooing is common in Asia, and it is another risk factor that might be addressed via education of at-risk populations [21].

China and India have a moderate prevalence of 1.9% and 1.5% respectively, but numerically HCV infects 44 million people. HCV epidemiology in China is uncertain, and prevalence and genotype distribution estimates are different across the country, with a lower rate in Yenan (Southwest) and the highest in north and central China, especially Henan and Hebei, but with a clear decrease in blood donors from those pooled before 1998 (12.87%) and after (1.71%) [33]. Genotype 1 is the most prevalent (69%), and the second is G2 (13%). India has similar mean HCV prevalence (1.5%), with parenteral transmission being the most significant risk factor due to mainly IDU and reuse of syringes. As reported for other regions, IDU transmission explains the high dominance of G3 (67%) over the rest, especially in the north and central regions (G3a is 80.2%), similarly to Thailand with a high risk of IDU HCV transmission. The presence of G4 and G6 in Indian populations could indicate a spread from the Middle East or Southeast Asia. G1 was common in Northeast Asia and in China, Korea, Japan, and Taiwan, while G6 was found in Vietnam and other Southeast Asian countries.

Large epidemiological studies have been performed in Japan. Regional differences, particularly a southwest-to-northeast prevalence gradient, have been observed and replicated in studies through time, with the highest prevalence in regions located in the southwest portion of the country. Historically, HCV has been transmitted through intravenous stimulant (methamphetamine) drug abuse among the youth during and after World War II, blood transfusions from paid blood donors, and injections using contaminated syringes and needles, particularly for the treatment of the *Schistosoma japonicum* infection, which was endemic in Japan before the introduction of intravenous antimony in 1921 [21]. The pattern of age-specific prevalence rates indicates historical transmission routes using unsanitized needles and blood transfusion, similar to what has been reported in Spain, Korea, and China, among others.

Taiwan is one of the countries with a higher HCV prevalence, especially in older age, because of intravenous injections used for minor conditions as well as inadequate equipment disinfection and reuse of syringes. A large number of studies report large geographical variation in HCV prevalence, with certain areas reporting a prevalence of over 30% and with differences that range from 2.6% to 30.9% in townships and from 0% to 90.5% in the villages of Tainan County. Genotypes G1 and G2 are dominant.

In Vietnam, blood transfusion remains the predominant risk factor, as almost all blood donors are paid. Lower prevalence rates are reported in the north compared to the south, and this is attributed to the longer use of IDU by those in the south. Tattoos, a history of hospitalization, and occupations other than farming were also reported as risk factors. Vietnam has the greatest concentration of G6 patients compared to other Asian countries (south China, Laos, Thailand, and Myanmar).

Genotype distribution

Understanding the distribution of HCV genotypes, which are now classified into six major genotypes and more than 50 subtypes, is very important as part of a molecular clue for the spread of HCV. These genotypes can differ up to 30% from each other in nucleotide sequence. Depending on the HCV genotype, the length of treatment can differ. G1b is less responsive to IFNα therapy compared to G2 and G3. It is therefore important to track the different genotypes of the HCV virus.

Genotypes G1a, G1b, G2a, G2b, and G3a are distributed globally and account for the majority of HCV infections worldwide. G1b is the most common genotype worldwide.

In North America and Northern Europe, G1a is the most common subtype, followed by G2b and G3a, while the most common subtype in Southern and Eastern Europe is G1b, followed by G2 and G3 [31]. Although G2a and G2b are relatively common in North America and Europe, G2c is found commonly in northern Italy [3, 20] and in one city (Cordoba) in Argentina (50%).

Genotype 1 is predominant in Latin America, with G1a dominating in Peru and Puerto Rico, and G1b dominating in Mexico, Venezuela, and Argentina.

G3a, which is endemic in Southeast Asia, has been encountered in Europe and the United States, and recent evidence indicates that G3a is currently the most prevalent genotype in IDU patients with an increasing trend

of global dissemination. This leads to the suggestion that this subtype may have originated in Southeast Asia and was introduced to Europe and North America by travelers [31].

In North Africa, similar to the situation in Eastern and Southern Europe, HCV subtype G1b seems to predominate. In Tunisia, G1b is largely the dominant subtype (79%), followed by G1a, G2a, G2b, G3a, and G4. In Morocco, G1b is the most prevalent (47% to 48%) but had a high circulation of G2 (29% to 37%). The pattern in Tunisia is reminiscent of that in Japan, where G1b accounts for 73%, followed by G2 (20%) and G1a (2%). Genotypes other than G1, G2, and G3 are actually more common among HCV-infected people in other parts of the world.

G1b is the dominant genotype in Asia–Pacific, particularly in Japan, South Korea, China, and Taiwan. G2a and G2b are also commonly distributed, in particular in Japan, South Korea, and southern Taiwan. G3 is highly prevalent on the Indian subcontinent and in Southeast Asia, Australia, and New Zealand [21, 22].

It is interesting to note that in the Philippines, HCV subtype distribution is similar to that seen in North America and Northern Europe, where G1a is the most common, followed by G1b, G2a, and G2b [22].

Other HCV genotypes such as G4, G5, and G6 are, at present, more geographically restricted. G4 is predominantly found in the Middle East and Africa. It is almost exclusive in Egypt and has a high prevalence in other countries of Central Africa such as Gabon, Nigeria, and Cameroon, but also in Tanzania. G4 has also been detected in Southern Europe (Italy) and is rising in Europe (detected in significant proportions in Spain, Greece, Switzerland, Portugal, France, Sweden, Poland, Germany, and the United Kingdom), the United States, and southern India, reflecting immigration patterns in those areas.

Genotype 5 was limited exclusively to South Africa, where it predominates, followed by G1, G2, G3, and G4, respectively. Lately, individuals infected with G5 have been sporadically detected in other countries; this was probably caused by immigration.

Genotype 6 is predominantly found in Southeast Asia. It has been isolated in Vietnam, Hong Kong, south China, Laos, Thailand, and Myanmar. Prevalence of G6 is not clear since classification using 5′ UTR, the most used target region, is unable to distinguish from G1.

Epidemiological studies have disclosed that the prevalence of HCV genotypes has evolved with time due to independent HCV outbreaks or changes in the predominant route of transmission. G1b has been associated with disease progression and risk of HCC development. Nevertheless, whether there exists intrinsic pathogenicity of HCV genotypes remains controversial.

Summary

The prevalence of HCV infection is approximately 2.3% of the world population, resulting in a global estimate of 160 million HCV-infected persons. Epidemiological data are the basis for the development of measures to prevent new infections, HCV screening and early treatments, to reduce the overall morbidity and mortality associated with HCV infection. In order to reduce the tremendous socioeconomic impact associated with HCV infection, reliable information regarding HCV prevalence and transmission routes should be constantly reviewed, since the epidemiological status of different regions is evolving. At present, the global estimates for the prevalence of HCV are still associated with some uncertainty, since information is biased toward specific population groups and thus underestimates real prevalence. Incomplete information is reported from many countries. Many infections are still undetected, and this should be taken into account to avoid iatrogenic transmission. More studies are needed at the national, regional, and local levels to estimate the real baseline of HCV epidemic programs to evaluate future prevention, educational, and control activities for each particular case, and to prevent high-risk procedures that in some cases involve cultural and educational barriers. Thus, strategies to decrease the spread of HCV infection must include, in addition to the education programs noted in this chapter, screening of the blood supply before transfusion, avoidance of paid blood donors, and, where politically feasible, needle exchange programs to decrease the incidence of new infections among IDU populations. Besides, improvement on more effective and globally available treatments will help reduce or elongate the period of chronicity and the number of patients requiring liver transplant, which has significantly increased 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 due to the lag (20 years or longer) between the onset of infection and the clinical manifestation of liver diseases.

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Chapter 18
The immune response to HCV in acute and chronic infection

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Summary

The propensity of hepatitis C virus (HCV) to cause chronic infection and ongoing liver disease represents a challenge to the immunologist and hepatologist alike. Critically the study of HCV has been hampered by the difficulty in studying individuals with acute HCV infection. In particular, many individuals may be asymptomatic or may not engage with healthcare facilities for social reasons. Currently, the only faithful animal model is the common chimpanzee, and researchers now have reduced access due to financial and political reasons. However, work is at hand to generate small-animal models by genetically manipulating mice to accept human tissue and human immune cells and support HCV replication. These studies may in the future add substantially to our knowledge of immunity to HCV. However, to date our knowledge has been gained predominantly from studies of individuals with chronic HCV infection. This has been supplemented by studies of acute HCV infection and also the work in the common chimpanzee that lends itself to longitudinal analysis of HCV infection. This chapter summarizes our current knowledge on how the immune system responds to HCV, and how HCV manages to survive the onslaughts of the innate and adaptive immune systems.

Introduction

HCV is a small enveloped positive-strand RNA virus; entry into the human body is via the parenteral route, thus avoiding the first filtering effects of the lymphoid system and initial exposure to lymph node–associated immune responses. The liver provides a uniquely tolerogenic environment in which the virus replicates. Other sites of HCV replication have also been proposed, including cells of the lymphoid system, especially B lymphocytes and cells of the monocyte or macrophage lineage including microglia. Entry into these cells may be facilitated by their possession of one or more of the receptors required for HCV entry, including CD81 and scavenger receptor B1. The presence of HCV in cells other than hepatocytes has been proposed as a mechanism for immune escape, and although HCV is associated with B cell lymphoma, there is no evidence that infection of extrahepatic sites leads to immunosuppression per se. Thus, overall, the immune response to HCV is likely to be governed predominantly by the initial hepatic infection with the consequent dichotomous outcome of clearance or chronicity. Consistent with this, individuals who have symptomatic acute infection within an associated hepatitis are more likely to clear HCV spontaneously than those who have asymptomatic infection. Thus, in the acute setting, the magnitude of the immune response may be positively correlated with the extent of inflammation and hepatitis.

The immune response to acute viral infections is characterized by a number of key interlinked processes: the initial cellular response to viral entry and replication,

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innate humoral and cellular responses, antigen processing and presentation, and adaptive humoral and cellular immune responses. In HCV infection, chronicity can be considered as the default outcome; therefore, all or most of these interlinked processes need to be optimized in order to effect clearance. Suboptimal responses may result from both host and viral factors, and in these cases there is an immunological "house of cards" in which one key element is absent and so a successful immune response is lost.

During acute HCV infection, chimpanzee studies have shown a profound induction of the type I interferon (IFN alpha [α] and IFN beta [β]) response [1]. Thus, as early as 48 hours post infection, there is an upregulation of a number of key IFN-stimulated genes, which persists until clearance but is also found in chronic HCV infection. Induction of type I IFN has also been seen in HAV infection but appears to be absent in HBV infection. Thus, it is not an inevitable hepatic response to viral infection but has some viral specificity [2]. This sets in motion a cascade of intracellular antiviral mechanisms, of paracrine effects to render neighboring cells more resistant to infection, and also signals to other components of the immune system to recruit inflammatory cells. The importance of this response to infection is demonstrated by the multiple mechanisms that HCV has evolved in order to counteract type I IFN signaling. Thus, even at the first step of infection, the virus is "working hard" to counteract the immune system.

Genetic considerations

Considerable insight into the immune response to HCV has been gleaned from genetic studies comparing individuals with different outcomes of infection. Thus, as genetic studies of resolution of HCV infection can be performed in a cross-sectional manner using DNA collected from individuals who have long resolved HCV infection, they are thus more straightforward to perform than cellular immune studies, which require accurate identification of individuals in the acute phase of infection. Genetic studies have been informative in that they can define associations and indicate productive avenues of research. However, they are rarely definitive by themselves, and functional investigations are required to define precise immunological mechanisms. Genetic studies may be especially influenced by ethnic diversity, and thus analysis of different populations for the same genetic polymorphism may give interesting insights into why outcome of infection is variable in different areas of the world.

Interferons

Consistent with observations on interferon signaling in the acute phase of HCV infection, polymorphisms in genes associated with these pathways such as 2–5 OAS, MxA, PKR, and TANK have been shown to influence the outcome of HCV infection either spontaneously or in the context of IFNα-based therapy (Figure 18.1) [3].

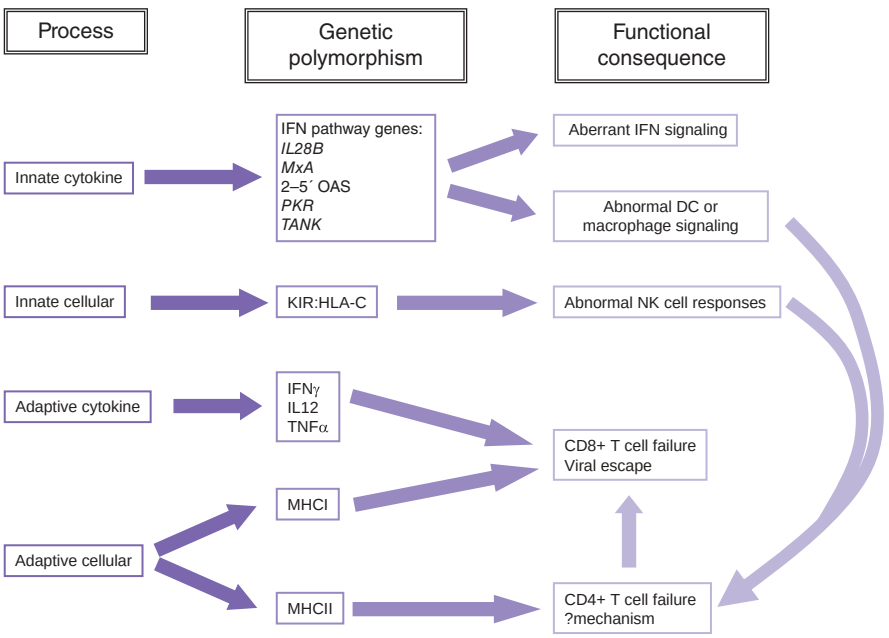

Figure 18.1 The interrelationship between genetic polymorphisms and functional abnormalities in HCV infections.

However, much interest has recently been generated in a related family of interferons, the type III interferons or interferon lambdas (IFNλs). These molecules have been relatively recently described, and their mechanisms of action and biological roles are not clearly identified. However, they share signaling pathways with the type I IFNs, with both families of IFNs signaling through JAK1, STAT1, STAT2, and Tyk2, indicating some mechanistic overlap. The strength of their association with HCV infection is profound, with four independent genomewide association studies (GWAS) from four continents demonstrating an association of the polymorphisms in the 5′ untranslated region (UTR) of the interleukin 28B (IL28B) (IFNλ3) gene with the outcome of HCV infection following treatment with pegylated interferon and ribavirin [4, 5]. The two single-nucleotide polymorphisms (SNPs) isolated were rs12979860 and rs809917, which both lie upstream of the start codon for the IL28B gene. The fundamental importance of the association of IL28B with HCV infection is demonstrated by the observation that this is found in four discrete populations and hence transcends ethnic diversity; the odds ratios of effect range up to 5, and there is an absence of discernible secondary targets in the analyses (i.e., this gene has p values of association more than 10 times smaller than the next target). Crucially, these same IL28B polymorphisms are also associated with spontaneous resolution of HCV infection, again in ethnically diverse populations including those derived from the United States, Europe, and Egypt [6]. Thus, in addition to transcending host diversity, this association transcends, at least to some extent, viral diversity, as genotype 4 HCV infection accounts for the vast majority of HCV infection in Egypt; it is relatively rare in the United States, where genotype 1 is most prevalent; and the SNP is also significant in Europe, where genotypes 1, 2, and 3 are found. Finally, it has also been shown to determine the outcome of HCV infection in individuals co-infected with HIV, and also in HCV infection following liver transplantation [7]. Thus, it is surely one of the strongest genetic associations in human immunology. Despite this extraordinary strength of association, the mechanism by which IL28B leads to clearance of HCV infection remains enigmatic. Transplantation studies have shown that both the donor and recipient IL28B genotypes contribute to the posttransplant outcome of treatment [8, 9]. This implicates both local effects due to IL28B secreted by the hepatocyte and also the potential for cells of the immune system such as macrophages or dendritic cells, the likely extrahepatic sources of this cytokine, to be contributing to this genetic effect. Based on the current pace of research in this area, one would anticipate that the riddle of IL28B and HCV will soon be solved.

Lymphocyte receptors and ligands

In addition to genetic studies identifying the role of the innate intracellular response as crucial in recovery from HCV infection, they have also identified a role of cells of the innate immune system, namely, natural killer (NK) cells. The responses of these cells are governed by a network of polymorphic immune receptors called killer-cell immunoglobulin-like receptors (KIRs). The combination of one of these KIRs (KIR2DL3) and its HLA-C ligands (specifically, the HLA-C molecules that form HLA-C group 1) is associated with both spontaneous and treatment-induced resolution of HCV infection [10, 11]. In those who spontaneously resolve HCV infection, this association was not found in those who were exposed to HCV through the receipt of blood products, only in those infected by other means, mainly intravenous drug usage and needlestick injuries. This has led to the hypothesis that NK cells are protective predominantly in those that receive relatively low inocula of HCV. This is a scenario that is reminiscent of murine cytomegalovirus infection, in which NK protection can be experimentally overcome by increasing the inoculating dose of virus.

These genes are also overrepresented in individuals who have been multiply exposed to HCV infection through high-risk behavior, but remain seronegative and aviremic – the so-called exposed uninfected population. However, the IL28B SNPs are not overrepresented in these individuals as compared to those with chronic HCV infection. These data imply that these two pathways are nonsynergistic in resolving HCV infection. Conversely the absence of protective KIR and IL28B, or the presence of the KIR (KIR2DS3) associated with a poor outcome following exposure to HCV, appears to be synergistically associated with chronic infection [12].

Major histocompatibility complex (MHC)

Adaptive immune responses are crucial to eradicate HCV infection, and these may be influenced by polymorphisms in the MHC. Classical MHC class I and class II molecules present viral peptides to T cells. In general, MHC class I presents peptides from the intracellular compartment. These peptides, 8 to 10 amino acids long, are derived from replicating virus within the hepatocyte and are displayed on the cell surface where they can be recognized by cytotoxic CD8 T cells. MHC class II is present mainly on cells of the immune system, including dendritic cells. These cells are involved in initiating the immune response to a viral infection. They sample the extracellular fluid, take up the virus, and present short viral peptides up to 20 amino acids long in the context of MHC class II. Dendritic cells are particularly impor-

tant due to their ability to express co-stimulatory molecules such as CD80 and CD86. The expression of such molecules with MHC class II is crucial to prime the naïve CD4+ T lymphocyte to mount an effective immune response. In addition, the nature of the immune response can be defined at this stage. Thus, secretion of cytokines such as IL12 induce CD4 T cells to secrete TH1-type cytokines that generate the CD8+ cytotoxic T cell response necessary for clearing infected hepatocytes. In addition to presenting viral peptides via MHC class II, dendritic cells have a unique property of cross-presentation. Thus, they are able to pick up viral particles from the extracellular fluid, process them into peptides 8–10 amino acids long, and then present them in the context of MHC class I. They can then stimulate naïve CD8+ T cells, and the co-localization of virus-specific CD8+ T cells with virus-specific CD4+ T cells can occur in the proximity of the dendritic cell, leading to the correct cytokine microenvironment for the generation of an effective antiviral T cell response. A number of genetic polymorphisms within these pathways have been shown to influence the outcome of HCV infection.

The MHC classes I and II are the most polymorphic gene system in humans, and there is also a substantial population diversity of these, which may in part account for ethnic diversity in the ability to clear HCV infection successfully. Diversity of these molecules is concentrated within the peptide-binding groove of the molecules so that different alleles present different viral peptides to the immune system. Thus, specific alleles of the MHC are associated with the outcome of HCV infection. For class I, these include HLA-A*3, HLA-B*27, and HLA-B*57, and for class II DQB1*0301 [13, 14]. While peptides derived from HCV for specific class I alleles have been clearly defined, this is less so for HLA class II.

Cytokine polymorphisms that influence adaptive immunity may affect the levels of cytokines that are secreted and thus determine the fate of infection. Polymorphisms within IFN gamma (IFNγ), tumor necrosis factor alpha (TNFα), and IL12 genetic regions have all been associated with the outcome of infection. If cytokine responses are attenuated, this may lead to the CD4+ and CD8+ T cell responses being inadequate or inappropriate and the immune response ineffective. Thus, host genetics can have a profound effect on both the magnitude and quality of the immune response to HCV infection.

Antibody responses

HCV infection is characterized by the presence of specific antibody to various viral proteins. Antibodies are generated by B lymphocytes, and it has been proposed that these cells can be infected by HCV, leading to the production of nonspecific antibody, cryoglobulins, B cell proliferation, and lymphoma. However, the majority of antibody produced is presumed to be directed against HCV.

Unfortunately, in the main, antibodies in HCV infection are directed against epitopes that are not present on the surface of the virion such as core and the nonstructural regions. Thus, they do not result in clearance of HCV from the circulation and hence are not neutralizing. Antibodies directed against the viral envelope proteins should theoretically bind to the virus and allow for clearance from the circulation. The envelope protein is the most exposed viral protein, and antibodies directed against this region of the virus are potentially protective. Indeed, in chimpanzee studies, passive transfer of neutralizing antibody directed against the envelope can provide partial protection against challenges with homologous virus, as can vaccination with envelope proteins that induce antibody [15]. However, in general, these effects can be overcome either by increasing the dose challenge or by challenge with a heterologous virus. Results obtained with a well-characterized and homogeneous group of young women infected by an HCV-contaminated anti-D immunoglobulin preparation containing the same virus inoculum (strain AD78) suggest the development of neutralizing antibodies in the early phase of infection in the majority of patients who are able to clear the infection spontaneously [16]. By contrast, a delayed induction of neutralizing antibodies was observed in patients with a chronic course of infection. This study supports an important role of an early neutralizing antibody response in HCV clearance. However, an antibody response is not a necessity for HCV resolution, as clearance is observed in the absence of neutralizing antibodies and even in hypogammaglobulinemic individuals [17].

Circumstantial evidence for a role of antibody also comes from the extensive sequence of the HCV envelope region. In individuals with chronic HCV infection, HCV circulates as a population of viral species called a "quasi-species." This diversity is driven by the rapid rate of mutation within the virus, due in part to the absence of proofreading capacity of the RNA-dependent RNA polymerase of the virus. Therefore, there are multiple copies of HCV produced within a single hepatocyte that may differ only slightly from each other. These variants are subject to both immune selection pressure and pressure related to the fitness of each individual virion to replicate, a "fitness cost." In particular, the E2 region of the virus contains a hypervariable region (HVR1) in which mutations may be well tolerated by the virus, and therefore it has a low "fitness cost"

compared to other regions of the virus. The variability of the virus at this region is driven by host antibodies that bind to specific viral variants, thus selecting them out and leaving the host with a pool of circulating virus that does not bind to potentially neutralizing antibody [18]. This enables the virus to remain one step ahead of the host, and so represents a potent mechanism of viral evasion from host immunity. Correlating the presence of this diversity with response to infection is a strong argument for the importance of antibody in determining the outcome of acute infection. Furthermore, HCV may avoid the antibody response by circulating as a complex with lipoproteins and also by using cell–cell transmission to avoid exposure to potential neutralizing Abs [19] .

Thus, it is likely that antibodies play at least a partial role in combination with cell-mediated immunity and the interferon response. However, the failure of individuals to generate a broadly neutralizing antibody, which is protective against rechallenge with heterologous strains of virus, has led to a skepticism about the potential for the development of a conventional HCV vaccine, in which individuals are immunized with virus envelope proteins, as is the case for many viral vaccines. It also led to questions of whether antibodies against HCV can be protective independent of a T cell immune response.

Dendritic cells and macrophages

These cells are crucial in shaping the immune response to infection and can determine the route that the immune response takes. Human dendritic cells (DCs) can be divided into two main lineages, myeloid and plasmacytoid. Myeloid DCs (mDCs) are the more prevalent in the peripheral blood and process antigen for presentation to T cells. Plasmacytoid dendritic cells (pDCs) are the major producers of interferons, especially type I IFNs. They may also be a source for the type III IFNs, which due to the genetic association of IL28B (IFNλ3) are currently receiving much attention. Secretion of these cytokines may have a direct antiviral effect but, more importantly, help to orchestrate a downstream immune response. Dendritic cells endocytose foreign antigens, including viral particles. Within the endocytic particle, the virus can be uncoated and expose innate immune sensors to viral products. Specifically, viral single-stranded RNA can encounter Toll-like receptor 7 (TLR7). This leads to the activation of the TLR signaling pathway, which results in the production of large amounts of type I interferon by pDCs. In addition, within myeloid DCs, TLR signaling can result in upregulation of MHC class II molecule, co-stimulatory molecules, and IL12. This key combination of outputs produces an optimal microenvironment for the generation of antiviral CD4 and CD8 T lymphocyte responses.

In chronic HCV infection, there is inconsistency in the reporting of DC phenotype and function. These include abnormalities in phenotype, antigen uptake, cytokine secretion, and expression of co-stimulatory molecules. These changes are most relevant in considering their relationship with T cells, and it has been reported that they have an impaired ability to stimulate the proliferation of antigen-specific T cells and hence generate a CD8 cytotoxic T cell response. This may be related to a diminished secretion of IL12 and increased production of IL10. DCs can be potently stimulated by ligands for Toll-like receptors (TLRs) called pathogen-associated molecular patterns (PAMPs). With respect to HCV, the GU-rich tract in the 3′ UTR of the virus binds to TLR7 and can hence stimulate IFNα secretion, especially from pDCs. Furthermore, the HCV core may stimulate mDCs to secrete IL10. However, the DCs in chronic HCV infection seem hyporesponsive. This is most likely a consequence of HCV infection, as opposed to a primary defect in host immunity, as in general individuals with chronic HCV are not immunosuppressed. How this defect arises is not clear. Whether HCV antigens can attenuate dendritic cell signaling in the same way as they interfere with TLR signaling in hepatocytes is a possibility, because the fulminant hepatitis C strain JFH1 can infect DCs *in vitro* and inhibit TLR signaling. However, HCV has not convincingly been shown to replicate in dendritic cells *in vivo*; therefore, this requires further investigation. It has also been shown that *in vitro* HCV can inhibit DC maturation and reduce their ability to respond to TLR agonists [20]. One alternative explanation may be the liver microenvironment, which in general is thought to be "tolerogenic." In particular, specialized cells that line the hepatic sinusoidal endothelium called liver sinusoidal endothelial cells (LSECs) make IL10 in response to foreign antigens. This cytokine polarizes CD4+ T cells to the TH2 phenotype and to secrete IL4, IL5, IL10, and IL13. This combination of cytokines induces B cell maturation and the production of antibodies, rather than the formation of a CD8+ cytotoxic T cell response, hence mitigating against clearance of infected hepatocytes and favoring chronic infection.

NK cells

The study of NK cells in the acute phase of HCV has revealed changes in NK cell phenotype and function that implicate them as a component in a successful immune response to HCV infection. In the acute phase of HCV infection, there is a skewing of NK cell subsets with an increase in $CD56^{bright}$ NK cells and an associated reduction in the $CD56^{dim}$ subset as compared to healthy individuals [21]. Subsequently, this normalizes in those

who clear HCV infection but not in those who remain chronically infected. In this phase, there is increased expression of the activating NKG2D, and in a separate study the activating receptor NKp30 has also been shown to be important. In one study, NK cells in the acute phase of HCV infection had an augmented production of IFNγ and enhanced degranulation responses. Consistent with the genetic studies, these degranulation responses were most marked on NK cells expressing the HLA-C1-specific KIR. In a second study of individuals followed longitudinally through HCV infection, the NK cells were more cytotoxic but less cytokine producing [22]. This was attributed to ongoing opiate use. However, they did observe a decline in the inhibitory receptor NKG2A in those individuals who resolved infection but not those who remained chronically infected.

The NK phenotype in the early phase of chronic HCV infection may well differ from that found in the conventional cross-sectional studies from which most of our knowledge about immune system responses to HCV comes. In these studies, there are alterations in NK cell numbers, subset, phenotype, and function. Overall in chronic HCV, NK cell numbers are depressed, and this is restored following successful treatment of infection. Consistent with observations in the acute phase of HCV infection, there is also a change in the relative proportions of the $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells in both the periphery and the liver [23]. In the periphery, there is skewing of the NK cell subset toward terminally differentiated CD56–CD16+ NK cells that in general are hypofunctional. In chronic HCV infection, these cells have a skewing in chemokine production toward MIP1β, and produce less IFNγ and TNFα. These cells also tend to express lower levels of activating NK cell receptors. However, the relative expression levels of the classical activating receptors NKG2D, NKp44, and NKp46 are controversial. Studies have found them both up- and downregulated in chronic HCV infection.

Functional changes in NK cells include polarization away from IFNγ secretion with possibly an increase in the production of type 2 cytokines including IL10 and TGFβ. This may skew the cytokine microenvironment away from the cytotoxic T cell responses required to eliminate HCV successfully and toward antibody production. Furthermore, NK cell cytotoxicity in general is thought to be maintained or increased, and the potential for NK cells to induce liver immunopathology is indicated by the upregulation of TNF-related apoptosis-inducing ligand (TRAIL) [24]. Critically increased TRAIL and also NKp46 expression has been found on intrahepatic NK cells in chronic HCV infection. Furthermore, ALT levels have been correlated with NKG2A, CD69, and CD107a expression.

One explanation for these findings of NK cells in chronic HCV infection is that they are driven by the cytokine microenvironment found in chronic HCV infection. This includes IFNα, possibly in the absence of sufficient levels of IL15. This may be due to abnormalities of the intrahepatic microenvironment or the lymphocyte compartment. Within the lymphocyte compartment are defined abnormalities of NK cells: DC cross-talk, in which activation of NK cells via the MIC-A/B axis and inadequate production of IL15 by DCs have been described [25].

T cell responses

The clearest association between outcome of HCV infection and immune responses has been shown for cellular immune responses. Indeed, several studies have shown that HCV elimination is associated with strong and sustained CD4+ and CD8+ T cell responses that target multiple epitopes within the different HCV proteins [26, 27]. Of note, with a delay of approximately 6–8 weeks, T cell responses become detectable and coincide with the onset of hepatitis. Recent data suggest that this is indeed due to delayed induction and not an impaired recruitment of specific CD8+ T cells to the liver [28]. After resolution of infection, these responses persist for decades and can last even longer than humoral responses. The strong link between cellular immune responses and outcome of infection is supported by the following findings:

- the temporal association between the onset of virus-specific CD8+ T cell responses and HCV clearance;
- the strong association between certain class I alleles that restrict specific dominant CD8+ T cell epitopes and spontaneous elimination of the virus [29];
- the strong antiviral effects of virus-specific CD8+ T cells observed *in vitro* using novel immunological models [30]; and, most importantly,
- the finding that CD8+ T cells control HCV replication *in vivo*.

Indeed, upon antibody-mediated depletion of CD8+ T cells, experimental infection of a chimpanzee led to HCV persistence until the CD8+ T cell response recovered and an HCV-specific CD8+ T cell response emerged [31]. Collectively, these results show that virus-specific CD8+ T cells are most likely the key effector cells in HCV control. While most studies have been focused on the analysis of IFNγ-producing CD8+ T cells, it is important to note that recent studies have also demonstrated the presence of HCV-specific IL17-producing CD8+ T cells that are characterized by high expression of CD161 and chemokine receptors such as CXR6 that are important for liver homing [32]. These cells might have a protective role in HCV infection, but additional studies are required to confirm this interesting hypothesis.

Next to CD8+ T cells, however, HCV-specific CD4+ T cells also contribute to HCV clearance. Several studies

in patients with acute HCV infection revealed that strong, multispecific, and sustained HCV-specific CD4+ T cell responses are associated with a self-limited course of infection. In addition, similar to the findings described for HLA class I, certain HLA class II alleles have been correlated with outcome of HCV infection. In strong support of the important role of CD4+ T cells, their depletion in previously protected chimpanzees led to HCV persistence and the emergence of CD8+ escape variants [33]. Finally, as has been described for CD8+ T cells, a recent study has reported the presence of IL17-producing CD4+ T cells in chronic infection, the role of which needs to be further defined [34].

Collectively, these findings clearly indicate that several components of the innate and adaptive immune responses are activated during acute HCV infection and most likely interact on several levels to eradicate the virus successfully in the few patients who clear the virus. In the majority of acutely HCV-infected patients, the virus is able to evade these combined immune responses and to persist. In the "Failure of immune responses" section, we will discuss several mechanisms of HCV evasion from these responses.

Failure of immune responses in acute and chronic HCV infection

Importantly, host immune responses are detectable in chronically HCV-infected patients. These responses are unable to control the virus but most likely contribute to ongoing liver disease. The mechanisms that contribute to the failure of these immune responses are similar in acutely and chronically infected patients, and will be discussed throughout this section (Table 18.1).

Evasion from innate responses

Importantly, HCV has developed several strategies to overcome innate responses. For example, it has been suggested that HCV interferes with establishing an IFN-induced antiviral state of the host cell [35]. Various studies have described a partial block of JAK/STAT signaling in different cell lines and even in transgenic mice expressing HCV proteins or in cells harboring a genomic HCV replicon. However, it is important to note that in cell culture, including various cell lines as well as primary human hepatocytes, HCV replication is highly sensitive to treatment with type I, II, or III IFNs [36]. It is, therefore, not clear whether modulation of IFN signaling by HCV occurs, and if so, whether it would suffice to explain the failure of IFN to clear HCV. Much better established is HCV interference with the induction phase of the antiviral response. It has been shown that the viral NS3/4A protease proteolytically cleaves central adapter molecules in two IRF3-activating antiviral pathways: MAVS in the cytosolic RLR pathway, and TRIF in the endosome-borne TLR3 pathway. For this reason, cells infected with HCV are impaired in their production of type I IFNs. Importantly, the observation that in HCV-infected Huh7 cells, MAVS is cleaved by the viral protease has also been confirmed in humans [37]. Attenuation of very early ISG expression by HCV might also be achieved through blocking RNA translation via PKR. In addition to the classical pathways of innate antiviral immunity described here, autophagy, an essential catabolic process of eukaryotic cells, is increasingly recognized as a key player in this defense. While this is generally looked at as another intrinsic antimicrobial defense strategy of the host, HCV might exploit this response for some early steps in its replication cycle.

Table 18.1 HCV evasion strategies.

Innate and adaptive immune response	Action	Possible evasion strategies (examples)
Interferons	• Induction of antiviral proteins	• HCV interference with induction of IFN response (cleavage of MAVS and TRIF) • Induction of autophagy
Antibody responses	• Partial protection and neutralization	• Evolution of viral quasi-species • HCV VHR1 as decoy for neutralizing antibodies
Denfritic cells and macrophages	• Antigen presentation and T cell priming	• Alteration in chronic infection?
NK cells	• Direct antiviral effects by cytolytic and noncytolytic functions	• HCV interference with NK cell activation • Decreased IFNγ production of NK cells
CD4+ T cells	• Provide help for CD8+ T cells and antibody responses	• Inhibition of CD4+ T cell priming • Impaired CD4+ T cell function
CD8+ T cells	• Direct antiviral activity by cytolytic and noncytolytic effector functions	• Emergence of viral escape mutations • Impaired CD8+ T cell function
Regulatory T cells	• Inhibit HCV-specific T cell responses	• Increased inhibitory activity during chronic HCV-infection
IL17 producing T cells	• Increased in the liver of chronically HCV infected patients; protective role?	Not known

Interestingly, two other groups showed recently that induction of autophagy by HCV might be directly involved in suppression of type I IFN production, as RIG-I stimulation in cells with a knockdown of key regulators of autophagy yielded significantly higher induction rates of IFNβ [38].

Evasion from NK cell responses

The overall killing capacity of NK cell in chronic HCV infection is not impaired but actually enhanced in chronically infected patients, most likely as a consequence of the microenvironment to which NK cells are exposed [39]. However, in addition to increasing the cytotoxic potential of NK cells, this may also lead to an increase in expression of the inhibitory receptor NKG2A. This receptor binds to the conserved HLA class I molecule HLA-E, expressed by all individuals. Interestingly, HCV is known to furnish a peptide that can upregulate HLA-E and hence inhibit NK cells, especially those expressing high levels of NKG2A.

There have also been reports indicating that HCV has the ability to interfere with the action of NK cells. For example, a recent study has suggested that NS5A-containing apoptotic bodies can trigger monocytes to produce increased amounts of IL10 and decreased levels of IL12. In consequence, this leads to a significant down-regulation of NKG2D on NK cells via TGFβ. Another proposed mechanism for HCV-induced NK cell inhibition is cross-linking of CD81 by the HCV envelope protein E2. It has been shown that engagement of this tetraspanin on the surface of NK cells exerted an inhibitory effect, leading to decreased cytotoxicity and IFNγ production. Nonetheless, this effect could be observed only at high concentrations of soluble E2 protein or HCV virions immobilized to the cell culture plate, but not by direct exposure of NK cells to infectious HCV particles (even at levels substantially higher than those found circulating in humans), implying that it is probably not physiologically relevant in HCV escape from the NK cell response [40].

Evasion from humoral immune responses

Multiple mechanisms for the failure of the humoral immune response have been suggested. For example, evolution of viral quasi-species within targeted epitopes may lead to escape from neutralizing antibodies [41]. HVR1 may also play a more general role in mutational escape by serving as a decoy for neutralizing antibodies, thus "protecting" other functionally important but less mutable epitopes. Interactions of HCV glycoproteins with high-density lipoprotein (HDL) and the scavenger receptor B1 (SCARB1) participating in HCV entry may protect from neutralizing antibodies. In addition, specific glycans on E2 also modulate cell entry and confer protection from neutralizing antibodies, and conformational changes or binding of nonneutralizing antibodies may prevent binding of neutralizing antibodies [42]. It has also been proposed that HVR-1 obstructs the viral CD81-binding site and conserves neutralizing epitopes. Interestingly, recent studies indicate that HCV may also evade neutralization by direct cell to cell transfer of the virus [43].

Evasion from T cell responses

Virus-specific T cell responses are also detectable during chronic HCV infection, where they probably contribute to the progression of liver disease [44]. Some patients with chronic HCV infection, however, lack strong and multispecific CD8+ T cell responses. In these patients, it is difficult to distinguish whether virus-specific CD8+ T cell responses were not primed initially (primary T cell failure) or were primed but disappeared quickly (T cell exhaustion). Results obtained from the early phase of acute HCV infection in chimpanzees and in healthcare workers infected via needlestick exposure support the hypothesis that at least in some patients, CD8+ T cells are not primed or are weakly primed during acute HCV infection [45]. Impaired priming of HCV-specific CD8+ T cells might be mediated by low numbers or functional impairment of antigen-presenting cells such as macrophages or dendritic cells. However, this issue remains controversial.

The mechanisms responsible for the failure of HCV-specific CD4+ and CD8+ T cell responses during chronic infection are not well defined, but viral escape and T cell dysfunction appear to be two important mechanisms that are discussed in detail in the next two sections (Figure 18.2).

Viral escape

HCV has an enormous replication rate (approx. 10^{12} virions per day) mediated by an RNA-dependent RNA polymerase lacking proofreading function. Thus, multiple virus variants co-circulate in an individual patient facilitating the selection of CD8+ T cell escape variants. The first evidence for an important role of viral escape was derived from studies of chronically infected patients and experimentally infected chimpanzees. Viral escape mutations seem to emerge during the acute phase of infection and are associated with the development of viral persistence. Similarly, in patients with acute HCV infection, viral escape from CD8+ T cell responses was also primarily detectable in patients developing chronic hepatitis C, but not in individuals who resolved infection [46]. Mutations outside of targeted CD8+ T cell

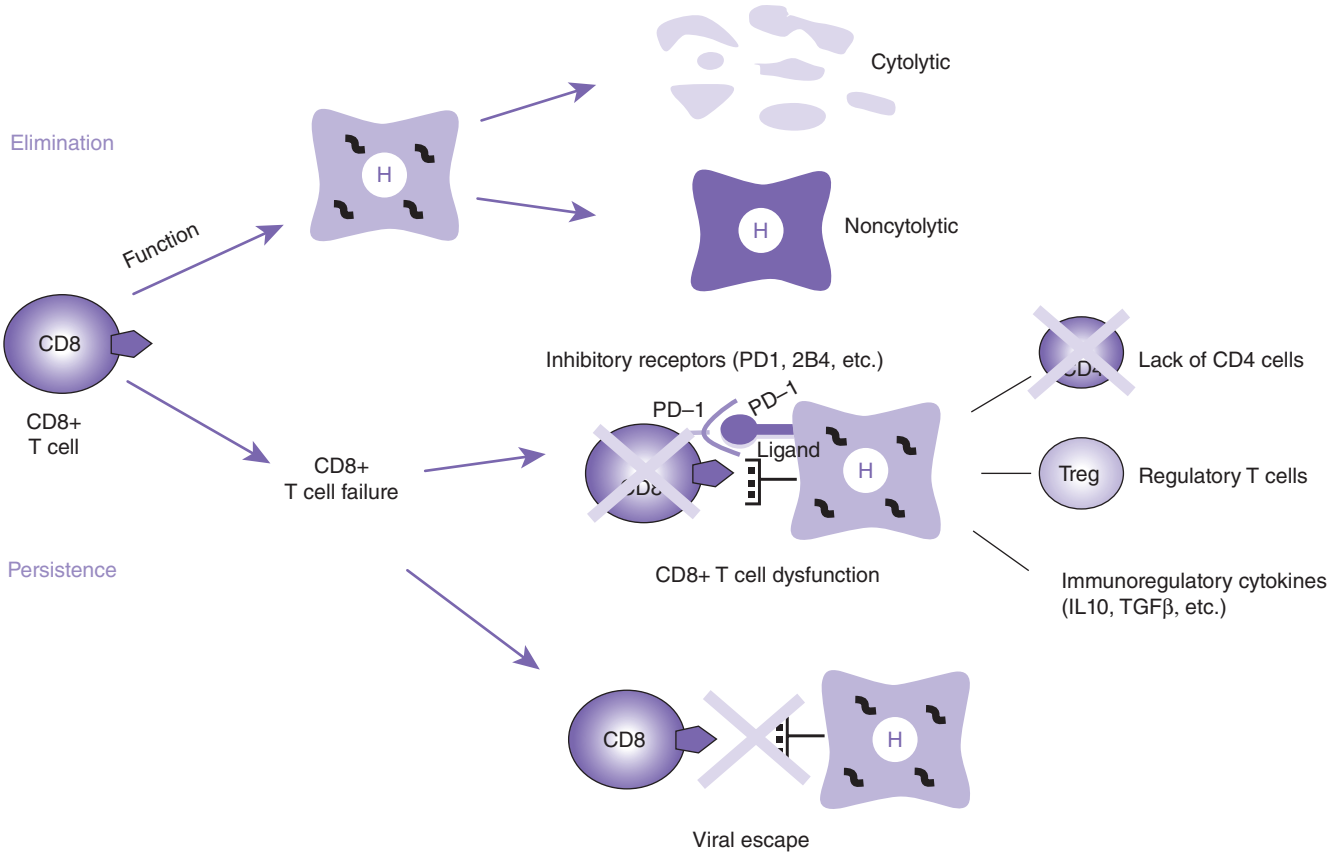

Figure 18.2 Elimination and escape from the CD8+ T cell response. Virus-specific CD8+ T cells can inhibit HCV replication by cytolytic (killing of infected hepatocytes) and noncytolytic (IFNγ-mediated) effector functions. Virus-specific CD8+ T cell failure is mediated by two major mechanisms: CD8+ T cell dysfunction and the emergence of viral escape mutations. Several pathways contribute to CD8+ T cell dysfunction, such as inhibitory receptors, lack of CD4+ T cell help, the action of regulatory T cells, and immunoregulatory cytokines. (Color plate 18.1)

epitopes corresponded to conversion to the consensus sequence, indicating that due to the lack of sufficient T cell–mediated selective pressure, HCV reverts to the fittest sequence, most likely represented by the consensus. This concept has been further supported by a study showing that the transmission of an HLA-B8-associated escape mutation to an HLA-B8 negative subject resulted in rapid reversion of the mutation [47]. These results were supported by a study using the cohort of Irish women described in this chapter; amino acid substitutions in known epitopes were directed away from consensus present in the inoculum in women having the HLA allele associated with that epitope, but toward the consensus in those women lacking the allele. These results support the concept of viral fitness cost, indicating that viral escape mutations are often associated with a reduced replicative capacity of the virus [48].

Based on the finding that immunodominant CD8+ T cell epitopes leave their viral footprint sequences in HCV genomes replicating during the chronic phase, virus genome-sequencing studies were performed in order to identify footprints of additional potential CD8+ T cell epitopes. These studies identified additional HLA allele-dependent polymorphisms and thus candidate CD8+ T cell epitopes. Importantly, the strongest association between specific sequence variations and an HLA allele in the study by Timm and coworkers was found in a viral region that was shown to contain an immunodominant HLA-B27-restricted CD8+ T cell epitope [49]. These results support the concept that CD8+ T cell–mediated pressure and viral escape are operative in a large fraction of patients.

Different molecular mechanisms are operating by which a certain mutation results in escape from a CD8+ T cell response. For example, mutations located at the HLA-binding anchors can strongly attenuate peptide binding to the HLA molecule. In contrast, mutations residing in the center of the epitope are more likely to

interfere with T cell receptor (TCR) recognition. Mutations in the flanking region may prevent proteasomal processing of the peptide precursor.

The factors promoting or limiting the emergence of viral escape are poorly understood. In the chimpanzee model, it has been shown that upon depletion of CD4+ T cells, in the acute phase of infection, viral escape from the CD8+ T cell response occurs and is associated with chronicity [33]. This finding has led to the hypothesis that viral escape within CD8+ T cell epitopes may be a consequence of insufficient CD4+ help. Other studies indicate that a limited TCR diversity might be associated with viral escape [50]. Of note, viral escape does not seem to occur in the context of dysfunctional CD8+ T cell responses. The strong association between specific HLA alleles and viral escape within an immunodominant HLA- restricted epitope indicates that the restricting HLA allele background also plays an important role in determining viral escape.

As mentioned here, another important determinant of viral escape is fitness cost, limiting the ability of the virus to tolerate a given escape mutation. Indeed, fitness cost might not only explain the occurrence of reversion after removal of T cell pressure or the absence of viral escape in specific CD8+ T cell epitopes, but also directly contribute to the protective effect of specific CD8+ T cell responses. This has been suggested for the protective HLA-B27 and HLA-A3 epitopes [51]. For instance, viral escape mutations affecting the main HLA-B27 anchor residues at positions 2 and 9 of the dominant HLA-B27 epitope impair HCV replication. This epitope is located within a region of NS5B that may be especially relevant for mediating viral fitness cost. Nevertheless, nearly all HLA-B27+ patients with persistent HCV genotype 1 infection display clustered viral sequence variations consistent with HCV escape mutations in this otherwise highly conserved region that, however, spare the HLA-binding anchor residues. This occurrence of clustered mutations is a striking finding, since viral escape mutations in HCV epitopes restricted by other HLA alleles usually affect only a single amino acid residue within the epitope [52]. Importantly, this clustering of mutations is required for efficient CD8+ T cell escape due to broad cross-recognition of viral variants. Taken together, these results clearly illustrate that escape from the immunodominant HLA-B27-restricted CD8+ T cell response is determined by counteracting factors: fitness cost and broad cross-recognition requiring clustered mutations. Thus, immune escape probably takes too long in most HLA-B27+ patients, providing the host immune system with enough time to clear the virus before escape can take place. Interestingly, a similar observation has been reported for HLA-A*03 that has been described to be protective in the Irish cohort of women infected with HCV genotype 1b from a single source [53]. Indeed, Merani and coworkers studied HLA class I–driven viral sequence diversity in this cohort, and the strongest association between any HLA class I allele and a sequence mutation was found within an HLA-A*03-restricted CD8+ T cell epitope in NS3 ($NS3_{1080–1088}$; amino acid sequence TVYHGAGTK) [54]. Similar to escape from the immunodominant HLA-B*27 epitope, viral sequence mutations within the HLA-A*03 epitope were clustered at positions 1087 and 1088 in most A*03-positive patients. A subsequent study revealed that HLA-A3-restricted CD8+ T cell responses indeed target these key epitopes and that two mutations are required to retain viral fitness [51]. Thus, clustered escape mutations occurring in both, the protective HLA-B*27 and HLA-A*03 restricted epitopes, argue for a complex pathway of viral escape requiring clustered mutations that may indeed be a major determinant of protective HLA class I alleles in HCV infection. It is important to note that viral escape is not limited to CD8+ T cell epitopes, and although escape mutations can occur in MHC class II–restricted epitopes, they are rarely found in chronically infected patients and chimpanzees. This implies that other mechanisms such as T cell dysfunction might be responsible for the deficient $CD4^+$ T cell responses associated with chronic HCV infection.

Although in the past few years, important insights into CD8+ T cell–mediated viral escape has been gained, we still do not know whether viral escape is a cause or consequence of HCV persistence. It is also important to note that viral escape is not a universal mechanism resulting from CD8+ T cell pressure. Indeed, combined immunological and virological studies suggest that viral escape occurs in 50–70% of targeted CD8+ T cell epitopes. Thus, other mechanisms such as CD8+ T cell dysfunction contribute to CD8+ T cell failure as well.

CD8+ T cell dysfunction

Several groups have suggested that CD8+ T cell dysfunction (e.g., the inability to secrete antiviral cytokines such as IFNγ or to proliferate in response to antigen contact) is a major determinant of viral persistence. This state of T cell dysfunction observed during chronic viral infections is characterized by an upregulation of inhibitory receptors, such as PD1 [55]. Indeed, in chronically HCV-infected patients, several groups have shown that a large fraction of HCV-specific CD8+ T cells also have high expression of PD1. These HCV-specific CD8+ T cells also display low levels of expression of CD127. Intrahepatic HCV-specific CD8+ T cells with high PD1 expression are prone to apoptosis [56]. Importantly, the impaired proliferative response of CD127-PD1+ HCV-specific CD8+ T cells to antigenic stimulation can be increased by blocking antibodies targeting PD1. These

findings strongly indicate that T cell exhaustion occurs during chronic HCV infection and that a blockade of inhibitory receptor pathways may represent a novel therapeutic strategy for the augmentation of T cell responses during HCV infection. However, the dysfunction of $CD127^-$ cells is not solely caused by inhibitory signals via PD1, since PD1 blockade alone was unable to restore the function of strongly inhibited HCV-specific CD8+ T cells in the liver. Of note, targeting the additional inhibitory receptor CTLA-4 resulted in an increase in T cell function [57]. These findings – together with the observation that PD1 expression on HCV-specific CD8+ T cells did not necessarily identify exhausted T cells during acute HCV infection – suggested that pathways other than PD1 play a role in the dysfunction of $CD127^-$ HCV-specific CD8+ T cells. For example, expression of the negative immune regulatory receptor Tim3 has been detected with HCV-specific CD8+ T cells in chronic infection, and its blockade may restore HCV-specific CD8+ T dysfunction [58]. Similarly, a recent study has suggested a role for 2B4 in HCV-specific CD8+ T cell dysfunction [59]. However, the relative contribution of different inhibitory receptors for HCV-specific CD8+ T cell dysfunction is not clear. Recently, $CD127^{low}$ HCV-specific CD8+ T cells were shown to co-express the inhibitory receptors 2B4, KLRG1, and CD160 in addition to PD1 in chronic HCV infection. Importantly, such a coexpression of multiple inhibitory receptors in addition to PD1 was also found with highly exhausted T cells during chronic LCMV infection. Indeed, in that model exhausted T cells required the simultaneous blockade of additional inhibitory pathways to restore antiviral function. In agreement with this finding, $CD127^-$ HCV-specific CD8+ T cells co-expressing multiple inhibitory receptors could be partly but not completely reinvigorated by PD1 blockade alone, indicating that targeting additional inhibitory pathways may be required to reverse the dysfunction of exhausted CD8+ T cells during HCV infection completely.

The mechanisms causing exhaustion of HCV-specific CD8+ T cells are still incompletely understood. Factors that might contribute to CD8+ T cell dysfunction are continuous antigen triggering, the lack of CD4+ T cell help, or the action of regulatory T cells or cytokines. These possibilities will be discussed in the rest of this section.

Lack of CD4 help

While CD8+ T cells are considered the major effector cells against viral pathogens, the successful elimination of HCV is probably highly dependent on sufficient CD4+ T cell help. It has been demonstrated in the LCMV mouse model that CD4+ T cell help is needed to sustain cytotoxic CD8+ T cell responses during chronic viral infection. However, in chronic hepatitis C, CD4+ T cell responses have been suggested to be weak or even absent and functionally impaired (e.g., due to secretion of low amounts of IL2). Importantly, by using de novo CD154 (CD40 ligand) expression in response to HCV antigens as a readout, we recently showed that virus-specific CD4+ T cells are not physically deleted but only functionally impaired [60]. Clearly, this impaired function of critical CD4+ T cell help might be a central determinant of CD8+ T cell dysfunction.

Suppression by regulatory T cells

Growing evidence suggests that regulatory T cells play a significant role in the suppression of virus-specific T cells. For example, in chronically HCV-infected patients, CD4+CD25+ T cells have been found at a higher frequency compared to individuals with resolved HCV infection or healthy controls. These regulatory T cells suppress the proliferation as well as IFNγ secretion of virus-specific CD8+ T cells *in vitro*. The suppression by CD4+CD25+ T cells was shown to depend on cell–cell contact but was independent from suppressive cytokines such as IL10 and TGFβ in some but not all studies [61]. Interestingly, the suppression was not restricted to HCV-specific CD8+ T cells, but also included CD8+ T cells specific for other viruses, such as Epstein–Barr virus and influenza [62]. However, specificity *in vivo* might be mediated by the enrichment of CD4+CD25+ T cells in the liver, where they might limit immunopathology in the chronic phase of HCV infection by blocking virus-specific CD8+ T cells by direct cell–cell contact. Of note, functional FoxP3+ CD4+ and HCV-specific FoxP3+ CD4+ T cells have been identified during the course of acute HCV infection. However, no specific correlation with the outcome of infection could be established in these studies.

Another type of regulatory T cell in HCV infection is virus-specific regulatory CD8+ T cells that express high levels of IL10. These regulatory T cells have been detected in the liver of HCV-infected individuals, and their suppression of virus-specific CD8+ effector T cells could be blocked by neutralizing IL10 antibodies [63, 64]. Indeed, the blockade of IL10 resulted in a stronger expansion of virus-specific CD8+ T cells supporting a biological and active role of IL10 in chronic HCV infection. It is also important to note that myeloid suppressor cells that can be induced by HCV may also contribute to CD8+ T cell dysfunction (e.g., through the production of reactive oxygen species).

Taken together, these combined results suggest that several mechanisms may contribute to HCV-specific CD8+ T cell dysfunction. However, the relative contribution of each of these different pathways needs to be clarified in future studies.

Finally, the specific micro-environment and architecture of the liver also need to be considered as major contributors to CD8+ T cell dysfunction. Indeed, recent research has documented multiple mechanisms by which immune responses in the liver are biased toward tolerance. For example, liver dendritic cell subsets as well as diverse subsets of unconventional antigen-presenting cells, such as liver sinusoidal endothelial cells or hepatic stellate cells, may play an important role in inducing immune suppression. It is also interesting to note that hepatocytes can also function as antigen-presenting cells and can activate CD8+ T cells. However, this seems to result in effector cells that are not fully functional, although this has not been shown to be the case in HCV infection.

Summary

Chronic HCV is a pressing clinical problem. Understanding the immune response to HCV is crucial to developing vaccine strategies and also to optimizing treatment strategies for individuals with HCV infection. Infection with HCV leads to a multifaceted immune response to a viral infection. Clearance has been variably associated with CD4 T cells, CD8 T cells, antibodies, and NK cells, not all of which are optimal in each individual. Synergism of these pathways is necessary for a successful immune response to HCV. However, not all need to be firing on all cylinders in order for an individual to clear infection, as redundancy, exemplified by the MHC, is a key feature of the immune system that has been driven by natural selection. Additionally, there is certainly a wide biological variability in specific immune response among individuals that clear HCV infection. Conversely, HCV, for such a small virus, appears remarkably well equipped to deal with our immune system as evidenced by the high rate of chronicity, and the many abnormalities found in immunocyte phenotype and function found in chronic HCV infection. Hitting the virus hard with direct acting antiviral drugs will be the key to clearing HCV infection in those chronically infected, and the role of the immune system in assisting these therapeutics will remain an important area for research. However, using our knowledge of HCV immunity to generate a successful vaccine for HCV will be a key challenge for current and future immunologists.

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Chapter 19
Animal models of hepatitis C virus infection

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Summary

The chimpanzee model has played a major role both before and after the discovery of hepatitis C virus (HCV), the etiologic agent of non-A, non-B hepatitis (NANBH). Chimpanzees were essential to prove that NANBH was indeed an infectious disease caused by a transmissible agent, and they provided an *in vivo* system for the biological amplification of the putative virus to obtain sufficient material for attempts at identification. It was from a chronically infected chimpanzee that a large amount of plasma was obtained for the molecular cloning of the HCV genome. The chimpanzee model has played a critical role not only in the initial discovery but also in the post-HCV era. Studies in chimpanzees were instrumental to investigate the host immune response elicited by HCV and to determine the natural history of hepatitis C. Finally, chimpanzees remain the only animal model to study the immunogenicity and efficacy of vaccine candidates against HCV. In this chapter, we review the role that nonhuman primate models have played before and after the discovery of the etiologic agent of NANBH. Furthermore, we discuss the utility of small-animal models that have been developed over the past 10 years for studies of HCV.

Introduction

The development of animal model systems has provided an invaluable resource for the study of human viral hepatitis [1]. Nonhuman primates have played an important role in furthering our understanding of hepatitis A virus (HAV) and hepatitis B virus (HBV) [1]. In contrast, much of our understanding of NANBH, as well as the discovery of its major causative agent, HCV [2], 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 [3–5], and the demonstration of serial transmission to other chimpanzees [6]. Chimpanzees were essential to the demonstration that the transmissible agent was filterable and had a lipid-containing envelope [7]. 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 etiological agent [2]. The first specific serological assay for HCV infection was not developed until 1989 [8]. This resulted from the partial cloning of the HCV genome after its recovery from plasma of an experimentally infected chimpanzee.

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The contribution of the chimpanzee model in the pre-HCV era

Prospective studies of transfusion-associated hepatitis (TAH) conducted in the United States and Europe in the 1960s and 1970s were instrumental in defining the natural history of NANBH (reviewed in [9]). 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 TAH cases. The discovery of NANBH led to a concerted effort to identify the causative agent. The chimpanzee 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 [10]. In addition, the chimpanzee was useful for evaluating the effectiveness of viral inactivation procedures. The host range of NANBH is remarkably limited. Attempts to transmit the disease to other nonhuman primate species with inocula of proven infectivity yielded negative or equivocal results [11].

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. 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 [10]. The clinical features of NANBH in chimpanzees closely reproduce those seen in humans [12]. The development of chronic hepatitis, a hallmark of NANBH, is also a common sequela in chimpanzees [9, 12]. 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.

In the absence of any reliable serological tests, the chimpanzee model was instrumental in establishing the duration of viremia 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 [4] and persisted at least until week 13 after inoculation (1 week after the peak ALT level) [6]. 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 NANBH agent in blood, in both humans and chimpanzees, could be obtained [6]. 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 [13]. The availability of the chimpanzee model permitted the determination of infectivity titers of sera containing NANBH [11, 14]. The results of these titration studies showed that the endpoint infectivity titer 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 had a high infectivity titre ($10^{6.5}$ mL each). Thus, the infectivity of NANBH viruses in serum is markedly lower than that commonly observed in HBV (10^8) [11] and hepatitis D virus (HDV) infection (10^{11}) [15]. 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.

The chimpanzee model has been crucial to determine most of the physico-chemical properties of the putative agent of NANBH. 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 titers of virus in clinical materials. 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 [16] or 1 : 2000 at 37 °C for 72 hours [17]. Infectivity could be abolished by heating at 100 °C for 5 minutes [17] or at 60 °C for 10 hours [16]. Complete inactivation of the agent was also achieved by heating at 60 °C for 30 hours after lyophilization [18], 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 [19]. Inactivation by exposure to lipid solvents, such as chloroform, indicated that the agent contained essential lipids, presumably in an envelope [7]. 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 [20]. The results of the biochemical and physical analyses suggested that the NANBH agent was an enveloped virus with a diameter of 30–60 nm.

The role of the chimpanzee model in the discovery of HCV

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 [21]. 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 titer of infectivity (10^6) were obtained for the molecular cloning of the viral genome [2]. Liters of plasma were pelleted to concentrate the virus particles; total RNA was extracted from the pellet and reverse transcribed into complementary DNA (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 [21]. 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" [21]. Only one positive clone was the result of this strenuous effort. 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 [21]. 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 [21]. Extensive molecular analysis has demonstrated that HCV is characterized by a high degree of genetic variability, a hallmark of RNA viruses. 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 that was merely based on exclusion into that of a specific disease, hepatitis C [8]. The application of this assay to clinical practice finally provided the best evidence that HCV is the major etiological agent of posttransfusion NANBH [22] as well as of community-acquired NANBH [23].

The contribution of the chimpanzee model in the post-HCV era

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. Thus, chimpanzees have represented an optimal source of controlled clinical material for studying the kinetics of viral replication and the relationship between viremia and the host immune response during the course of acute and chronic HCV infection [24]. 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 viremia, which in most cases becomes detectable within 1 week after inoculation, long before other markers can be detected (Figure 19.1) [25–29]. 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 [29]. During the acute phase, the peak viral titer ranges from 10^5 to 10^7 genome copies per mL (Figure 19.1). 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 [3, 14]. The severity of hepatitis, as measured by the ALT levels, does not appear to correlate with the infectivity titer of the inoculum [14]. In self-limiting HCV infection of chimpanzees, the viremia is transient and lasts for a variable period, ranging from 10 to 38 weeks [26–28] but occasionally longer [24].

The humoral response to primary HCV infection may be concomitant or delayed with respect to the clinical onset of the disease, and it is highly variable (Figure 19.1). 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 viremia and the time of antibody seroconversion, or between the infectivity titer and the time of seroconversion [26, 28]. The time interval between inoculation and antibody seroconversion ranges from a few weeks to several months. 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 [26, 28]. The shortest interval from the onset of hepatitis to antibody seroconversion has been observed using the third-generation assay, which detects antibodies against several HCV proteins, including core [30].

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

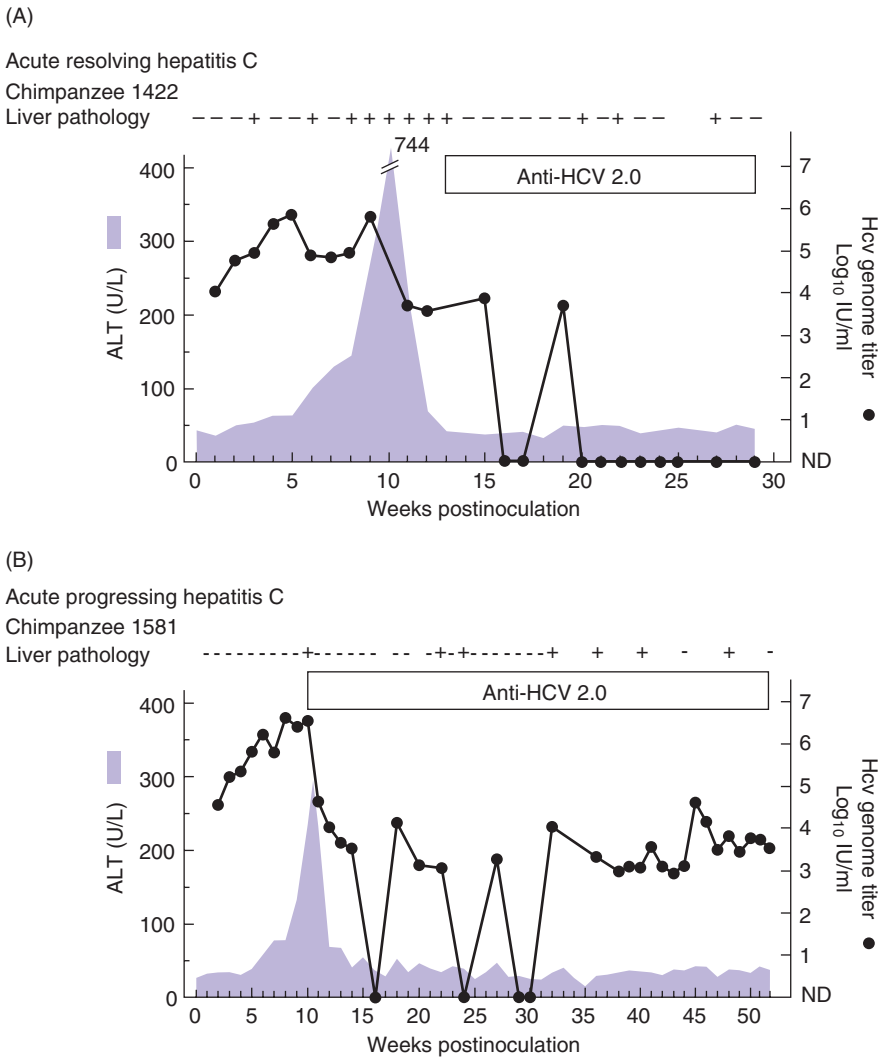

Figure 19.1 Patterns of antibody response to hepatitis C virus (HCV) and HCV viremia in (A) a chimpanzee with acute resolving hepatitis C; and (B) a chimpanzee with acute progressing hepatitis C. The gray area indicates the values of serum alanine aminotransferase (ALT). Liver pathology indicates necroinflammatory changes rated as negative or positive. Viral titers measured in Monitor 2.0 are indicated. Samples found negative in a sensitive RT- nested PCR assay are shown as not detected (ND). (Source: Modified with permission from Sakai A, Takikawa S, Thimme R *et al.* J Virol 2007;81:7208–7219 [50])

may be of value in predicting the outcome of HCV infection. The initial antibody pattern was not significantly different between animals that developed chronic HCV infection and those that cleared the viremia. Thus, the detection of serum HCV RNA is the only currently available test to monitor outcome of the disease. HCV RNA is also the crucial marker for establishing an early diagnosis of primary HCV infection. In fact, although the introduction of the second- and third- generation antibody assay has considerably narrowed the seronegative window, there is still a serologically silent period during which HCV RNA 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 [25–28]. The virological and serological profiles of chronic HCV infection differ from those seen in acute hepatitis. Persistent viremia is associated with the presence of antibodies against structural and nonstructural proteins, both of which increase in parallel with the progression of the hepatitis and persist at high titers as the disease continues [28]. A correlation was not observed between the pattern of HCV viremia and the ALT profile during long-term follow-up [25, 27, 28]. Persistent viremia 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. 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 [27, 31].

Infection with HCV is a major cause of chronic hepatitis, cirrhosis, and eventually hepatocellular carcinoma (HCC) in humans [31]. Chimpanzees can be readily infected with HCV, resulting in either acute or chronic infection. However, despite the high genetic homology between chimpanzees and humans, some aspects of HCV infection differ profoundly between the two species. Acute hepatitis C in chimpanzees is usually milder than in humans, and the immune response against HCV, particularly the humoral response against E1 and E2 glycoproteins, is remarkably weaker. The rate of chronicity is similar. When a series of chimpanzees inoculated with HCV (strain H77) were analyzed, two-thirds of them were found to develop chronic HCV infection (Purcell RH, unpublished data). One of the most important differences between humans and chimpanzees regards the development of fibrosis, which is a typical feature of chronic hepatitis C in humans whereas it is usually not detected in chronically HCV-infected chimpanzees. Whether this reflects differences in the degrees of inflammation and fibrogenesis between the two species remains to be established. However, it is important to underline that chimpanzees are rarely followed for more than a decade, and therefore it is difficult to exclude whether fibrosis may develop also in chimpanzees after several decades. While cirrhosis and HCC are the most frequent long-term sequelae of chronic hepatitis C in humans, cirrhosis has never been reported in chimpanzees, and HCC has been thus far reported in only one chimpanzee experimentally infected with serum obtained from a patient with chronic NANBH. Indeed, many chimpanzees chronically infected with HCV have been followed for more than 12 years without detecting HCC [32]. This is not surprising, however, as the life span of chimpanzees is only slightly less than that of humans.

Monoclonal HCV infection in chimpanzees

The generation of infectious cDNA clones of HCV represented an important advance in HCV research, providing new insights into the mechanisms of HCV pathogenesis and paving the way for the development of novel HCV culture systems [33, 34]. Infectious cDNA clones of HCV have been generated for strains of genotypes 1a, 1b, 2a, 3a, and 4a. Demonstration of the infectivity of such wild-type clones depended on being able to perform intrahepatic transfections of chimpanzees [35]. Such 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 [24]. 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 [30, 33, 34, 36, 37]. 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. Thus, monoclonal infection of chimpanzees provides a suitable model for studying the viral and host factors that determine the outcome of acute HCV infection [24, 30]. A study of acute hepatitis C in 10 chimpanzees experimentally infected with the same monoclonal H77 virus showed that irrespective of the outcome, there was a significant decrease in virus titer after peak levels were reached. All 10 animals had similar levels of interferon gamma (IFNγ) induction in the liver, which coincided with the initial decrease in virus titer and with the development of hepatitis. However, only four animals were able to clear the infection [30]. Sequential sequence analysis of HCV genomes recovered from four animals with monoclonal HCV H77 infection that persisted showed a higher mutation rate during the acute phase [38, 39]; in one of these studies, it was found that most acute phase-coding mutations were in CTL-targeted epitopes. These findings indicated that during acute hepatitis C, virus evolution was driven primarily by positive selection pressure exerted by CD8+ T cells.

The ability to test infectivity of molecular HCV clones in chimpanzees permitted functional analysis, in which the role of genomic regions believed to harbor important determinants of HCV pathogenesis, or relevant targets for drug development, can be determined. In a first study, Yanagi *et al.* [40] analyzed the importance of various elements of the 3′ UTR of HCV. Subsequently it was shown that *p7* and various enzymatic gene regions, such as the *NS3* protease, *NS3* helicase, and the *NS5B* polymerase, were critical for HCV [41, 42]. The biological role of the HVR1 was directly investigated by Forns and colleagues [43], 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 titers of viremia and delayed antibody seroconversion. However, coinciding with the appearance of five amino acid changes, including two changes in E2, the viral titers increased progressively and remained at a relatively high level throughout follow-up. 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. Finally, the role of adaptive mutations in the HCV replicon system [44, 45], 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* [46].

HCV and fulminant hepatitis

Before the discovery of HCV, putative NANBH agents were thought to account for 30–40% of all cases of fulminant NANBH. However, the evidence hitherto accumulated suggests that HCV rarely causes fulminant hepatitis [47]. An etiologic link between HCV and fulminant NANBH was established by longitudinal analysis of serum samples collected from a patient enrolled in a prospective study of transfusion-associated NANBH [48]. Several attempts have been made to transmit hepatitis from plasma of patients with fulminant NANBH to animal models, but they have been unsuccessful [14]. 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) (Ch 1422) [49]. 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 30 chimpanzees infected with different HCV strains. The animal developed antibodies to HCV and eventually cleared serum HCV RNA. However, when a second animal (Ch 1581) was infected using plasma from Ch 1422 and a third one (Ch 1579) was infected with RNA transcripts from a consensus cDNA clone of HC-TN [50], 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 liver inflammation, as commonly seen in the chimpanzee model. Although the course of viremia was similar, Ch 1579 resolved the infection, whereas Ch 1581 became persistently infected. Thus, the unusual severity observed in Ch 1422 did not breed true in the other two animals. Virulence depends upon a complex interplay between the virus and the host.

Interactions among hepatitis viruses in chimpanzees

The chimpanzee 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 [51]. HCV superinfection does interfere with HBV synthesis, as evidenced by suppression of both HBV DNA and HBsAg serum levels [51, 52]. Using PCR, it has been possible to investigate the effect of chronic HBV infection on the replication of HCV [28]. One chimpanzee, a chronic carrier of HBsAg, was inoculated with HCV-positive serum from a patient with posttransfusion 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. This unusual pattern of HCV viremia may be the consequence of an inhibitory effect of HBV infection on HCV replication. The animal developed a transient anti-HCV antibody response, which is likely the consequence of the short duration of HCV viremia. The animal remained HBsAg-positive throughout the study. Replication of HBV was suppressed during superinfection of this chimpanzee with HCV. Thus, there may be a reciprocal inhibition of replication in dual infections by HBV and HCV. Although the short duration of HCV viremia 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 [28]. This animal developed acute NANBH that progressed to chronicity. 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.

Lack of protective immunity against re-infection or superinfection with HCV

After the discovery of HCV, extensive seroepidemiological studies have conclusively shown that this virus is the major cause of both posttransfusion and community-acquired NANBH [8, 22, 23], 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 re-infection with the same virus.

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 [53]. The patterns of viremia 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 posttransfusion 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. Viremia was transient in four animals but became chronic in one. All chimpanzees seroconverted to HCV. Each rechallenge of a convalescent chimpanzee resulted in the reappearance, within 1–3 weeks, of serum HCV RNA, although the duration of viremia during rechallenge was shorter (range 1–10 weeks) than that seen during primary infection (range 11–17 weeks) (Figure 19.2). Although there was less biochemical

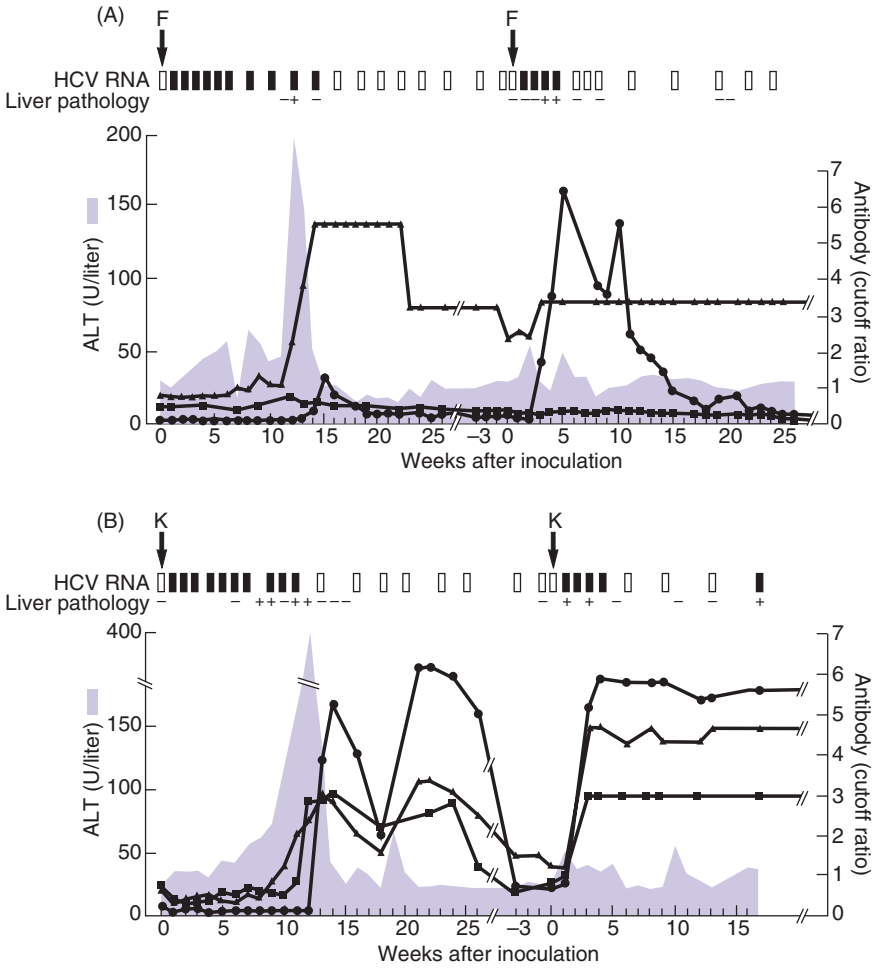

Figure 19.2 Course of HCV infection in (A) chimpanzee 963, rechallenged with the homologous HCV strain; and (B) chimpanzee 793 with a heterologous HCV strain. The gray areas indicate the values of serum ALT. The arrows indicate the time of challenge and the strain used for inoculation. Open bars indicate 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 cutoff ratio represents the ratio between the absorbance value for the test sample and that for the assay cutoff; values above 1 were considered positive. (Source: Modified with permission from Farci P, Alter HJ, Govindarajan S *et al.* Science 1992;258:135–140 [53])

evidence of hepatitis after 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 (Figure 19.2). Genetic analysis of the HVR1 demonstrated that the recurrence of viremia was not due to reactivation of the original challenge strain, but to infection with the HCV strain used for rechallenge. 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.* [54], who documented the reappearance of HCV viremia in chimpanzees rechallenged with either homologous or heterologous strains of HCV. Other studies subsequently confirmed that chimpanzees that had resolved a first episode of acute experimental HCV infection again develop viremia when rechallenged with either the homologous or heterologous viruses of the same major genotype [36, 55, 56]. As previously observed, however, the duration of viremia is in most cases significantly shortened following re-infection, and biochemical evidence of hepatitis can be observed in only a few cases, suggesting a partial protection. Transient infection was also documented following heterologous rechallenge with a different major genotype [57]. Evidence that HCV may cause more than one episode of acute hepatitis was obtained in polytransfused thalassemic children [58].

The availability of monoclonal virus made possible *bona fide* homologous rechallenge studies, which demonstrated that re-infection is not the result of the emergence of immune escape variants [24, 36]. Strikingly, even in this "best-case scenario" for the host, re-infection 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 [59]. In one animal, rechallenge resulted in transient viremia lasting 2 weeks associated with cellular immune responses, but in a second animal HCV persisted despite vigorous anamnestic intrahepatic T cell responses. In the former animal, repeated rechallenges eventually resulted in persistent infection. The animal did not develop neutralizing antibodies during follow-up.

Inoculation of both HCV antibody-positive and HCV antibody-negative human immunoglobulin preparations failed to prevent HCV infection in chimpanzees, when administered intravenously 1 hour after virus challenge [60]. 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-exposure administration of hepatitis C immune globulin can markedly prolong the incubation period of acute hepatitis C, but not prevent or delay HCV infection.

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. The first evidence of superinfection was obtained by Kao *et al.* [61], who documented a second episode of posttransfusion 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 [49]. The superinfection event was again transient, lasting only for 2 weeks. Similar evidence has been obtained in the animal model [62]. Okamoto *et al.* demonstrated that chimpanzees chronically infected with HCV genotype 1b can be superinfected with genotype 1a and subsequently 2a [62]. The superinfecting strain may either replace the original virus or transiently coexist 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.

Neutralizing antibody response against HCV

One of the most important issues related to the immune response elicited by HCV is the fact that the majority of infected immunocompetent individuals fail to clear the infection. Although HCV infection induces strong adaptive immune responses, several lines of evidence suggest that the virus has developed numerous strategies to avoid immune recognition [63]. One of the most effective mechanisms of immune evasion is the remarkable genetic diversity of HCV [64], which circulates in infected individuals as a population of different but closely related genomes termed a quasi-species [65]. 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 clearance of HCV and in the long-term control of HCV infection. They provide the best correlate of protection against several viral infections in vaccinated individuals. One of the major challenges in studying NAbs against HCV, and their role, has been the lack of efficient and reproducible *in vitro* systems for the propagation of this virus in cell culture. However, the development over the past few years of various *in vitro* assays based on infectious retroviral

pseudoparticles (HCVpp) [66, 67] and virus-like particles [68] bearing the HCV envelope glycoproteins E1 and E2, as well as, more recently, the development of a cell culture infectious HCV (HCVcc) [69, 70] expressing the envelope from different HCV strains [71], have provided new tools to characterize the breadth and duration of neutralizing antibody responses and their role in determining the outcome of HCV infection.

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 [72]. However, 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 posttransfusion NANBH (H77). The infectivity of the inoculum had been previously titrated in chimpanzees [14]. 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 [25]. Both plasmas had high titers of antibodies against both structural and nonstructural HCV proteins, including anti-envelope 1 (E1) and E2 antibodies. The virus–plasma mixtures were incubated overnight at 4 °C, and then the residual infectivity was tested by intravenous inoculation of seronegative chimpanzees. 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.

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 multiple 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*. These *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 contribute to 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 1970s had indeed documented a reduced incidence and severity of posttransfusion NANBH in subjects treated with serum immunoglobulins [73–75]. 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 [76]. Epidemiological and laboratory studies suggested that the exclusion might have removed NAbs and hence compromised the safety of the resulting immunoglobulin product. 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 [77]. 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 hypervariable region 1 as a major neutralization domain of HCV

Several lines of evidence indicate that the most variable region of the entire HCV genome, the HVR1 of E2 [78], is a critical target of NAbs. Conclusive evidence that the HVR1 is a critical target of NAbs was obtained in a study of HCV neutralization conducted in chimpanzees using a hyperimmune rabbit serum raised against a synthetic HVR1 peptide [79]. This study also provided the first *in vivo* model for the emergence of neutralization escape mutants because the anti-HVR1 antiserum neutralized the predominant variant present in the quasi-species, which bore the sequence used for immunization,

but it 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 HVR1 region is believed to play an essential role in HCV infection [64]. 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 [80, 81]. In contrast with the pattern seen in humans are the observations made in chimpanzees infected with HCV molecular clones [33, 34], which demonstrated that chronic HCV infection may develop even in the absence of a viral quasi-species in the inoculum [37] and without the emergence of mutations within the HVR1. These data may appear difficult to reconcile with the evidence accumulated in humans. However, there are some differences between humans and chimpanzees. Despite similar HCV RNA levels, the HVR1 region in infected chimpanzees undergoes very little sequence variation compared with humans [82, 83], suggesting that chimpanzees exert a weaker positive selective pressure on this region.

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. Although research in chimpanzees has been recently severely restricted [84], the chimpanzee model will undoubtedly be critical in attempting vaccine development as well as for defining the role of immunoglobulins in preventing HCV infection and for testing the efficacy of passive immunoprophylaxis.

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 [63]. 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 viremia.

In experimentally infected chimpanzees, unlike naturally infected humans, liver tissue can be collected frequently during the acute phase of hepatitis. The chimpanzee model has played a major role in the study of cellular immunity to HCV.

Small-animal models for the study of HCV infection

Small-animal models represent the only alternative to research in chimpanzees since other primates are not susceptible to HCV (Table 19.1). However, only T cell– and B cell–deficient mice (SCID mice) engrafted with human hepatocytes yield robust experimental HCV infection [85, 86]. Susceptibility to HCV infection requires a high-quality implant resulting in a chimeric liver that is 70–80% of human origin [87].

Following inoculation with different HCV variants originating from infected patients [85–87] and cell cultures [86, 88, 89], or intrahepatic transfection with RNA transcripts from molecular HCV clones [89], a rapid increase in HCV titers can be observed with genome titers of $>10^7$ IU/ml within 2–4 weeks, and with efficient passage to naïve mice [86, 89]. Finally, long-term infection for up to 10 months is possible [85, 86]. Human liver chimeric mouse models permit infection with isolates of the six major HCV genotypes and important subtypes [90]. The obvious shortcoming is that these mice models do not permit studies of the adaptive immune response against HCV, limiting their use in studies of immunity and pathogenesis. However, this model has contributed to research on virus neutralization, virus–receptor interactions, novel therapeutics, and the innate host responses (discussed further in this chapter). Finally, studies in this model have been used to evaluate the *in vivo* relevance of functional studies with recombinant HCV in Huh7 cell-based culture systems [88].

The human liver chimeric mouse model has been successfully used to test antibody-based strategies to prevent HCV infection. Proof-of-concept studies demonstrated that IgG purified from a genotype 1a–infected patient efficiently neutralized the homologous HCV, but only partially neutralized heterologous HCV strains [91]. Thus, all mice loaded with the patient IgG (at 1 mg/g) were protected against subsequent challenge with homologous HCV, whereas mice challenged with other HCV genotypes had only partial protection. Given the extensive heterogeneity of HCV, the relatively low cross-genotype neutralization efficacy clearly demonstrates the challenges ahead for the development of a broadly effective HCV vaccine. Therefore, it was of interest that human liver chimeric mice infused with HCV E2-specific human monoclonal antibodies, directed against a conserved epitope, were protected against heterologous HCV challenge [92]. There has also been an interest in determining whether antibodies against host–cell HCV receptors could prevent HCV infection. For example, human liver chimeric mice loaded with monoclonal antibodies blocking CD81 or SR-BI were protected against subsequent HCV

Table 19.1 Animal models in hepatitis C virus (HCV) research.

	 Chimpanzees	 uPa-SCID mice	 FRG mice	 AFCS-hu HSC/hep mice	 Rosa26-Fluc mice	 Rat – immune-tolerized	 Tree shrew
	Only complete model	Engraftment with human hepatocytes	Engraftment with human hepatocytes	Engraftment with human hepatocytes and leukocytes	Genetically humanized with HCV co-receptor molecules	Engraftment with human hepatocytes cells	Nonprimate mammal
Complete viral life cycle	Yes	Yes	Yes	No	No	Yes	Yes
Steps in viral life cycle	Replication*, infection, virus production	Replication*, infection, virus production	Infection, virus production	Infection, intrahepatic replication	Infection-intrahepatic bioluminescence expression, entry only	Infection, virus production	Infection, virus production
HCV genotypes	1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a	1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a	1a, 2a, 3a	1a	Not relevant	1	1b, 2c, 3b, 6
Culture viruses	JFH1, J6/JFH1	JFH1, J6/JFH1	JFH1	None studied	JFH1-based recombinants modified to express CRE recombinase*	None studied	None studied
Viremia levels	High (4–6 $\log_{10}$ IU/mL)	High (5–7 $\log_{10}$ IU/mL)	High (5–7 $\log_{10}$ IU/mL)	None detectable	None detectable	Low	Low
Host responses	Innate, adaptive	Innate (gene expression)	Innate (None studied)	HCV specific cellular responses	Not relevant	Not relevant	Not relevant
Liver pathogenesis	Acute, chronic*	Not relevant	Not relevant	Hepatitis, liver fibrosis	Not relevant	Not relevant	Liver fibrosis – cirrhoses
Therapeutics							
Interferon-based treatment	No effect	Antiviral effect	Antiviral effect	Not relevant	Not relevant	None studied	None studied
Direct-acting antiviral (DAA) agents	Protease/ polymerase	Entry, protease, NS5A, polymerase	None studied	Not relevant	Not relevant	None studied	None studied
Host factor directed drugs	miR-122 antagonist	Cyclophillin inhibitor, and others	Cyclophillin inhibitor	Not relevant	Not relevant	None studied	None studied
Passive immuno prophylaxis							
Polyclonal human anti-HCV	Delayed viremia	Cross-genotype protection	None studied	None studied	None studied	None studied	None studied
Monoclonal human anti-HCV	None studied	Cross-genotype protection	None studied	None studied	Protective (anti-E2)	None studied	None studied
HCV receptor blocking antibodies	None studied	Protective (anti-CD81, anti-SR-BI)	None studied	None studied	Protective (anti-CD81)	None studied	None studied
Vaccines							
Therapeutic	Only available model	Not relevant	Not relevant	Not relevant	Not relevant	Not relevant	Not relevant
Prophylactic	Only available challenge model	Not relevant	Not relevant	Not relevant	Neutralizing antibodies	Not relevant	Not relevant

For each indicated model, specific characteristics on utility and applications are indicated. For all small animal models, there are areas of research that are not currently feasible, indicated with "not relevant." (Reproduced from [104] with permission.)

* Tested by intrahepatic transfection with RNA transcripts from full-length HCV clones.

** Activates a cellular reporter permitting bioluminescence imaging.

*** Chimpanzees are not necessarily good models of chronic liver disease and HCV-associated hepatocellular carcinoma. (Source: Bukh J. Gastroenterology 2012;142:1279–1287 [104])

challenge with different genotypes [93]. Whether anti-HCV or anti-receptor antibodies identified in this model could be used in immunoprophylaxis or in immunotherapy remains to be determined.

Recently, the effectiveness of novel HCV drugs has been studied in the human liver chimeric mouse model. However, this mouse model most likely has limitations in testing the safety and effectiveness of therapeutic strategies targeting host factors, such as locked nucleic acid (LNA)-modified oligonucleotide directed against microRNA 122, shown to be effective against HCV in chimpanzees [94].

Treatment of human liver chimeric mice with different directly acting antivirals (DAAs), including the protease inhibitor telaprevir, now licensed for treatment of HCV genotype 1–infected patients, resulted in decreased viremia levels, but resistance mutants were selected [95, 96]. Thus, the findings observed with DAA in the human liver chimeric mouse model seem to mirror findings in HCV-infected patients. Finally, the human liver chimeric mouse model is also being used for initial testing of novel approaches to inhibit HCV infection, including drugs affecting viral entry [97].

Due to the immunodeficient background required for transplantation with human hepatocytes in the human liver chimeric mouse model, studies of adaptive immune responses and immunopathogenesis are not possible. However, this mouse model does permit studies of innate immune responses. Gene expression profiling studies in mice transplanted with human hepatocytes from different donors, but inoculated with the same HCV strain (genotype 1a), demonstrated a differential response, including innate immune responses [98]. However, such responses did not affect the HCV titers.

Other immunodeficient animal models of HCV infection have been reported, such as mice with heterotopic human liver grafts. The model yielded only low-level viremia, but it has been used to evaluate the antiviral potential of an anti-HCV human monoclonal antibody and a DAA [99]. In another recent model, mice were genetically modified to support engraftment of human hepatocyte progenitor cells and hematopoietic stem cells, resulting in human hepatocyte repopulation of the liver and reconstitution with human leukocytes [100]. In about 50% of cases, inoculation with high-titer HCV-infected patient sera resulted in the detection of viral genomes in the liver, but HCV could not be detected in blood. Mice with detectable liver HCV RNA had increased liver infiltration of human immune cells with evidence of hepatitis. Finally, HCV-specific T cell responses were detected in the liver of HCV RNA–positive mice with evidence of liver fibrosis. Thus, this model could represent an *in vivo* model to study adaptive immunity and pathogenesis of HCV infection.

There have been several attempts to develop small immunocompetent animal models of HCV infection. The tree shrew (*Tupaia belangeri*) has been suggested as a model of HCV infection [101]. However, it yields low and variable infection rates and HCV titers. Development of persistent infection and evidence of chronic liver disease have been reported. Another suggested model is the immunotolerized rat transplanted with human hepatoma cells. Following inoculation with HCV-infected sera, HCV antigen and replicating HCV RNA could be detected in human liver cells, and increasing viremia levels were detected during 8–12 weeks of follow-up. In addition, infected rats showed signs of hepatitis [102]. The most recent immunocompetent animal model of HCV infection is a genetically humanized mouse expressing the human cellular receptors required for HCV entry [103]. In this model, which has generated a lot of excitement, a subpopulation of liver cells of the mice is infected with recombinant adenoviruses expressing critical human receptors [103]. This permits HCV entry into mouse liver cells; detection of HCV entry was accomplished by using a genetically modified HCV that directed the expression of a readily detectable reporter signal within the liver. However, there was no apparent evidence for HCV replication in the liver, and HCV could not be detected in blood. Apparently, entry into humanized liver cells occurred via known HCV entry pathways. The model was used for passive immunoprophylaxis studies and to test for protection by a recombinant vaccine inducing neutralizing anti-HCV antibodies.

In summary, the human liver chimeric mouse model is well established for HCV infection, drug testing, and passive immunoprophylaxis studies, although it is not suitable for studies of adaptive immune responses. All the current mouse models have severe limitations, and the only reliable and efficient animal model of HCV infection remains the chimpanzee [104]. New models using complex approaches to study specific steps in the viral life cycle in small animals hold promise for the development of more advanced humanized models. However, they are restricted in their utility for studying HCV infection and natural history, as well as for testing new therapeutics and prophylactic vaccines.

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Chapter 20
Extrahepatic manifestations of hepatitis C virus infection

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Summary

Hepatitis C virus (HCV) can replicate outside the liver and therefore cause extrahepatic manifestations (EHMs). Patients can present with rheumatological, neurological, nephrological, or dermatological symptoms. EHMs are classified according to their prevalence in HCV-infected patients and their pathogenic link to HCV. Lymphoproliferative disorders such as mixed cryoglobulinemia represent the most closely related EHMs of HCV. For several EHMs, especially in those with a pathogenic link, elimination of HCV is associated with clinical improvement. Therefore, antiviral therapy is considered as the first treatment option; however, achievement of sustained virological response may not always result in clinical response and antiviral therapy may lead to an exacerbation of extrahepatic symptoms. Different EHMs require different alternative therapeutic strategies, for example treatment with rituximab in patients with severe mixed cryoglobulinemia syndrome. The management of HCV-associated EHMs is complex and necessitates a team of experts in different medical specialties.

Introduction

Patients with HCV infection may present with a variety of symptoms that are not necessarily related to liver disease such as fatigue, arthralgia, myalgia, or Sicca-like syndrome. These symptoms can be related to EHMs of HCV infection.

Studies have reported that up to 40–74% of patients with HCV infection develop at least one EHM during their lifetime [1]. EHMs might even be the first clinical manifestation of HCV infection. Therefore, knowledge of extrahepatic diseases associated with chronic hepatitis C may help diagnosis at stages before severe liver damage has occurred. In addition, EHMs of hepatitis C can dramatically affect the overall prognosis of the disease. Thus, specific management of EHMs is essential.

In this chapter, we will discuss diseases that have frequently been reported to be associated with HCV infection and the specific management they require. Some EHMs are certainly caused by HCV, while for others a causal relation with HCV infection is controversial and in some cases is based on just single-case reports.

Zignego *et al*. [2] defined four categories of EHMs of HCV infection depending on the level of evidence for an association. Category A includes manifestations with a certain association proven by epidemiological data and results concerning the pathogenesis. EHMs in category B are characterized by epidemiological evidence only, whereas in category C an association is controversial and still needs to be confirmed by additional data. Category D is used for diseases for which a link to hepatitis C has been reported in just a few cases.

Figure 20.1 gives an overview of possible EHMs, how they are linked, directly or indirectly to HCV infection, and the grade of evidence for an association according to the categories defined by Zignego *et al*. [2].

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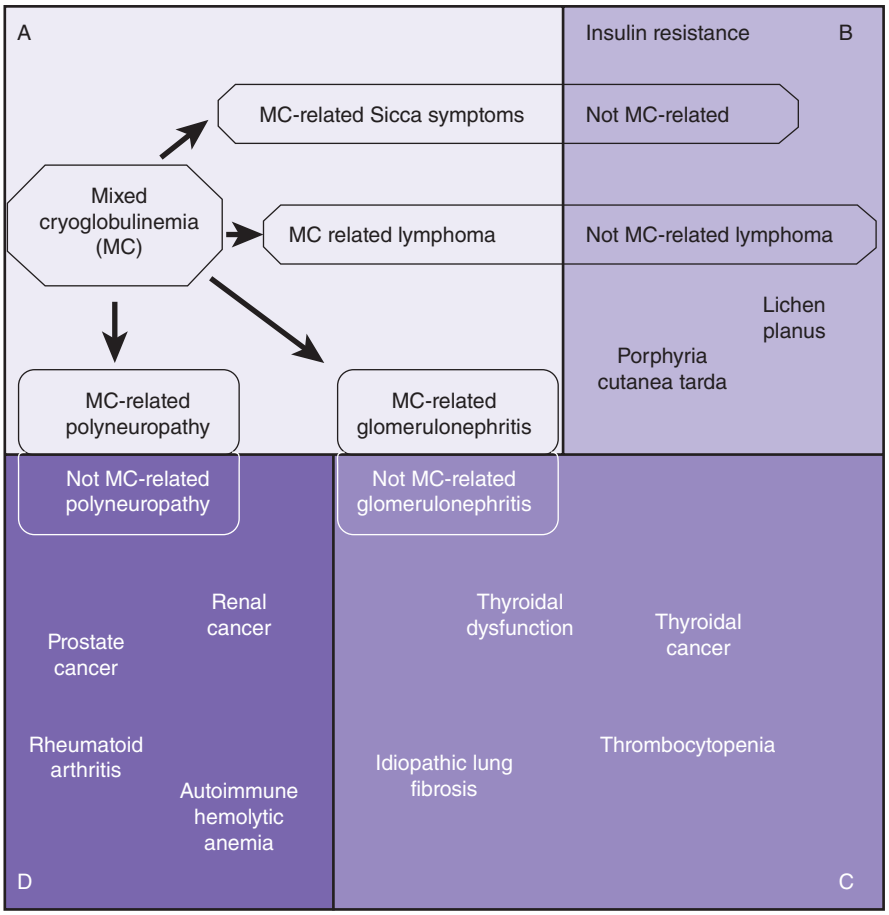

Figure 20.1 Classification of extrahepatic manifestations (EHM) of hepatitis C virus (HCV) infection adapted from Zignego *et al*. [2]. The four big squares represent the four different categories defined by Zignego *et al*. Each EHM of HCV infection has been placed according to the category to which it belongs. EHMs that can also occur as part of mixed cryoglobulinemia (MC) syndrome have been allocated to more than one category. If they appear MC related, they belong to category A. Otherwise, a different category has been chosen depending on the level of evidence for an association with HCV infection independent from MC.

Mixed cryoglobulinemia

Cryoglobulinemia is defined by the presence of specific immunoglobulins so called "cryoglobulins" in the blood. The characteristic feature of these immunoglobulins is the precipitation and formation of immune complexes at temperatures lower than 37 °C.

In general, cryoglobulinemia is divided into three types according to the monoclonal or polyclonal character of the responsible immunoglobulins. In type I immunoglobulins, either IgG or IgM are of monoclonal origin. Type II and III are so-called mixed cryoglobulinemias (MC) because in both there is at least one polyclonal component in the precipitating immunoglobulins. Type II usually contains a polyclonal IgG and a monoclonal IgM element with rheumatoid factor (RF) activity. In type III, all cryoglobulins are polyclonal [2, 3].

Type I is found in lymphatic malignancies, especially B cell lymphomas. MC, on the other hand, can appear in different kinds of diseases, but there is the strongest association by far with HCV infection. Approximately 60–96% of MC is caused by HCV, and about 19–55% of the patients with HCV infection have detectable cryoglobulins [3, 4]. Whether the frequency of MC is higher in HCV genotype 2 infection is controversial. The three types of cryoglobulinemia and their characteristics are summarized in Table 20.1.

Pathogenesis

Cryoglobulins are produced by B cells. HCV infection in turn can lead to clonal B cell expansion followed by cryoglobulinemia. A couple of possible pathways have been discussed.

Chronic hepatitis C as a chronic infection is accompanied by a continuous stimulation of the immune system, which might be a reason for increased lymphocyte proliferation. This is associated with an increased risk of

Table 20.1 Cryoglobulins modified according to Ferri *et al*. [10].

Type	Immunologic composition	Associated diseases
Cryoglobulinemia Type I	Monoclonal Ig (mostly IgM)	Different lymphoproliferative disorders
Mixed cryoglobulinemia Type II	Monoclonal component with RF activity (mostly IgM) and polyclonal Ig (mainly IgG)	Mainly HCV, Less frequently other infections, lymphoproliferative disorders, autoimmune diseases, or essential MC
Mixed cryoglobulinemia Type III	Polyclonal Including a polyclonal Ig with RF activity (mostly IgM)	Mainly HCV, Less frequently other infections, lymphoproliferative disorders, autoimmune diseases, or essential MC

HCV: hepatitis C virus; MC: mixed cryoglobulinemia; and RF: rheumatoid factor.

chromosomal aberrations, which can lead to lymphoproliferative disorders [5].

Besides this indirect role as a trigger factor for the immune system, it has been shown that HCV is not only a hepatotropic but also a lymphotropic virus. HCV RNA is able to replicate in lymphatic cells, and the presence of HCV RNA in lymphocytes is a risk factor for lymphoproliferative disorders like lymphoma or cryoglobulinemia [5].

One specific pathway, through which HCV is suggested to increase proliferation of B cells, is the interaction of the HCV envelope protein E2 and the B cell surface protein CD81. CD81 is upregulated in patients with HCV infection. However, in patients with hepatitis C–related MC, even higher levels of CD81 have been observed [6]. Binding of E2 on CD81 leads to B cell activation as well as proliferation and causes hypermutation of the immunoglobulin gene in B cells [6, 7]. Mutations in E2 may help to explain why not all patients develop MC, since Hofmann *et al*. showed that specific mutation patterns in the E2 gene correlate with the presence of MC [8].

Chromosomal translocation 14,18 resulting in overexpression of the anti-apoptotic gene *bcl*-2 plays a crucial role in MC. Frequency of translocation 14,18 is significantly higher in HCV-infected patients with MC compared to patients with chronic hepatitis C but without MC. An even higher prevalence of this translocation can be found in cases of MC with monoclonal components (MC II) or non-Hodgkin lymphoma (NHL) [5, 6].

Cryoglobulinemia results in a deposition of immunoprecipitants comprising pathological IgG and IgM in the endothelium. This leads to small-vessel damage, with leukocytoclastic vasculitis, which is the basis for the clinical presentation [3].

Clinical presentation

In general, in HCV-infected patients, just the presence of cryoglobulins does not significantly influence the natural course of hepatitis C [9]. The majority of patients with detectable cryoglobulins are asymptomatic or show only mild symptoms. About 3–30% of the cases of MC, depending on different studies, present with clinical signs [3, 4]. The presence of cryoglobulins as well as clinical symptoms accompanied by characteristic histological or serological markers is called MC syndrome. Thus, in total, only 1–15% of the patients infected with HCV develop MC syndrome. It is still not completely understood why just a small percentage of the patients with MC are symptomatic, although several risk factors have been reported, including high levels of cryoglobulins and MC type II [3].

Table 20.2 Frequency of clinical symptoms in patients with mixed cryoglobulinemia syndrome [10, 11].

Clinical symptom	Frequency in patients suffering from mixed cyroglobulinemia syndrome in %
Weakness	89–100
Purpura	91–98
Arthragia	83–98
Peripheral polyneuropathy	36–80
Sicca-symptoms	36–53
Raynaud's syndrome	34–48
Nephropathy	30–36
Lymphoma	7.5–10
Arthritis (non-erosive)	7–10

Weakness: Weakness was documented as a symptom without differentiation from fatigue. Nephropathy: Either definite membranoproliferative glomerulonephritis [10] or just any case of proteinuria, increased serum creatinine, glomerulonephritis, or progressive renal failure [11]. Lymphoma: Either only B cell NHL [10] or predominantly B cell NHL (17 of 21 patients) [11].

The variety of symptoms that can appear in MC syndrome is shown in Table 20.2. The traditional triad, which was named after Meltzer who first described MC, consists of the most frequently documented symptoms: weakness, purpura caused by systemic vasculitis, and arthralgia [10, 11].

Polyneuropathy

Polyneuropathy can be found in up to 80% of the patients with hepatitis C–related MC syndrome. It is

most likely caused by a vasculitis of small epi- and endoneural vessels.

Polyneuropathy more frequently presents as mixed, axonal, mainly sensory peripheral neuropathy. Although most of the time hepatitis C–associated polyneuropathy occurs as a part of MC syndrome, there are also case reports of a clinical manifestation of polyneuropathy independent from MC [2, 7].

Lymphoma

As described in this chapter, the influence of HCV on the lymphatic system results in increased lymphocyte proliferation. After rearrangement of the proto-oncogene *bcl*-2, causing prolonged B cell survival, more mutagenesis might happen affecting different proto-oncogenes and anti-oncogenes. As a result, lymphatic malignancies appear.

It has been mentioned that HCV RNA can be found in lymphatic cells, and it has been suggested that an infection of lymphatic cells by HCV may contribute to the development of lymphatic malignancies. There might exist some unknown indirect pathways since a direct insertional oncogenesis can definitely be excluded due to the fact that HCV RNA cannot be integrated into host DNA since the virus does not contain an enzyme with reverse transcriptase activity [5].

Some authors have described MC, especially type II (with a monoclonal component), as a stage just before lymphoma. Indeed, about 7.5–10% of patients with HCV-related MC type II develop a B cell NHL at some point [2, 10]. Other types of lymphatic malignancies can appear as well in patients with HCV infection, but the most frequent HCV-related lymphomas are B cell NHLs [2, 6]. Although lymphoma appears more often in patients with MC and HCV infection, the prevalence of lymphatic malignancies also seems to be increased in patients with chronic hepatitis C without cryoglobulins [2, 7].

Sicca-like symptoms

Sicca-like symptoms, confirmed by abnormal Schirmer and Saxon test results, can be found in up to 50% of patients with HCV infection, and there seems to be an association with mixed cryoglobulinemia syndrome similar to other lymphoproliferative diseases. But there seems to be no definite association with a classic Sjögren syndrome concerning a correlation of Sicca-like symptoms with the presence of characteristic antibodies, including those against Ro, La, and alpha-fodrin [12].

Nephropathy

Involvement of the kidney is a frequent and feared complication, as its management can be difficult. HCV-related nephropathy can occur with but also without MC. In MC, renal involvement significantly increases mortality. Therefore, it is an important prognostic factor [11]. Response to antiviral therapy is significantly impaired in patients with renal involvement [13]. Reduced renal function also interferes with ribavirin therapy. Thus, early diagnosis and treatment of renal impairment are essential. Possible laboratory findings include hematuria, anemia, proteinuria (which can be accompanied by edema), immunodeficiency, and thrombophilia, as well as elevated levels of serum creatinine. Renal involvement can also result in hypertension.

Membranoproliferative glomerulonephritis is the most common renal manifestation of HCV-related MC. Most of the patients are asymptomatic or present with proteinuria and microhematuria. Intraglomerular deposits of RF, HCV antigen, and antibody are responsible for kidney damage. Approximately 15% of the patients with membranoproliferative glomerulonephritis due to MC develop severe renal failure, which requires dialysis [2, 3, 7].

Diagnosis

Diagnosis of MC syndrome is based on serological and histological findings as well as characteristic clinical symptoms. Useful serological markers are complement factor C4, RF, HCV antibodies, and HCV RNA indicating HCV infection and detection of cryoglobulins.

For the detection of cryoglobulins, a specific procedure should be followed. Before separating serum from cellular components of the blood, the blood sample has to be kept warm at 37 °C. Afterward, serum needs to be incubated at 4 °C for about 1 week. Finally, Western blot or comparable methods can be used as the final step [10, 11]. In approximately 60–70%, RF is detectable in the serum of patients with MC. RF is a nonspecific marker as it can be found in several autoimmune diseases and even in healthy individuals. But since the measurement of cryoglobulins may not be easily accessible, RF can be a useful screening marker. Beside markers that can be found in the serum, clonal B cell infiltrates in liver or bone marrow can be identified by immunohistochemical methods. Biopsy can reveal a leukocytoclastic vasculitis [10].

Based on the parameters mentioned here, Ferri *et al*. designed a classification, which can be used for the diagnosis of MC syndrome. A modified version of this classification for the diagnosis of HCV-associated MC syndrome is presented in Figure 20.2 [10, 11].

Treatment

Understanding the pathogenic pathway of MC syndrome in HCV infection leads to therapeutic strategies.

Diagnostic categories	Diagnostic marker	Major or minor
Serological and pathological markers	Cryoglobulins	Major
	Rheumatoid factor and/or leukocytoclastic vasculitis and/or clonal B cell infiltrates in liver and/or bone marrow	Major
	Low C4	Minor
Clinical symptoms	1. Skin ulcers 2.Chronic hepatitis 3. Membranoproliferative glomerulonephritis 4. Peripheral polyneuropathy	
	Two of the above-mentioned clinical signs and/or purpura	Major
	One of the above-mentioned clinical signs	Minor

All three major criteria: "definite" MC syndrome.
At least one major or two minor criteria: "incomplete or possible" MC syndrome.

Figure 20.2 Proposed algorithm for a diagnosis of mixed cryoglobulinemia syndrome, modified according to Ferri *et al*. [10].

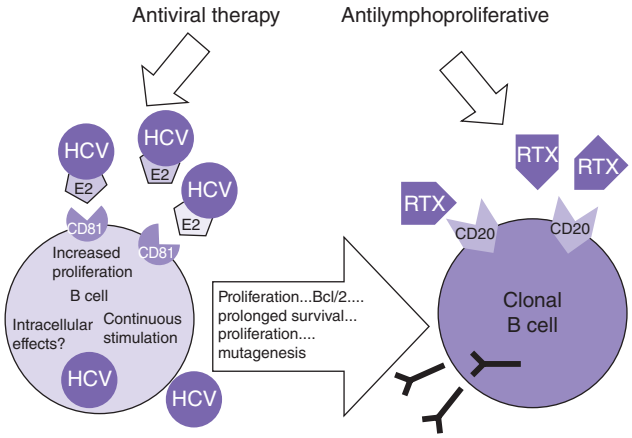

Figure 20.3 Pathogenic mechanisms and resulting therapy targets of hepatitis C–related mixed cryoglobulinemia syndrome. Different mechanisms are involved in hepatitis C virus (HCV)-induced B cell proliferation. A direct signaling pathway is initiated by the binding of the HCV envelope protein E2 to the B cell surface protein CD81. Continuous stimulation of the immune system and intracellular effects of HCV have also been suggested to have a certain impact. Increased proliferation results in mutagenesis and finally in clonal B cells that produce cryoglobulins (black antibodies). As a result of this pathogenic model, therapeutic options are antiviral therapy to remove HCV or inhibition of B cell proliferation by the antilymphoproliferative anti-CD20 antibody rituximab (RTX).

MC is caused by clonal B cell expansion triggered by HCV. As a result, there are basically two different therapeutic targets: one is to eliminate the triggering cause, which is HCV itself. Another very effective option is to interfere further downstream by inhibiting B cell proliferation, as it has been well established in several other lymphoproliferative disorders (Figure 20.3).

Antiviral treatment

The primary goal should be the elimination of HCV. In the past, standard antiviral therapy of hepatitis C was based on two drugs, interferon alpha (IFNα) or pegylated IFN (PEG-IFN) and ribavirin (RBV), a fact that has changed dramatically over the last few years. Many direct-acting antiviral agents (DAAs) are in clinical development. Two HCV protease inhibitors (PIs), boceprevir and telaprevir, have already been approved by the US Food and Drug Administration and the European Medicines Agency for the treatment of patients with HCV genotype 1 [14]. DAAs will certainly have a great impact on therapeutic strategies for EHMs of hepatitis C as well. However, to this point there are no data available for this subgroup of patients, and this still has to be investigated in clinical trials. Thus, in this chapter, we will focus on the data for treating MC syndrome with PEG-IFN and RBV.

The antiviral efficacy of IFN in hepatitis C has been well known for several years. However, the history of IFN in the treatment of cryoglobulinemia started even earlier. Because of its antiproliferative effect, IFN was used in the management of MC even before the discovery of HCV itself [15]. Later, Misiani *et al*. nicely demonstrated the close relation between clinical and biochemical improvement and the underlying decrease in HCV RNA level after a 24-week course of IFN. In this study, during treatment HCV RNA was undetectable in 60% of patients with hepatitis C–associated MC syndrome. HCV RNA absence was accompanied by a significant decrease of cryoglobulins-precipitates, transaminases, IgM level, RF, and serum creatinine, indicating an improvement of renal function as well. In contrast, these improvements were not observed in the 40% of patients who showed no antiviral response, marked by persistent detection of HCV RNA in serum. These results demonstrated the essential role of antiviral therapy for the management of MC syndrome. HCV relapse was associated with a reappearance of typical

Table 20.3 Different therapy regimens for hepatitis C–related mixed cryoglobulinemia syndrome.

Author	Patient number	Therapy regimen	VR/ SVR %	CR/SR %	Comment
Misiani (1994) [16]	27	Interferon alpha (IFNα) 1.5 MIU 3/w (1 w) then 3 MIU 3/w (23 w)	56/0	56/0	CR = CCR + PCR
Calleja (1990) [29]	18	IFNα 3MIU 3/w 12 months	56/28	56/28	CR = CCR + PCR
	13	IFNα 3MIU 3/w 12 months + ribavirin 1200 mg/d	69/54	69/54	8 nonresponders and 5 relapsers
Mazzaro (2005) [18]	18	Peg-IFNα 1.0 μg/kg/w + ribavirin 1000–1200 mg/d (48 w)	83/44	89/44	
Sansonno (2003) [26]	20	Rituximab 375 mg/m^2/w (4 w)	ND	80/60	HCV-RNA increase
Visentini (2011) [27]	24	Rituximab 250 mg/m^2/w (2 w)	ND	79/25-46 Only 3 patients with CCR	6 patients have not yet finished follow-up Stricter criteria for CCR No increase of HCV-RNA
Saadoun (2008) [30]	16	Peg-IFNα 1.5 μg/kg/w + ribavirin 600–1200 mg/d (12 m) + rituximab 375 mg/m^2/w (4 w)	69/50	CCR: 63/50 + PCR = 31	Group consists of 11 nonreponders and 5 relapsers
Saadoun (2010) [25]	55	Peg-IFNα 1.5 μg/kg/w + ribavirin 600–1200 mg/d (48 w)	SVR: 60	CCR: 73 + PCR: 24/18	
	38	rituximab 375 mg/m^2/w (4 w) followed by peg-IFNα 1.5 μg/kg/w + ribavirin 600–1200 mg/d (48 w)	SVR: 58	CCR: 74 + PCR:24/18	
Dammacco (2010) [24]	15	Peg-IFNα 1.5 μg/kg/w + ribavirin 1000–1200 mg/d (48 w)	67/>13	33/13 + PCR: 33	Long follow-up 36 month
	22	Peg-IFNα 1.5 μg/kg/w + ribavirin 600–1200 mg/d (48 w) + rituximab 375 mg/m^2/w (4 w) followed by two 5 monthly infusions	77/>46	55/46 + PCR: 23	Strict criteria for complete response

CCR: Complete clinical response; CR: clinical response; PCR: partial clinical response; SR: Sustained clinical response; SVR: sustained virological response; VR: virological response.

clinical symptoms of MC syndrome [16]. The association between virological response and a significant improvement of clinical and immunological signs related to MC has been confirmed in several studies using different antiviral regimens afterward (Table 20.3) [2, 3]. Beside the documented benefits for patients treated with IFN, there are also contrary reports, in which an exacerbation of cryoglobulinemia-associated vasculitis after treatment of hepatitis C with IFN or PEG-IFN was observed [17].

Also, RBV seems to be beneficial for hepatitis C–related MC, as even RBV monotherapy may have a positive influence on clinical symptoms, serum levels of transaminases, and viral load without full eradication of HCV [2].

In recent years, there have also been studies using combination therapy of PEG-IFN and RBV, the recent standard treatment for chronic hepatitis C (Table 20.3). For example, Mazarro *et al*. documented 18 patients suffering from hepatitis C–related MC syndrome who were treated with PEG-IFN and RBV for 48 weeks. End-of-treatment virological response was 83%. All of these patients showed a clinical improvement, most of them a complete response, and only two exhibited a partial response after therapy. One-half of the patients classified as virological responders failed to achieve a sustained virological response (SVR) as HCV RNA could be detected 6 months after the end of treatment. In all of these patients, recurrence of HCV RNA was accompanied by relapse of clinical symptoms. In contrast, all patients with SVR showed a sustained clinical response as well, confirming the importance of antiviral therapy for the management of hepatitis C–related MC syndrome [18].

Interestingly, in two patients clinical symptoms of MC disappeared although these patients were classified as virological nonresponders. This fact might be caused by the antiproliferative effect of IFN, which has been discussed in this chapter [18]. In some cases, despite the absence of virological response, it may be worthwhile to continue therapy with IFN or PEG-IFN if there is an improvement of MC-related symptoms. We, for example,

published a case report of a 65-year-old female with hepatitis C–related MC presenting with rapidly progressive severe polyneuropathy. Because of her severe polyneuropathy, the patient was not able to walk on her own. We started antiviral therapy with PEG-IFN/RBV, but HCV RNA levels did not decline after 3 months, which normally requires therapy to be stopped. However, we decided to continue treatment according to the decrease of cryoglobulin level and improvement of clinical symptoms detectable since 4 weeks after the start of therapy. During continued therapy, the patient recovered the ability to walk for more than 50 meters on her own [19]. Thus, although there is a strong link between virological and clinical response, there are obviously patients with clinical response but not virological response.

In contrast, there have also been reports that patients can suffer from persistence of MC syndrome despite achieving SVR [20]. Co-infection with Epstein–Barr virus or an underlying lymphatic malignancy may contribute to the persistence of MC after elimination of HCV. Other studies discussed occult HCV infection as an explanation for persistence of MC [20]. However, after achievement of SVR, an occult HCV infection seems to be unlikely as late relapse is rare [21]. According to the pathogenic pathways triggered by HCV interaction with B cells resulting in mutations or even lymphomas, it is also possible that irreversible mutagenic changes might be responsible and HCV might not be necessary as a trigger for clonal expansion at some point. As a result, alternative strategies are needed for patients with persisting MC after viral elimination. Alternatives are also necessary for nonresponders to antiviral therapy, as in these cases symptoms of MC might remain as well. In view of the pathogenic mechanism, a possible therapeutic option can be found further downstream by directly inhibiting the underlying clonal B cell expansion (Figure 20.3).

Inhibition of clonal B cell expansion with rituximab

A concept to counter clonal B cell expansion is by treating it with the anti-CD20 monoclonal antibody rituximab (RTX). The success and safety of RTX in hepatitis C–associated cryoglobulinemia have been confirmed under different circumstances [22]. Patients show high response rates in terms of clinical presentation and immunological markers like cryoglobulin level. Ferri *et al*. were able to demonstrate the beneficial effects of RTX in patients with MC syndrome, of whom 92% were infected with HCV. In this multicenter study, 87 patients with cryoglobulinemic vasculitis were treated. Besides impressive success in terms of clinical and immunological signs, renal improvement was also remarkable. 95% of the patients with nephropathy showed a clinical response, and 50% achieved a complete clinical response. For peripheral neuropathy, a response could be observed in 70%, and for NHL in five out of six cases. During therapy and in a follow-up period of 6 months, mostly moderate side effects at a frequency of 21% appeared. The most frequent adverse effects attributed to RTX were infusion-related reactions (5%) and infections (5%). Three patients had a severe adverse event and had to quit the study [23].

Recent data suggest that the best therapeutic result can be achieved by a combined therapy consisting of an aggressive antiviral therapy (including PEG-IFN and RBV) and an inhibition of B cell proliferation by using RTX.

Dammacco *et al*. treated 22 patients with a triple therapy of PEG-IFN, RBV, and RTX, and 15 patients with a dual therapy of PEG-IFN and RBV only (Table 20.3). After one year, therapy was stopped. At the end of therapy, 54.5% of the patients who received the triple therapy had achieved a complete response, which was significantly higher than 33.3% in the group treated with dual therapy. Criteria for a complete response were strict in this study, as a complete clinical, immunological, and virological response and the disappearance of B cell clonalities were required. Three years after the end of therapy, 17.7% of the patients treated with RTX showed a relapse of MC syndrome. Conversely, there was a relapse rate of 60% in the group with PEG-IFN/RBV [24]. Positive effects of the triple therapy were also published by Saadoun *et al*., who compared in a prospective nonrandomized cohort study 38 patients treated with RTX for a month on a weekly basis before starting with PEG-IFN/RBV for 48 weeks with a control group of 55 patients receiving PEG-IFN/RBV only. In patients treated with triple therapy, the time until clinical improvement was on average 3 months shorter. Patients who received triple therapy also benefited in terms of cryoglobulin clearance. Renal outcome measured by GFR, serum creatinine, proteinuria, and hematuria were significantly better after therapy that included RTX, resulting in a twofold higher rate of complete clinical response in terms of renal involvement (81% vs. 40%). However, in contrast to the findings of Dammacco *et al*., there was no difference in overall clinical response in the group that received RTX regarding MC syndrome. Both groups had similar rates of total clinical response (73.7% in triple therapy vs. 72.7% without RTX), and there was no difference in the percentage of relapse after therapy (18.8% in triple therapy vs. 18.9% without RTX). An important observation is that RTX did not impair SVR (60% in triple therapy vs. 57.9% without RTX). Triple therapy resulted in additional adverse effects caused by RTX, such as serum sickness and a higher risk of infection. Similar to the results of monotherapy with

RTX, these additional side effects were moderate, and no patient had to discontinue therapy [25].

In a meta-analysis of 279 patients with MC syndrome treated with RTX, the majority of adverse effects were classified as moderate as well. Nevertheless, seven cases of severe adverse events possibly related to RTX were identified, and three patients died. Increased risk of infections has to be considered when RTX is used [23]. In addition, HCV viral load might increase, particularly if high-dose RTX (375 mg) is used as monotherapy [26]. Although RTX does not have any significant adverse impact on liver function or SVR, liver function and viral load should be carefully monitored. One study used 250 mg RTX and did not observe an increase of HCV RNA, but the clinical response rate appeared to be lower compared to other studies (Table 20.3) [27]. Importantly, hepatitis B serology has to be performed before treatment with RTX. Hepatitis B surface antigen (HBsAg)-positive and even HBsAg-negative but hepatitis B core antibody (anti-HBc)-positive individuals can develop hepatitis B reactivation, which may lead to fulminant hepatitis. In those cases, preemptive therapy with nucleos(t)ide analogs should be given until 12 months after the end of RTX treatment [28].

Other

Corticosteroids may be considered in cases of severe complications of MC syndrome like renal involvement or neuropathy, especially in patients who did not respond to other therapies. The role of cyclophosphamide is mainly restricted to severe cases and in combination with plasmapheresis. Plasmapheresis should be considered in very severe and life-threatening situations. It is quick and effective in removing cryoglobulins. A benefit can be achieved by a low-antigenic-content diet, which requires a low concentration of alimentary macromolecules. This diet should be considered as supportive treatment; however, since no severe side effects have been observed, it can widely be considered [22].

Conclusion

According to the data published so far, first-line therapy of hepatitis C–related MC syndrome should be the elimination of HCV as it cures the underlying disease. This can be achieved by standard antiviral therapy for hepatitis C using PEG-IFN/RBV or, in the future, by adding DAAs such as HCV PIs. A significant advance of the new PIs, telaprevir and boceprevir, in the treatment of hepatitis C genotype 1 has been proven [14]. It is likely that there will be a positive effect on HCV-related cryoglobulinemia as well [29]. Despite the lack of data on patients with MC, triple therapy including either telaprevir or boceprevir can be considered in patients with MC because sustained elimination of HCV RNA offers the best chance of permanent clinical response in patients with MC syndrome.

RTX is an effective and safe therapeutic option. In patients with mild MC syndrome, additional therapy with RTX may not be necessary due to the risk–benefit ratio. In severe cases of MC, where patients require a rapid improvement, or in cases with renal involvement, where prognosis is significantly impaired, additional therapy with RTX before or simultaneously with antiviral treatment might be the first choice. Patients who do not tolerate antiviral therapy can be symptomatically treated with RTX as well. In cases of nonresponse to antiviral treatment, continuing IFN if well tolerated can be considered according to the improvement of MC syndrome. Nevertheless, RTX might be a better alternative under these circumstances [30].

Other options are mainly limited to life-threatening situations.

Treatment of lymphoma

HCV-infected patients suffering from B cell NHL should be treated depending on the grade of malignancy. For indolent lymphomas, antiviral therapy (maybe accompanied by RTX) seems to be sufficient in many patients. In these cases, elimination of HCV can lead to a complete regression of lymphoma as it has been observed in mucosa-associated lymphoid tissue (MALT) caused by *Helicobacter pylori*. For aggressive lymphomas, chemotherapy is needed first. Afterward, antiviral treatment can be considered [2, 6, 7].

Insulin resistance as an extrahepatic manifestation

The liver plays a crucial role in glucose and fat metabolism. Diseases of the liver obviously have a negative impact on metabolic homeostasis. Patients with liver cirrhosis present more frequently with insulin resistance (IR) leading to type 2 diabetes mellitus (T2D). But on top of that, HCV infection seems to be an additional risk factor for metabolic disorders [31].

Epidemiology

In 1994, Allison *et al*. first described a significantly higher prevalence of T2D in patients with hepatitis C–related liver cirrhosis compared to patients with liver cirrhosis of other etiologies. Afterward, the association of hepatitis C with T2D was discussed controversially. There were several studies published like the work of Hui *et al*. reporting a markedly increased rate of T2D and IR in patients with HCV infection. Hui *et al*. compared 121 individuals with HCV infection and 137 without HCV

infection. Significantly higher levels of fasting insulin, C peptide, and homeostatic model assessment insulin resistance (HOMA-IR) could be found in the HCV-positive population. Furthermore, IR has been identified as an independent predictive factor for the progression of liver fibrosis in chronic hepatitis C [32, 33].

In contrast, a few published studies such as one by El-Serag *et al*., who investigated a large number of cases, could not detect a higher prevalence of diabetes in patients with HCV infection [34].

In 2008, a meta-analysis of 34 studies was published by White *et al*. demonstrating a markedly increased risk of the development of diabetes due to HCV infection. This meta-analysis included retrospective as well as prospective studies. Risk of diabetes was significantly higher in patients with HCV infection than in patients infected with hepatitis B [35]. Overall epidemiologic data indicate an association between IR, T2D, and chronic hepatitis C. It has even been suggested that the level of HCV RNA might correlate with T2D [31, 33].

Besides an epidemiologic correlation, a major argument for a link between hepatitis C and T2D or IR is based on data indicating a significant improvement of IR and glucose tolerance in patients with hepatitis C after successful antiviral treatment [31, 36].

Pathogenesis

According to the results described in this chapter, an association between HCV and IR and consequently T2D seems to be very likely. There also exist some hints for possible pathogenic mechanisms, but the interaction of HCV and glucose metabolism is still not completely understood. Factors that seem to play a crucial role are tumor necrosis factor alpha (TNFα), peroxisome proliferator-activated receptors (PPARs), suppressors of cytokine signaling (SOCSs), and mammalian target of rapamycin (mTOR), depending on the involved HCV genotype.

In 2004, Shintani *et al*. reported results of experiments with transgenic mice expressing HCV core protein. The transgenic mice had higher insulin levels and worse IR compared to control mice. Additionally, diabetes could be induced by a high-fat diet, whereas this had no effect on control mice. Interestingly, insulin sensitivity could be restored by treatment with an anti-TNFα antibody, demonstrating an important role of TNFα in the development of HCV-associated T2D [37]. Although there is obviously a direct effect of the HCV core antigen on the development of IR, the pathogenic pathways still remain unclear. One pathway seems to be via SOCS3, which antagonizes the insulin-induced phosphorylation and therefore activation of insulin receptor substrate 1 (IRS1). SOCS3 can be induced by insulin and TNFα. It provides negative feedback and is expressed in elevated levels in obesity. HCV core is able to induce SOCS3 as well, leading to an increased ubiquitination and a decreased expression of IRS1 and IRS2. Several studies suggest that the HCV genotype does have a great impact on the molecular mechanisms involved in the development of IR and T2D, affecting both pathogenic pathways and clinical presentation. HCV genotype 1b, for example, seems to activate mTOR. In contrast, HCV genotype 3a decreases expression of PPAR-γ. Both result in downregulation of IRS1, leading to impaired insulin sensitivity. Phosphatidylinositol 3-kinase (PI3-kinase) has been suggested to be involved too, since it can activate IRS1 via phosphorylation [33, 36].

Despite the lack of a clear mechanism, IR can be considered as a specific EHM of HCV infection according to several epidemiological studies, the higher risk in hepatitis C than in other liver diseases like hepatitis B, the various pathogenic pathways initiated by the HCV core protein, and the beneficial effect of antiviral therapy. Therefore, T2D and IR may at least belong to group B as defined by Zignego *et al*. [2].

Diagnosis

Diagnosis of T2D is established using a glucose tolerance test or other tests such as HbA1c. Diagnosis of IR without increased levels of fasting serum glucose but hyperinsulinemia can be difficult. The gold standard for the assessment of IR is the hyperinsulinemic euglycemic clamp test. But since this is a time-consuming and demanding procedure, it is not an appropriate method for daily clinical usage. An alternative and widely used option is HOMA-IR. For calculation of HOMA-IR, the product of fasting plasma insulin measured in μU/l and fasting plasma glucose measured in mmol/l is needed.

$$\text{HOMA-IR} = (\text{fasting plasma glucose} \times \text{fasting plasma insulin})/22.5$$

The HOMA-IR score achieves a high correlation with the hyperinsulinemic euglycemic clamp test but is easier to perform. The typical HOMA-IR score for a normal individual is supposed to be 1. However, a range of values are found in the healthy population, which makes it difficult to define a cutoff for abnormal HOMA-IR results indicating IR or T2D. According to the World Health Organization, IR is defined by a HOMA-IR level above the 75th percentile of the population. But the range of individual HOMA-IR scores varies in different populations. As a result, a lot of diverse cutoff levels have been used. This is further complicated by altered laboratory techniques impairing the comparability of HOMA-IR measured in different centers. In patients

with chronic hepatitis C, a valid cutoff for IR seems to be a HOMA-IR score of more than 4 [38].

Clinical presentation and impact of insulin resistance in chronic hepatitis C

IR and the resulting T2D can cause many problems mainly related to angiopathy. Key examples are polyneuropathy, peripheral vascular occlusive disease, stroke, and heart attack. In patients with hepatitis C, other additional complications of IR and T2D can be observed. These additional complications will be discussed in this chapter.

The link between insulin resistance, steatosis, and fibrosis

HCV infection can lead to steatosis and non-alcoholic steatohepatitis (NASH). Especially in HCV genotype 3 infection, part of the reason for an increased prevalence of steatosis is due to a direct impact of HCV. A direct viral effect has been shown in a mouse model by overexpression of the HCV core protein, which caused steatosis. Other interesting interactions between lipid metabolism and HCV have been revealed in the past, including lipid droplets that apparently are important for viral replication. The accumulation of lipid droplets is facilitated by a phenylalanine instead of tyrosine at position 164 in HCV core protein, as it is characteristic for HCV genotype 3 [39].

However, a major risk factor for the development of NASH is IR and T2D, which, as discussed in this chapter, can be triggered by HCV infection as well. Steatosis might be considered as a hepatic manifestation of the metabolic syndrome. The presence of steatosis or NASH aggravates the prognosis of patients with HCV infection dramatically. There is a significant correlation between severity of steatosis and liver fibrosis in patients infected with HCV genotype 1 [40]. In contrast, in patients infected with HCV genotype 3, steatosis is not linked to liver fibrosis [39].

In chronic hepatitis C, T2D plays a vital role in the development of liver fibrosis. Some data might also suggest that IR (regardless of the presence of diabetes) may be a predictive factor for the occurrence of esophageal varices [36].

In patients with chronic hepatitis C, comorbidity with T2D results in an increased risk of hepatocellular carcinoma (HCC) compared to HCV infection without diabetes [33, 36]. This might be linked to the association between IR and steatosis and resulting oxidative stress, since Ohata *et al*. identified steatosis as an independent and significant risk factor for HCC in patients with HCV infection [41]. However, the mitogenic potential of insulin may contribute to the development of HCC as well [36].

Impact of insulin resistance on antiviral therapy

A very interesting as well as complicating issue is the adverse impact of IR on response to antiviral therapy. Insulin resistance has been identified as a negative predictor for SVR in HCV genotypes 1–4 [42]. Furthermore, an impaired virological response is related not only to IR but also to other markers of metabolic syndrome like obesity, hypertension, and steatosis [43].

Because of the consequences of comorbidity mentioned here, it is not surprising that IR is associated with an increased mortality in patients with chronic hepatitis C [36]. Therefore, diagnosis and treatment of IR and its complications are important. The consequences of IR and diabetes in chronic hepatitis C and possible therapeutic targets are shown in Figure 20.4.

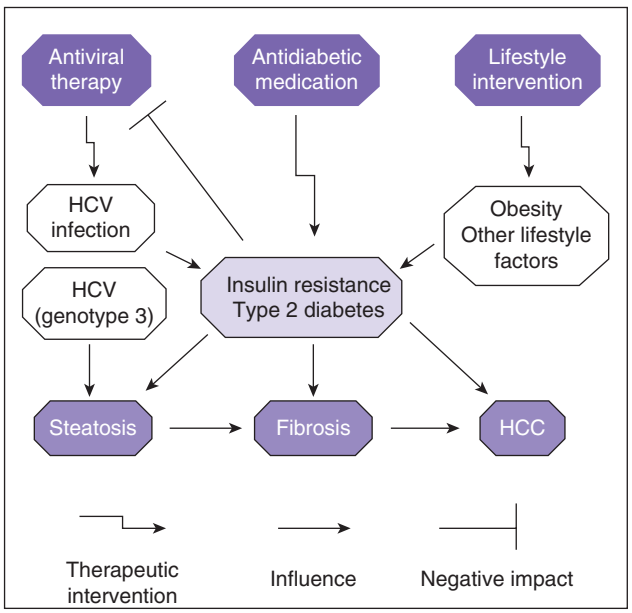

Figure 20.4 Impact of insulin resistance and possible therapeutic targets in chronic hepatitis C. Risk factors for the development of insulin resistance (white boxes) include obesity and a certain lifestyle as well as the hepatitis C virus (HCV) infection itself. In patients infected with HCV, insulin resistance (light purple box) is associated with several adverse effects (boxes in mid-purple), including a lower response rate to antiviral therapy, hepatic steatosis, faster progression of liver fibrosis, and a higher prevalence of hepatocellular carcinoma (HCC). Hepatic steatosis can also be affected directly by HCV (genotype 3). To prevent the adverse effects of insulin resistance in chronic hepatitis C, it is rational to remove the risk factors of insulin resistance (obesity and HCV) or to treat insulin resistance directly. Possible therapeutic interventions (dark purple boxes) are lifestyle intervention, antidiabetic drugs, or antiviral therapy.

Treatment

A successfull eradication of HCV leads to an improvement of IR and T2D. However, considering the adverse influence of IR and associated metabolic syndrome on the success rate of antiviral therapy, it might be rational to treat IR first. Different therapeutic strategies have been investigated.

Lifestyle intervention

Although HCV infection is an independent risk factor for IR and T2D, some advice concerning lifestyle changes that are useful in T2D can be given to patients with hepatitis C–related diabetes as well. Lifestyle changes include a low-fat Mediterranean diet, loss of weight in case of obesity, and moderate physical exercise. The mentioned lifestyle changes do have a great beneficial impact on IR, and in addition they improve general health condition. It has been shown that exercise and diet alone can lead to a remarkable improvement of hepatitis C–related IR, as well as a significantly higher chance of SVR to subsequent antiviral therapy. Diet and exercise can also decrease serum levels of ALT as well as steatosis and fibrosis [33, 36].

Role of antidiabetic drugs

The molecular mechanisms through which HCV leads to IR involve downregulation of PPAR-γ, suggesting that use of pioglitazone may be a good option. But controversial results have been published. Several studies have investigated the use of pioglitazone in combination with PEG-INF/RBV. All studies observed beneficial effects on IR. However, a positive impact on SVR is controversial. Some studies documented increased rates of SVR, [44], whereas a recent well-performed, randomized study including 150 patients could not detect any difference in terms of SVR in patients treated with a triple therapy, including pioglitazone and PEG-IFN/RBV compared to PEG-IFN/RBV alone [45]. Furthermore, pioglitazone often leads to an increase of body weight and may cause hepatotoxic side effects, making its use difficult in patients with chronic hepatitis.

First-line therapy of T2D associated with obesity and metabolic syndrome is the biguanide metformin. It is possible that metformin may reverse metabolic syndrome and improve SVR to hepatitis C treatment.

The Spanish TRIC-1 study aimed to determine if SVR can be improved by adding metformin to standard antiviral therapy. Included patients were infected with HCV genotype 1; they had pathological IR but no clinical diabetes. IR improved in the group treated with metformin and PEG-IFN/RBV compared to the group treated with PEG-IFN/RBV and placebo. Per protocol, SVR was achieved in 67% of the patients who received metformin compared to 49% in the control group. This result was very close to statistical significance, suggesting that a bigger observed population would have been needed and metformin might be a useful therapy option [46]. Metformin can cause lactic acidosis in patients with liver diseases, although this is extremely rare except in patients with very advanced disease.

A recent retrospective study of a huge cohort in Taiwan by Lai *et al*. revealed the ability of antidiabetic drugs, especially metformin but also pioglitazone, in reducing the risk for HCC in patients with T2D. However, this beneficial effect was not specific for the HCV-infected subpopulation. For sulfonylurea, they detected a positive impact that was not significant [47]. However, there are results suggesting that sulfonylurea as well as exogenous insulin may increase the incidence of HCC in chronic hepatitis C [48].

Conclusion

Treatment of IR and related metabolic disorders is a crucial but difficult part of the management of hepatitis C. Lifestyle intervention should be considered for every patient as it does not have any adverse side effects but improves metabolic syndrome, general health condition, as well as response to antiviral therapy. Furthermore, antidiabetic medication might be useful as well. Due to lack of definitive data, insulin sensitizers should be used with caution and preferably only in the setting of clinical trials.

Preliminary data suggest that IR is not predictive for SVR using triple therapy with telaprevir and PEG-IFN/RBV [49].

Dermatological diseases

Some dermatological diseases may be associated with HCV infection. Porphyria cutanea tarda (PCT) and lichen planus (LP) are most likely related to HCV infection. For other disorders, like polyarteritis nodosa, the link is less clear. Several dermatological manifestations occurred in single-case reports only [50]. In this section, we will focus on the most common dermatologic manifestations, which are PCT, LP, and MC-related purpura (as discussed in this chapter).

Porphyria cutanea tarda

Epidemiology

In patients with HCV infection, prevalence of PCT is about 0.77–1%, which is approximately 12 times higher than in the general population [34]. In studies with cohorts of more than 50 patients with PCT, HCV RNA

is detectable in 8–90%. A meta-analysis by Gisbert *et al*. revealed an average rate of 57% of HCV RNA positivity in patients with the sporadic form of PCT [51]. In terms of comorbidity of chronic hepatitis C and PCT, there are huge regional differences. Stölzel *et al*. reported a comorbidity of only 8% after investigation of 111 patients with PCT in Germany [52], whereas in the Czech Republic average results were nearly three times higher. In the United States, Gisbert *et al*. found in their meta-analysis a prevalence of 66%, but the strongest association can be found in Italy (83%) and Japan (85%) [51]. Genetic and environmental features may contribute to these regional differences.

Pathogenesis

Malfunction of uroporphyrinogen decarboxylase, either hereditary (familial form) or acquired (sporadic form), has been identified as the main reason for the development of PCT. In the sporadic form that is associated with HCV infection, impaired activity appears only in the liver.

Clinical presentation

Patients with PCT present with increased sensitivity to light, resulting in multiple lesions predominantly on light-exposed skin like the face or back of the hands. There might appear superficial erosions or even subepidermal bullae as well as hypertrichosis, hyperpigmentation, and dystrophic calcifications [53].

Diagnosis

Diagnosis is based on clinical signs and laboratory findings. Laboratory findings are due to impaired porphyrin metabolism. In the urine, increased levels of uroporphyrins I and III, heptacarboxyporphyrins, or other acetic-acid substitute porphyrins might appear, whereas porphobillinogen is not affected. Isocoproporphyrins and heptacarboxyporphyrins can be detected at increased concentrations in feces. In the serum, uroporphyrin can be measured in elevated levels. Some patients with PCT show increased ferritin levels as well as transferrin saturation, indicating an iron overload, and mild siderosis appears in the majority of patients. This has led to the suggestion that a liver iron overload might be part of the pathogenic pathway [53]. After diagnosis of PCT, testing for HCV infection should be performed.

Treatment

In HCV-associated PCT, the first step should be protection from exposure to sun. In severe cases, chloroquine or iron depletion can be considered. But hepatotoxic side effects must be considered, and iron depletion may interfere with antiviral therapy as anemia regularly occurs during treatment with PEG-IFN/RBV, and even more often if a PI is used as well. The need for antiviral therapy should be made based on the stage of liver disease or the presence of other EHMs of HCV infection [14, 50, 53].

Oral lichen planus

LP is an inflammatory mucocutanous disease with persistent hyperkeratosis.

Epidemiology

The link between oral LP and hepatitis C is controversial. Although a meta-analysis suggested an overall increased prevalence of HCV infection in patients with LP, huge regional differences have been revealed in single studies [54]. Data from Italy or Japan, for example, contrast with studies from Northern Europe [50].

Diagnosis

Diagnosis of LP relies on typical clinical appearance. Biopsy should be performed to confirm diagnosis and to exclude oral malignancies [55].

Clinical presentation

Multiple mucosal lesions of different forms are the characteristic clinical appearance of LP. They may be reticular, erythematous (atrophic), or erosive. These lesions can be painful [55].

Treatment

The role of antiviral therapy remains unclear. There is a case report by Doutre *et al*. suggesting that IFN can be helpful [56]. However, IFN has also been reported to be a causative factor for LP [57]. Treatment of oral LP includes intensive hygienic oral care, or, in symptomatic patients, topical immunosuppressive drugs (steroids) should be considered [55].

Other EHMs

Other observed disorders that have been suggested to be associated with hepatitis C are thyroid disorders, thrombocytopenia, atherosclerosis, extrahepatic malignancies, MC-negative rheumatoid arthritis, autoimmune hemolytic anemia, idiopathic lung fibrosis, and cardiomyopathy. For all of them, an association with

HCV infection is uncertain due to controversial results or a lack of evidence. Some of these manifestations are discussed in this section.

Another important extrahepatic feature of chronic hepatitis C is an involvement of the central neural system (CNS). Patients often present with fatigue. Approximately 20–80% of HCV-infected individuals develop fatigue at some point independent of the stage of hepatitis. Other possible signs of CNS involvement can be depression or cognitive impairment [58]. Weissenborn *et al*. identified increased rates of depression as well as impaired attention in 30 HCV-infected patients with only a mild degree of liver disease compared to uninfected controls [59]. The effect of hepatitis C on the CNS is a potentially serious complication, as a lot of patients are involved and it markedly impairs quality of life. For more detailed discussion on HCV infection and the brain, please refer to Chapter 21.

Thyroid disorders

It is controversial whether HCV infection affects the thyroid gland. But there are some reports, including a meta-analysis, suggesting an increased prevalence of thyroid disorders in patients with chronic hepatitis C [60]. Antonelli *et al*. detected a significantly increased prevalence of hypothyroidism as well as autoimmunity marked by antithyroglobulin and antithyroperoxidase antibodies in patients with chronic hepatitis C (especially in female patients) compared to both healthy and HBV-infected patients. Subclinical hypothyroidism can be found in 2–13% of the patients with chronic hepatitis C. Female gender and antithyroperoxidase antibodies were identified as major risk factors for the development of hypothyroidism [60]. Thyroid disorders can also be aggravated or even caused by antiviral therapy with IFN, and can persist after end of treatment [61]. Thus, thyroid malfunction can markedly complicate antiviral therapy. Therefore, monitoring thyroid hormones before and during IFN treatment is essential. Management can include thyroid hormone replacement or, in certain situations, discontinuation of IFN therapy.

Atherosclerosis

Whether HCV promotes the risk of atherosclerosis has not been elucidated yet. An increased prevalence of T2D, on the one hand, but a protective pattern of serum lipid proteins, on the other hand, associated with HCV infection make it difficult to predict the overall outcome [62]. Ishizaka *et al*. found a significantly increased prevalence of HCV infection in patients with carotid-artery plaques (3.7% vs. 1.7%) [63]. In contrast, a recently published study analyzing a large population in Egypt suggests that these two factors balance the risk, resulting in an equal prevalence of atherosclerosis in HCV-infected and HCV-negative patients. However, when matching the groups by ordinary cardiovascular risk factors, an increased risk in HCV-infected patients appeared [64].

Extrahepatic malignancies

HCV infection has been reported to be a risk factor for the development of some extrahepatic malignancies. The increased prevalence of lymphomas, for example, has already been discussed in this chapter.

There may also be an association of hepatitis C with breast cancer in young patients and with prostate carcinoma [65, 66]. Other malignancies that have been suggested to be related to HCV infection are primary renal cell carcinoma [67] and thyroid papillary cancer [2]. So far, evidence is weak, and data have to be confirmed in larger studies.

Summary

The variety of disorders seen in patients with HCV infection is huge, and it often remains uncertain whether a disease has an etiological association with HCV infection and can therefore be defined as an EHM of HCV.

EHMs of HCV infection appear frequently and can markedly impair quality of life, complicate the management of hepatitis C, or even significantly worsen the overall prognosis of the disease. As a result, it is essential for physicians to be aware of EHMs, to make proper diagnosis, and to institute appropriate treatment. Patients presenting with disorders that often appear with HCV infection, like mixed cryoglobulinemia or lichen planus, should be tested for hepatitis C.

Despite the diversity of diseases, antiviral treatment is helpful in many cases, especially if there is a strong association between that condition and HCV infection, as has been shown in MC and IR. Therefore, antiviral therapy should in general be considered as the first treatment option, and new DAAs will certainly improve the success rate in upcoming years. In some cases, it might even be rational to continue an IFN-based antiviral regimen despite a virological failure if there is clinical response. IFN-associated side effects and potential exacerbation of extrahepatic symptoms have to be considered. In some conditions, antiviral therapy is not sufficient and EHMs require additional, more specific therapeutic strategies, for example RTX in severe cases of MC syndrome.

In conclusion, EHMs of HCV infection necessitate multidisciplinary management involving not only hepatologists but also sometimes rheumatologists, hematologists, neurologists, nephrologists, and dermatologists.

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We apologize that we could not cite every original paper due to space restrictions.

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Chapter 21
Central nervous system complications of hepatitis C virus infection

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Summary

Chronic hepatitis C virus (HCV) infection is a major cause of liver morbidity, and hepatic encephalopathy is the clearest example of central nervous system (CNS) involvement as a result of virus-induced cirrhosis. However, there is accumulating evidence to suggest a direct effect of HCV infection on CNS function, which can occur at an early stage of chronic infection prior to the development of cirrhosis and unrelated to encephalopathy. In this chapter, we review the current evidence from neuropsychological, brain-imaging, and virological studies that support a direct link between HCV infection and cerebral function and propose a model for HCV infection of the CNS.

Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of liver-related morbidity and mortality with hepatic fibrosis, cirrhosis, and hepatocellular carcinoma 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 cryoglobulinemia 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]. There has been interest in the possibility of a link between chronic HCV infection and cerebral dysfunction, occurring at an early stage of the infection, prior to the development of cirrhosis and unrelated to cryoglobulinemia. HCV RNA has been detected in the CNS, and recent data show that the virus may infect brain endothelial cells. In this chapter, we review the current evidence for a link between CNS dysfunction and HCV infection, and examine the possibility of direct infection within the brain.

CNS involvement in HCV-related vasculitis

Chronic HCV infection accounts for between 80% and 98% of cases of mixed cryoglobulinemia [6], a condition characterized by the presence of immune complexes between mono- or polyclonal immunoglobulin (Ig) M (rheumatoid factor) and polyclonal IgG. These complexes precipitate at low temperatures *in vitro* and

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contain anti-HCV IgG, HCV RNA, and rheumatoid factor [6]. The prevalence of cryoglobulinemia 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 cryoglobulinemia, and, most importantly, case mix; cryoglobulinemia 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 cryoglobulinemia 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 hemi- or tetraparesis [9, 10], dysarthria [11], encephalopathy [5, 12, 13], dizziness [14], and communicating hydrocephalus [15]. The main pathophysiologic mechanisms are ischemia or hemorrhage due to diffuse or segmental vasculitis of the small cerebral vessels [4]. Ischemic lesions may be detectable with cerebral magnetic resonance imaging as hyperintensities, most commonly in the white matter.

Nonspecific 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 labeled as having cryoglobulinemia syndrome. No relationship has been found between the presence of cryoglobulins and the 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 nonspecific 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 necessarily imply causality, since there are many associated factors that 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 in this chapter.

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 to those with chronic hepatitis B 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 that have shown significant improvements in 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 studies discussed here did not blind their subjects to HCV polymerase chain reaction (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 patients' knowledge of their response. Other studies have demonstrated an association between depression and other comorbidities 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 in this section, since fatigue is a multidimensional symptom and is influenced by multiple interrelating social, behavioral, 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 interferon alpha [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, 45–54]. 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 either to 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 on 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 to patients with liver disease of 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 infection with HIV, 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 that were related to measurements of fatigue severity [45]. 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]. McAndrews and colleagues [50] applied strict exclusion criteria in an attempt to minimize confounding factors and showed minimally poorer learning

efficiency in a small proportion of HCV patients compared to controls, with only 13% considered clinically impaired. Other measures assessing attention and speed of processing were not impaired in this study. Fontana and colleagues studied a patient cohort with advanced liver fibrosis who were enrolled in the HALT-C trial and found that 33% were cognitively impaired on a composite score of 10 neuropsychological tests, with verbal recall and working memory being the most affected domains [51]. A significant proportion (38%) had cirrhosis, however, which constitutes a significant confounder as working memory is known to be negatively affected by advanced liver disease and hepatic encephalopathy. A recent Irish study of a small, homogeneous cohort of iatrogenically infected women, of whom nine had spontaneously cleared HCV, showed that PCR-positive women had impaired memory and attention compared to normal controls and the PCR-negative group [52]. Huckans and colleagues [53] attempted to control for substance use disorder (SUD) and showed that HCV+/SUD– patients performed worse than the HCV–/SUD– control group in verbal learning and attention domains. Their results suggest that HCV infection was associated with cognitive impairment in the absence of a history of SUD.

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 detected only 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 noncirrhotic and compensated cirrhotic groups were enrolled after HCV infection was diagnosed at blood donation. Hence, these groups were positively selected for good health, since 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 to a healthy control group, rather than defining impairment by comparison to population norms. Perry and colleagues [54] recently reviewed the studies in this field and concluded that the combination of evidence from neuropsychological, neurophysiologic, and neuroimaging studies provided "strong support for the presence of an attentional deficit mediated by frontal subcortical circuitry in a group of patients infected with HCV." 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 [55] and are consistent with the involvement of subcortical brain systems [56]. This raises the question of whether HCV may have an additive adverse effect on cognition in patients infected with HIV.

Several studies have now documented greater neurocognitive impairment in patients co-infected with HIV–HCV on measures of executive and overall cognitive functioning [57–63], while a smaller number of studies failed to show any additional impact of HCV infection. Liver fibrosis progresses more rapidly in HIV–HCV co-infection, and an assessment of cirrhosis is necessary to exclude minimal hepatic encephalopathy. Aronow and colleagues [57] studied patients with late-stage HIV infection; 31 tested positive for HCV and were compared to 31 randomly selected HIV mono-infected subjects from the same cohort. Subjects co-infected with HIV–HCV showed greater neurocognitive impairment on a composite scoring system, but there were no statistically significant differences between the groups on individual tests. Two published studies from the Manhattan HIV Brain Bank cohort [58, 59] enrolled HIV-positive subjects with advanced HIV and applied a broad range of neuropsychological tests to a group of 67 patients co-infected with HIV–HCV and a group of 49 well-matched patients mono-infected with HIV. High levels of cognitive impairment were reported across all measured domains in both groups. Co-infected patients were more impaired in executive functioning and more likely to meet diagnostic criteria for AIDS dementia. The authors concluded that there was a detectable impact of HCV co-infection on neurocognitive functioning, despite the advanced stage of HIV disease of their cohort. These studies used biochemical parameters only to assess liver disease, which do not reliably detect cirrhosis. Furthermore, only serological tests were used to diagnose HCV infection, and subjects with cleared infection may have been included. Hilsabeck and colleagues studied 195 individuals infected with HIV, of which 25 were HCV co-infected. Eighty percent of the group co-infected with HCV–HIV were classified as impaired, compared to 69% of the group mono-infected with HIV [60]. This difference was largely due to psychomotor slowing in the co-infected patients, while there were no significant differences between the groups in the other cognitive domains tested. Again, the degree of underlying liver disease was also not controlled for in this study, and co-infected patients had a significantly higher rate of previous alcohol dependence and drug use. Letendre and colleagues [61] did attempt to control for drug use in a cross-sectional analysis of a cohort of 526 prospectively recruited subjects, of which 239 were infected with HIV; they considered the impact of HCV infection and methamphetamine use. Twenty-one percent of the cohort were HCV infected. HCV-infected subjects performed worse on neurocognitive assessment, independent of HIV status and methamphetamine use. Multivariate analysis showed that infection with HIV, infection with HCV, and methamphetamine use were all independently associated with worse cognitive performance. Higher plasma HCV RNA levels were

associated with impaired memory, supporting a link between viral replication and brain injury. Martin and colleagues [62] identified an additive effect on cognitive function of co-infection over mono-infection with either virus only, in 159 drug users with known HIV and HCV serostatus (but no controls for cirrhosis), and there is a possibility that the additional effect of HCV infection on cognitive performance was mediated by minimal hepatic encephalopathy rather than a CNS effect of HCV. A number of other studies have identified positive associations between HIV–HCV co-infection and neurocognitive impairment.

In contrast, a retrospective study from the AIDS Clinical Trials Group Longitudinal Linked Randomized Trials (ALLRT) found that active HCV infection did not exacerbate clinically significant neurocognitive dysfunction or peripheral neuropathy in individuals with controlled HIV infection [63]. In summary, some reports support an association of HCV infection with increased cognitive impairment in HIV infection, but the impact of liver cirrhosis and drug use has not been adequately studied.

Brain imaging

Magnetic resonance (MR) techniques provide one of the most important tools for quantitative analysis of composition and structure. Importantly, the technique is noninvasive, 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 [64]. 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–choline and glycerophosphoryl–choline that 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 [65]. Semiquantitative 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 [66]. This pattern has been reported only with cirrhosis, usually complicated by hepatic encephalopathy and, in patients with hyperammonemia, due to urea cycle diseases.

To date, there have been six cerebral ^{1}H MRS studies in patients infected with HCV but without cirrhosis [40, 50, 67–70], and these studies are summarized in Table 21.1. 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 to both normal controls and patients with chronic hepatitis B infection [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 to 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 [40], patients who performed worse on neurocognitive testing had significantly higher basal ganglia Cho:Cr ratios, although there were no significant associations between MRS metabolites and specific cognitive functions. 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 that were significantly associated with impairments in working memory [68].

Weissenborn and colleagues used MRS to study 30 HCV-infected patients with normal liver function who had cognitive testing [69]. They found decreased NAA:Cr ratios in occipital gray matter compared to healthy controls. In a further study, elevations in white and gray matter mI have also recently been described by the same group [70], and, in light of an inverse relationship between fatigue and cerebral metabolites, they invoked a theory of cerebral compensatory change. McAndrews and colleagues undertook MRS analysis on their cohort of 37 HCV-infected patients with minimal hepatitis [50]. They found elevations in cerebral Cho and reductions in NAA in voxels that were localized to the central white matter. These findings are in keeping with the results from both Weissenborn's group and our own center. However, none of the studies used exactly the same MR parameters for data acquisition or the same methods of data analysis, and so it is difficult to draw firm conclusions other than that definable MRS-measurable metabolic abnormalities exist in a proportion of HCV-infected patients with minimal or absent liver disease.

These MRS findings are qualitatively different from those encountered in hepatic encephalopathy. However,

Table 21.1 Studies employing proton magnetic resonance spectroscopy (MRS) in patients with chronic hepatitis C; metabolic abnormalities compared to healthy controls are shown for different brain regions.

Reference	Brain region	MRS metabolite abnormality	Relationship with psychometric assessment	MRS protocol	Method of analysis
[67]	BG Frontal WM	↑Cho/Cr ↑Cho/Cr	Not assessed	PRESS TR/TE 1500/135 ms	Marconi proprietary software
[40]	BG Frontal WM	↑Cho/Cr ↑Cho/Cr	Higher BG Cho/Cr in patients impaired in > 2 tasks on cognitive battery	PRESS TR/TE 1500/135 ms	Marconi proprietary software
[69]	Occipital GM	↓NAA/Cr	No association found	STEAM, TR/TE/ 1500/18 ms	GE proprietary software
[50]	BG Central WM	↑Cr ↑Cho, ↓NAA	No associations found	PRESS TR/TE 2000/30 ms	LC model with quantitation
[68]	Frontal WM	↑*m*I/Cr	Correlation with working memory reaction time	PRESS TR/TE 1500/40 ms	AMARES algorithm in MRUI
[60]	BG Parieto-occipital WM	↑Cho, ↑Cr, ↑NN ↑Cho, ↑GG	Inverse correlation between fatigue and WM and BG metabolites	STEAM TR/TE 1500/30 ms	LC model with quantitation

BG: basal ganglia; Cho: choline; Cr: creatine; GG: glutamate plus glutamine; *m*I: *myo*-Inositol; ms: millisecond; NAA: N-acetyl-aspartate; NN: combined NAA plus *N*-acetyl-aspartyl-glutamate; TE: echo time; TR: repetition time; WM: white matter.

they mirror those described in HIV infection [71], in which subcortical gray matter and frontal white matter are the principal sites of the metabolic abnormalities [72, 73]. In HIV, the MRS 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 [56, 74]. In early HIV disease, the most common finding is increased mI/Cr in the frontal white matter [75, 76]. Elevated Cho/Cr and decreased NAA/Cr in both the white matter and basal ganglia have also been reported, even in asymptomatic patients [71–73, 77]. In advanced HIV disease, these metabolic abnormalities are all commonly reported [78]. The interpretation of the MRS data has been consistent with neuropathological findings (i.e., neuronal loss) (reduced NAA) and CNS inflammation (increased mI and Cho) [73, 78]. The parallels between the reported MRS results in HIV and HCV infection suggest that, similar to the CNS effects of HIV infection, neuroinflammation may also be a feature in chronic HCV infection and may underlie the neurocognitive deficits observed in a proportion of patients.

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 patient chronic HCV infection [79]. Although cirrhotic patients were not excluded, subgroup analyses were made. HCV-infected patients had delayed P300 peak latencies and reduced amplitudes compared to age-matched healthy subjects. Seventeen percent 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 [80].

Microglial and brain macrophage activation in HCV infection has also been assessed with positron emission tomography (PET), using a ligand that binds to the peripheral benziodiazepine receptor (PK11195) [81]. Taylor-Robinson's group demonstrated increased ligand binding in the caudate nucleus of HCV-infected patients using PK11195 in a pilot PET study, recruiting patients with histologically mild liver disease. The findings, which correlated with viremia, suggest the presence of neuroinflammation and underline the potential similarity of neuropathological processes in HIV and HCV infection. Weissenborn and colleagues examined the effect of HCV infection on neurotransmission [82]. Using a combination of (18)F-fluoro-deoxy-glucose PET and single-photon emission computed tomography (SPECT) in 15 patients with minimal liver disease, they demonstrated reduced glucose metabolism in the limbic association cortex, and in the frontal, parietal, and superior temporal cortices, which correlated with reduced dopamine transporter availability. The authors conclude

that alterations in dopaminergic neurotransmission may be an important mechanism of cognitive impairment in HCV infection.

In summary, there is now accumulated evidence using advanced imaging techniques to demonstrate metabolic, inflammatory, and neurochemical changes within the CNS in HCV infection, including in patients without significant liver disease.

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 1×10^{12} virions are produced daily by the infected liver, this corresponds to a virion production rate of only 50 particles per hepatocyte per day, if it is assumed that a liver contains 2×10^{11} hepatocytes and 10% are infected [83]. 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 reverse transcription PCR (RT-PCR) [84]. The rapid degradation of viral RNA is especially relevant when analyzing postmortem 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 titers 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 since 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 and macrophages, and dendritic cells [85–94]. These studies presuppose that different HCV variants are tropic to different cellular compartments, as is the case for HIV [95, 96]. 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 phagocytosed by peripheral blood mononuclear cells (PBMCs), and that the mere presence of different viral sequences does not prove viral replication. The genomic region most commonly studied has been the hypervariable region 1 (HVR1) within the envelope protein E2. Sequencing of HVR1 from multiple clones from different sites has generated conflicting results regarding the relative complexity of the quasi-species distribution in liver and serum and the presence of extrahepatic replication [88, 97, 98].

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) [99]. This element consists of three stem-loop structures (domains II, III, and IV) and a pseudo-knot, and is recognized by ribosomes, thus allowing translation of the viral polyprotein in a cap-independent manner [100, 101]. Although this region is the most invariant of the HCV genome, it does exhibit a quasi-species distribution in human serum [102]. 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 [102–105]. Furthermore, the effect of these mutations may be either to enhance or to abrogate translational efficiency in different cell lines, suggesting that interactions between specific cellular factors and the IRES may be important in determining cellular tropism [102–105]. 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 [106].

Several groups have reported nucleotide substitutions in the HCV IRES, which are associated with lymphoid replication [86, 103, 107, 108]. 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, and G to A at position 243), which were not detected in the wild-type inoculum [107]. In a subsequent experiment, these mutations increased IRES translational activity in lymphoid cell lines [103]. 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 [108], monocytes, and macrophages [109], and the A107, A204, and A243 polymorphisms have been detected in monocyte-derived dendritic cells [105].

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 [110, 111]. 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 [112]. Indeed, soon after the discovery of HCV, the negative strand was widely reported to be present in serum and PBMCs [112–115]. However, the strand specificity of these early studies was questioned in subsequent work [116, 117]. Experimental methods were adapted to increase strand specificity, including the use of rTth, a thermostable enzyme, and higher temperatures in the PCR cycle [118, 119], allowing a 10000-fold discrimination between the detection of the correct and incorrect strands [120].

Although the initial reports that HCV readily replicates in substantial numbers of PBMCs and serum have been questioned [119], 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 [118, 121, 122] and immunocompromised individuals [109, 123]. Other studies have demonstrated negative-strand HCV in bone marrow and hematopoietic progenitor cells [124, 125]. It has 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 harbor low levels of replicating strains [94]. In one 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 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. However, another study has not shown this, in the absence of stimulation [126].

Detection of HCV RNA in the CNS

The above techniques have 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 out of six patients, who died of complications of chronic HCV [127]. In one of these patients, the A204 and 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 the subcortical white matter and cerebral cortex from two patients who died after liver transplantation for chronic HCV infection [128]. In these two HIV negative patients, the A204 and A243 polymorphisms were present in both tissue and serum sequences.

The same group has also analyzed cell pellets from cerebrospinal fluid, obtained from HIV-positive patients with meningitis or cerebral toxoplasmosis [129]. In three patients, the A107, A204, and A243 polymorphisms were detected in cerebrospinal fluid (CSF) cell pellets and circulating PBMCs. Genotyping by sequencing of the NS5b region revealed different genotypes in the CSF pellets and PBMCs compared to serum in two out of three cases (serum 3a, PBMC/CSF cells 1a; serum 1a, PBMC/CSF cells 4c; and serum 1b, PBMC/CSF cells 1b). Although the authors suggest that their results show that HCV neuro-invasion could be related to trafficking of infected leucocytes across the blood–brain barrier by a mechanism analogous 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 or toxoplasmosis.

In another autopsy study that 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 out of three patients [130]. 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 and 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 favor CNS infection. The translational efficiencies of the brain-derived IRES sequences were generally reduced compared to 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 and A243 mutations showed preserved translational efficiency in HuH7 but reduced efficiency in CHME3 cells. These data suggest that IRES polymorphisms, including A204 and A243, may be important as a viral strategy of reduced translation to favor latency in the CNS as well as other extrahepatic sites, such as dendritic cells [105]. A number of other quasi-species studies support the notion of HCV replication within the CNS [131–133].

More recently, attention has shifted to which cells within the CNS may be permissive to HCV replication. Laser capture microdissected microglia and astrocytes from human postmortem samples yielded HCV RNA [134, 135]. Furthermore, infected microdissected microglial cells expressed significantly increased levels of proinflammatory cytokines IL1, TNFα, IL12, and IL18, consistent with microglial activation, suggested by the imaging studies discussed in this chapter [136]. Somewhat surprisingly, immunohistochemical studies using antibodies to HCV nonstructural proteins 3 and 5 have demonstrated positive staining in human brain and have also localized HCV to microglia and astrocytes [134, 136, 137]. 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 [138, 139]. Although it has been postulated that HCV-infected monocytes might cross the blood–brain barrier to introduce the virus to microglia, there are no data to support this mechanism [67].

Microglia and astrocytes do not express all of the cell surface receptors that are required for direct viral entry [140]. Recent screens of neural lineages for a cell culture system that might support HCV entry and replication revealed two permissive neuro-epithelioma cell lines that also expressed all of the cellular molecules required for virus entry: scavenger receptor B-I (SR-BI), tetraspanin CD81, and tight junction proteins claudin-1 and occludin [140, 141]. The presence of these receptors on undifferentiated tumor cell lines raises the possibility that other nonhepatocyte cells may be permissible to HCV entry and infection *in vivo*. This possibility is strengthened by the recent demonstration by the same group that brain microvascular endothelial cell (BMEC) lines support entry of and infection by cell culture–derived HCV (HCVcc), showing a spreading infection and release of particles that were subsequently infectious for Huh7 hepatoma cells [142]. Immunochemical staining of human brain sections revealed that microvascular endothelium expresses all four receptors required for HCV entry and that HCV entry was restricted to brain-derived endothelial cells, with endothelial cells isolated from liver sinusoids or umbilical vein endothelium failing to support HCV infection. In contrast to Huh7 cells that sustained persistent infection, BMEC infection declined after 120 hours in parallel with cytopathic effects, suggesting an acute, lytic infection. Infected BMEC displayed apoptosis and reduced endothelial barrier activity, leading the authors to propose that HCV may disrupt blood–brain barrier integrity, allowing influx of peripheral cytokines, viruses, and immune cells. In this way, HCV endothelial infection could ultimately lead to an altered immune environment within the CNS with resultant microglial activation.

Conclusions

The possibility of a cerebral effect of HCV has been investigated at different levels: neuropsychological, metabolic, imaging, 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. 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 importance. 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 not yet known. A small pilot study [143] using brain proton MRS and cognitive assessments before, during, and after standard antiviral therapy showed significant CNS metabolic changes toward normal levels in those patients who had an SVR. These findings were interpreted as improvement in cerebral immune activation associated with HCV clearance. Patients achieving SVR also showed significant improvements in verbal learning, memory, and visuospatial memory, which were not seen in treatment nonresponders. This study was small and did not have a healthy control group to allow conclusions on the clinical significance of the observed changes, and therefore it needs to be validated in a larger sample. Conversely, another treatment study reported increased rather than decreased basal ganglia Cho/Cr and NAA/Cr in patients with SVR and no improvement in cognitive measures [144], which would suggest that viral clearance is not associated with improved cognitive function or cerebral metabolite concentrations seen on MRS. However, it might be that a long wash-out phase after the end of treatment is required to mitigate the neurological effects of IFNα treatment itself [145]. Indeed, there is emerging evidence that gains in quality of life and symptoms occur only sometime after cessation of treatment [16]. Consistent with this is the recent report from Kraus, which demonstrated significant improvements in working memory, attention, and vigilance at least one year after the end of treatment in sustained virological responders [146]. No improvements were seen in nonresponders.

The demonstration of altered cerebral metabolism using ^{1}H MRS again supports the central biological hypothesis. However, the findings are not highly spe-

cific for any pathological process. Elevations in choline-containing compounds have been reported not only in cerebral HIV infection but also in bipolar depression [147] and chronic fatigue syndrome [148]. Although it is postulated that increased cerebral Cho:Cr ratios may be due to altered membrane fluidity in activated microglia, as in HIV infection [149], 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 the reversibility of the observed effects but, again, could be complicated by the neurotoxicity of IFNα.

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. Recent data from Fletcher and colleagues [142] provide a potential mechanism for viral entry to the CNS, through altered blood–brain barrier permeability as a consequence of brain endothelial infection (Figure 21.1). Blood–brain barrier breakdown might allow entry of inflammatory cytokines, viral particles, and other neurotoxic substances that could potentiate neurological symptoms and activate microglial cells and astrocytes. Furthermore, there may be interactions between neurotoxic viral proteins, as suggested in a recent report where HCV core protein potentiated HIV-1 neurotoxicity in a mouse model [135].

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 [150], which may have an upregulatory role in neuroinhibitory pathways in the brain. Activated microglia also release excitatory amino acids, which can induce neuronal apoptosis through a process known as excitotoxicity [151], and are potent producers of neurotoxins such as nitric oxide. These processes may be amplified by the release of cytokines and chemokines [152, 153] and recruit 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 [154] or, independently, in the absence of HCV infection of the CNS. Although

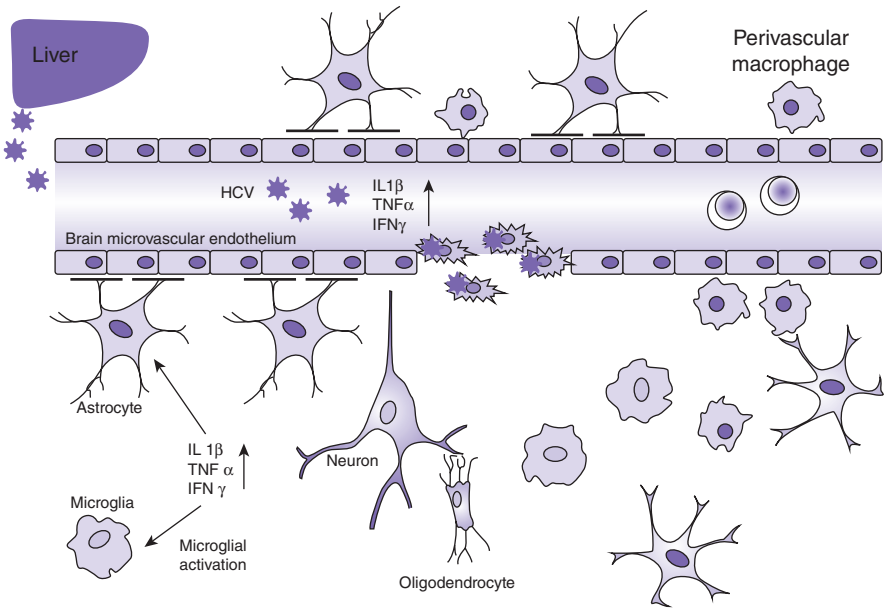

Figure 21.1 A proposed model for hepatitis C infection of the central nervous system. HCV virions released by the liver reach the blood–brain barrier (BBB) and infect brain microvascular endothelial cells, which express all of the receptors required for viral entry, including LDL-R and apolipoprotein E [142]. Apoptosis and disruption of the BBB ensue [142], allowing entry of viral particles, inflammatory cytokines, immune cells, and other substances that may result in microglial activation within the CNS. Low-level immune activation within the brain may explain to some degree the neuropsychological symptoms and cognitive impairments observed in some patients [68, 81]. 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 [130]. 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 that may be less responsive to therapy [18]. (Source: Fletcher NF, McKeating JA. J Viral Hepat 2012;19:301–306 [159]).

cytokines are large peptides, animal studies have demonstrated the passage of cytokines across the blood–brain and blood–spinal cord barriers [155]. Peripherally derived cytokines may also be passively transported into the brain at sites where there is blood–brain barrier breakdown. Intracerebral cytokines have been associated with immunological, neurochemical, neuroendocrine, and behavioral activities [156], probably through the pathways described here. It is clear that treatment with IFNα is associated with depression and complaints of memory impairment and cognitive slowing [157], 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 IL1, IL6, and TNF and patients' fatigue in chronic HCV infection [158], although no study exists to date on circulating cytokine levels and cognitive function in chronic HCV infection.

The mechanisms discussed in this chapter remain hypothetical. Clinical, imaging, and further virological studies may ultimately answer these questions.

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Chapter 22
In vitro replication models

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Summary

In 1989, Choo and coworkers were the first to succeed in the molecular cloning of the hepatitis C virus (HCV), the causative agent of a liver disease that at that time was called non-A, non-B hepatitis [1]. This discovery paved the way for the implementation of diagnostic tests and set the stage for the development of antiviral therapies. The latter requires a profound understanding of the viral replication cycle, for which the availability of robust and adequate cell culture models is an essential prerequisite. Their development has been an exhaustive adventure that took many years until systems became available that recapitulated authentic viral RNA replication, and ultimately, the complete viral replication cycle in cultured human liver cell lines. In this chapter, we will summarize the various *in vitro* replication models available for studies of HCV. For ease of understanding, we start off with a brief introduction to the viral replication cycle. A more detailed description of this aspect is given in Chapter 16.

Genome organization and replication cycle of HCV: a 2-minute course

HCV has a single-stranded, positive-sense RNA genome with a length of about 9.6 kb. It contains two highly structured nontranslated regions (NTRs) that flank a single long open reading frame (ORF). The 5′ NTR contains a type III internal ribosome entry site (IRES) mediating translation of the ORF in a cap-independent manner. Activity of the IRES appears to be influenced by microRNA (miRNA)-122 that binds at two sites close to the 5′ end of the HCV genome and enhances RNA stability as well as RNA replication (for a review, see [2]). The 3′ NTR is composed of a variable region, a polypyrimidine tract, and the 98 nucleotide long highly conserved 3′ terminal X-tail (Figure 22.1A). Both the 5′ NTR and the 3′ NTR contain RNA elements that are required for replication (for a review, see [3]).

The HCV ORF encodes a polyprotein of ~3000 amino acids that is cleaved by viral and host cell proteases into 10 different products (Figure 22.1A). The structural protein's core, envelope protein 1 (E1), and E2 are located within the amino-terminal part of the polyprotein, whereas the nonstructural (NS) proteins NS2 to NS5B are encoded in the remainder. Polyprotein cleavage is mediated by various strategies, and cleavage products are associated with intracellular membranes (Figure 22.1B). Additional HCV proteins are generated by ribosomal frame shifting or internal translation initiation and have therefore been designated F proteins or p8 minicore, respectively [4–6]. However, their *in vivo* relevance is unclear.

The infectious hepatitis C virion is a lipoprotein-like particle consisting of at least the genomic RNA, the core protein, the two envelope proteins E1 and E2, and multiple copies of apolipoprotein E [7, 8] (Figure 22.2). The core protein and the RNA genome form the inner structure of the virus particle. The two envelope glycoproteins, eventually in concert with apolipoprotein E, mediate attachment and entry into the host cell. The viroporin p7 might act as an ion channel contributing by some unknown mechanism to assembly (reviewed in

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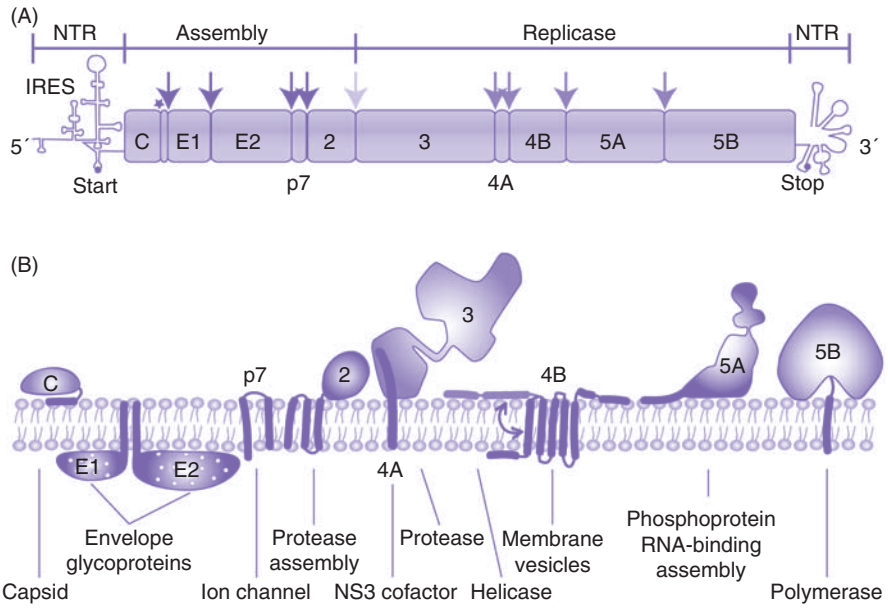


Figure 22.1 Schematic representation of the HCV genome organization and membrane topology of viral proteins. (A) The HCV genome is flanked at the 5′ and 3′ ends by highly structured nontranslated regions (NTRs). The polyprotein is indicated by the shaded bar. Cleavage sites are indicated by arrows, with different shading referring to involved proteases. Black arrows: host cell signal peptidase; light gray: NS2–3 protease; dark gray: NS3 protease; *: signal peptide peptidase; IRES: internal ribosome entry site. The positions of the start and stop codons of the large open reading frames are indicated with dots. (B) Schematic representation of the proposed membrane topologies of HCV proteins. A schematic of a membrane bilayer and proposed transmembrane domains of the HCV proteins are shown. Functions ascribed to individual proteins are indicated at the bottom.

[9]). NS2 is a bifunctional molecule: it is required for HCV assembly, probably by acting as an organizer that mediates interactions between the RNA-containing replicase and the envelope proteins; and NS2 is responsible for autocatalytic cleavage of the NS2–NS3 junction (reviewed in [10]). NS3 is also a bifunctional molecule. It contains in the amino-terminal domain a serine-type protease that, after association with its co-factor NS4A, cleaves most of the NS proteins (Figure 22.1A). The same protease plays an important role in counteracting the innate immune response against HCV by cleaving important signaling molecules such as MAVS (mitochondrial antiviral signaling protein) and TICAM-1/TRIF (Toll-like receptor 3 adaptor molecule) [11, 12]. Two additional enzymatic activities, RNA helicase and nucleoside triphosphatase, reside in the carboxy-terminal two-thirds of NS3 (reviewed in [3]). Alterations of intracellular membranes, in particular membranous vesicles, are induced by NS4B. Accumulations of these vesicles, designated the membranous web, are thought to be the site of viral RNA synthesis (for review, see [13]). NS5A is a dimeric zinc-binding metalloprotein [14] that is tethered to intracellular membranes via an amino-terminal amphipathic helix (Figure 22.1B). In addition, this protein localizes to lipid droplets where assembly of HCV particles might initiate. NS5A is required for both RNA replication and assembly of infectious virus particles and is thought to regulate both processes in a phosphorylation-dependent manner (reviewed in [3]). The membrane-associated NS5B protein is the RNA-dependent RNA polymerase (RdRp). Like other viral RdRps, it contains a "GDD" amino acid sequence motif comprising the active site, and it is composed of finger, thumb, and palm domains [15] (Figure 22.1B).

A brief summary of the HCV replication cycle is given in Figure 22.2. Infection starts by binding of the envelope glycoproteins E1 and E2 to different host cell molecules that either "trap" virus particles on the cell surface (e.g., heparan sulfate and low-density-lipoprotein receptor [LDLR]) or are essential for virus entry (CD81, Occludin, Scavenger receptor class B type I [SR-B1], and Claudin-1) (reviewed in [16]). Two additional cell surface molecules, the cholesterol-uptake receptor Niemann–Pick C1-like 1 (NPC1L1) [17] and the epidermal growth factor receptor (EGFR), contribute to HCV entry [18]. Virus spread occurs either via extracellular particles entering cells in a clathrin-dependent manner or via cell-to-cell spread, which appears to have different entry molecule requirements. Upon release of the

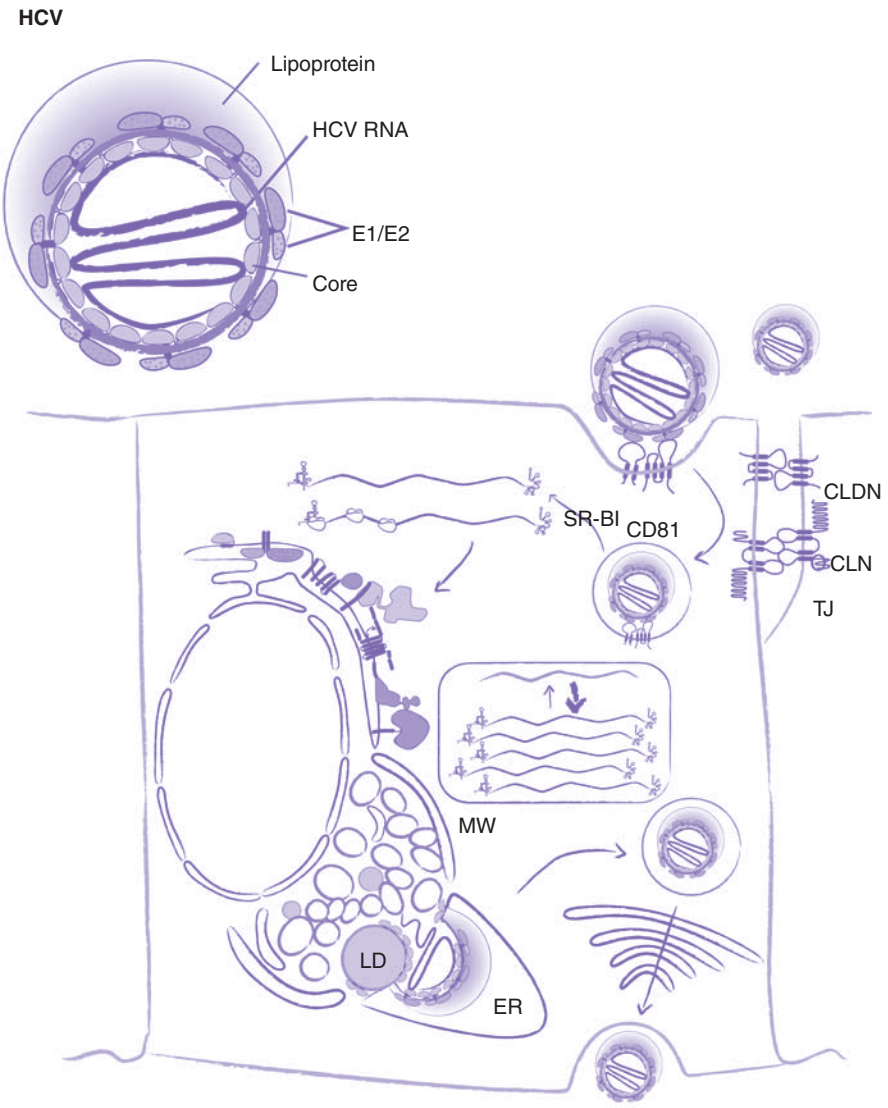


Figure 22.2 Schematic of the HCV particle and the viral replication cycle. HCV particles are thought to consist of a nucleocapsid that is associated with the RNA genome, the viral envelope containing E1/E2 complexes, and a lipoprotein shell containing, in addition to high amounts of triacylglycerol and cholesterol esters, apolipoprotein E [7]. HCV enters via receptor-mediated endocytosis, for which at least four entry molecules (e.g., SR-B1: scavenger receptor B1; CLDN: claudin-1; and OCLN: occludin) residing on the cell surface and eventually also in tight junctions (TJ) are required. Genomic RNA is liberated into the cytoplasm, and after translation and processing viral proteins it induces the formation of the membranous web (MW). Most likely within the MW, minus-strand RNA (gray wavy line) is synthesized and serves as a template for the production of excess amounts of plus-strand RNAs. They are used for translation or packaged into the nucleocapsid. After budding into the lumen of the rough endoplasmic reticulum (rER), which is tightly coupled to lipid droplets (LD), the progeny virus is presumably transported via the Golgi complex to the cell surface, where the transport vesicle membrane fuses with the plasma membrane to release the virus particle.

RNA genome into the cytoplasm, the polyprotein is translated at the rough endoplasmic reticulum (rER). After or during polyprotein cleavage, the membrane-associated replication complex forms, catalyzing 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. Alternatively, plus-strand RNA progeny is used for the assembly of infectious virus particles via a process that is tightly linked to cytosolic lipid droplets and the very-low-density lipoprotein (VLDL) pathway (reviewed in [19]).

HCV cell culture systems

Several systems have been developed to study the HCV replication cycle, or parts thereof, in cultured cells. In this chapter, we will focus on the most relevant ones, which are described in detail throughout the rest of this section.

The HCV pseudoparticle (HCV_{pp}) system

A system that greatly facilitates studies of HCV entry is the HCV pseudoparticle (HCV_{pp}) system [20] (Figure 22.3). It exploits the fact that retroviral particles can incorporate heterologous envelope glycoproteins, including the envelope proteins E1 and E2 of HCV,

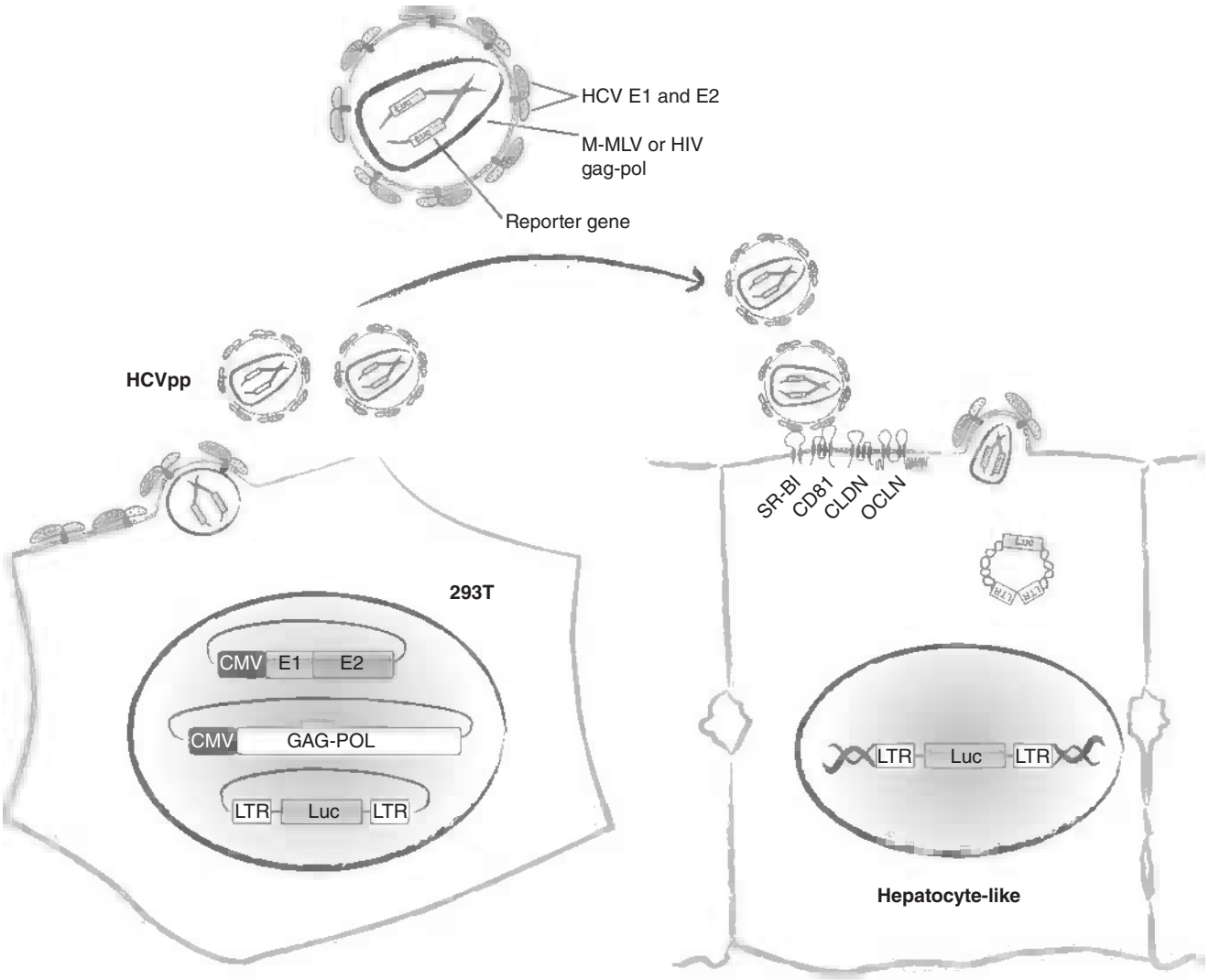


Figure 22.3 Experimental approach for the production of HCV_{pp}. Producer cells (293T) are co-transfected with three different plasmids: (1) the retroviral packaging plasmid composed of the human cytomegalovirus (CMV) promoter and the coding region for the structural (Gag) and the enzymatic proteins (Pol); RNA generated from this construct lacks the packaging signal and therefore is not incorporated into the retroviral nucleocapsid; (2) the transfer vector composed of the two long-terminal repeats (LTRs), the RNA packaging signal (not shown), and an expression cassette containing the CMV promoter (not shown) upstream of a marker gene such as the firefly luciferase (Luc); and (3) the plasmid for expression of the HCV envelope proteins E1 and E2. Upon transfection of 293T cells, the marker gene-encoding RNA is packaged into the retroviral nucleocapsid, which acquires upon release its envelope into which native E1/E2 complexes are incorporated. HCV_{pp}-containing culture supernatant is used for infection of target cells (e.g., Huh7 cells). Pseudoparticle entry is governed by E1/E2 and thus depends on the same set of entry molecules used by authentic HCV particles. Upon reverse transcription, the cDNA of the transfer vector is integrated into the genome of the target cell. Successful transduction of the reporter gene, which is a marker for successful infection, can then be measured by appropriate reporter assay.

without loss of functionality and that the specificity of infection with these "pseudotyped" retroviral particles is mediated by the heterologous envelope. Thus, HCV_{pp}s contain E1 and E2 in their envelopes and either retroviral or lentiviral nucleocapsids. HCV_{pp}s are generated by co-transfection of 293T cells with three different plasmids (Figure 22.3): firstly, the packaging construct coding for the structural (*Gag*) and polymerase (*Pol*) proteins of the corresponding retro- or lentivirus; secondly, a viral transfer vector encoding an easy measurable reporter gene; and, thirdly, an expression vector encoding the two HCV envelope glycoproteins E1 and E2. Upon inoculation of a panel of different target cells with HCV_{pp}s, a preferential tropism for liver-derived cell lines or primary hepatocytes was observed showing that hepatotropism is encoded, at least in part, in the envelope glycoproteins [20]. Furthermore, infection can be blocked with antisera from HCV-positive patients or with monoclonal antibodies directed against E2, thus demonstrating that HCV_{pp}s carry functional E1 and E2 complexes that are responsible for HCV entry into cells.

By using the HCV_{pp} system, it was demonstrated that CD81, an E2-binding molecule, is an important component of the receptor complex but is insufficient to confer entry of the pseudoparticles into the cell [20, 21]. Hsu and colleagues also studied the role of two further HCV receptor candidates, namely, SR-B1 and the LDLR [21]. 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 low-density lipoprotein (LDL) is not associated with the particles that were generated in 293T cells, which is at variance with authentic HCV particles (discussed further in this chapter). Nevertheless, HCV_{pp}s have been instrumental to confirm the existence of HCV neutralizing antibodies [20, 22]. Importantly, the HCV_{pp} system was the key for the discovery of two additional HCV receptors, Claudin-1 and Occludin [23, 24], and to identify the auxiliary role of the EGF receptor for HCV entry [18].

Transfection of cell lines with replicons

HCV is a plus-strand RNA virus, and by definition its genome is infectious. Therefore, transfection of permissive cells with a functional HCV genome should initiate a complete replication cycle. For this reason, numerous attempts have been made to set up such culture systems. One prerequisite is the availability of cloned infectious HCV genomes. However, in spite of their existence, productive replication in cells transfected with these RNAs was in most cases not detectable or was too low for detailed studies (reviewed in [25]). For instance, Aizaki and colleagues [26] transfected 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, and culture supernatant transferred to naïve FLC4 cells initiated a second round of infection. Although these results indicated the production of infectious HCV particles in transfected cells, this culture system is complicated and not commonly available, and the overall yields of HCV are very low.

In an attempt to overcome these limitations, Lohmann and coworkers constructed subgenomic selectable HCV replicons [27]. They were derived from a genotype 1b consensus genome, designated Con-1, which was cloned from the liver of a chronically infected patient who had undergone liver transplantation. The overall structure of the first-generation bicistronic replicons is depicted in Figure 22.4. 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*) that 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 were generated by the proper positioning of the T7 RNA polymerase promoter at the 5′ end and by introducing the ribozyme (Rib) of the hepatitis D virus and the terminator sequence for T7 RNA polymerase at the 3′ end of the replicon sequence, respectively. After electroporation into cells of the human hepatoma cell line Huh7, replicons were translated, and viral proteins built up the replicase that mediated amplification of the replicon RNA. Since it encoded the neomycin phosphotransferase, cells became resistant to G418 only when the replicon amplified to a sufficiently high level, whereas cells with low replication levels or nontransfected cells were eliminated (Figure 22.4). Given the power of this positive selection, it became possible to establish Huh7-derived cell lines containing high amounts of self-replicating HCV RNA [27].

Thorough analyses of these cell lines revealed that, on average, one cell contained 1000–5000 plus-strand RNA molecules per cell, whereas the amount of negative-strand RNA was ~10-fold lower, thus qualifying the latter as a replication intermediate. Consistent with the inhibitor profile of the NS5B RdRp, replicon RNA synthesis was not affected by actinomycin D, whereas synthesis of cellular RNAs was blocked. Viral proteins could easily be detected (e.g., after metabolic radiolabeling and immunoprecipitation or by immunofluorescence analyses). These results demonstrated the high efficiency of the replicon system as compared to all other cell culture systems available at that time.

Subsequent studies conducted in numerous laboratories confirmed the initial observation and led to the construction of modified replicons with different readout systems for HCV replication as well as improved replication capacity (for a review, see [28]).

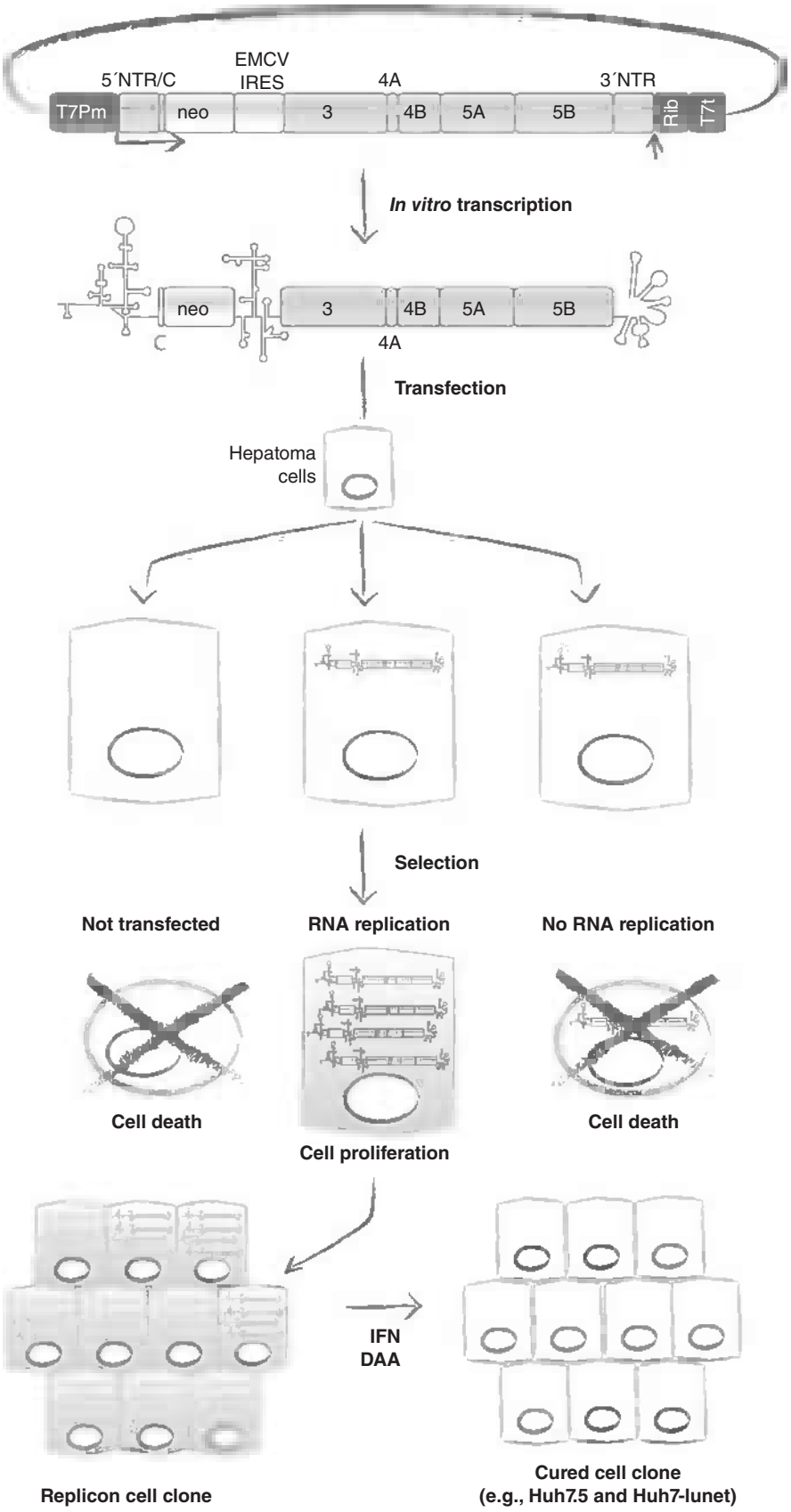

5´NTR/C
EMCV IRES
4A
3´NTR
T7Pm
neo
3
4B
5A
5B
Rib
T7t
In vitro transcription
neo
3
4B
5A
5B
4A
Transfection
Hepatoma cells
Selection
Not transfected
RNA replication
No RNA replication
Cell death
Cell proliferation
Cell death
IFN
DAA
Replicon cell clone
Cured cell clone
(e.g., Huh7.5 and Huh7-lunet)

Figure 22.4 Experimental strategy to generate stable replicon cells and "cured" cell clones. Schematic illustration of the method used to establish subgenomic replicon-harboring Huh7 cells. The DNA construct (top) consists of a T7 RNA polymerase promoter (T7Pm), the HCV 5′ NTR, the selection marker neomycin phosphotransferase (*neo*), the IRES of the encephalomycarditis virus (EMCV-IRES), the NS3-to-NS5B coding sequence, and the 3′ NTR. To allow synthesis of *in vitro* transcripts with the correct 3′ end, the ribozyme (Rib) of the hepatitis D virus and the terminator sequence for T7 RNA polymerase (T7t) have been inserted. Synthetic replicon RNA molecules are transfected via electroporation into the human hepatoma cell line Huh7. Upon selection with G418, only those cells that support efficient RNA replication will survive and can be expanded to replicon cell clones. Cells that either were not transfected or do not support efficient RNA replication are eliminated. Huh7 cell clones with higher HCV permissiveness can be generated by elimination of replicons from established cell clones using treatment with interferon (IFN) or an HCV-specific drug. Examples of such "cured" cell clones with enhanced permissiveness are Huh7.5 and Huh7-Lunet cells.

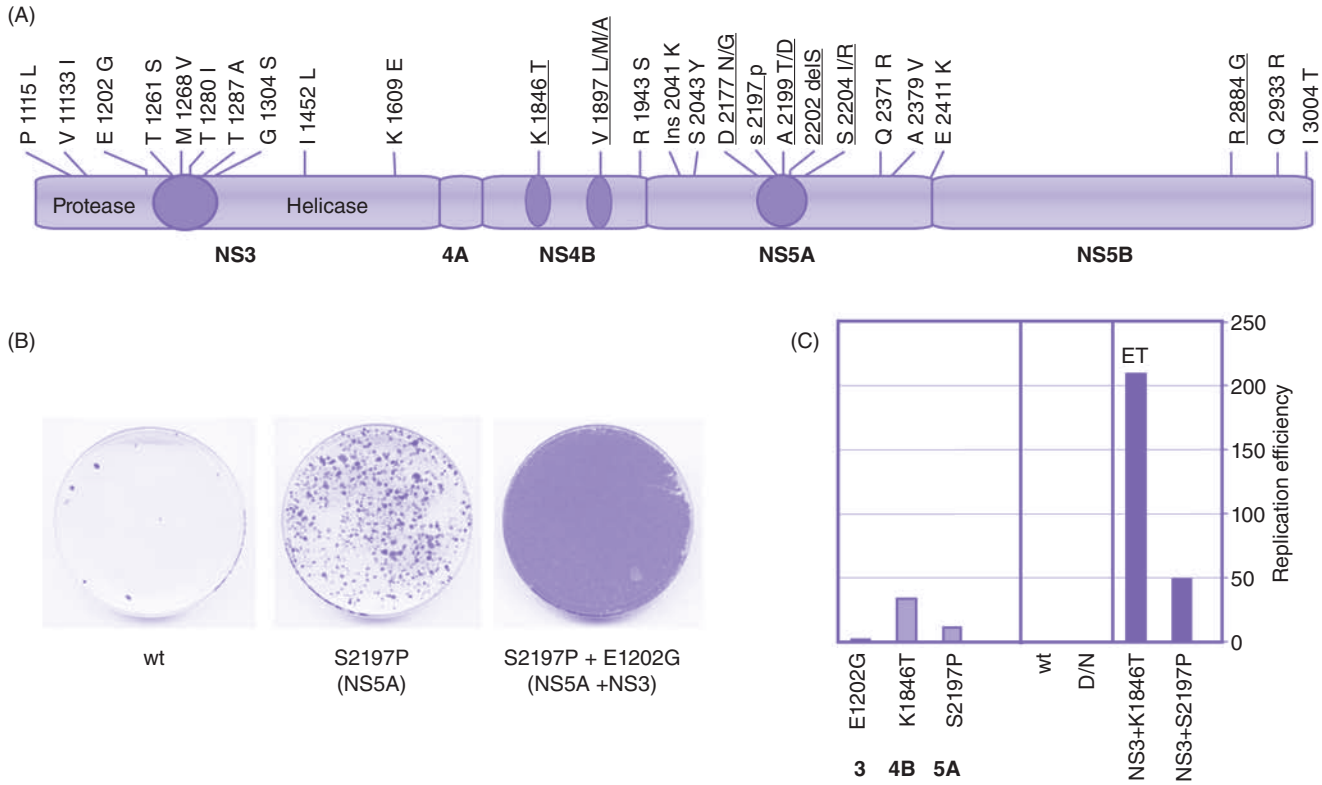


Figure 22.5 Schematic representation of cell culture adaptive mutations and their impact on HCV RNA replication. (A) Amino acid substitutions that were found to be conserved in 26 independently selected replicon cell clones are indicated. Four different "hot spots" of adaptive mutations in NS3, NS4B, and NS5A were identified (filled ovals). Mutations identified as the only conserved alteration in at least one cell clone are underlined. The impact of individual and combined adaptive mutations on (B) colony formation efficiency or (C) relative luciferase expression 48 hours after transfection of replicons is specified at the bottom. Initial letter refers to the amino acid of the wild-type sequence, numbers refer to amino acid positions in the Con-1 polyprotein, and subsequent letter refers to the substituting amino acid residue. D/N: replication deficient mutant; wt: Con-1 replicon without adaptive mutations; ET: the most adapted Con-1 replicon [54].

The role of cell culture adaptation in establishing the HCV replicon system

A drawback of the first-generation replicons was their low efficiency. Only a low number of G418-resistant cell colonies were obtained even after transfection of large amounts of synthetic replicon RNA. However, within an established replicon cell clone, the copy number of viral RNA was high. This paradox was solved by the identification of replication-enhancing mutations in the HCV-coding region that were conserved in the majority of replicon RNAs present in a given cell clone (reviewed in [29]) (Figure 22.5A). These mutations that cluster in distinct regions of the replicase increase the number of G418-resistant cell colonies, which is indicative of enhanced viral RNA

replication (Figure 22.5B). Taking advantage of this discovery, highly efficient replicons were developed that can be analyzed in transient replication assays. For instance, replicons were constructed in which the selectable marker *neo* has been replaced by easily detectable reporter genes like the one encoding the luciferase of the firefly (Figure 22.5C). In this setting, RNA replication is measured by using luciferase assays, and it was shown that luciferase activity closely correlates with replicon RNA levels.

In an attempt to turn the subgenomic replicon system into a cell culture model supporting virus production, genomic replicons have been engineered that encode the complete HCV polyprotein and that contain replication-enhancing mutations in order to achieve sufficiently high RNA amounts. However, in none of these attempts could virus production be achieved [30, 31]. An explanation to these questions emerged from a study addressing the *in vivo* infectivity of an HCV Con-1 genome containing replication-enhancing mutations [32]. Two mutations, one residing in NS3 and one in NS5A, were introduced into the genome, and RNA transcripts were inoculated into the liver of a chimpanzee. In three independent attempts, no productive infection could be established [32]. When a chimpanzee was inoculated with a Con-1 genome containing a single replication-enhancing mutation in NS5A, the animal became viremic, but with a delay of about 1 week as compared to wild type [32]. Importantly, all HCV genomes isolated from the chimpanzee inoculated with the mutant had reverted to wild type, which indicates that replication-enhancing mutations lead to attenuation *in vivo*. Along these lines, the HCV-N isolate (also genotype 1b) has low *in vivo* infectivity [33], but replicons derived from this isolate are highly efficient in cell culture [34]. This could be attributed to a four amino acid insertion within the NS5A protein that is responsible for the inherent ability of the HCV-N isolate to replicate efficiently without further adaptive mutations [35].

A more recent analysis of replication-enhancing mutations revealed that the attenuation is due to interference of these mutations with the production of infectious virus particles [36]. Transfection of Huh7 cells with a wild-type Con-1 genome gave rise to HCV particles with proven *in vivo* infectivity [36]. In contrast, replication-enhancing mutations reduced virus release to various extents. The strongest impairment was found with replication-enhancing mutations located in the NS3 helicase (E1202G and T1280I) as well as NS5A (S2204R), whereas a highly adaptive mutation in NS4B (K1846T) still allowed virus production, albeit at reduced amounts. Importantly, virions released by the K1846T mutant in NS4B were infectious both in cell culture and *in vivo*, but replication in cell culture was too low for detailed analyses [36].

The paradigm that genotype 1–derived isolates require particular mutations for efficient replication in cell culture that, in turn, affect virion production was a major roadblock for the establishment of a fully permissive cell culture system. However, in 2003 Kato and colleagues described a particular genotype 2a isolate designated JFH1 (because it was cloned from the serum of a Japanese patient with fulminant hepatitis). This isolate replicated in cell culture with unprecedented efficiency and did not require replication-enhancing mutations [37].

A side-by-side comparison of Con-1 and JFH1 replicons with identical architecture revealed a much faster kinetic of minus-strand synthesis for JFH1, indicating that this isolate encoded an exceptionally efficient replicase [38]. In fact, subsequent studies pointed to the NS5B RdRp as a major determinant: Murayama and colleagues determined the replication efficiency of chimeric replicons composed of sequences of JFH1 and the closely related J6 strain [39]. Both isolates are infectious in the chimpanzee, but only JFH1 replicates efficiently in cell culture [40]. By generating chimeric replicons between these two isolates, Murayama and coworkers could show that the coding regions encompassing the NS3 helicase and NS5B as well as the 3′ NTR are the main determinants for efficient JFH1 replication, with NS5B being the most important single factor [39]. Analogous results were obtained with chimeras between JFH1 and the genotype 1b isolate Con-1 [41]. The molecular mechanisms by which NS5B of JFH1 confers high replication competence are not known. It might be due to particular interactions between NS5B and other viral or host cell proteins, *cis*-acting elements residing in the NS5B coding region, or the enzymatic activity of the RdRp itself. Support for the latter option is given by the observation that the RdRp of JFH1 appears to have a much higher specific activity *in vitro* as compared to the enzyme of the Con-1 isolate [41]. This is probably due to the higher capability of JFH1 NS5B to initiate RNA synthesis *de novo* [42], which is the expected mode of initiation of RNA synthesis *in vivo*. In fact, the JFH1 enzyme has a very closed conformation and is ideally suited for *de novo* initiation of RNA synthesis [42]. Mapping studies identified several amino acid residues located in the palm, thumb, and C-terminal subdomain of NS5B that contribute to enhanced *de novo* RNA synthesis. Most critical is residue Ile-405 in the thumb domain of the polymerase, which strongly stimulates replication in cell culture by enhancing overall *de novo* RNA synthesis [43]. A high-resolution structural comparison of the JFH1 and J6 enzymes indicated a clear correlation between a closed-thumb conformation of the RdRp and efficient *de novo* RNA synthesis. Thus, the JFH1 polymerase, the key enzyme of the viral replicase, seems to have adopted a unique conformation

that allows exceptionally efficient RNA replication while retaining the ability to produce infectious virus particles. In the beginning of 2013 three papers were published reporting the establishment of efficient replicons derived from genotype 3 and 4 HCV isolates [86–88]. Like genotype 1 replicons also these replicons need adaptive mutations for efficient RNA replication in cell culture. The availability of robust genotype 3 and 4 replicons is an important addition to the available set of genotype 1 and 2 replicons and will be of great value for basic HCV research as well as for the development of better treatment options.

The HCV_{cc} system: production of infectious HCV particles

With the availability of the JFH1 isolate, it became possible to establish a fully permissive HCV cell culture system (by analogy to the pseudoparticle system called the HCV_{cc} system). This system follows a principle that was not successful for all other HCV isolates: transfection of Huh7-derived cells with *in vitro* transcripts corresponding to authentic HCV genomes, and careful analysis of cells and culture supernatants for viral RNA replication, protein expression, and virus production [44] (Figure 22.6). Indeed, culture supernatant of JFH1-transfected cells contained infectious HCV with a median density of ~1.15 g/ml. E2-containing virus particles with an average diameter of 50–65 nm were detected. Their infectivity could be neutralized with CD81-specific antibodies, thus demonstrating the importance of this entry molecule for HCV infection.

A drawback of the JFH1 isolate (Figure 22.7A) is its inherently poor assembly competence (i.e., virus titers achievable with this genome are rather low). For this reason and motivated by the lack of HCV_{cc} systems for other HCV isolates and genotypes, intra- and intergenotypic chimeras were generated (Figure 22.7B and 22.7C). The first one was composed of the Core-to-NS2 coding region derived from the J6 isolate fused to the NS3–NS5B replicase region of JFH1 [45] (Figure 22.7B). Titers of infectious HCV achievable with this intragenotypic chimera are much higher as compared to the JFH1 wild type. Importantly, these viruses are infectious *in vivo*, and virus produced *in vivo* is infectious for Huh7 cells, thus fulfilling Koch's postulates [46]. A slightly different construct design has been used by Pietschmann and coworkers, who identified a particular cross-over site (designated the "C3" position), right after the first transmembrane segment of NS2, as most suitable for highly assembly-competent JFH1-derived chimeric genomes [47] (Figure 22.7C). Most efficient is the so-called JC1 chimera giving rise to infectivity titers that are ~1000-fold higher as compared to the JFH1 wild type, although RNA replication is not affected [47]. Another way to increase assembly competence of JFH1 is continuous passage in Huh7 cells. This leads to the selection for genomes containing particular mutations in the structural or nonstructural region that enhance assembly competence dramatically (for a review, see [28]). This adaptation strategy has also been exploited to increase the assembly competence of multiple intergenotypic chimeras, allowing the production of viruses of the seven known HCV genotypes.

Although the JFH1 system has transformed HCV research, a drawback is that all studies rely on this particular virus isolate, and it is unclear how applicable obtained results are for other HCV genotypes. To overcome some of these limitations, JFH1-derived variants have been generated that are chimeric with respect to the main targets for direct-acting antivirals (DAAs): the NS3/4A protease and NS5A (Figure 22.7D and 22.7E) [48–51]. Another approach that was pursued by Yi and colleagues was the adaptation of the genotype 1a isolate H77 to cell culture in a way so that a genome was obtained that replicates to a high level but retains at least some competence for virus production. In fact, it was possible to generate a particular variant called H77-S that replicates to a level comparable to that of JFH1 [52]. However, the specific infectivity of H77-S particles is significantly lower (5.4×10e4 vs. 1.4×10e2 RNA copies per focus-forming unit for H77-S vs. JFH1, respectively). Nevertheless, the infectivity of H77-S particles could be blocked with CD81-specific antibodies or by the sera of genotype 1a–infected individuals, arguing that these particles are authentic [52].

Very recently, Li and coworkers were able to generate non-JFH1 HCV genomes of gt2a and 2b that replicate with high efficiency in human hepatoma cells and that support virus production [53]. By using domain swapping between J6 and JFH1 as well as continued passage in cultured cells, the authors identified six mutations scattered throughout the polyprotein and increasing replication and virus production by several orders of magnitude. Importantly, insertion of a subset of these mutations into the distantly related HCV isolate J8 (gt2b) allowed selection for an adapted J8 variant containing nine mutations. This variant replicated efficiently and supported the production of infectious virus, albeit at reduced titers as compared to the adapted J6 mutant [53].

Host cell permissiveness

Apart from the virus isolate, the host cell itself contributes substantially to efficiency of HCV replication. It was shown that within a given population of naïve Huh7 cells, only a subpopulation is capable of supporting high-level RNA replication (reviewed in [29]). When replicon-harboring cells were treated with IFNα or a

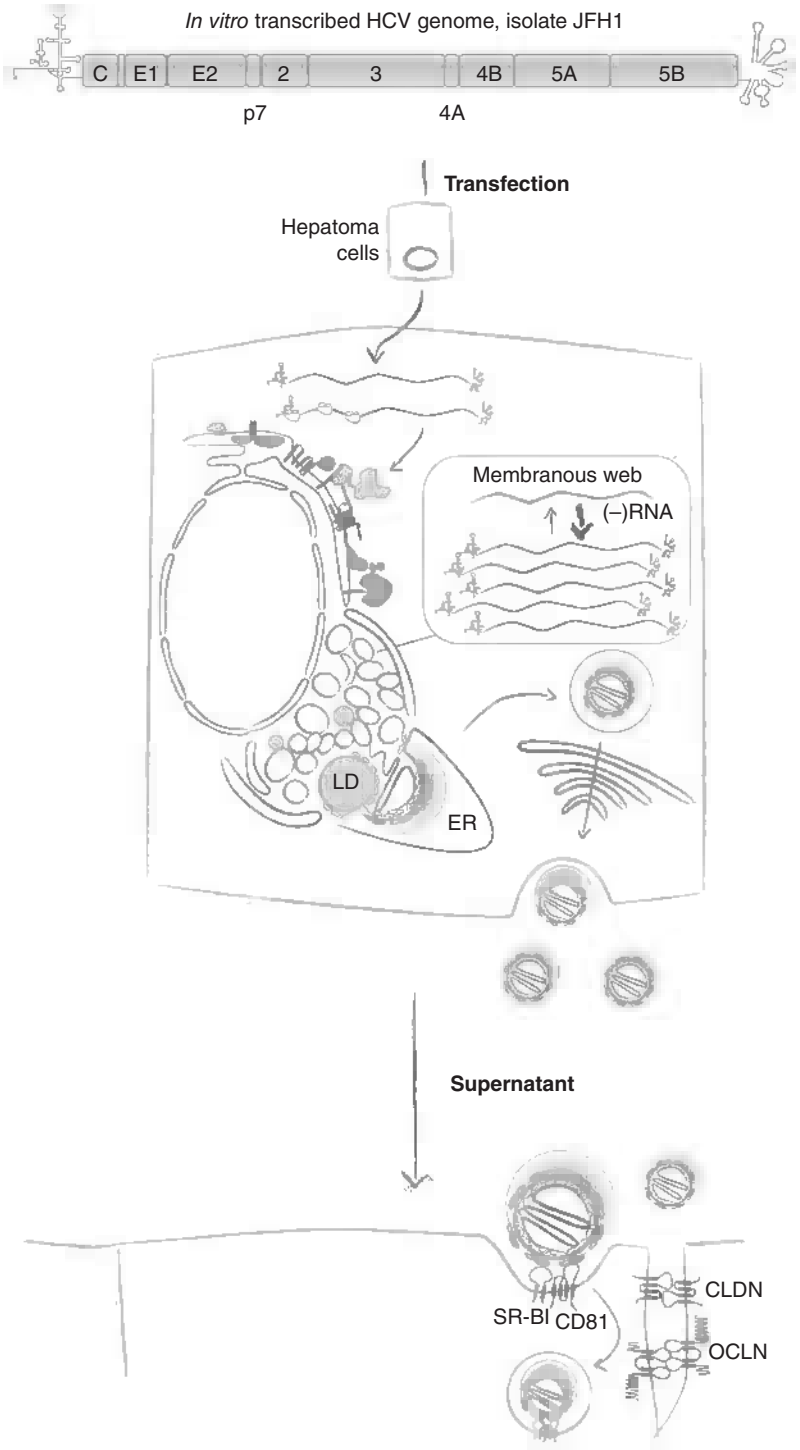
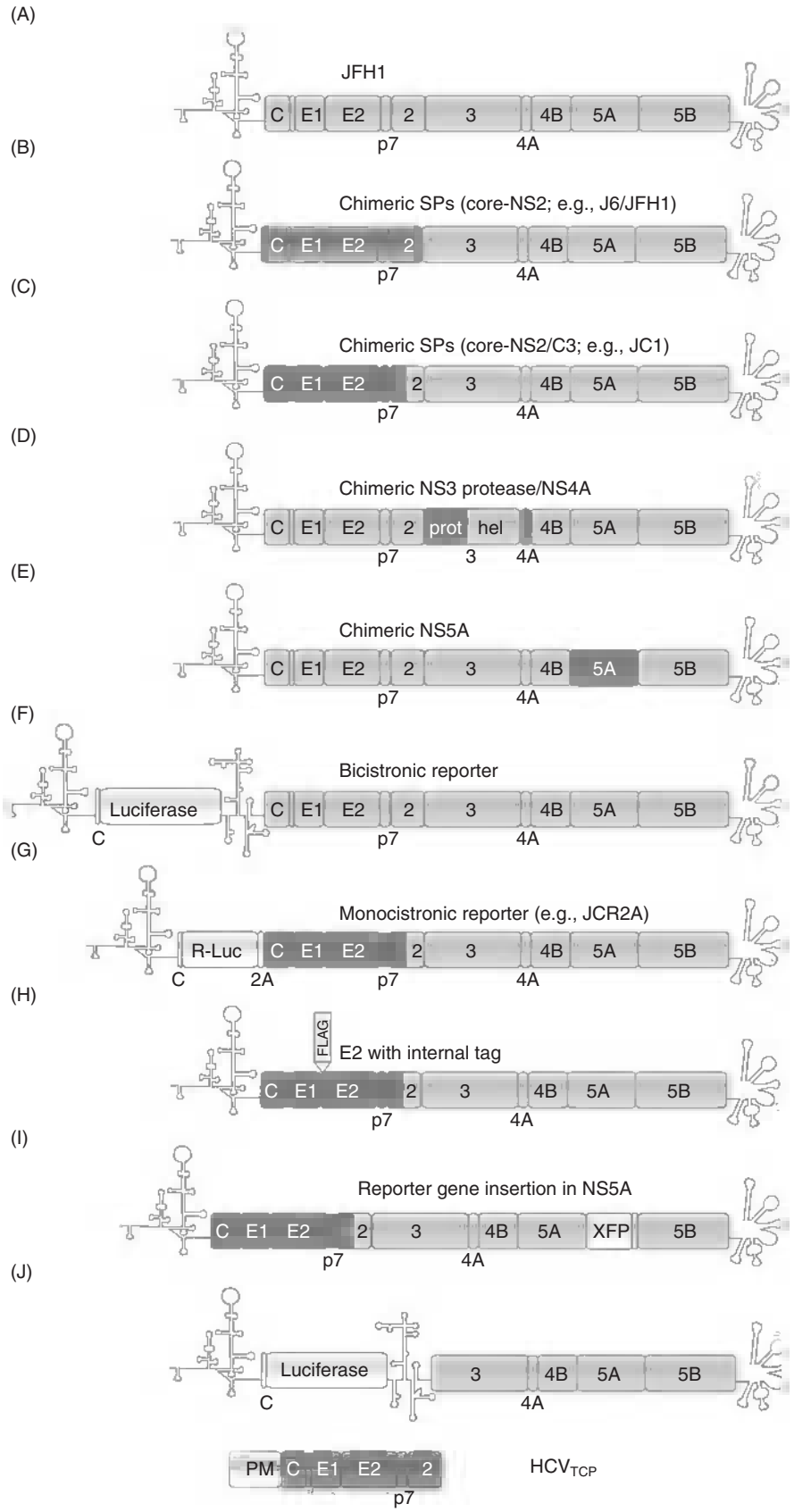


Figure 22.6 Schematic of the HCV_{cc} system. *In vitro* transcribed genomic HCV RNA from the JFH1 isolate is transfected into Huh7 cells. Transfected viral RNA initiates the replication cycle leading to the production of infectious HCV particles. Cell-free supernatant from the producer cell can be transferred to naïve target cells to initiate infection.

Figure 22.7 Structure of viral genomes derived from the JFH1 isolate. (A) Schematic representation of the JFH1 genome [44]. (B) Chimeric JFH1 genome in which the region encoding Core to NS2 has been replaced by the analogous region of another virus [45, 82]. (C) Analogous JFH1 chimera, but with a "cross-over" site (C3 position) located right after the first transmembrane segment of NS2 [47]. A J6–JFH1 chimera generated in this way has the highest assembly efficiency and is called "JC1." (D) A chimeric JFH1 genome in which the NS3 protease domain and the NS4A cofactor have been replaced by the analogous regions of other HCV isolates [48, 49]. (E) A chimeric JFH1 genome in which the NS5A coding region has been replaced by the analogous region of other HCV isolates [51]. (F) Design of a bicistronic reporter virus genome [83]. (G) Design of a monocistronic reporter virus genome based on the JC1 chimera. The shown construct is called JCR2A. It contains a 2A protease cleavage site to allow separation of the reporter from the amino-terminus of the core protein [84]. (H) Design of a fully replication competent JC1 genome containing an affinity tag fused to the amino-terminus of E2 to allow capture of HCV particles [55]. (I) Design of a fully functional HCV genome based on the JC1 chimera and containing a NS5A with an in-frame insertion of a fluorescent protein (XFP) [85]. (J) Design of a selectable subgenomic replicon and the corresponding helper cassette providing the assembly factors in *trans* [80]. This system allows production of HCV_{TCP} particles. PM: promoter.


(A)
JFH1
C E1 E2 2 3 4B 5A 5B
p7 4A
(B)
Chimeric SPs (core-NS2; e.g., J6/JFH1)
C E1 E2 2 3 4B 5A 5B
p7 4A
(C)
Chimeric SPs (core-NS2/C3; e.g., JC1)
C E1 E2 2 3 4B 5A 5B
p7 4A
(D)
Chimeric NS3 protease/NS4A
C E1 E2 2 prot hel 4B 5A 5B
p7 3 4A
(E)
Chimeric NS5A
C E1 E2 2 3 4B 5A 5B
p7 4A
(F)
Bicistronic reporter
Luciferase
C E1 E2 2 3 4B 5A 5B
C p7 4A
(G)
Monocistronic reporter (e.g., JCR2A)
R-Luc C E1 E2 2 3 4B 5A 5B
C 2A p7 4A
(H)
FLAG
E2 with internal tag
C E1 E2 2 3 4B 5A 5B
p7 4A
(I)
Reporter gene insertion in NS5A
C E1 E2 2 3 4B 5A XFP 5B
p7 4A
(J)
Luciferase
3 4B 5A 5B
C 4A
PM C E1 E2 2
p7
HCV$_{TCP}$

selective inhibitor to remove the replicon, the resulting so-called cured cells supported HCV replication more efficiently than naïve control cells (Figure 22.4). This result suggests that the selective pressure used to generate a replicon cell clone leads not only to selection for replication-enhancing mutations in the viral RNA, but also to selection for more permissive cells. Different levels of permissiveness were also observed between various passages of naïve Huh7 cells originating from the same cell stock. These differences (up to 100-fold) were not due to variations in IRES-dependent translation or RNA stability [54].

In addition to Huh7 cells, replicon cell clones derived from several liver and nonliver cell lines of human or mouse origin have been described, including replicon cell clones derived from the cell lines HuH6, HeLa, Hepa1–6, and 293 (reviewed in [29]). With the discovery of JFH1, stable replicon cell clones in HepG2 and IMY-N9 cells as well as the mouse hepatic cell line Hep56.1D could be established [55]. Accumulating evidence suggests that either the lack of essential host cell factors promoting HCV replication or the presence of restriction factors limiting HCV replication in a given cell line determine permissiveness (for a review, see [19]). For instance, the human hepatoma cell lines Hep3B and HepG2 do not express sufficient amounts of miR-122 [56, 57]. However, ectopic overexpression of this essential HCV dependency factor rendered the cells permissive for viral replication. In addition, overexpression of CD81 that is limiting in HepG2 cells made these cells also susceptible to HCV infection [57]. Another example is apolipoprotein E, which is expressed in low amounts in many human and mouse liver cell lines. These cells support the production of infectious HCV only poorly, but virus titers can be enhanced dramatically upon ectopic expression of this apolipoprotein[55]. Activation of an antiviral response such as the one triggered by IFN is another reason limiting HCV replication in many cells. Interestingly, Huh7 cells that are most permissive for HCV have a very much impaired innate immune response, and in the case of one particular cell clone designated Huh7.5, this defect can be ascribed, at least in part, to a particular mutation in the RIG-I gene [58]. Another particular cell clone is Huh7-Lunet, which was also generated by "curing" of a replicon cell clone and supports HCV replication to very high levels [59].

Although these hepatoma cell lines are of great help to study the basic principle of the HCV replication cycle, they do not properly mimic virus–host cell interactions and, thus, pathogenesis. For this reason, several groups established cell lines based on the immortalization of human hepatocytes. Several such liver cell lines have been developed and tested for HCV permissiveness (reviewed in [29]). Among these are (1) PH5CH human liver cells, which were immortalized with the simian virus (SV) 40 large T antigen; (2) the human liver cell line FLC4 that can be cultured in a three-dimensional radial-flow bioreactor; (3) and HuS-E and HuS-T cells, which were immortalized by transduction of human telomerase reverse transcriptase and E6/E7 of human papilloma virus or SV40 large T antigen. In the case of the latter, HCV permissiveness was improved by attenuating the IFN response. Nevertheless, in all cases HCV replication is very low, thus precluding detailed analyses of the viral replication cycle.

Host range of HCV replication

The primary host cell for HCV is the human hepatocyte. However, this host range is not strict, and nonhuman as well as nonhepatic cells have been identified supporting HCV replication. For instance, Fletcher and colleagues were able to demonstrate that two human brain endothelial cell lines, hCMEC/D3 and HBMEC, support HCV entry and replication [60], although virus titers attained with these cell lines are ~100-fold lower as compared to Huh7-derived cells.

A few studies have described stable replication of subgenomic HCV replicons in mouse cell lines [55, 61, 62]. However, the number of cell clones supporting stable replication of HCV RNA is extremely low and requires long-term selection. More importantly, mutations conferring adaptation of such HCV replicons to mouse cells have not been detected. Instead, the procedure appears to select for more permissive cells capable of sustaining long-term HCV replication. Transient replication that mimics more closely an infection is virtually undetectable in these cells, although ectopic expression of miR-122 and knockout of interferon regulatory factor 3 or protein kinase R appears to increase RNA replication [63, 64]. Thus, HCV replication might be limited both by intrinsic and induced restriction factors, and by low expression or mouse–human incompatibility of host dependency factors. Incompatibility has been studied for HCV entry, and it was shown that CD81 and Occludin are used in a highly species-specific manner; rodent orthologs support HCV entry only very poorly [24, 65]. Moreover, Claudin-1 and SR-BI appear to contribute to species specificity, albeit to a lower extent [66, 67]. Interestingly, incompatibility does not apply to assembly, because expression of mouse apolipoprotein E in Hep56.1D mouse hepatic cells is sufficient to produce infectious HCV particles [55].

Infection of primary cells

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 or chimpanzees with HCV-positive patient sera (for a review,

see [68]). Since HCV replication in these cell cultures is usually low, highly variable, and donor dependent, several improvements have been made to increase efficiency. One obvious improvement is the use of HCV_{cc} particles that can be generated in rather large quantities and that represent a well-defined and homogeneous inoculum. In addition, new protocols have been established that increase the efficiency of HCV replication in primary human hepatocytes (PHHs) (e.g., [69, 70]). Finally, it appears that the IFN response triggered in primary cells, but not in Huh7 cells, limits HCV infection. For instance, Andrus and colleagues expressed paramyxoviral V proteins in human fetal liver cells and observed a 10–100-fold higher HCV replication as compared to control cells [71]. Since the V proteins are known to antagonize IFN induction and antiviral signaling, these results suggest that the IFN response limits HCV replication in IFN-competent primary cells.

Although hepatocytes are considered as primary target cells of HCV, peripheral blood mononuclear cells (PBMCs) as well as B and T cell lines appear to support HCV replication as well, albeit with very low efficiency (reviewed in [29]). Analysis of the cellular subsets supporting HCV replication identified T and B lymphocytes. In addition, the susceptibility of human T lymphocytes to *in vitro* infection with patient-derived HCV and productive replication in these cells has been shown [72, 73]. The factors determining HCV lymphotropism are not known, but CD5, in addition to CD81, appears to be an important factor for HCV entry into T cells [74]. Interestingly, T cells could not be infected with JFH1, which indicates that HCV_{cc}s do not mimic all aspects of HCV tropism. This might be due to the intense imprinting of HCV particles with host lipids and lipoproteins, which is much more intense *in vivo* as compared to HCV_{cc} produced in Huh7-derived cells [75].

Applications of HCV cell culture systems

Although the HCV_{cc} system is suitable to study every aspect of the viral replication cycle, it has several drawbacks, such as (1) the limitation to essentially one virus isolate (JFH1), (2) the limitation to use predominantly Huh7-derived cells in order to achieve high-level replication, and (3) the need to work in a BSL3 laboratory. For these reasons, HCV_{pp} and HCV replicons are still valid tools. For instance, the HCV_{pp} system has been instrumental to identify HCV entry molecules. This became possible because HCV_{pp}s allow studies of viral entry into cells that do not support HCV replication. After entry, all subsequent steps are governed by the retroviral or lentiviral vector, and therefore entry can be measured (e.g., by expression of an integrated reporter gene for which HCV replication is not required). In the case of HCV replicons, the availability of replication-enhancing mutations and highly permissive cell lines allowed the establishment of transient replication systems. Here HCV replication is measured, for example by expression of a reporter gene that has been stably integrated into the replicon or by using live cell imaging in the case of replicons expressing a fully functional NS5A–GFP fusion protein (reviewed in [29]). Importantly, replicons encode all prime targets for DAAs (NS3 protease, NS5A, and NS5B RdRp) and have been instrumental for validation of rationally designed compounds, for optimization of antiviral substances, and for screening. In fact, inhibitors targeting NS5A have been identified by using replicon screens, and these compounds show great promise in clinical trials [76]. Finally, resistance mutations identified in patients treated (e.g., with the recently approved protease inhibitors boceprevir and telaprevir) have also been detected with replicons, thus validating this culture system [77].

Another application of the replicon system is the study of T cell responses. By using Huh7-derived cells containing a stable replicon and transduced with a particular human leukocyte antigen (HLA) allele, Jo and coworkers could show that CD8+ T cells suppress HCV replication by a noncytolytic IFNγ-dependent mechanism [78]. These results underscore the great flexibility of the replicon system.

Apart from HCV replicons, the HCV_{cc} system has also been improved in many ways. This includes bicistronic and monocistronic luciferase reporter viruses applicable for high-throughput screens or detailed kinetic analyses, genomes expressing modified HCV proteins suitable for live cell imaging, and genomes expressing tagged E2 allowing affinity purification of HCV particles (reviewed in [25]) (Figure 22.7H). In addition, Jones and colleagues developed a system suitable for monitoring productive HCV infection. This system is based on the transduction of target cells with a reporter construct expressing a fusion protein that is composed of a fluorescent protein, a nuclear localization signal (NLS), and the carboxy-terminal fragment of MAVS that contains the cleavage site for the NS3 protease as well as the transmembrane segment [79]. In transduced cells that have been productively infected with HCV, the NS3 protease cleaves the MAVS fragment of the fusion protein, thus liberating the fluorescent protein that, due to its NLS, accumulates in the nucleus. Monitoring HCV infection by fluorescence relocalization does not require fixed, lysed, or processed cells and therefore allows real-time visualization of HCV infection in live cells, including primary human hepatocytes [79].

Taking advantage of the highly efficient JC1 genome, Steinmann and coworkers created a HCV transcomplementation system supporting production of infectious HCV particles containing a subgenomic replicon and

therefore initiating only one round of infection [80] (Figure 22.7J). This so-called HCV_{TCP} (transcomplemented particles) system is based on a packaging cell line stably expressing the "assembly factors" (Core to NS2). The cell line is transfected with a subgenomic NS3-5B replicon encoding, for example, a reporter gene. This RNA is incorporated into virus particles that can be used for infection of target cells. Depending on the integrated reporter gene, HCV replication can be determined, for example, by luciferase assay or, in the case of GFP-tagged replicons, by live cell imaging [80]. Owing to its flexibility and low biosafety requirements, this system is instrumental for characterizing key functions of HCV assembly [55, 81].

Conclusion

In the last few years, significant progress has been made in the development of HCV cell culture models. Most important was the establishment of a system supporting the complete viral replication cycle in cultured cell lines. Nevertheless, there is still much work to be done. One obvious gap is the lack of HCV isolates beyond JFH1 that replicate with high efficiency and support virus production. Another need is for more authentic cell lines that are suitable for studies of HCV–host cell interaction that goes beyond viral replication itself and mimics, among others, intrinsic antiviral defense and pathogenesis. Given the strong link between HCV infection and chronic liver disease, most notably hepatocellular carcinoma, these systems will be indispensable to decipher the complex etiology of these pathogenetic events.

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Chapter 23
Natural history of chronic HCV infection and non-invasive assessment of hepatic fibrosis

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Summary

The natural history of hepatitis C virus (HCV) is highly variable from one infected individual to another. Once chronic infection is established, prognosis mainly depends on the amount and progression of liver fibrosis and the risk of developing cirrhosis. Liver biopsy, traditionally considered as the reference standard for staging of fibrosis, has been challenged over the past decade by the development of novel non-invasive methodologies. These methods rely on two distinct but complementary approaches: (1) a "biological" approach based on the levels of serum biomarkers of fibrosis; and (2) a "physical" approach based on the measurement of liver stiffness using transient elastography. The use of non-invasive methods to assess liver fibrosis in chronic HCV has resulted in a significant decrease in the need for liver biopsy. However, these methods will likely not completely abolish the need for liver biopsy, and they should rather be employed as an integrated diagnostic algorithm with liver biopsy.

Natural history of chronic HCV infection

As mentioned, the natural history of chronic HCV infection is highly variable from one infected individual to another. Some authors view the disease as inexorably progressive, with a high probability of developing over time cirrhosis and its complications, portal hypertension, hepatocellular carcinoma (HCC), liver failure, and death. Others regard chronic hepatitis C as a benign disease, remaining clinically silent for decades, with the majority of infected individuals not progressing to cirrhosis and not dying from the disease. Disagreements probably derive from differences in the studies' design and the populations studied [1]. Efforts to determine HCV's natural history have been hampered by the fact that the onset of HCV infection is usually silent and its course is prolonged, as long as several decades. Acute infection leads to chronic infection in the majority of persons; and spontaneous clearance of viremia, once chronic infection has been established, is rare. Most chronic infections will lead to hepatitis and some degree of fibrosis. Severe complications and death usually occur only in persons with cirrhosis, which is estimated to develop in 15% to 20% of those infected within 20 years [2]. Therefore, several decades of follow-up evaluation are required to determine the outcome of HCV infection.

Hepatic fibrosis staging and progression

Current understanding of HCV infection has been advanced by the concept of hepatic fibrosis progression [3]. Hepatic fibrosis is the final result of a wide variety

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Table 23.1 Scoring systems for liver fibrosis in chronic hepatitis C.

Fibrosis stage	Knodell (5)	Ishak (5)	Metavir (7)
No fibrosis	0	0	0
Fibrosis of some portal areas without septa	1	1	1
Fibrosis of most portal areas without septa	1	2	1
Portal fibrosis with few septa	3	3	2
Septal fibrosis without cirrhosis	3	4	3
Incomplete cirrhosis	4	5	4
Cirrhosis	4	6	4

of liver injury that results in the deposition in the liver of scar tissue (i.e., excess extracellular matrix). It is a dynamic process, characterized by a preponderance of fibrogenesis (the excess synthesis and deposition of extracellular matrix) over its removal (fibrolysis) [4]. With ongoing liver damage, fibrosis may progress to cirrhosis, which is characterized by a distortion of the liver vasculature and architecture, and the major determinant of morbidity and mortality in patients with chronic hepatitis C. Therefore, an estimate of fibrosis progression represents an important surrogate endpoint for assessing the outcome of HCV infection.

Histologic evaluation of a liver biopsy specimen has traditionally been used for staging of fibrosis, based on semiquantitative scoring systems, including the Knodell score [5], the Ishak's score [6], and the METAVIR scoring system [7] (Table 23.1). The intra- and interobserver reproducibility of the METAVIR scoring system has been shown to be good (kappa = 0.80 among pathologists) for both activity and fibrosis staging. However, liver biopsy has several limitations that should be acknowledged. It is an invasive procedure associated with transient pain, anxiety, and discomfort in around 30% of cases [8, 9], and rare but potentially life-threatening complications (hemorrhage in 0.3% of cases and mortality in 0.01%) [10]. The accuracy of liver biopsy to assess fibrosis has also been questioned, in relation to sampling errors and intra- and interobserver variability that may lead to over- or understaging. The size of the biopsy specimen, which varies between 10 and 30 mm in length and between 1.2 and 2 mm in diameter, represents 1/50 000 of the total mass of the liver and so carries substantial sampling error. For instance, it has been shown that only 65% of 15 mm biopsies and 75% of 25 mm biopsies were correctly staged [11] and that cirrhosis diagnosis may be missed on a single blind liver biopsy in 10% to 30% of cases [12]. Also, a difference of at least one fibrosis stage between the right and left lobes has been reported in around 30% of cases [13]. Increasing the length of liver biopsy decreases the risk of sampling error [14]. Finally, apart from the characteristics (sample size) of the liver biopsy, the degree of experience of the pathologist (specialization, duration of practice, and academic practice) may also have an influence on interobserver agreement [15].

Poynard *et al*. [3] have suggested that there are variable rates of fibrosis progression with three different populations of "rapid," "intermediate," and "slow" fibrosers. Using the median fibrosis progression rate (stage at the first biopsy divided by duration of infection) in untreated patients, the median expected time to cirrhosis was 30 years. One-third of patients had an expected median time to cirrhosis of less than 20 years, and another one-third would never progress to cirrhosis or would not progress to cirrhosis for at least 50 years. There was a strong correlation of fibrosis stages, almost linear, with age at biopsy and duration of infection. This correlation was not observed with activity grades. Fibrosis stage was the best marker of ongoing fibrogenesis [3]. Although it was initially assumed that fibrosis progression occurred at a linear rate over time, evidence indicates that the rate appears to accelerate with time, even after controlling for age [16].

Approaches used to estimate fibrosis progression

As discussed in this chapter, published estimates of fibrosis progression and time to cirrhosis are dependent on study design and the patient population investigated. Different approaches have been used, including retrospective and prospective studies and a combination of these two approaches, retrospective-prospective cohort studies. Retrospective studies have often been derived from large tertiary referral centers and may reflect ascertainment bias because individuals with advanced liver disease are more likely to be referred. In such studies, cirrhosis was reported in 17% to 55% (mean: 42%) of cases, HCC in 1% to 23%, and liver-related deaths in 4% to 55% [1]. Conversely, a more optimistic view was seen in prospective studies: cirrhosis developed in 7% to 16% (mean: 11%), HCC in 0.7% to 1.3%, and liver-related death in 1.3% to 3.7%. However, the failing of the latter studies was that their duration of follow-up evaluation was relatively short, ranging between 8 and 16 years, far too short a time to determine the outcome accurately. A compromise approach comes from retrospective-prospective studies in which the precise time of past infection with acute hepatitis C can be defined retrospectively and the subjects subsequently followed prospectively. Using this methodology, progression to cirrhosis was observed in 0.3% to 5.9% (mean: 2.1%), HCC in 0%, and liver-related

death in 1.0% to 2.1% [1]. It should be stressed, however, that studies with low rates of progression involved mainly cohorts of young females infected after exposure to contaminated immunoglobulins with low alcohol intake.

Efforts have been made to reconcile these data. To gain a clearer understanding of the rate of progression to cirrhosis in HCV infection, Freeman *et al*. performed a systematic review including 57 studies [17]. These studies could be divided into four groups based on the methods of recruitment: (1) cross-sectional studies involving patients referred to tertiary care centers (liver clinic series, n = 33), (2) longitudinal posttransfusion hepatitis studies (posttransfusion cohorts, n = 5), (3) cross-sectional surveys of persons newly diagnosed at blood donor screening (blood donor series, n = 10), and (4) longitudinal community-based studies (community-based cohorts, n = 9). Estimates of progression to cirrhosis after 20 years of chronic HCV infection were 22% (95% CI, 18–26%) for liver clinic series, 24% (11–37%) for posttransfusion cohorts, 4% (1–7%) for the blood donor series, and 7% (4–10%) for the community-based cohorts (Figure 23.1). These results suggested that the community-based cohort studies most closely representing HCV-infected populations in industrialized countries (young adulthood HCV infection, with injecting drug use being the predominant mode of HCV infection, and a significant proportion with normal ALT levels) were the most representative basis for estimating progression in the general population.

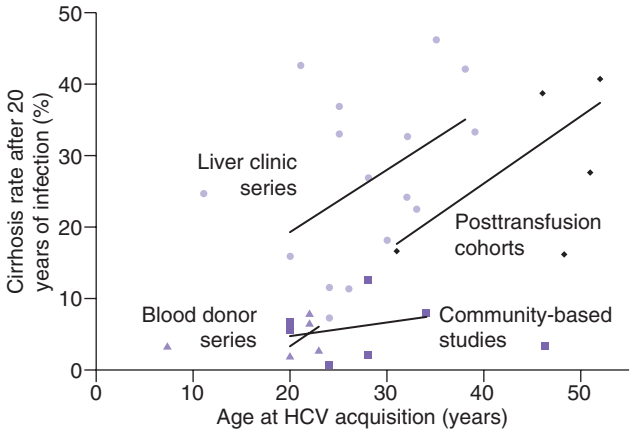

Figure 23.1 Modeled impact of the age at infection on the rate of progression to cirrhosis for each study category. Individual dot points correspond to cirrhosis prevalence after a 20-year HCV infection, assuming linear disease progression for each study. Only studies that gave information on the age at HCV infection were included. Solid lines represent the modeled impact of age at HCV acquisition within each study category. (Source: Adapted from Freeman AJ, Dore GJ, Law MG, *et al.* Hepatology. 2001;34(pt 1):809–816 [17])

In a more recent meta-analysis [18], taking into account 111 studies including the 57 previous ones (n = 33121 HCV patients), Thein *et al*. found that the estimated prevalence of cirrhosis at 20 years after the infection was 16% (14–19%) for all studies, 18% (15–21%) for cross-sectional or retrospective studies, 7% (4–14%) for retrospective-prospective studies, 18% (16–21%) for studies conducted in clinics, and 7% (4–12%) for studies conducted in the community. Two recent community-based long-term follow-up studies suggested that the risk of HCC increased with HCV viral load, ALT levels, and HCV genotype 1 [19, 20].

Cumulatively, these studies do suggest that there is a variable rate of disease progression to cirrhosis and its complications, likely because of multiple factors.

Factors associated with hepatic fibrosis progression

Several factors have been shown to be associated with fibrosis progression rate, including age at infection, duration of infection, male gender, alcohol consumption, smoking, HIV co-infection, histological activity, and more recently metabolic factors such as steatosis, insulin resistance, and obesity.

Age at infection and duration of infection

Age at infection has been shown to correlate with fibrosis progression, even after controlling for duration of infection [3, 21]. Patients infected after the age of 40 have a higher risk of fibrosis progression than those infected before, whatever their initial fibrosis stage. The role of aging in fibrosis progression could be related to higher vulnerability to oxidative stress, or to a reduction in immune capacities.

Male gender

Males are at higher risk of fibrosis progression than females [3, 21]. When metabolic factors and alcohol consumption are taken into account, the association between male gender and fibrosis is reduced but persists [22]. Estrogens are thought to be involved because of their modulatory effect on fibrogenesis, as suggested by clinical [23] and experimental studies [24].

Alcohol consumption

The role of alcohol consumption has been well established for daily intake greater than 40 or 50 g per day [25, 26]. The impact of low-level alcohol consumption remains unclear thus far. A reduction of and/or abstinence from alcohol is likely beneficial in patients with chronic hepatitis C.

Smoking

Smoking has been suggested as an independent predictor for fibrosis progression in patients with chronic hepatitis C, even after controlling for alcohol consumption [27, 28]. The precise mechanisms underlying the association have not been clarified. Daily cannabis smoking may also be an independent predictor of fibrosis progression [29, 30].

Coffee

Coffee consumption may reduce the risk of hepatic fibrosis or disease progression [31, 32]. The mechanisms underlying the potential hepatoprotective effects of caffeine in patients with chronic hepatitis C remain to be determined. It has been suggested that caffeine intake may decrease fibrosis progression by reducing necroinflammatory liver injury [33]. However, further study is required to validate this hypothesis.

HIV co-infection

Several studies have demonstrated that patients co-infected with HCV and HIV have more rapid fibrosis progression than HCV mono-infected patients, even after taking into account age, sex, and alcohol consumption [34, 35]. On average, nearly one-half of the patients co-infected with HIV–HCV have developed cirrhosis after 25 years of HCV infection [36].

The CD4 count appears to be a major factor determining the rate of HCV disease progression in patients co-infected with HIV–HCV: for instance, in the D:A:D (Data Collection on Adverse Events of Anti-HIV Drug) study that prospectively followed 23 441 HIV-1-infected individuals, the relative risk of liver-related death was 6.7 times higher if the CD4 count was $<200/mm^3$ compared with a CD4 count $>200/mm^3$ [37].

Histological activity

Several studies have shown that the degree of activity on the initial biopsy was highly predictive of progression to cirrhosis [38, 39]. Also, patients with persistently normal liver enzymes have lower rates of fibrosis progression and are less likely to develop cirrhosis than those with elevated ALT [40].

Metabolic factors: steatosis, insulin resistance, and obesity

The contribution of metabolic factors has been increasingly recognized over the last few years. Steatosis is common in chronic hepatitis C (50–65%) but mild in most patients. Schematically, two distinct forms of steatosis can be found: viral induced, related to a direct cytopathic effect of HCV in genotype 3–infected patients; or metabolic, related to classical metabolic factors in non–genotype 3–infected patients. Steatosis, whether of metabolic or viral origin, contributes to disease progression in chronic hepatitis C [41, 42]. The association of steatosis with fibrosis, which is strongest in genotype 3 infections, is independent of age, sex, and alcohol consumption [43]. Finally, viral-induced steatosis has been shown to disappear in patients who achieve SVR [44, 45].

Diabetes mellitus and insulin resistance have also been shown to be independently associated with more severe fibrosis [22, 46]. The observed association between high Body Mass Index (BMI) and fibrosis is confounded by underlying relationships with steatosis and diabetes. Lifestyle modifications leading to weight reduction and insulin sensitization may improve the outcome of obese and/or insulin-resistant patients with chronic hepatitis C.

Other factors

Factors such as iron overload and co-infection with schistosomiasis or hepatitis B virus have also been reported to be associated with fibrosis progression [40]. Finally, it should be stressed that some factors such as age at infection, duration of infection, or male gender cannot be modified, whereas others such as alcohol consumption, smoking, HIV co-infection, or metabolic factors (steatosis, insulin resistance, and obesity) can be modified [40].

Non-invasive assessment of hepatic fibrosis

The limitations of liver biopsy, as well as the availability of virologic tools for determining genotypes and viral load and new antiviral drugs, have rapidly reduced the use of liver biopsy in management of patients with chronic hepatitis C and fostered the development of novel non-invasive methodologies. When assessing hepatic fibrosis in chronic hepatitis C, there are two critical endpoints: (1) the presence of significant fibrosis (i.e., Metavir $F \geq 2$ or Ishak $F \geq 3$), which is an indication for antiviral treatment; and (2) the presence of cirrhosis (i.e., Metavir $F = 4$ or Ishak $F = 5$–6), which is an indication for specific monitoring of complications related to portal hypertension and to the increased risk of developing HCC [47, 48].

Currently available non-invasive methods

Among the currently available non-invasive methods, there are two distinct approaches: (1) a "biological"

approach based on the levels of serum biomarkers of fibrosis, and (2) a "physical" approach based on the measurement of liver stiffness using transient elastography (TE) [49]. Although complementary, these two approaches are based on different rationales and concepts: TE measures liver stiffness related to elasticity, which corresponds to a genuine and intrinsic physical property of liver parenchyma, whereas serum biomarkers are combinations of several not strictly liver-specific blood parameters optimized to mimic fibrosis stages as assessed by liver biopsy.

Biological approach: serum biomarkers of liver fibrosis

Initially, individual "direct" markers, reflecting either the deposition or the removal of extracellular matrix in the liver such as hyaluronate, and "indirect" markers including simple routine blood tests such as the Prothrombin Index, platelet count, and AST–ALT ratio have been evaluated for their ability to assess liver fibrosis, mainly in chronic hepatitis C [50]. Although these individual markers are useful for the diagnosis or exclusion of cirrhosis, they have limited accuracy for the diagnosis of significant fibrosis [51]. The development of more sophisticated scores combining "direct" and "indirect" markers using a mathematical formula, for which the FibroTest® (Biopredictive, Paris, France) has been the pioneer [52], has substantially improved diagnostic accuracy. Several other scores [53–66] have been proposed since (summarized in Table 23.2), some of which are protected by patents and commercially available.

Table 23.2 Currently available serum biomarkers to assess liver fibrosis in patients with hepatitis C.

- Fibrotest® (Biopredictive, Paris, France) patented formula combining α-2-macroglobulin, γGT, apolipoprotein A1, haptoglobin, total bilirubin, age, and gender
- Forns Index = 7.811 − 3.131 × ln(platelet count) + 0.781 × ln(GGT) + 3.467 × ln(age) − 0.014 × (cholesterol)
- AST-to-platelet ratio (APRI) = AST (/ULN) / platelet (10^9/L) × 100
- FibroSpectII® (Promotheus Laboratory Inc., San Diego, USA) patented formula combining α-2-macroglobulin, hyaluronate, and TIMP-1
- MP3 = 0.5903 × log PIIINP (ng/ml) − 0.1749 × log MMP-1 (ng/ml)
- Enhanced Liver Fibrosis score® (ELF) (iQur Ltd, Southampton, UK) patented formula combining age, hyaluronate, MMP-3, and TIMP-1
- Fibrosis Probability Index (FPI) = 10.929 + (1.827 × LnAST) + (0.081 × age) + (0.768 × past alcohol use*) + (0.385 × HOMA-IR) − (0.447 × cholesterol)
- Hepascore® (PathWest, University of Western Australia, Australia) patented formula combining bilirubin, γGT, hyaluronate, α-2-macroglobulin, age, and gender
- Fibrometers® (BioLiveScale, Angers, France) patented formula combining platelet count, prothrombin index, AST, α-2-macroglobulin, hyaluronate, urea, and age
- Lok Index = −5.56 − 0.0089 × platelet (10^3/mm^3) + 1.26 × AST:ALT ratio = 5.27 × INR
- Gotebörg University Cirrhosis Index (GUCI) = AST × prothrombin-INR × 100 / platelet
- Virahep-C model = −5.17 + 0.20 × race + 0.07 × age (years) + 1.19 ln (AST [IU/L]) − 1.76 ln (platelet count [103/mL]) + 1.38 ln (alkaline phosphatase [IU/L])
- Fibroindex = 1.738 − 0.064 × (platelets [10^4/mm^3]) + 0.005 × (AST [IU/L]) + 0.463 × (gamma globulin [g/dl])
- FIB-4 = age (years) × AST [U/l]/(platelets [10^9/l] × (ALT [U/l])1/2
- HALT-C model = −3.66 − 0.00995 × platelets (103/mL) + 0.008 × serum TIMP-1 + 1.42 × log (hyaluronate)

Diagnostic performances of serum biomarkers

Diagnostic performances for significant fibrosis and cirrhosis of biomarkers are summarized in Table 23.3. To date, FibroTest® and the Aspartate Aminotransferase Platelet Ratio Index (APRI) have been the most extensively studied. In a meta-analysis performed by the developer [67], which pooled 6378 subjects (with an analysis of individual data in 3282) with both the FibroTest® and biopsy (3501 HCV), the mean standardized area under the receiver operating characteristic curve (AUROC) for diagnosing significant fibrosis was 0.84 (95% CI: 0.83–0.86) and for cirrhosis was 0.85 (0.82–0.87). Another meta-analysis [68] analyzed results from 6259 HCV patients from 33 studies; the mean AUROC values for APRI in diagnosis of significant fibrosis and cirrhosis were 0.77 (standard error [SE] = 0.012) and 0.83 (SE = 0.013), respectively.

In the largest study (n = 913 HCV patients) that prospectively compared the most popular patented tests (FibroTest®, Fibrometre®, and Hepacore®) with the nonpatented test (APRI), the AUROC values for significant fibrosis ranged from 0.72 to 0.78 with no significant differences among scores [69]. In patients with cirrhosis, the AUROC values were higher, ranging from 0.77 to 0.86, and differences were again not significant. Although nonpatented tests such as the Forns Index, FIB-4, and APRI perform less well, they do not incur additional costs, are easy to calculate, and available almost everywhere.

Limitations of serum biomarkers

Although biomarkers have high applicability (>95%) and interlaboratory reproducibility [70, 71], none are liver specific – their results can be influenced by comorbid conditions, and they require a critical interpretation of results. For instance, FibroTest® and Hepascore®

Table 23.3 Diagnostic performance of serum biomarkers of fibrosis for significant fibrosis (F ≥ 2) and cirrhosis (F4) in patients with hepatitis C.

Biomarkers	Etiologies	Year	Patients (*n*)	F ≥ 2 (%)	F4 (%)	Cutoffs	AUROC	Se (%)	Sp (%)	CC (%)
FibroTest® [52]	HCV	2001	339	80		>0.48	0.87	75	85	46
Forns Index [53]	HCV	2002	476	26		<4.2 >6.9	0.81	30–94	51–95	45
APRI [54]	HCV	2003	270	50		≤0.5 >1.5	0.80	41–91	47–95	44
					17	<1.0 ≥2.0	0.89	57–89	75–93	72
FibroSpectII® [55]	HCV	2004	696	52		>0.36	0.83	77	73	75
MP3 [56]	HCV	2004	194	45		<0.3 >0.4	0.82	35–65	85–96	NA
ELF® [57]	Mixed	2004	1021	40		0.102	0.78	87	51	NA
					12	NA	0.89	NA	NA	NA
FPI [58]	HCV	2005	302	48		≤0.2 ≥0.8	0.77	42–85	48–98	40–49
Hepascore® [59]	HCV	2005	211	57		≥0.5	0.82	63	89	92
					16	>0.84	0.89	71	89	NA
Fibrometer® [60]	Mixed	2005	598	56		NA	0.89	80	84	82
Lok Index [61]	HCV	2005	1141		38	<0.2 ≥0.5	0.81	40–98	53–99	52
GUCI [62]	HCV	2005	179		12	>0.1	0.85	80	70	NA
ViraHep-C [63]	HCV	2006	398	37		≤0.22 >0.55	0.83	51–90	54–90	52
Fibroindex [64]	HCV	2007	360	50		≤1.25 ≥2.25	0.83	30–40	97–97	35
FIB-4 [65]	HCV	2007	847		17	<1.45 >3.25	0.85	38–74	81–98	68
HALT-C model [66]	HCV	2008	512		38	<0.2 ≥0.5	0.81	47–88	45–92	48

AUROC: area under ROC curve; CC: correctly classified as true positive and negative; HBV: chronic hepatitis B; HCV: chronic hepatitis C; NA: not available; Se: sensitivity; Sp: specificity.

produce false-positive results in patients with Gilbert's syndrome or hemolysis, because these patients have hyperbilirubinemia [72]. Similarly, acute hepatitis can produce false positive results in APRI, Forns Index, FIB4, or Fibrometer® tests, which all include levels of aminotransferases.

Physical approach: measurement of liver stiffness

Transient elastography

Principle and reproducibility

TE (FibroScan®, Echosens, Paris, France) has been proposed for the measurement of liver stiffness [73]. Briefly, an ultrasound transducer probe is mounted on the axis of a vibrator. Vibrations of mild amplitude and low frequency are transmitted by the transducer, inducing an elastic shear wave that propagates through the underlying tissues. Pulse–echo ultrasound acquisitions are used to follow the propagation of the shear wave and to measure its velocity, which is directly related to tissue stiffness. The stiffer the tissue, the faster the shear wave propagates. TE measures liver stiffness in a volume that approximates a cylinder of 1 cm wide and 4 cm long, between 25 mm and 65 mm below the skin surface. This volume is at least 100 times bigger than a biopsy sample and is therefore far more representative of the hepatic parenchyma. TE is painless, rapid (less than 5 minutes), and easy to perform at the bedside or in the outpatient clinic. The results are immediately available and expressed in kilopascals (kPa), corresponding to the median value of 10 validated measurements, and range from 2.5 to 75 kPa [74], with normal values around 5.5 kPa [75].

TE reproducibility has been shown to be excellent for both interobserver and intra-observer agreement, with intraclass correlation coefficients (ICCs) of 0.98 [76, 77]. However, interobserver agreement was significantly reduced in patients with lower degrees of hepatic fibrosis or hepatic steatosis, increased BMI [76], or liver stiffness values <9 kPa [77].

Diagnostic performances of transient elastography

The two index studies suggesting the role of TE in the assessment of liver fibrosis have been conducted in patients with chronic hepatitis C [78, 79]. Many other groups have confirmed these results since [69, 80–82]. As shown in Table 23.4, TE more accurately detects cirrhosis (AUROC values: 0.87–0.98, with correct classification ranging from 87% to 94%) than significant fibrosis (AUROC values: 0.75–0.91, with correct classification from 57% to 83%).

It should be stressed, however, that despite high AUROC values, a substantial overlap of liver stiffness values was observed between adjacent stages of hepatic fibrosis, particularly for lower fibrosis stages. Several meta-analyses [83–86] have confirmed the better diagnostic performance of TE for cirrhosis than for fibrosis, with mean AUROC values of 0.94 and 0.84, respectively

Table 23.4 Diagnostic performance of transient elastography for significant fibrosis (F ≥ 2) and cirrhosis (F4) in patients with hepatitis C.

Authors	Etiologies	Year (n)	Patient (%)	F ≥ 2 (%)	F4 (%)	Cutoffs (kPa)	AUROC	Se (%)	Sp (%)	CC
Castera *et al.* [78]	HCV	2005	183	74		7.1	0.83	67	89	73
					25	12.5	0.95	87	91	90
Ziol *et al.* [79]	HCV	2005	251	65		8.6	0.79	56	91	68
					19	14.6	0.87	86	96	94
Arena *et al.* [80]	HCV	2008	150	56		7.8	0.91	83	82	83
					19	14.8	0.98	94	92	92
Lupsor *et al.* [81]	HCV	2008	324	65		7.4	0.86	76	84	79
					21	11.9	0.94	87	91	90
Degos *et al.* [69]	HCV	2010	913	62		5.2	0.75	90	32	57
					14	12.9	0.90	72	89	87
Zarski *et al.* [82]	HCV	2012	382	47		5.2	0.82	97	35	64
					14	12.9	0.93	77	90	88

HCV: chronic hepatitis C; HBV: chronic hepatitis B; AUROC: area under the ROC curve; Se: sensitivity; Sp: specificity; CC: correctly classified as true positive and negative; and NA: not available.

[85]. In a meta-analysis of 40 studies (32 papers and eight abstracts), sensitivity and specificity values were 0.83 and 0.89 for patients with cirrhosis and 0.79 and 0.78 for patients with significant fibrosis. However, only nine studies (comprising 1364 patients) had acceptable standards for liver biopsy and TE, which limits the conclusions. It will therefore be important to perform meta-analyses of data from individual patients.

Applicability and limitations of TE

The interpretation of TE results should be always in the hands of an expert clinician and should be made together with information regarding patient demographics, disease etiology, and essential laboratory parameters as well as careful observation of the manufacturer's recommendations (number of valid shots ≥10, success rate [the ratio of valid shots to the total number of shots] ≥60%, and interquartile range [IQR, reflecting the variability of measurements] less than 30% of the median liver stiffness measurement [LSM] value [IQR/LSM ≤30%]) [74]. Although TE reproducibility has been shown to be excellent, its applicability may not be as good as that of biomarkers. Indeed, in our experience on more than 13 000 examinations over 5 years, LSMs were not interpretable in nearly one in five cases (failure to obtain any measurement in 4% and unreliable results not meeting manufacturer's recommendations in 17%) [87]. The principal reasons were obesity, particularly increased waist circumference, and limited operator experience. These results emphasize the need for adequate operator training and for technological improvements in obese patients such as the introduction of the XL probe [88].

Finally, as the liver is an organ wrapped in a distensible but non-elastic envelope (Glisson's capsule), additional space-occupying tissue abnormalities, such as edema and inflammation, cholestasis, and congestion, may interfere with LSM independently of fibrosis. The risk of overestimating liver stiffness values has been reported in cases of ALT flares in patients with acute viral hepatitis or exacerbation of chronic hepatitis B as well as in cases of extrahepatic cholestasis or congestive heart failure [89].

Novel methods to measure liver stiffness

Several other liver elasticity–based imaging techniques are being developed, including 2D acoustic radiation force impulse imaging (ARFI) and 3D magnetic resonance elastography (MRE). ARFI involves mechanical excitation of tissue using short-duration (~262 µsec) acoustic pulses that propagate shear waves and generate localized, µ-scale displacements in tissue. The shear-wave velocity (expressed in m/s) is measured in a smaller region than in TE (10 mm long and 6 mm wide), but can be chosen by the operator. The major advantage of ARFI is that it can be easily implemented on a modified commercial ultrasound machine (Acuson 2000 Virtual Touch™ Tissue Quantification, Siemens Healthcare, Erlangen, Germany). However, ARFI values, in contrast to TE values, have a narrow range (0.5–4.4 m/sec). This limits definitions of cutoff values for patient management decisions.

MRE uses a modified phase contrast method to image the propagation characteristics of the shear wave in the liver. Elasticity is quantified by MRE (expressed in kPa) using a formula that determines the shear modulus, which is equivalent to one-third of the Young's modulus used with TE. The theoretical advantages of MRE include its ability to analyze almost the entire liver and its applicability to patients with obesity or ascites. However, MRE cannot be performed in livers of patients

with iron overload because of signal-to-noise limitations, and it is too costly and time-consuming to use in routine practice.

There are only limited data on the accuracy of ARFI and MRE. Preliminary results [90] indicate that the accuracy of ARFI is similar to that of TE. However, most studies are based on small samples of heterogeneous populations and did not always use liver biopsy as reference. A single pilot study of MRE (96 patients) suggested that MRE might be more accurate than TE in diagnosis of significant fibrosis [91], but validation is required.

Comparison and combination of approaches

Because performances of TE and serum biomarkers have been shown to be equivalent for the diagnosis of significant fibrosis [69, 78, 82], the use of either method could depend on local availability. Strategies that combine two serum biomarkers [92, 93], or TE and serum biomarkers [78, 94, 95], have been proposed to increase diagnostic accuracy in patients with hepatitis C. The advantage of combining two unrelated methods, such as TE and biomarkers, over the combination of two biomarkers is that TE provides more direct measurement of the liver structure than biomarkers, and there is no relationship between the applicability of TE (success rate and interquartile range) and that of a biomarker [94]. Also, the combination of TE and serum biomarkers might be more effective than the combination of two biomarkers for detecting significant fibrosis (with a significantly greater number of liver biopsies avoided) [96, 97].

In that respect, the use of either TE or patented biomarkers (FibroTest®, Fibrometer®, and Hepascore®) was recommended as the first step in assessing fibrosis stage in treatment-naïve patients with hepatitis C without comorbidities, after an independent systematic review by the French Health Authorities [98], and recently endorsed by the European Association for Study of Liver (EASL) clinical practice guidelines [47]. However, with the development of direct-acting antiviral therapies (DAAs), particularly with next-generation DAAs or interferon-free regimens, which produce higher rates of sustained virologic response, discriminating between fibrosis stages F0–F1 and ≥F2 might not be relevant in determining treatment indications.

TE appears to be best suited for cirrhosis screening, because it has a higher level of performance than biomarker assays [69, 95, 99]; combining TE with biomarkers does not increase diagnostic accuracy [82, 95, 99]. However, the applicability of TE is lower (80% vs. 95% for biomarker assays), and the performance levels of these diagnostics might not differ for intention-to-diagnose analysis [82].

Monitoring disease progression

Non-invasive methods can be used to identify patients with cirrhosis who are at risk of disease progression. Measurements of liver stiffness might be used to assess clinical outcomes because they correlate with the severity of liver disease [100], according to a retrospective study conducted in a single center. This study provided the first "proof of concept" that liver stiffness is a prognostic factor for patients with cirrhosis. Furthermore, liver stiffness correlates with portal pressure (based on hepatic venous pressure gradient [HVPG] measurements), which accurately predicts clinical events, as well as with the presence of esophageal varices [101]. However, the diagnostic accuracy of TE (specificity below 60%) is too low for identification of patients with esophageal varices in clinical practice. As for biomarkers, in a large-scale multicenter comparative study, the combination of the Lok Index and Forns Index had the best diagnostic performance for high-risk esophageal varices (large esophageal varices, those with red signs, or decompensated cirrhosis), avoiding endoscopy in around one-third of patients [102]. When compared with serum biomarkers, TE did not perform better for the detection of esophageal varices and large esophageal varices [99]. In summary, non-invasive methods, particularly TE, can identify patients most likely to develop clinically significant portal hypertension, but are not sufficient to identify patients with esophageal varices in clinical practice to replace endoscopy for surveillance of varices [101]. Apart from portal hypertension, a correlation between liver stiffness values and the risk of HCC has also been reported in a large cohort of Japanese patients with chronic hepatitis C [103].

Taken together, these results suggest that TE could be used to discriminate rapidly among patients at different stages of compensated cirrhosis, and place them in different risk categories. Also TE can be easily repeated during the follow-up of patients except in those with ascites.

Finally, the potential of non-invasive methods for predicting clinical outcomes and survival seems to be greater than that of liver biopsy [104–107], probably because the non-invasive tests measure ongoing pathophysiological processes and functions that a biopsy cannot.

Conclusion

The natural history of hepatitis C is highly variable from one infected individual to another. Once chronic infection is established, prognosis mainly depends on the amount and progression of liver fibrosis and the risk of developing cirrhosis.

Significant progress has been made over the past decade regarding assessment of hepatic fibrosis in patients with chronic hepatitis C, highlighting the fact that there is no perfect method. On the one hand, there is increasing awareness that liver biopsy is an imperfect gold standard. On the other hand, an increasing number of non-invasive tests are now available for which TE, Fibrotest®, and APRI are the most extensively validated. As a matter of fact, these tests are already widely used in routine clinical practice in France, resulting in a significant decrease in the need for liver biopsy [108]. However, it is likely that non-invasive methods will reduce but not completely abolish the need for liver biopsy [109]. We believe that liver biopsy and non-invasive methods, particularly TE, should be employed as an integrated system to allow a more efficient and convenient management of patients with chronic hepatitis C [110].

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Chapter 24
Hepatitis C and hepatocellular carcinoma

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Summary

Hepatitis C virus infection is one of the major causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). The genetic structure, genomic organization, and molecular biology of HCV, including the viral life cycle, as well the epidemiology, transmission, and natural course of HCV infection, have been studied in great detail. The continuous improvement of antiviral therapy and the most recent advances suggest that an effective treatment of most patients with chronic HCV infection may become reality in the near future.

Introduction

Hepatitis C virus (HCV) infection [1] is one of the major causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). The genetic structure, genomic organization, and molecular biology of HCV, including the viral life cycle, as well the epidemiology, transmission, and natural course of HCV infection have been studied in great detail. The continuous improvement of antiviral therapy and the most recent advances suggest that an effective treatment of most patients with chronic HCV infection may become reality in the near future.

HCV infection can be acute and self-limiting (<20%) or result in chronic hepatitis (>80%). The mechanisms underlying the high rate of chronic HCV infection include viral factors, such as genetic diversity or quasispecies, mutations resulting in escape from immune recognition, and others, as well as host factors, such as an interleukin 28B (IL28B), renamed interferon λ 4 (INF x 4) gene polymorphism [2], the absence of specific HLA-DRB1 and DQB alleles, the lack of an HCV-specific CD4 T cell response, low titers of neutralizing antibodies targeting HCV structural proteins or viral entry into hepatocytes, and others. Factors predicting disease progression include male gender, acquisition of HCV infection later in life, co-infection with hepatitis B virus (HBV) or HIV, alcohol use, and liver steatosis. Among patients with chronic hepatitis C, 20–30% will develop liver cirrhosis over a period of 20–30 years. Liver cirrhosis can be associated with severe clinical complications, such as ascites, gastrointestinal bleeding, or hepatic encephalopathy, as well as HCC development, even in the face of only few or mild clinical signs and symptoms. In the following, the clinical, molecular, and viral pathogenesis of HCV-associated HCC development will be discussed in some detail.

Clinical aspects of HCV-induced HCCs

HCC incidence has increased over the last several decades, with chronic HCV infection being a major risk factor [3, 4]. Indeed, in patients with HCC, the prevalence of anti-HCV positivity ranges between 30% and 80%, depending on the geographic region. HCV-associated HCCs develop almost exclusively in patients with liver cirrhosis (>90%) at an annual rate of about 2%, yielding a 5-year cumulative incidence of about 10%. Apart from chronic HCV infection, other risk factors for HCC development such as HBV infection, obesity in men, diabetes mellitus, heavy alcohol use, hereditary hemochromatosis, or porphyria may coexist in patients with chronic hepatitis C and may in part increase the HCC risk.

Liver cirrhosis clearly is the major clinical risk factor for HCC development and, therefore, must be considered as a premalignancy. Conversely, prevention of liver cirrhosis by successful antiviral treatment of chronic hepatitis C effectively reduces HCC incidence [5, 6]. However,

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once liver cirrhosis has developed, the HCC risk persists to some degree despite successful antiviral treatment [7].

Laboratory parameters suggestive for liver cirrhosis are elevated bilirubin, prolonged partial prothrombin time, reduced albumin levels, and decreased platelet counts. The aminotransferase levels only poorly correlate with liver histology: patients with repeatedly normal ALT levels almost always show histological evidence of chronic inflammation, albeit minimal or mild. The best clinical predictor of hepatitis C progression is the degree of inflammation and fibrosis in liver biopsies: while patients with mild inflammation and no fibrosis have only a 1% annual risk of progression to liver cirrhosis, in patients with severe inflammation and bridging fibrosis, the annual risk is about 10%, resulting in a 10-year cumulative incidence of liver cirrhosis of about 100% [3, 4].

Liver inflammation, fibrosis, and HCC development

HCC development is closely related to liver fibrosis, with about 90% of HCCs arising in cirrhotic livers. HCCs in general develop in response to chronic liver injury in the setting of cell damage and chronic inflammation, resulting in two divergent cellular responses: regeneration and fibrosis [8]. Cell damage and chronic inflammation may contribute to the malignant transformation of hepatocytes directly through telomere shortening and inactivation of cell cycle checkpoints [9], release of reactive oxygen species (ROS), or altering paracrine signaling in the cellular environment. Myofibroblast activation at the same time may result in liver fibrosis that is promoted by the epithelial-mesenchymal transition (EMT) and accompanied by changes in extracellular matrix (ECM) composition [10] as well as in the activity of nonparenchymal cells. These changes may generate a growth-promoting, anti-apoptotic environment, among others, by sequestration of growth factors by the ECM and reduced tumor cell surveillance by natural killer (NK) and natural killer T (NKT) cells [8, 11, 12]. In addition, altered purinergic signaling as well as bacterial translocation and Toll-like receptor 4 (TLR4) signaling may be involved in hepatocarcinogenesis and HCC promotion [13, 14].

Molecular hepatocarcinogenesis and HCV infection

HCC development has been associated with numerous genetic and epigenetic changes of hepatocytes and of nonparenchymal liver cells (the "microenvironment"), independent of the etiology of the underlying liver disease [12]. These changes include mutations of oncogenes, tumor suppressor genes, as well as DNA methylation, histone modifications, and the expression of microRNA (miRNA) species [15, 16, 17]. Further, genomewide association studies (GWAS) have identified single-nucleotide polymorphisms (SNPs) that may be involved in hepatocarcinogenesis and may eventually contribute to the definition of the HCC risk of an individual patient with liver cirrhosis ("personalized hepatology") [18].

The molecular pathogenesis of HCC development in patients with chronic HCV infection remains ill defined [19, 20, 21]. The mechanisms underlying the progression of HCV infection to HCC usually take many years or decades to develop. Genomics, transcriptomics, and proteomics have identified many genetic and epigenetic alterations associated with HCC development. However, the changes of gene expression identified in tumor cells are very heterogeneous, raising the question of whether specific, still-unidentified changes at early preneoplastic stages trigger the transformation process and whether differentiated hepatocytes or stem cells are at the origin of HCC [22, 23].

HCV genotypes

A correlation between HCV genotypes and the risk of HCC development is still controversial. In a large cross-sectional study and a case–control analysis [24], including 593 patients with chronic hepatitis C, 166 patients with cirrhosis and HCC, and 219 patients with cirrhosis without HCC, HCV genotype 1b was more prevalent among patients with cirrhosis and HCC compared to patients with cirrhosis but without HCC (<0.01). HCV genotype 1b was, next to age and male gender, significantly associated with the risk of HCC development in patients with liver cirrhosis. In addition, in a pairwise comparison of patients with liver cirrhosis with or without HCC, matched by age, sex, and Child–Pugh class of liver cirrhosis, HCV genotype 1b was overrepresented in patients with cirrhosis and HCC. HCV genotype 1b significantly increased the risk of HCC development in cirrhosis, independent of gender, age, and Child–Pugh class. Similarly, a prospective study consecutively including a cohort of anti-HCV-positive patients with liver cirrhosis revealed a significantly higher HCC risk in patients infected with HCV genotype 1b compared to patients infected with other HCV genotypes. The same cohort was reexamined 10 years later after a prospective follow-up for up to 17 years [5]. Multivariate analysis again identified a significantly higher risk of HCC development in patients with cirrhosis infected with HCV genotype 1b as compared to those infected with HCV genotype 2a/c ($p = 0.0001$). A dominance of genotype 1b was also observed in a case–control study in patients with HCV-related HCC.

In several other studies, however, no significant association between the HCV genotype and the HCC risk of patients with cirrhosis could be demonstrated. For example, in the EUROHEP cohort study including 255 Caucasian patients with HCV-related cirrhosis, the

5-year cumulative HCC incidence was 6% for HCV genotype 1b, 5% for genotype 2, and 4% for other genotypes [25]. Similarly, prospective studies from Italy and Germany including anti-HCV- and HCV RNA–positive patients did not identify the genotype as a risk factor for HCC development. Another prospective study from France, including 668 chronically HCV-infected patients with no clinical evidence of decompensation, referred to a single institution with a follow-up of 40 months, again did not identify a particular HCV genotype as a risk factor [26–27]. Taken together, in view of these controversial data, the contribution of the HCV genotype to the risk of HCC development remains unclear.

Pro-carcinogenic factors

In chronic HCV infection, pro-carcinogenic factors are steatosis, oxidative stress, and insulin resistance (IR). Thus, chronic hepatitis C shares many similarities with non-alcoholic fatty liver disease (NAFLD), which may lead to non-alcoholic steatohepatitis (NASH) and HCC. In NAFLD, chronic excess of nutrients causes endoplasmic reticulum (ER) stress and leads to increased steatosis and IR. These phenomena are intrinsically linked, amplify each other, and induce inflammation, which in turn favors hepatocarcinogenesis. The accumulation of fat in the liver has been known to trigger the recruitment of inflammatory cells and to increase the levels of prooxidants. Reactive oxygen species (ROS) directly act on essential biomolecules and induce hepatotoxicity. ROS also indirectly activate redox-sensitive transcriptional cascades, which trigger the production of cytotoxic, proinflammatory, and fibrogenic mediators. Both steatosis and ER or oxidative stress have causal roles in the development of IR by molecular pathways that are only beginning to be elucidated [28]. In IR, insulin receptor substrate (IRS)-induced signaling is frequently downregulated or blocked by various mechanisms. Apart from ER stress and fatty acid metabolites, IR can also be triggered by peptide mediators and cytokines secreted by adipose tissue and/or the liver. Finally, IR and oxidative stress aggravate steatosis by stimulating lipid synthesis, increasing the transfer of fatty acids from adipose tissue to the liver, and inhibiting lipid secretion by interference with mitochondrial function. In conclusion, a complex interplay between steatosis, ER or oxidative stress, and IR, whose underlying molecular mechanisms remain largely undefined, can lead to chronic liver inflammation, apoptosis, and fibrogenesis, which are central to the development of liver cirrhosis and HCC in patients with chronic hepatitis C. Increased cell proliferation in a setting of oxidative stress leads to the accumulation of DNA damage, which may result in chromosome and gene instability that forms the genetic basis for the malignant transformation of hepatocytes. In addition, recent data clearly indicate that epigenetic changes, including miRNAs, are also involved in hepatocarcinogenesis.

Table 24.1 Hepatocarcinogenesis and HCV infection or protein (Source: Tsai WL, Chung RT. Oncogene 2010 Apr 22;29(16):2309–2324 [20]).

Oncogenic pathways	HCV infection or HCV protein
Inflammation, fibrosis, and cirrhosis	Immune response to HCV infection
Oxidative stress	HCV core, NS3, and NS5A
ER stress	HCV core, and NS5A
Ca^{2+} signaling	HCV core, and NS5A
Androgen signaling	HCV core
Raf–MAPK	HCV core
TGFβ	HCV core
p53	HCV core, NS3, and NS5A
Wnt–β-catenin	HCV core and NS5A
Oncogene activation	HCV core, E2, NS3–NS4A, and NS5A
Genomic instability	HCV core, NS3, and NS5A

HCV proteins

So far, it has not been possible to correlate the expression of specific HCV proteins with HCC development. HCV does not integrate into the host genome and has a predominantly cytoplasmic life cycle [29]. Hepatocarcinogenesis, therefore, must involve several indirect mechanisms, including the interplay between chronic inflammation, steatosis, fibrosis, and oxidative stress and their pathological consequences. However, several HCV proteins have been shown to have direct oncogenic effects and to upregulate mitogenic processes (Table 24.1).

Direct interactions of different HCV proteins with host cell factors have been shown to lead to changes in cellular signaling cascades involved in regulation of cell metabolism and division, and seem to be sufficient to induce hepatocarcinogenesis [30]. Overall, it is thought that the synergism between chronic inflammation and direct virus–host cell interactions triggers the malignant transformation of hepatocytes. The requirement for such a synergism would also explain the slow "multistep" transformation process that underlies human HCC development. A considerable time lag between HCV infection and the development of cirrhosis and HCC is common and may explain the heterogeneity of genetic and epigenetic alterations observed in different HCCs [31, 32].

By modulating gene transcription and translation as well as posttranslational events, the HCV proteins interfere with innate immunity to favor viral persistence and liver inflammation. They alter cell signaling, apoptosis, membrane physiology, and protein trafficking, and they induce oxidative stress, genomic instability, as well as

malignant transformation. Among the HCV proteins core, NS3, NS4B, and NS5A have all been shown to have transforming potential when transiently or stably expressed in cell culture, or in transgenic mice expressing the different viral proteins or the HCV polyprotein [19, 20, 21].

The HCV "core protein" is a highly conserved, basic protein that multimerizes, probably in conjunction with microtubules [33], to form the viral nucleocapsid and to package the viral RNA genome. It is localized at the cytoplasmic surface of the ER and on lipid droplets. The latter observation is likely related to the induction of liver steatosis observed in HCV-infected patients as well as in transgenic mice overexpressing HCV core [34]. Core has also been shown to localize to the outer membranes of mitochondria [35] and is involved in changes of apoptosis and lipid metabolism as well as in malignant transformation. Among many interactions with cellular factors, core has been shown to induce ROS production via interaction with heat shock protein Hsp60 [36], to bind the tumor suppressor proteins p53 [37], p73 [38], and pRb [39]. Core also inhibits the expression of the cyclin-dependent kinase (CDK) inhibitor p21/Waf [40]. p21 is a transcriptional target of p53 and blocks the cyclin–CDK complexes involved in cell cycle control and tumor formation. Core induces activation of the Raf1–MAPK pathway [41, 42], protects cells from serum starvation and growth arrest, and drives cells into proliferation. NFκB transcription has been shown to be activated [43, 44, 45] and repressed [46] by HCV core. HCV core has also been shown to activate the Wnt–β-catenin pathway, which controls cell proliferation, DNA synthesis, and cell cycle progression [47]. Furthermore, HCV core variants have been shown to interact with Smad3 and to inhibit the TGFβ pathway [48]. TGFβ signaling not only controls cell proliferation, differentiation, and apoptosis but also stimulates liver regeneration and fibrogenesis through its actions on ECM. TGFβ levels are frequently increased in patients with chronic HCV infection and correlate with the degree of fibrosis [49]. Finally, HCV core protein associates with cellular membranes [50, 51] and lipid vesicles [51], binds to apolipoprotein II [52, 53], and reduces microsomal triglyceride transfer protein activity [53], resulting in impaired assembly and secretion of very low-density lipoproteins, steatosis, and oxidative stress. These *in vitro* findings are likely to be relevant for HCV pathogenesis because transgenic mice expressing HCV core protein also develop steatosis [53, 51] and HCCs [54, 30].

Overexpression of E2 inhibits eIF2α phosphorylation by the double-stranded RNA (dsRNA)-activated protein kinase (PKR) or the ER stress–signaling kinase PERK [55, 56]. Similarly, overexpression of NS4A, NS4B, or NS4A–NS4B has been reported to induce an ER stress–mediated unfolded protein response, to reduce ER-to-Golgi trafficking, to inhibit protein synthesis, and to cause cytopathic effects [57, 58, 59]. NS2 has been shown to inhibit the cellular pro-apoptotic molecule CIDE-B [60] and to downregulate transcription [61].

"NS3-4A serine protease" has been reported to interact with p53 to repress p21 function, to block activation of the transcription factors IRF3 and NFκB, and to antagonize innate antiviral defenses by interfering with RIG1-, MDA5-, and TLR3-mediated signal transduction [62, 63]. Indeed, RIG1 inactivation has been shown to render Huh7 cells permissive to HCV replication [64].

NS5A has been shown to induce EMT [65] and to interact with the geranylgeranylated cellular protein FBL2 [66], an F-box motif containing protein that is probably involved in targeting cellular proteins of still unknown identity for ubiquitylation and degradation. A number of studies suggest that NS5A is also involved in interferon resistance [67], and one possible mechanism may be its ability to induce expression of the type I interferon antagonist IL8 [68]. In addition, NS5A has been described to contain an "interferon sensitivity–determining region" (ISDR) that presumably mediates inhibition of PKR, an activator of innate immunity [69]. The accumulation of mutations in this region is thought to correlate with antiviral treatment efficacy. Importantly, overexpression of NS5A has been reported to induce a number of effects in cells, including oxidative stress; activation of signaling pathways such as STAT3, PI3K, and NFκB; and altered transcriptional regulation, including p21 [70, 71] and pRB [72]. Other NS5A interaction partners include apoliporotein A1, the major protein found in HDLs; the tumor suppressor p53; Grb2, an adaptor protein involved in mitogen signaling; SRCAP, an adenosine triphosphatase (ATPase) that activates cellular transcription; karyopherin β3, a protein involved in nuclear trafficking; Cdk1/2, and Fyn, Hck, Lck, and Lyn, Src family kinases [73, 67, 74, 75, 76]. It has also been reported that NS5A expression in the context of the HCV polyprotein results in the inhibition of the transcription factor Forkhead as well as in the phosphorylation and inactivation of the GSK3, leading to accumulation of β-catenin and stimulation of β-catenin-dependent transcription [77]. Finally, NS5A-dependent activation of upstream binding factor, a Pol I DNA-binding transcription factor, which occurs as a result of upregulation of both cyclin D1 and CDK4, leads to enhancement of rRNA transcription activation [78].

Genetic changes

HCCs are genetically heterogeneous tumors. This is not unexpected, given the number of etiological factors implicated in its development, the complexity of hepatocyte physiology, and the advanced stage at which HCCs are usually diagnosed. GWAS of genetic altera-

tions occurring in HCC revealed two major mechanisms of hepatocarcinogenesis. In the first, genetic alterations are acquired in the context of elevated oxidative stress caused by the vicious circle between chronic inflammation, IR, and steatosis; in the second, transformation is induced by β-catenin mutations that dysregulate the Wnt pathway [79]. So far it has not been possible to correlate chromosome instability with a consistent pattern of proto-oncogene activation in HCC, but several growth factor–signaling pathways are frequently affected, including signaling from insulin-like growth factor (IGF), hepatocyte growth factor, Wnt, TGFα–EGF, and TGFβ [80, 31]. The interplay between these pathways and their respective roles and contributions to HCC development remain to be elucidated, however. One of the most commonly affected pathways involved in cell cycle check control is the p53 pathway, which limits cell survival and proliferation in response to telomere shortening and oncogene activation in order to ensure genome integrity. Loss of p53 by, for example, deletion, mutation, degradation, or direct inhibition during progression of chronic hepatitis C to cirrhosis likely results in proliferation of hepatocytes with shortened telomeres or chromosomal damage and to predispose one to hepatocarcinogenesis. Indeed, p21 expression, a downstream target of p53 that blocks cell entry into the S phase, is increased in cirrhosis when presumably significant numbers of hepatocytes with genome damage have accumulated, but is lost in premalignant liver lesions and HCC [81]. Besides p53, the retinoblastoma (Rb) pathway is another major checkpoint that limits cell proliferation in response to DNA damage, telomere shortening, and oncogene activation. In human HCC, the Rb pathway is defective in more than 80% of cases [82]. Moreover, gankyrin, an inhibitor of p53 and Rb checkpoint function, is overexpressed in the vast majority of HCCs [83]. Expression of insulin-like growth factor 2 (IGF2) is frequent and thought to be an early event in hepatocarcinogenesis, present in more than 60% of dysplastic nodules and HCC [84]. The IGF2 receptor impairs cell proliferation by promoting degradation of the IGF2 mitogen and by activation of TGFβ signaling [85]. β-catenin pathway activation is very common in hepatocarcinogenesis and is detectable in more than 50% of HCCs. It can directly induce hepatocyte transformation without the need for multiple genetic-epigenetic alterations [86]. β-catenin activation is mainly induced by β-catenin gene mutations and/or Wnt-signaling pathway alterations [82]. Normally, the Wnt–β-catenin pathway is involved in cell growth and proliferation as well as developmental control and cell adhesion. Cellular levels of β-catenin are tightly regulated by proteasome-dependent degradation. In this context, a recent study demonstrated that HCV-induced miR-155 expression promotes hepatocyte proliferation and tumorigenesis by activating Wnt signaling [87]. Further, a recent high-resolution characterization of a HCC genome from a patient with chronic HCV infection identified new mutation patterns, intrachromosomal rearrangements, and fusion genes as well as a genetic heterogeneity within the HCC [88].

Epigenetic changes

In addition to the dysregulation of the pathways discussed here, HCV infection has been shown to be associated with epigenetic changes that are likely to contribute to hepatocarcinogenesis. HCV-induced ROS have been shown to activate histone deacetylase in a fashion similar to that of hydrogen peroxide and to cause hypoacetylation of histones [89]. Hypomethylation of the IGF2 locus in hepatitis C cirrhosis has been shown to predict HCC development [90]. Another study reported the hypermethylation of p16, p15, p14, pRB, and the PTEN promoters in patients with sustained viral response in comparison to nonresponders [91]. Finally, increased hTERT DNA levels have been found to predict HCC development [92].

GWAS and SNPs

There is increasing evidence for genetic variations in several loci that are associated with an increased HCC risk of patients with chronic HCV infection. Recent examples are the DEPDC5 locus on chromosome 22 [93] and the 5′ flanking region of MICA on chromosome 6 [94]. Apart from the role of such loci for the development of HCV-related HCC, these SNPs may contribute to the definition of the HCC risk of the individual patient with chronic HCV infection.

HCV infection and malignancies other than HCC

Apart from HCC, HCV is also a well-established risk factor of lymphoproliferative syndromes such as type II mixed cryoglobulinemia [95]. Increasing evidence further suggests a contribution of HCV infection to the development of malignant lymphoma. In this context, two meta-analyses identified a 5–10-fold increased risk of B cell non-Hodgkin lymphoma (B-NHL) in anti-HCV-positive individuals [96, 97]. In most studies, the odds ratio of developing B-NHL for HCV-infected patients ranged between 2:1 and 4:1. Interestingly, there is a striking geographical and demographic variation in the correlation between chronic HCV infection and B-NHL development, with a relatively high risk in Southern Europe but not in Northern Europe or North America [96–97]. This finding may have several causes, such as different HCV prevalence in these geographic regions, different control populations, and different methods of HCV detection.

A recent European multicenter case–control study addressed the association between HCV infection and lymphoma in 1807 patients with newly diagnosed lymphoid malignancies and 1788 healthy controls. HCV infection was detected in 53 patients with lymphoma (2.9%) and in 41 control subjects (2.3%) (odds ratio: 1.52, CI: 0.93–2.15). In sub-analyses, HCV prevalence was associated with diffuse large B cell lymphoma but not with chronic lymphocytic leukemia or follicular, Hodgkin, or T cell lymphoma [97–98]. A large, population-based Scandinavian case–control study including 2819 lymphoma patients and 1856 healthy controls documented a modest but significant association between HCV infection (defined as HCV RNA positivity) and NHL (odds ratio: 1.7 [0.2–16.2]) [97, 99]. Also, in a prospective study from Japan, the risk of NHL development among patients with chronic HCV infection was increased [97, 100]. Further, a retrospective study in US veterans identified a 20–30% increased risk of NHL development in HCV-infected patients [97, 101].

Overall, the available epidemiological data suggest an increased risk of lymphoma development in HCV-infected patients. Further studies are needed, however, to identify the mechanisms underlying HCV-related lymphoma development, including the contributing host and viral factors [97–98]. Interestingly, clinical data show a regression of lymphoma after successful treatment of HCV infection, supporting the concept of HCV infection as a cause of lymphoma development in humans [97, 102].

HCV infection has also been linked to the development of intrahepatic cholangiocarcinoma (ICC). A cohort study from Japan, for example, showed a relatively high ICC incidence in 600 patients with HCV-related cirrhosis compared to the estimated incidence in the general population [97, 103]. A case–control study including 625 patients with ICC aged 65 years and older and 90 834 controls found a higher prevalence of HCV infection in ICC patients (adjusted odds ratio, 7.4; $p < 0.0001$), suggesting that HCV infection is indeed a risk factor for ICC development [97, 104]. Similarly, in a hospital-based study performed in the United States, ICC patients had a higher prevalence of anti-HCV antibodies than controls (6.0% versus 0.8%, $p = 0.01$) [97, 105]. Finally, a recent cohort study including 146 394 HCV-infected and 572 293 HCV-uninfected patients who received care at US Veterans Affairs healthcare facilities observed a twofold elevated risk of ICC in patients with chronic HCV infection [97, 106]. It remains unclear, however, whether these associations are independent from the underlying liver disease or cirrhosis.

Conclusions and perspectives

Our current view is that the mechanism of HCV-induced HCC is multifactorial. However, because of the lack of adequate models, it has been difficult to demonstrate the specific roles of HCV proteins and the liver microenvironment in the malignant transformation of hepatocytes. To identify and characterize these mechanisms, primary human hepatocyte cultures supporting chronic HCV infection would be most useful to examine the accumulation of transforming events, leading to the selection of transformed cells after several cell passages. An immunocompetent animal model, susceptible to chronic HCV infection, would be important to analyze not only the different viral proteins but also the liver micro-environment involved in HCC development (including IR, steatosis, oxidative stress, cytokine expression in response to HCV expression, liver regeneration, fibrosis, etc.). The recent discovery of cellular co-receptors required for virus entry and the better understanding of the molecular biology of HCV replication should open new avenues to address these important questions.

While chronic inflammation and liver regeneration resulting from immune-mediated liver cell death most likely contribute to the malignant transformation of hepatocytes and HCC development in patients with chronic HCV infection, a direct role of HCV proteins remains elusive. *In vitro* studies have shown that HCV expression may interfere with cellular functions that are important for cell differentiation and cell growth. Their relevance for HCC development in patients, however, needs to be confirmed in models as well as patients.

One of the challenges for the future is to define precisely gene signatures as well as epigenetic markers, including miR species that allow one to predict the HCC risk of the individual patient with chronic hepatitis C ("personalized hepatology") [107].

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Chapter 25
Treatment of hepatitis C

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Summary

The goal of hepatitis C virus (HCV) therapy is viral eradication to prevent complications of chronic infection. The sustained virologic response rate for pegylated interferon alpha (PEG-IFNα) plus ribavirin (RBV) has been only about 50% in patients infected with genotype 1 HCV, the most prevalent genotype in Europe and North America. Therapy has been revolutionized recently by the development and approval of direct-acting antiviral agents (DAAs). Triple therapy with PEG-IFN/RBV and a protease inhibitor has supplanted PEG-IFN/RBV alone as the new standard of care (SOC) for genotype 1 HCV infection. This chapter provides an overview on chronic hepatitis C treatment, including dosing regimens, response rates, stopping rules, side effects, drug resistance development, and medication interactions. A perspective on novel agents in clinical development and interferon-sparing regimens concludes this chapter.

Introduction

Chronic infection with HCV constitutes a major global health concern with up to 200 million people infected worldwide [1]. Chronic hepatitis C (CHC) is a leading cause of liver disease with cirrhosis and hepatocellular carcinoma as potential sequelae [2]. The goal of HCV therapy is to prevent these complications through viral eradication. For the past decade, CHC has been treated with a combination of PEG-IFNα and RBV [3–6]. Viral eradication rates have remained suboptimal over this time period, particularly in patients with genotype 1 HCV, which is responsible for around 60% of infections worldwide. Sustained virologic response (SVR) rates for genotype 1 CHC are approximately 40–50% following 48 weeks of PEG-IFN/RBV therapy, and they are even lower in patients of African descent, those with HIV co-infection, or those with high viral load or advanced fibrosis. A significant step forward in the quest for host factors that mediate response to interferon therapy was the recent discovery of a group of single-nucleotide polymorphisms (SNPs) in the region of the *IL28B* (type 3 lambda interferon) gene through genome-wide association studies [7]. Today, the *IL28B* genotype at the rs12979860 locus is the most powerful baseline predictor of SVR in genotype 1 caucasian patients treated with PEG-IFN/RBV, with SVR in about 70% of patients with the favorable CC genotype of the *IL28B* rs12979860 polymorphism compared to 25–30% in those with the CT or TT genotype.

Therapy has been revolutionized recently by the development and approval of DAAs. In contrast to the nonspecific antiviral activity of IFN and RBV, DAAs are designed to inhibit viral proteins involved in the HCV life cycle. The complexity of the viral machinery allows for numerous potential targets, including the NS3/4A serine protease, NS5A replication complex protein, NS5B RNA-dependent RNA polymerase (RdRp), and

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NS4B and NS3 helicase proteins. The first two approved DAAs are telaprevir (Incivek™) and boceprevir (Victrelis™), both ketoamide inhibitors of the NS3/4A protease, indicated for use in patients with genotype 1 CHC. They mark the beginning of an extraordinary new era in HCV therapy. The coming years are expected to bear witness to newer DAAs with simplified dosing regimens and/or lower toxicity that, when used in combination, may lead to viral eradication in most CHC patients undergoing treatment [8]. Indeed, recent proof-of-concept studies have vindicated the hope held for years by many investigators that HCV can be eradicated without IFN [9, 10].

HCV treatment, objectives, and outcome

The goal of anti-HCV therapy is viral eradication. The recommended treatment in all HCV genotypes (discussed in this chapter) is the combination of PEG-IFN and RBV for 24 to 48 weeks, depending upon the genotype, with sustained virologic response in about 50% of genotype 1 and 80% of genotype 2 and 3 HCV-infected patients (Figure 25.1A). Two pegylated interferons are licensed for CHC treatment – PEG-IFNα-2a (Pegasys™) with a 40-kd branched polyethylene glycol (PEG) molecule covalently linked to the IFNα-2a molecule, as well

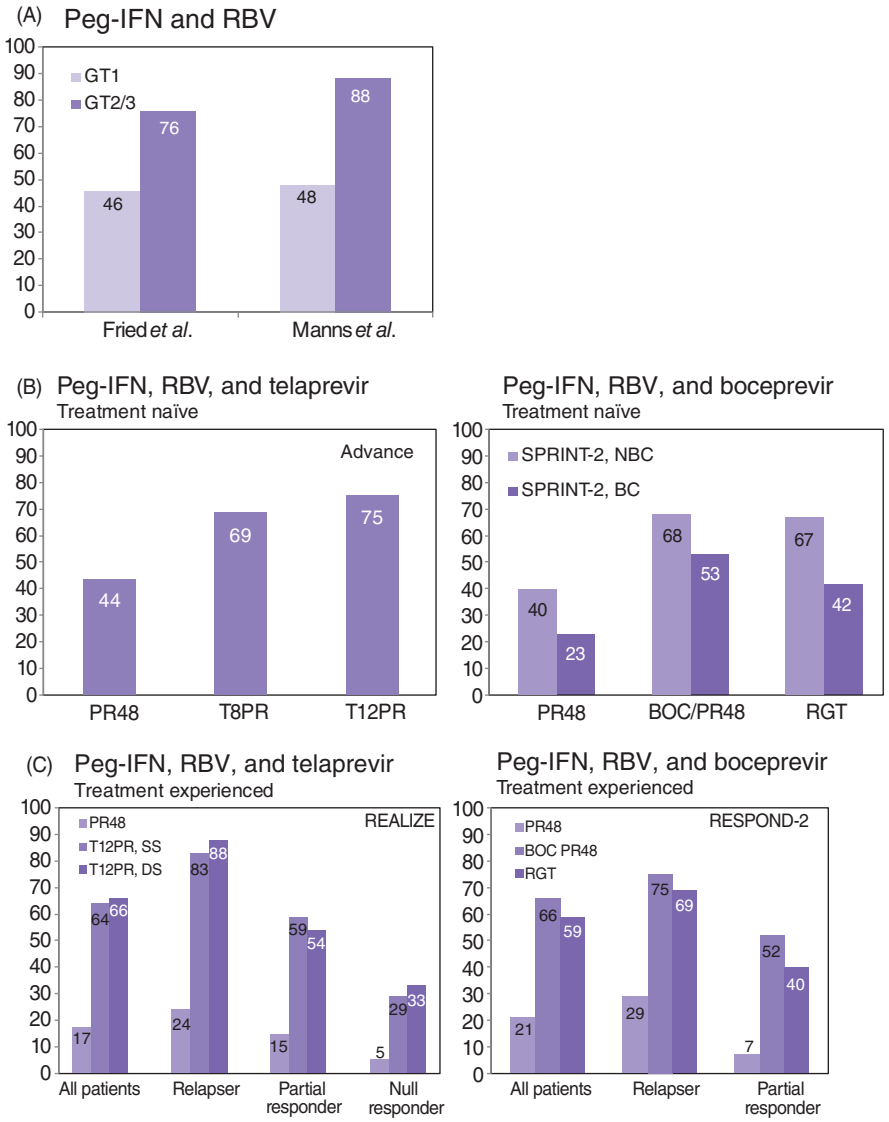

Figure 25.1 (A) SVR rates from pivotal PEG-IFN/RBV trials for genotypes 1 and 2/3, as well as for (B) PEG-IFN/RBV + PI in treatment-naïve and (C) treatment-experienced patients. BC: black cohort; DS: delayed start; GT: genotype; NBC: nonblack cohort; PR: PEG-IFN/RBV; RGT: response-guided therapy; SS: simultaneous start.

as PEG-IFNα-2b (Peg-Intron™) with a 12 kd linear PEG moiety covalently linked to an IFNα-2b molecule. The dosage is different between the two PEG-IFNs. In genotype 1 CHC, PEG-IFNα-2a is administered at a once-weekly fixed dose of 180 µg via subcutaneous injection, together with RBV (Copegus™) 1000 mg or 1200 mg daily, according to body weight (≤75 kg or >75 kg, respectively). For genotypes 2 and 3, a lower dose of Copegus (800 mg daily) is used. PEG-IFNα-2b subcutaneous injection is dosed according to body weight (1.5 µg/kg/week). RBV (Rebetol™) is dosed according to body weight with 800 mg for patients <65 kg, 1000 mg for 65–85 kg, 1200 mg for 85–105 kg, and 1400 mg for >105 kg. Standard duration for PEG-IFN/RBV therapy is 48 weeks in genotype 1 and 4, and 24 weeks in genotypes 2 and 3. Due to the limited worldwide prevalence of genotypes 5, 6, and 7, a recommendation for dosing and treatment duration for these genotypes cannot be made. Preliminary data suggest that 48 weeks of treatment are superior to 24 weeks in HCV genotypes 4 and 6 [11].

The treatment duration can be individualized by the virological response pattern, the so-called response-guided therapy (RGT). Response is measured by a surrogate virological parameter that describes virus kinetics under treatment. Using sensitive polymerase chain reaction (PCR) or transcription mediated amplification (TMA)–based assays with a lower limit of detection of 10–50 IU/mL, the response patterns are labeled according to their timing relative to treatment. SVR, or virus eradication, is defined as the absence of HCV RNA 24 weeks after treatment discontinuation. Undetectable HCV RNA at the end of treatment (EOT) is referred to as EOT response (ETR). It does not predict the likelihood for an SVR but is mandatory for it to occur. Rapid virological response (RVR) is defined as negative HCV RNA at week 4, and extended rapid virological response (eRVR) is defined as undetectable HCV RNA at week 4 and at week 12. The presence of RVR and eRVR is associated with a high likelihood of achieving an SVR. Early virological response (EVR) is defined as ≥2 log HCV RNA decline or a complete absence of HCV RNA at week 12, and it is the most accurate predictor for treatment failure if not achieved. Treatment failure is due to nonresponse or breakthrough on antiviral treatment, as well as relapse upon treatment completion. Nonresponders are differentiated into null and partial responders, with the partial responder showing a ≥2 log HCV RNA decline at week 12 but never achieving undetectable HCV RNA, whereas null responders fail to achieve a decline of ≥2 log HCV RNA after 12 weeks of treatment. Relapse is defined as the reappearance of HCV RNA upon treatment discontinuation and after ETR was documented, whereas viral breakthrough is defined as the reappearance of HCV RNA while still on antiviral treatment [11].

Genotype 1 CHC patients on PEG-IFN/RBV who had a low viral load at baseline and who achieved an RVR can be successfully treated with a shortened treatment duration of only 24 weeks. SVR after 24 weeks of PEG-IFN/RBV was achieved in 89% of such patients. Patients with HCV genotype 2 or 3 infection and RVR on PEG-IFN/RBV are also eligible to shorten their treatment duration. SVR was achieved in 62–94% for 12 to 16 weeks of treatment, which is comparable to SVR rates of 70–95% for 24 weeks of treatment. The ACCELERATE trial [12], however, raised controversies regarding shorter treatment duration in genotype 2 and 3 infection, demonstrating superiority of the 24-week arm over the 16-week arm even for patients who achieved RVR. This was potentially due to different RBV dosing in the different trials, a fixed dose (800 mg) in the ACCELERATE trial versus weight-based (1000–1200 mg) dosing in most other trials. Thus, treatment of only 12 or 16 weeks in patients with genotype 2 or 3 infection who achieve RVR and are intolerant to PEG-IFN/RBV is possible, but relapse rates may be higher. In contrast to the shortened treatment duration for rapid responders, extended therapy for 72 or 48 weeks in genotypes 1 and 2/3, respectively, should be considered in those patients who are slow to respond to PEG-IFN/RBV, that is, not achieving negative HCV RNA at week 12 but doing so by week 24. The higher SVR was primarily due to the lower relapse rates observed for slow responders when treated over an extended period. High-dose PEG-IFN for patients with delayed response was generally not successful in improving SVR. High-dose RBV with 1600–3600 mg daily showed impressive SVR of 90% in a pilot study with 10 genotype 1 CHC patients with a high baseline viral load >800 000 IU/mL. However, high-dose RBV is associated with significant safety issues (e.g., anemia requiring growth factor in all patients, as well as blood transfusion in two). Thus, higher PEG-IFN or RBV doses are not recommended as a first-line strategy [11] (Table 25.1A–B).

The new standard of care in chronic hepatitis C treatment

The NS3/4A serine protease is required for HCV polyprotein processing and, together with the NS3 helicase, is involved in infectious virus production. Since the approval of the ketoamide NS3/4A protease inhibitors (PIs) telaprevir and boceprevir by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA), triple therapy with a PEG-IFN/RBV backbone and a ketoamide PI has supplanted the use of PEG-IFN/RBV alone as the new standard of care (SOC) for genotype 1 CHC.

Table 25.1A Response-guided therapy with PEG-IFN/RBV in genotype 1.

HCV RNA				
Baseline	TW4	TW12	TW24	Treatment duration*
LVL	<12–15 IU/mL	Negative	Negative	24 weeks
HVL	<12–15 IU/mL	Negative	Negative	48 weeks
LVL/HVL	>12–15 IU/mL	Negative	Negative	48 weeks
		<2 log decline		Stop
		≥2 log decline and/positive	Positive Negative	Stop 72 weeks

Table 25.1B Response-guided therapy with PEG-IFN/RBV in genotypes 2 and 3.

HCV RNA			
Baseline	TW4	TW12	Treatment duration*
LVL	<12–15 IU/mL	Negative	16 weeks
HVL	<12–15 IU/mL	Negative	24 weeks
LVL/HVL	>12–15 IU/mL	Negative	24 (LVL)–48 (HVL) weeks
		<2 log decline	Stop
		≥2 log decline and/positive	48 weeks

*No treatment shortening in patients with, for example, cirrhosis and other negative predictors for SVR. LVL: HCV RNA <600 000–800 000 IU/mL; HVL: HCV RNA >600 000–800 000 IU/mL; PEG-IFN/RBV: pegylated interferon-alpha/ribavirin; TW: treatment week.

Triple therapy combining the PEG-IFN/RBV backbone with a ketoamide NS3/4A protease inhibitor

Telaprevir (Incivek™)

Telaprevir is a selective peptidomimetic NS3/4A PI that forms a covalent, reversible enzyme-inhibitor complex. Its efficacy in combination with PEG-IFN/RBV in both treatment-naïve and treatment-experienced patients with genotype 1 CHC was established in the multicenter phase IIb PROVE 1, PROVE 2, and PROVE 3 trials [13–15]. Through these early trials, superior response rates were observed with the addition of telaprevir to PEG-IFN/RBV, driven by high rates of RVR and low rates of relapse. Ribavirin was found to be an essential component of these treatment regimens. The concept of response-guided therapy (RGT), in which treatment duration is determined by viral response early in the course of therapy, was introduced for PI-based triple therapy.

A phase III study in treatment-naïve genotype 1 CHC patients was carried out in the pivotal ADVANCE study [16], which compared telaprevir-based regimens to PEG-IFN/RBV in 1088 patients. Patients were randomized to one of three treatment arms: T8PR, which is telaprevir 750 mg q8h + PEG-IFNα-2a/RBV for 8 weeks followed by an additional 16/40 weeks of PEG-IFN/RBV; T12PR, which is telaprevir + PEG-IFN/RBV for 12 weeks followed by an additional 12/36 weeks of PEG-IFN/RBV; and PR48, which is PEG-IFN/RBV for 48 weeks. Patients in the telaprevir arms who achieved undetectable HCV RNA at weeks 4 and 12 (eRVR) were treated for a total of 24 weeks, whereas those who did not achieve eRVR were treated for 48 weeks total.

Significantly higher SVR rates were observed in both telaprevir arms as compared to control with 75% and 69% in the T12 and T8 groups achieving SVR, respectively, compared with 44% in the PR48 group ($p < .0001$) (Figure 25.1B). Reanalysis using FDA-requested revised criteria (i.e., counting SVR at week 12 post treatment as SVR when a 24-week follow-up was not available, and counting patients with HCV RNA between the lower limit of detection and lower limit of quantitation at week 24 of posttreatment follow-up as SVR) resulted in SVR rates in the package insert of 79%, 72%, and 46%, respectively. Relapse rates were observed to be 9% in each telaprevir arm and 28% in the control arm. In the now-approved T12PR regimen group, 58% of patients achieved an eRVR, of whom 89% went on to SVR. In African-American patients, addition of telaprevir increased SVR rates to 62% and 58% in the T12PR and T8PR groups, respectively, from 25% in the PR group. Similarly, a significantly improved SVR rate (62%) was also observed in patients with bridging fibrosis or cirrhosis in the T12PR group versus control (33%).

The ILLUMINATE study firmly established the foundation for RGT in treatment-naïve patients receiving telaprevir-based regimens [17]. All patients were treated with 12 weeks of telaprevir + PEG-IFN/RBV with those achieving an eRVR subsequently randomized to either 12 or 36 additional weeks of PEG-IFN/RBV. Sixty-five percent of the 540 study patients achieved eRVR. The 24-week telaprevir-based regimen was non-inferior to the 48-week regimen with SVR rates of 92% versus 88%, respectively. High SVR rates were achieved with RGT in patients with eRVR regardless of race, ethnicity, as well as the presence or absence of advanced fibrosis. However, data at present are insufficient to support RGT in cirrhotic patients, since of the few patients with cirrhosis enrolled in the ILLUMINATE trial, there was a trend toward higher SVR rates in patients with eRVR who were treated for 48 weeks.

Table 25.2 Telaprevir prescribing guidelines.

Treatment-naïve and prior relapsers			
HCV RNA*	PEG-IFN/RBV/TPV triple therapy	PEG-IFN/RBV dual therapy	Treatment duration
Undetectable at weeks 4 and 12 Detectable (1000 IU/ml or less) at week 4 and/or 12	First 12 weeks First 12 weeks	Additional 12 weeks Additional 36 weeks	24 weeks 48 weeks
Prior partial and null responders			
	PEG-IFN/RBV/TPV triple therapy	PEG-IFN/RBV dual therapy	Treatment duration
All patients	First 12 weeks	Additional 36 week	48 weeks

*Discontinue all medications if HCV RNA > 1000 IU/ml at week 4 or 12 or if HCV RNA is detectable at week 24. Patients with cirrhosis who have undetectable HCV RNA at week 4 and 12 of PEG-IFN/RBV + telaprevir may benefit from an additional 36 weeks of PEG-IFN/RBV (48 weeks total). PEG-IFN/RBV: pegylated interferon-alpha/ribavirin; TPV: telaprevir.

The phase III REALIZE trial evaluated telaprevir-based therapy in treatment-experienced patients [18]. Six hundred sixty-two genotype 1 relapsers, partial responders, and null responders of prior PEG-IFN/RBV therapy were randomized to receive either 48 weeks of PEG-IFN/RBV (PR48, control group) or one of two telaprevir regimens: PEG-IFN/RBV for 4 weeks followed by telaprevir 750 mg q8h + PEG-IFN/RBV for 12 weeks followed by PEG-IFN/RBV for an additional 32 weeks (delayed-start arm), or telaprevir 750 mg q8h + PEG-IFN/RBV for 12 weeks followed by PEG-IFN/RBV for 36 weeks (simultaneous-start arm). Among relapsers, the SVR rate was 88% in the telaprevir delayed-start arm and 83% in the simultaneous-start arm compared to 24% in the control group (Figure 25.1C). For prior partial responders, the SVR rate was 54% and 59% in the delayed- and simultaneous-start arms, respectively, versus 15% in controls. Finally, 33% and 29% of null responders achieved SVR in the delayed- and simultaneous-start arms, respectively, versus 5% in control. While both telaprevir-based regimens were superior to control, the lead-in (delayed-start) regimen did not significantly improve SVR rates over simultaneous start. Subgroup analysis demonstrated that presence of cirrhosis minimally affected SVR rates in telaprevir-treated relapsers, while cirrhotic partial responders and null responders had significantly decreased SVR rates. SVR was achieved in 14% of null responders and 44% of partial responders with bridging fibrosis or cirrhosis.

Telaprevir is approved for the treatment of genotype 1 CHC in oral tablets at a dose of 750 mg to be taken every 8 hours with food (>20 g fat) in combination with PEG-IFN/RBV. The three-drug regimen is given for 12 weeks, followed by RGT of either 12 or 36 additional weeks of PEG-IFN/RBV depending on viral response and prior treatment status. For treatment-naïve patients and prior relapsers, those with eRVR are eligible for truncated therapy with 12 more weeks of PEG-IFN/RBV. The exception to this guideline is treatment-naïve patients with cirrhosis, which despite eRVR should have 36 additional weeks of PEG-IFN/RBV. All prior partial and null responders should receive 12 weeks of the three-drug regimen, followed by 36 weeks of PEG-IFN/RBV. Stopping rules for discontinuation of the entire regimen include HCV RNA >1000 IU/mL at week 4 or 12, as well as detectable HCV RNA at week 24 [19] (Table 25.2).

Boceprevir (Victrelis™)

Boceprevir is another selective peptidomimetic NS3/4A PI that forms a covalent but reversible enzyme-inhibitor complex. The phase IIb SPRINT-1 trial [20] demonstrated improved viral eradication rates with the addition of boceprevir to PEG-IFN/RBV and also suggested a potential role for a 4-week lead-in period of PEG-IFN/RBV prior to the initiation of boceprevir to achieve optimal SVR rates and to minimize the rates of virologic resistance with the three-drug regimen. The inclusion of a low-dose RBV arm demonstrated not only the importance of including RBV but also the need for initial dosing at previously established levels.

The phase III SPRINT-2 trial studied boceprevir in combination with PEG-IFNα-2b and RBV in 1097 (938 nonblack and 159 black) treatment-naïve genotype 1 HCV patients [21]. All patients received a 4-week lead-in of PEG-IFN/RBV and were then randomized to receive placebo + PEG-IFN/RBV for an additional 44 weeks,

boceprevir 800 mg TID + PEG-IFN/RBV for an additional 44 weeks, or RGT with boceprevir 800 mg TID + PEG-IFN/RBV for an additional 24 weeks, followed by 20 more weeks of PEG-IFN/RBV if serum HCV RNA was detectable at any time during weeks 8–24. All patients with detectable HCV RNA at week 24 were discontinued from the study. Nonblack and black patient cohorts were randomized separately.

In the nonblack cohort, the SVR rate in the control arm was 40% and significantly higher in both boceprevir groups: 68% ($p < .0001$ vs. control) in the boceprevir/PEG-IFN/RBV 48-week treatment group, and 67% ($p < .0001$ vs. control) in the RGT group (Figure 25.1B). The SVR rate in the control arm of the black cohort was lower (23%), but also was significantly improved in the boceprevir groups at 53% ($p = .004$ vs. control) in the boceprevir/PEG-IFN/RBV 48-week treatment group and 42% ($p = .04$ vs. control) in the RGT group. High responsiveness to PEG-IFN/RBV in the nonblack cohort as evidenced by a ≥1 log decline in HCV RNA during the lead-in period was strongly predictive of SVR in all groups as compared to those with <1 log decline (82% vs. 39% in the boceprevir/PEG-IFN/RBV 48-week treatment group, 82% vs. 29% in the RGT group, and 52% vs. 5% in the control group).

The phase III RESPOND-2 trial studied boceprevir-based regimens in treatment-experienced patients [22]. Prior nonresponders in the study were "partial" responders, that is, they had experienced a ≥2 log reduction in HCV RNA by week 12 of prior therapy but had persistent viremia at week 24, or those who relapsed at EOT and thus not did achieve SVR. Control and experimental arms were similar to those of SPRINT-2 except that the period of triple therapy was longer in the RGT group: PEG-IFN/RBV for 48 weeks (control), a 4-week lead-in of PEG-IFN/RBV followed by either boceprevir/PEG-IFN/RBV for 44 weeks or RGT with boceprevir/PEG-IFN/RBV for an additional 32 weeks if serum HCV RNA was undetectable at week 8, or boceprevir/PEG-IFN/RBV for an additional 32 weeks followed by 12 more weeks of PEG-IFN/RBV if serum HCV RNA was detectable at week 8. Patients with detectable HCV RNA at week 12 were discontinued from the study.

SVR rates were significantly higher in both the boceprevir/PEG-IFN/RBV 44-week group (66%) and RGT group (59%) as compared to control (21%) (Figure 25.1C). Prior relapsers had higher SVR rates than prior partial responders in all arms, as expected. Those patients with a ≥1 log decline in HCV RNA at the end of the 4-week lead-in period of PEG-IFN/RBV had the highest SVR rates of 79% when followed by boceprevir/PEG-IFN/RBV for 44 weeks and 73% in the RGT group. Those patients in the boceprevir groups with <1 log decline in HCV RNA during the lead-in period had significantly higher SVR rates than similar patients in the control group (34% and 33%, respectively, vs. 0% in the control group).

Boceprevir is approved for the treatment of genotype 1 CHC in oral tablets at a dose of 800 mg every 8 hours to be taken with food in combination with PEG-IFN/RBV. All patients are to receive a 4-week lead-in of PEG-IFN/RBV followed by the addition of boceprevir in combination with PEG-IFN/RBV. The definition of RVR, which is HCV RNA undetectable 4 weeks after the addition of boceprevir or 8 weeks from the start of treatment, is different from that of telaprevir. Treatment duration is determined by RGT based on the HCV RNA level at treatment weeks (TW) 8, 12, and 24. For treatment-naïve patients with undetectable HCV RNA at TW8 and TW24, three-drug therapy is terminated at TW28. In treatment-naïve patients with detectable HCV RNA at TW8 and undetectable HCV RNA at TW24, the three-drug regimen is completed through TW36 (not TW28, as in the SPRINT-2 trial), followed by PEG-IFN/RBV alone through TW48. In previous partial responders or relapsers with undetectable HCV RNA at TW8 and TW24, the three-drug regimen is continued through TW36. Analogous to slower responders among treatment-naïve patients, those with detectable HCV RNA at TW8 and undetectable HCV RNA at TW24 are treated with the extended course of the three-drug regimen through TW36 followed by PEG-IFN/RBV alone through TW48. Treatment is determined to be futile if HCV RNA is >100 IU/mL at TW12 or detectable at TW24, at which points all three drugs should be discontinued. The week 12 stopping rule in the label is different from that in the phase III clinical trials, where futility was different between treatment-naïve (no futility rule at 12 weeks) and treatment-experienced patients (HCV RNA detectability at 12 weeks). A uniform stopping rule was introduced after the phase III trials based on the observation that patients with HCV RNA ≥100 IU/mL at TW12 are unlikely to achieve an SVR. Package instructions state that RGT was not studied in null responders, and a total of 48 weeks of treatment is recommended for these patients, those with poor IFN responsiveness at week 4, and those with compensated cirrhosis whether treatment naïve or experienced [23] (Table 25.3).

Some important differences are to be considered about RGT and futility between boceprevir and telaprevir

For boceprevir, all therapy should be discontinued if HCV RNA is ≥100 IU/mL at week 12 or ≥10–15 IU/mL at week 24. RGT is recommended for treatment-naïve noncirrhotic patients but not for those with cirrhosis. Based on kinetic modeling and different from the phase III clinical trials, the FDA recommends a boceprevir

Table 25.3 Boceprevir prescribing guidelines.

	HCV RNA*		
	TW8	TW24	Treatment regimen
Treatment-naïve	Undetectable	Undetectable	Complete three-drug regimen at TW28
	Detectable	Undetectable	Continue three-drug regimen through TW36, then continue PEG-IFN/RBV through TW48
Prior partial responders or relapsers	Undetectable	Undetectable	Complete three-drug regimen at TW36
	Detectable	Undetectable	Continue three-drug regimen through TW36, then continue PEG-IFN/RBV through TW48

*All patients receive 4 weeks of PEG-IFN/RBV lead-in. Discontinue all meds if HCV RNA ≥100 IU/ml at TW 12 or if HCV RNA is detectable at TW24. Previous null responders and patients with compensated cirrhosis should receive 4 weeks of PEG-IFN/RBV followed by an additional 44 weeks of PEG-IFN/RBV + boceprevir through week 48. TW: treatment week; PEG-IFN/RBV: pegylated interferon-alpha/ribavirin.

triple therapy for 32 weeks for slow responders not meeting RGT criteria, preceded by the 4-week lead-in and followed by a 12-week PEG-IFN/RBV treatment. For telaprevir, all therapy should be discontinued if HCV RNA is ≥1000 IU/mL at week 4 or 12, and/or ≥10–15 IU/mL at week 24. RGT is recommended in treatment-naïve patients and prior relapsers with an eRVR and without cirrhosis.

Challenges in the management of chronic hepatitis C patients treated with the new standard of care

Challenges related to protease inhibitors

Adverse events and toxicity management

Adverse events are more frequent in patients treated with PIs plus PEG-IFN/RBV than those treated with PEG-IFN/RBV alone. The toxicity of PI-containing triple regimens is considerable and requires early recognition and management.

Adverse events related to telaprevir were mostly rash, anemia, pruritus, nausea, diarrhea, and anorectal complaints, mandating treatment discontinuation in the ADVANCE trial [16] in approximately 10% (in the telaprevir groups) versus 7% in the PR48 group. Rash was reported in 56% of patients receiving telaprevir but was mild to moderate (grade 1/2) in more than 90%, while progression to grade 3 (involving >50% of the body surface area) was reported in 4–6% of patients. Grade 1/2 rash can be treated with emollients, moisturizers, and topical corticosteroids, whereas the prescribing information mandates immediate telaprevir discontinuation for grade 3 rash [24]. Stevens–Johnson syndrome and drug rash with eosinophilia and systemic symptoms were both observed in less than 1% of patients but require immediate discontinuation of all antiviral medication. Telaprevir was associated with anemia in 36% of patients (hemoglobin decrease to below 10 g/dL) with an incremental decline in hemoglobin during the period of dosing (1.0–1.5 g/dL relative to PEG-IFN/RBV alone), and 14% developed severe anemia with hemoglobin below 8.5 g/dL. Diarrhea was found in approximately 32% of patients treated with telaprevir and anorectal complaints in 29% in the telaprevir-treatment arms versus 7% in controls [8].

For boceprevir, SPRINT-2 [21] demonstrated similar therapy discontinuation rates secondary to adverse events across all treatment arms, that is, 16% in the PEG-IFN/RBV group, 16% for the lead-in + boceprevir/PEG-IFN/RBV group, and 12% for RGT. Adverse events related to boceprevir were anemia, neutropenia, nausea, and dysgeusia. Anemia was found in 49% of patients treated with boceprevir despite permission to use erythropoietin during the trial. Severe anemia was reported in 9%. Ribavirin dose reduction secondary to anemia was required more often in patients receiving boceprevir than controls (21% vs. 13%). Treatment discontinuation due to anemia was rare (2% vs. 1%, respectively). RBV dose reduction in the management of anemia did not adversely affect SVR rate compared to those managed with erythropoietin. Dysgeusia is common in boceprevir treatment but rarely drug limiting [8].

Drug–drug interactions (DDIs)

Telaprevir as a potent inhibitor of CYP3A4 is contraindicated in patients concurrently taking medications that are also highly dependent on CYP3A clearance, in which elevated concentrations are associated with serious adverse events. Noteworthy inclusions on this list include but are not limited to lovastatin, simvastatin,

ketoconazole, and alfuzosin. Telaprevir increases the systemic exposure to oral midazolam ninefold, whereas intravenous dosing resulted in only 3.4-fold higher systemic exposure. No data are available for propofol, which is a substrate and inhibitor of CYP3A4. Boceprevir is a potent inhibitor of CYP3A4 and CYP3A5, though its major metabolic pathway is via alpha-ketoreductase. It is therefore contraindicated for concurrent use with medications that are also highly dependent on CYP3A clearance. It is also contraindicated in concurrent use of medications that strongly induce CYP3A to avoid loss of efficacy [8]. Systemic hormonal contraception is allowed as concomitant medication but may not be as effective in women taking boceprevir or telaprevir.

Pharmacokinetic data from healthy volunteers are available on the impact of PIs on plasma levels of calcineurin inhibitors (CNIs), cyclosporine, and tacrolimus. Significantly higher levels of both immunosuppressant agents were found when co-administered with telaprevir. Tacrolimus is boostered by telaprevir to plasma levels 70-fold higher, while cyclosporine was less affected [25].

Antiretroviral therapy (ART) can lead to DDIs with telaprevir and boceprevir. Studies indicate that PI plasma levels are significantly changed by ritonavir-boosted HIV PIs and vice versa. Significantly higher plasma levels were observed for tenofovir (TDF) when given together with telaprevir but not when used in combination with boceprevir [25]. No DDIs were observed for the HIV integrase inhibitor raltegavir and PIs.

The drugs listed as contraindicated or capable of potential interactions for both PIs overlap considerably. Clinicians are advised to consult regularly the list of contraindicated drugs and the more extensive list of drugs with potential interactions, available in the package insert. Helpful information is also available from several Web resources (e.g., www.hep-druginteractions.org).

Resistance to direct antivirals and monitoring

The use of DAAs raises concerns regarding the development of resistant viral variants. Replication of HCV occurs rapidly with approximately 10^{12} new virions produced each day in the typical infected individual and is prone to replication errors, which spontaneously produces resistant variants. Each patient is infected with a viral quasi-species "cloud" composed of genetically distinct but closely related viral genomes. The resistance-associated amino acid variants (RAVs) are usually less fit in terms of replication and/or infectious virus production and therefore present in much smaller quantities than wild-type virus.

In vivo resistance to PIs has been observed in multiple clinical studies. Telaprevir monotherapy selects for RAVs less than one week after starting therapy. Ongoing therapy may select for the emergence of additional resistant variants and/or a viral population increasingly rich in RAVs; however, these often have impaired replicative fitness. Similarly, boceprevir monotherapy leads to the selection of RAVs that show cross-resistance to telaprevir. Concomitant PEG-IFN and RBV administration is clearly important in terms of reducing virologic breakthrough, as demonstrated in PROVE 2 where breakthrough occurred in only 2% of patients treated with triple therapy as opposed to 26% of those treated with PEG-IFN/telaprevir. The PROVE studies demonstrated that while RAVs might emerge with DAA treatment, they do not necessarily impact the risk of virologic breakthrough in the setting of combination therapy with PEG-IFN/RBV. RAVs develop more frequently in genotype 1a patients as opposed to genotype 1b patients, as evidenced by analysis in SPRINT-2 and RESPOND-2 (RAVs present in 48–58% genotype 1a versus 41–48% genotype 1b in patients not achieving SVR, respectively), which likely accounts for the slightly lower SVR rate in genotype 1a patients. Six amino acid positions in the NS3 protease domain are frequently found with RAVs against ketoamide PIs (e.g., V36A/M, T54A/S, V55A, R155K/Q/T, A156S/T/V, and V170A). However, multiple additional low-frequency variants have been identified that might also contribute to PI resistance.

Though not common, RAVs are detectable at baseline in up to 5% of treatment-naïve patients, more commonly in genotype 1a (5.8%) than 1b (1.4%), as genotype 1b has a higher genetic barrier to resistance. The clinical significance of baseline RAVs remains unclear, with early evidence suggesting no significant impact on SVR rates in patients treated with telaprevir combination therapy in PROVE 1 and 2 [13, 14], or with boceprevir combination therapy in SPRINT-2 [21].

RAVs tend to regress over weeks to months following DAA cessation, allowing wild-type virus to re-emerge and leading to undetectable RAVs in over 80% of patients 2 years upon EOT. The time to reversal to wild type is shorter in genotype 1b than genotype 1a HCV. Because of their intrinsic unresponsiveness to interferon and consequent higher chances of treatment failure with PIs, prior null responders appear to be at greatest risk for selecting RAVs and present a population for which "quad regimens" with a PEG-IFN/RBV backbone and two DAAs, or multiple DAAs with a high barrier to resistance, will likely provide significant increments in efficacy. At present, individualized treatment decisions in null responders, weighing the patient's stage of liver disease, prior tolerance of therapy, and individual motivation, are required. Strict adherence to stopping rules is essential, and PI dose reduction is strictly prohibited to minimize the emergence of RAVs and evolution of resistant variants with improved viral fitness due to compensatory second-site mutations.

Challenges related to the PEG-IFN/RBV backbone

In the registration trials for PEG-IFNα-2a and PEG-IFNα-2b plus RBV, almost all patients had adverse events and 10–14% had to discontinue treatment due to adverse events. Depression is a common adverse event; however, DDIs become increasingly complex in patients concurrently using PI-based triple therapy and CYP3A-dependent antidepressants (e.g., citalopram). Decrease in blood counts is common and often requires dose adjustments. However, neutropenia is not associated with an increased incidence of serious bacterial infection in noncirrhotic patients. The use of RBV is complicated by varying degrees of hemolysis in at least one-third of individuals. RBV dose adjustments to 600 mg daily are needed in 9–15% of patients when hemoglobin falls below 10 g/dL or shows a drop of more than 2 g/dL within 4 weeks on therapy and does not appear to impact SVR. RBV cessation is appropriate when hemoglobin goes below 8.5 g/dL. Growth factors to treat therapy-associated anemia, such as erythropoietin or darbepoietin, are costly and have not been shown to improve SVR rates. Preexisting thrombocytopenia, such as autoimmune thrombocytopenia (ITP, or Werlhof disease), can be managed with orally active thrombopoietin-receptor agonists, such as eltrombopag or romiplostin. Eltrombopag allowed successful antiviral therapy with PEG-IFN/RBV in patients with baseline platelet counts between 20 000 and 70 000 mm^3 but can increase the risk of thromboembolism. Routine use of growth factors is not recommended, and dose reduction of PEG-IFN and/or RBV remains the primary action for managing cytopenias [11].

PEG-IFN modifies the immune response and may induce or aggravate autoimmune disorders, such as autoimmune hepatitis, psoriasis, or sarcoidosis. Individuals with active autoimmune disease are not recommended to undergo antiviral therapy with PEG-IFN/RBV, except in situations where simple drug therapy (e.g., thyroid replacement) is all that is required to control the autoimmune disorder. Immunosuppressive agents can cause HCV RNA titers to increase, which may reduce the chance of SVR. The management of CHC in individuals with autoimmune liver disease requires careful assessment of the benefit-to-risk ratio.

RBV is cleared by the kidney and should be used with caution in patients with renal disease or failure, which can exacerbate hemolysis. Patients with CHC and advanced kidney disease (GFR <60 mL/minute) not undergoing hemodialysis can be treated with reduced doses, that is, PEG-IFNα-2a (135 μg/week) or PEG-IFNα-2b (1 μg/kg/week) plus RBV (200–800 mg/day). Dialysis patients should consider standard IFN or low-dose PEG-IFNα-2a (135 μg/week), and RBV at very low doses with careful monitoring for anemia [11].

RBV is a known teratogenic agent, whether it is taken by the male or the female in a partnership. Safe contraception by the patient and his or her sexual partner is absolutely required while under treatment and for 6 months afterward, because of the prolonged RBV half-life. Hormonal contraceptive may be less effective in women receiving PI-based triple therapy.

Any evidence of hepatic decompensation precludes treatment with a PEG-IFN/RBV backbone, except in rare circumstances just prior to liver transplant, and should be carried out at a liver transplant center.

Who should be treated for chronic hepatitis C?

Regardless of the serum alanine aminotransferase level, treatment decisions should be individualized based on the liver disease severity, potential serious treatment side effects, the presence of comorbidities, and the likelihood for SVR upon treatment. Studies on the natural history of HCV indicate that about 55–85% of individuals with acute HCV remain chronically infected. Spontaneous HCV clearance was found more frequently among infants, adolescents, and young women than among elderly people. The risk of developing cirrhosis ranges from 5% to 25% over a period of 25–30 years versus 1–3% in women and particularly children infected at a young age. Progression to cirrhosis is influenced by confounders such as alcohol consumption, immunosuppression, diabetes, hepatic steatosis, iron overload, and HIV–HCV co-infection. The risk of hepatic decompensation and development of hepatocellular carcinoma (HCC) in patients with HCV-related liver cirrhosis is approximately 30% over 10 years or 1–3% per year. Liver biopsies had been the gold standard to assess the degree of liver fibrosis, which can be scored with staging systems (e.g., Ishak or Metavir). Non-invasive techniques, such as transient elastography, will not completely replace liver biopsies but help in reducing its need. Extrahepatic disease manifestations (e.g., mixed cryoglobulinemia or MALT [mucosa-associated lymphoid tissue] lymphoma) are indicators for anti-HCV therapy regardless of the fibrosis stage of the liver. Characteristics of persons for whom therapy is widely accepted are as follows: age 18 years or older; HCV RNA positive; significant fibrosis (≥F2 or higher); bilirubin <1.5 g/dL, INR <1.5, albumin >3.4 g/dL, and platelet count >75 000 mm^3; no evidence of hepatic decompensation, ascites, or encephalopathy; hemoglobin >13 g/dL (male) and >12 g/dL (female), ANC ≥1500/mm^3; and creatinine <1.5 mg/dL [11].

Factors influencing response to antiviral therapy

Pretreatment predictors of response should be considered in choosing the best available strategy, optimiz-

ing and adjusting the pretreatment setting, advising patients on their chances of achieving SVR, and thus enhancing their compliance and adherence to the treatment regimen.

Nonadjustable factors

HCV genotype and viral load

The genotype is the major viral factor that determines the likelihood of achieving SVR following a complete course of antiviral therapy. In general, SVR rates are higher in genotype non-1 infected patients, specifically in genotypes 2 and 3. Studies containing PEG-IFN/RBV indicate that patients most likely to respond to therapy are those infected with genotype 2 and, to a slightly lower degree, those with genotype 3 HCV. The majority of individuals worldwide are infected with less responsive genotypes, namely, genotypes 1 and 4. Boceprevir and telaprevir are approved for only genotype 1 CHC; however, preliminary data demonstrate moderate antiviral activity for telaprevir against genotype 2 and mild activity against genotype 4, whereas no relevant activity was shown against genotype 3 HCV [26]. Antiviral compounds with broad genotype coverage are in drug development. Besides the genotype, the viral load is predictive for treatment response, with <600 000 IU/mL showing higher SVR rates with PEG-IFN/RBV. In immunosuppressed individuals, the viral load can be very high, as observed in HIV–HCV co-infected or post–liver transplant patients. The high viral titer combined with poor tolerance to antiviral therapy for HCV reduce their likelihood of SVR. The baseline viral load plays a less important role for triple therapy with PI.

Host genetics

Genetic markers influence the natural course of an HCV infection as well as its response to therapy and can aid in clinical decision making. SNPs around the *IL28B* gene on chromosome 19 were found to be involved in spontaneous HCV clearance and PEG-IFN/RBV-induced virus eradication. They may also have an influence on liver fibrosis and inflammation in CHC. The *IL28B* gene related to those SNPs codes for interferon-lambda-3 (IFNλ-3). The rs12979860 SNP is recommended as the best genetic marker in predicting response to PEG-IFN/RBV with the CC genotype associated with a twofold increase in SVR on PEG-IFN/RBV in genotype 1 CHC. Genotypes 2 and 3 seem to be less impacted by *IL28B* genotype. The frequency of rs12979860 varies among diverse genetic ancestor groups and partly explains the differences in SVR observed among them, particularly the low SVR in African Americans. Underlying mechanisms are unclear but may involve patterns of IFN-stimulated genes (e.g., IFNγ-inducible-protein 10 [IP10]) in infected hepatocytes. Early studies on the relationship between *IL28B* genotypes and PI-based regimens indicate that in treatment-naïve patients, the largest increment in SVR relative to PEG-IFN/RBV occurs in patients with a T allele at the rs12979860 locus (CT or TT). Patients with the CC genotype have minimal to moderate increments in SVR, but are highly likely to require only 24–28 weeks of therapy. In treatment-experienced patients, in whom the CC genotype is less frequent than in the general population, *IL28B* is less of a differentiator for prediction of response. *IL28B* genotyping is recommended when it is likely to influence decision making to initiate antiviral therapy. There are insufficient data, however, that support genotyping for treatment decisions toward PI-based versus non-PI-based regimens, or to decide upon the duration of therapy. Other host factors, such as inosine triphosphatase genotypes, predict ribavirin-induced anemia but not SVR.

Hepatic fibrosis

The greater the degree of hepatic fibrosis, the lower the response to PEG-IFN/RBV-based antiviral therapies, which is due to an impaired interferon response in the liver.

Patient age

Younger age (<40 years) is a positive predictor of antiviral response for PEG-IFN/RBV.

Adjustable factors

Liver steatosis is associated with lower SVR rates if not related with genotype 3 HCV infection, which showed the same SVR rates irrespective of the presence or absence of steatosis. The mechanisms underlying the poor response to PEG-IFN-based regimens in patients with steatosis are unknown, but are likely related to insulin resistance. The glucose metabolism is directly affected by HCV infection, potentially leading to insulin resistance or diabetes with subsequent acceleration of liver fibrogenesis. However, the administration of insulin sensitizers to PEG-IFN/RBV (e.g., pioglitazone) [27] has failed to show improved SVR rates. In light of the possible DDIs for antidiabetic drugs with PIs in the new SOC, the early management of insulin resistance should focus primarily on lifestyle changes that contribute positively to treatment outcome (e.g., reduction of body weight or alcohol consumption). Another factor frequently found in CHC is excess serum or hepatic iron, which is associated with poor response to PEG-IFN-based therapy. Improved clinical disease courses and higher SVR rates were reported for patients with iron overload following an iron reduction therapy [28].

Vitamin D deficiency is another adjustable factor that was recently shown to lower SVR on PEG-IFN/RBV treatment. Its supplementation was reported to increase the SVR rate in PEG-IFN-based regimens, and vitamin D levels were complementary to *IL28B* in predicting SVR. The exact mechanisms underlying these observations are yet to be defined [29].

The adjustable factor that has the greatest effect on the therapy outcome remains the patient's ability to maintain full-dose or close to full-dose therapy for the prescribed duration. It has been calculated from PEG-IFN/RBV trials that patients who are able to maintain at least 80% of the PEG-IFN dose required and 80% of the RBV dose required have an 80% chance of achieving an EVR, which determines the subsequent likelihood of an SVR. Patients who maintained 80% of their prescribed PEG-IFN dose, but less than 80% of the RBV dose, showed EVR below 60%. EVR was only 70% if 80% of the PEG-IFN dose was not achieved but 80% of RBV was achieved. The worst outcome was observed when less than 80% were achieved for both, PEG-IFN and RBV, with EVR rates of only 30%. Both DAA dosing and strict adherence to DAA-containing regimens are of particular importance to prevent the development of RAVs with subsequent treatment failure. Only limited data are available on that issue, and research on the pharmacokinetics and trough levels of DAAs in real-life settings has to be awaited.

Many factors play a role in facilitating adherence to treatment, such as early and prompt management of adverse events, but also the time of therapy initiation needs to be optimal for the patient (e.g., social events, family planning, and job).

Special patient populations

End-stage liver disease

Patients with CHC-related compensated liver cirrhosis (Child–Pugh A) are eligible for treatment containing a PEG-IFN/RBV backbone, whereas this backbone should be considered with great caution in decompensated liver cirrhosis. SVR rates are lower and the risk of developing RAVs is higher in cirrhotic patients, especially when they are previous null responders. PEG-IFN/RBV treatment in cirrhotic patients can lead to serious adverse events, specifically severe bacterial infections or hepatic decompensation. Hematologic adverse events are more frequent in cirrhotic patients, particularly in those with decompensated disease.

Liver and other solid-organ transplant

End-stage liver disease due to CHC is the major indication for liver transplantation in Europe and North America. HCV recurrence post transplantation is the rule, often with rapidly progressive disease in the grafted liver. Recurrence can be prevented when HCV RNA is undetectable before liver transplant. Thus, with careful supervision and dose modification, some patients can benefit from pretransplant treatment. Only limited data are available on anti-HCV treatment in heart and lung recipients with conflicting data on the short-term outcome. Interferon is successfully used for the treatment of HCV re-infection after liver transplant, with minimal or no increase in rejection. Treatment should be initiated only after demonstration of recurrent liver disease using liver biopsy and should be performed only at tertiary centers. Many DDIs are possible for immunosuppressant agents and PIs in the novel SOC (as discussed in this chapter).

HIV and HCV co-infection

Liver-related mortality in individuals with HIV–HCV co-infection has become more common 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. The rate of liver disease progression in co-infected individuals is almost double that seen with HCV infection alone. The APRICOT trial showed SVR rates of 29% for PEG-IFNα-2a/RBV in genotype 1 CHC, whereas genotypes 2 and 3 showed SVR in about 62% of patients. The recommended treatment for HIV–HCV co-infected is PEG-IFN/RBV for 48 weeks. Interim data for triple therapy with telaprevir in co-infected patients at weeks 4, 12, and 24 were similar to those for HCV mono-infected individuals. Triple therapy of co-infected patients with boceprevir achieved negative HCV RNA at 12 weeks after treatment completion (SVR12) in 61% versus 27% in the PEG-IFN/RBV arm. The use of anti-HCV PIs needs caution and close clinical monitoring because of the potential DDIs with antiretroviral therapy (ART) for HIV infection (as discussed in this chapter). Clinical trials evaluating the safety and efficacy of boceprevir or telaprevir in patients under ART are ongoing. RBV can cause severe anemia in patients with HIV taking zidovudine (AZT). It also inhibits inosine-5-monophosphate dehydrogenase, which potentiates didanosine (ddI) toxicity, with fatal lactic acidosis reported for some patients receiving RBV and ddI concurrently.

African-American patients

The *IL28B* CC genotype is found in substantially lower frequency in patients of African-American ancestry,

which explains in part their poor SVR rates observed in previous PEG-IFN/RBV trials. They are also less likely to clear HCV naturally, potentially contributing to the higher HCV prevalence in that patient population. Genotype 1 CHC in patients with African-American descent should be treated with the new SOC, giving the higher SVR rates observed irrespective of the patient ethnicity. Constitutional baseline neutropenia is common in African Americans but is not related to higher rates of serious infections or adverse events on PEG-IFN. Baseline ANC <1500 mm^3 is not an exclusion criterion for treatment with PEG-IFN, and on-treatment dose reduction for neutropenia needs careful assessment, giving the frequent poor IFN responsiveness in patients with African-American ethnicity. In general, the management of African-American patients should not differ from that of Caucasian patients.

Children and adolescents

Children are shown to have minimal progression of liver disease over 5–20 years. Nevertheless, significant disease can occur. The challenge is to identify appropriate candidates for treatment. Children age 2–17 with CHC should be considered for therapy using the same criteria as in adults. PEG-IFNα-2b (60 µg/m^2/week) plus RBV (15 mg/kg/day) is approved for 24 weeks in genotype 2/3, 48 weeks in genotype 1/4, as well as 48 weeks for genotype 3 with a baseline viral load >600 000 IU/mL with overall 65% SVR [30]. PEG-IFNα-2a (180 µg/1.73 m^2, weekly) plus RBV (15 mg/kg/day) for 48 weeks is approved in genotype 1 CHC, achieving 53% SVR [31]. Boceprevir and telaprevir are not yet recommended in children or adolescents younger than 18 years of age because of unknown safety and efficacy issues.

Ongoing drug users

These patients can vary from the stable patient on a supervised methadone (L-polamidone) or heroin program to the erratic patient who injects from time to time and continues to share needles and syringes with others. Delivery of care to the latter is a considerable challenge and requires substantial resources. Treatment with PEG-IFN/RBV for CHC can be considered even for illicit drug users, as well as for patients on a methadone maintenance program. The new SOC is not approved for illicit drug users and patients on methadone. Plasma levels of methadone are reduced when used together with telaprevir, whereas no data are available for concurrent use of methadone and boceprevir. A prerequisite condition for treatment initiation is that they are willing and able to follow the treatment regimen closely and perform safe contraception. They should receive specialist support by psychiatrists and drug abuse counseling services.

Management of patients with advanced liver disease who fail to achieve SVR on a protease inhibitor–based regimen

Given the preliminary results from trials of quadruple therapy regimens and regimens combining multiple classes of DAAs, there is still potential to achieve viral cure even in patients who fail on a PI-based triple therapy. These patients should be referred to tertiary centers and preferably enrolled into clinical trials.

Novel agents in clinical development

Strategies in current clinical trials focus on the evaluation of different combinations of DAAs and host-targeting agents (HTAs), with or without RBV. Some pharmacological prerequisites are important to combine multiple DAAs, that is, at least additive antiviral efficacy and no cross-resistance, and careful ascertainment of possible DDIs and overlapping safety issues. The INFORM-1 trial provided a proof of principle that a PEG-IFN-free DAA combination therapy in treatment-naïve and -experienced genotype 1 CHC patients can effectively suppress HCV replication [9]. When combining the macrocyclic NS3/4A PI danoprevir with the nucleoside analog NS5B polymerase inhibitor mericitabine (R7128), the strongest antiviral activity was observed in treatment-naïve patients at highest dose levels, and similar responses were also achieved in previous null responders [8, 32] (Figure 25.2).

Other trials assessed the combination of a NS3/4A PI with a nonnucleoside polymerase inhibitor. The SOUND-C1 trial demonstrated potent antiviral activity in treatment-naïve genotype 1 HCV patients, combining BI 201335 with the nonnucleoside polymerase inhibitor BI 207127 and RBV. Higher response rates were observed in genotype 1b compared to genotype 1a with RVR rates for dual DAA plus RBV from 73% to 100% depending on the DAA dosing [33]. The SOUND-C2 trial confirmed the potent antiviral activity of this combination; however, the RBV-sparing arm showed lower response rates than other arms of the trial. In another study, patients were randomized to 4-week dual, triple, or quadruple therapy, combining the PI GS-9256 and the nonnucleoside polymerase inhibitor GS-9190 with or without RBV or PEG-IFN/RBV. The range for RVR was from 100% in quadruple, to 38% in triple, and only 7% in the dual therapy, combining only the two DAAs [8, 32]. The very low response rate in the latter group was related to the frequent occurrence of RAVs, highlighting the important role of PEG-IFN/RBV to prevent the

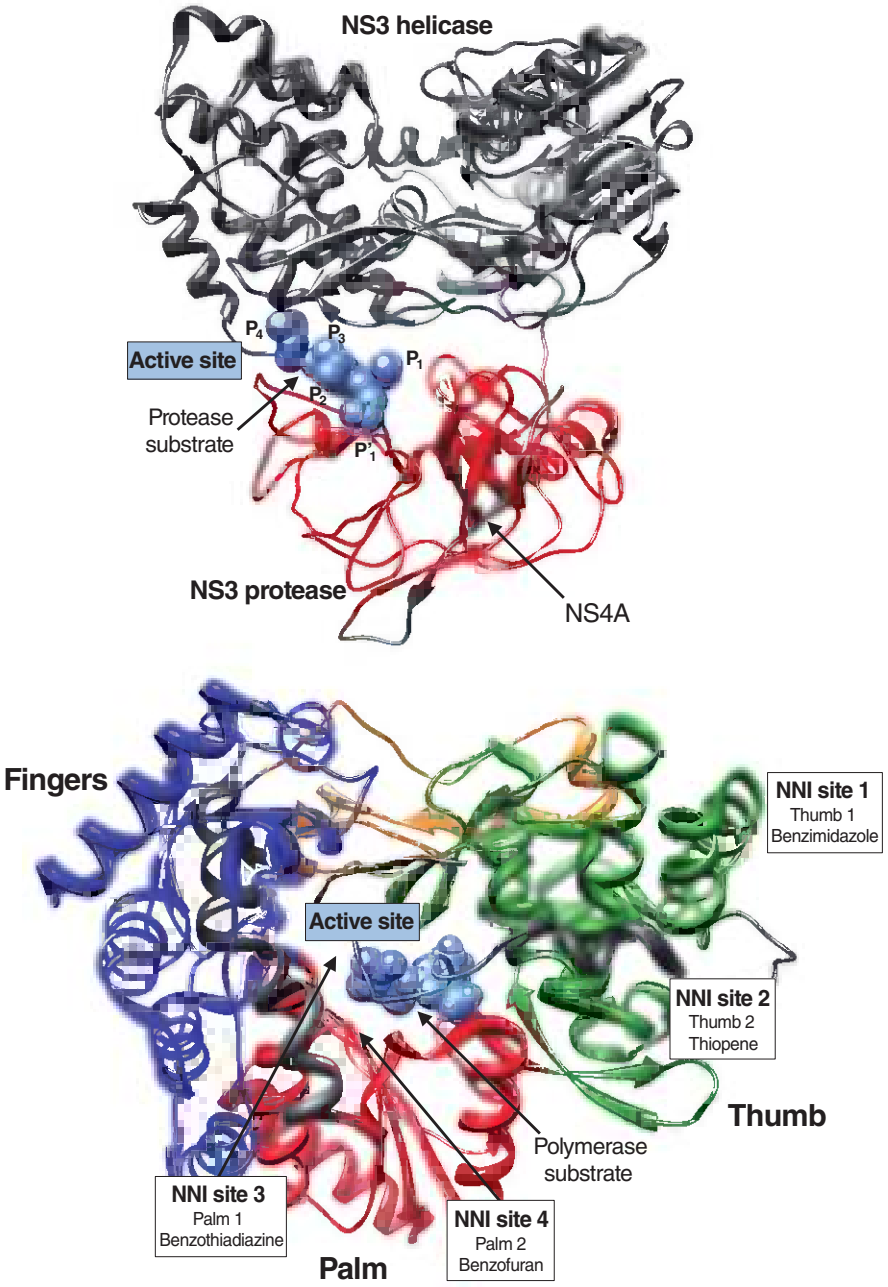

Figure 25.2 NS3 protease-helicase and NS5B polymerase structure and molecular target sites. Top: Protease active site with natural substrate of proteolysis. Bottom: Polymerase palm, thumb, and fingers in different gray with active site bound inhibitor and NNI sites 1 to 4 (Source: Welsch C, Jesudian AB, Zeuzem S *et al.* Gut 2012;61(Suppl 1):136–146 [8]). (Color plate 25.1)

emergence of resistance and virologic breakthrough in DAA-containing regimens. The ZENITH trial assessed antiviral activity of telaprevir and the nonnucleoside polymerase inhibitor VX-222 in dual, as well as with RBV in triple or PEG-IFN/RBV in quadruple, combination for treatment-naïve genotype 1 CHC. The quadruple therapy showed no viral breakthrough and was associated with high antiviral activity. Thirty-eight percent to 50% of the patients were able to undergo only 12 weeks of therapy, with 82–93% achieving SVR12 [8, 32].

In a different approach, genotype 1 CHC, treatment-experienced null responders were randomized to receive an all-oral combination of a NS5A inhibitor and a PI, daclatasvir (BMS-790052) and asunaprevir (BMS-650032) respectively, or a combination of the two DAAs with

PEG-IFN/RBV for 24 weeks. The DAA-alone group showed an overall viral breakthrough rate of 55%, with only two of nine genotype 1a CHC patients but both genotype 1b–infected patients achieving SVR [10]. The quadruple therapy with the PEG-IFN/RBV backbone was highly effective, with all patients achieving SVR12 and 9 of 10 patients achieving SVR24 [10].

Nucleos(t)ide analogs against the highly conserved active site of NS5B have an equal antiviral activity against different genotypes and a high barrier to resistance [8, 32]. Data from the ELECTRON trial in treatment-naïve noncirrhotic adults showed 100% SVR in genotype 2/3 CHC with the nucleotide analog GS-7977 plus RBV for 12 weeks. The first interferon-free regimen containing a NS5A inhibitor, daclatasvir, plus GS-7977 for 24 weeks, achieved >95% SVR in genotype 1/2/3 treatment-naïve patients [34].

The key lesson from data reported in previous trials containing DAAs is as follows – high antiviral efficacy and high barrier to resistance are indispensable requirements for all oral PEG-IFN-free regimens to avoid early treatment failure. Naturally occurring polymorphisms in DAA target structures may be of clinical importance, as some variants may provide *a priori* for escape from DAAs, and second-site compensatory mutations that enhance the fitness of resistant viruses emerging on therapy are likely to occur [35]. We expect complex patterns of RAVs with varying and yet unknown escape mechanisms from DAAs in future antiviral treatment. The lead-in concept with PEG-IFN/RBV was specifically designated to suppress such kind of RAVs before starting on a direct antiviral. However, the majority of studies have shown no effect of a PEG-IFN/RBV lead-in phase in reducing breakthrough or relapse rates, as demonstrated in REALIZE [18], Silen-C1, and Silen-C2 [8, 32].

A way to circumvent genotype-dependent antiviral activity is inhibitors targeting host cell structures relevant for replication of HCV in hepatocytes. These host target agents tend to have a high barrier to resistance. The cyclophilin A-binding molecule alisporivir is among the most advanced, with genotype 1 to 4 coverage. A phase II clinical trial demonstrated the superiority of once-daily alisporivir plus PEG-IFN/RBV in achieving SVR in genotype 1 CHC with 76% SVR compared to 55% in the PEG-IFN/RBV control arm, but this compound is currently on hold due to serious adverse events. Another host target agent is miravirsen (SPC3649), an oligonucleotide miR-122 targeting drug, which is a liver-specific microRNA that HCV requires for RNA replication [36]. A proof-of-concept study in treatment-naïve genotype 1 CHC patients, receiving once-weekly subcutaneous miravirsen injection without PEG-IFN/RBV, demonstrated dose-dependent, prolonged reductions in HCV RNA that continued to fall after the end of treatment with a mean HCV RNA decline from baseline to week 10 of –2.71 $\log_{10}$ [32].

Interferon-sparing regimens and future perspectives

Naturally existing variants are of substantial relevance to the success of DAA-containing regimens [37], but may be of limited clinical significance at present as they are likely to be suppressed by the PEG-IFN/RBV backbone. For all-oral therapy, PEG-IFN/RBV needs to be replaced by another backbone with a low chance of resistance development. If any need for interferon in future CHC treatment remains, PEG-IFNλ may be an interesting drug as it shows similar antiviral activity and an improved safety and tolerability profile compared with PEG-IFNα. Regimens applicable across all HCV genotypes are needed. Most DAAs in phase III trials are restricted for genotype 1 HCV. Several practical issues need to be improved for future-generation antivirals, in particular DDIs, toxicity and overlapping safety issues, dosing, as well as futility rules, which restrict their applicability at present [32].

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Chapter 26
Development of anti-HCV drugs

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Summary

The treatment of chronic hepatitis C virus (HCV) infection has undergone dramatic changes recently. Treatment advances have been enabled by an enhanced understanding of the viral life cycle. Novel anti-HCV drugs fall broadly into two categories: direct acting antivirals (DAAs) and host-targeted antivirals (HTAs). The first of these to enter the clinic are agents that block the HCV NS3/4A protease: telaprevir and boceprevir. Several other classes of DAAs are under development, including inhibitors of the NS5B RNA-dependent RNA polymerase (nucleos(t)ide and nonnucleoside inhibitors) and agents that target the viral NS5A protein. Also under investigation are drugs that block host factors required for the viral life cycle, such as antagonists of cyclophilin A and micro-RNA-122. These new classes of drugs have been identified because of a foundation of knowledge of underlying protein structure and activity, but also by using unbiased discovery approaches, and have resulted in an ever-enlarging armamentarium against HCV.

Introduction

Chronic infection with hepatitis C virus (HCV) is the second most frequent cause of liver-related death across the globe; it is estimated to affect 2% of the world's population, accounting for 180 million individuals. Following exposure to HCV, spontaneous clearance occurs in only approximately 20% of individuals, and chronic HCV infection may progress to cirrhosis, end-stage liver disease, and hepatocellular carcinoma (HCC). In the United States, seroprevalence studies estimated that 3.2 million individuals are currently living with chronic hepatitis C, and as the population ages, hepatitis C–related morbidity and mortality have steadily risen; as of 2007, HCV had surpassed HIV as a cause of mortality in the United States [1]. The number of Americans known to harbor infection will doubtless rise when the recommendation by the Centers for Disease Control (CDC), of the US Department of Health and Human Services (DHHS), to screen all individuals of the "baby boomer" generation is implemented. Thus, highly effective and tolerable treatment of chronic HCV has great public health importance. The goal of treatment is to achieve viral clearance, or sustained virologic response (SVR), which is defined as the absence of HCV RNA 24 weeks after the completion of therapy – this endpoint has been associated with long-term clearance of virus, reduced rates of HCC, and improved all-cause mortality [2].

Therapy for chronic hepatitis C has long been restricted to the combination of two nonspecific antiviral agents: interferon alpha (IFNα) and ribavirin (RBV). For the past decade, pegylated IFNα (PEG-IFNα) supplanted IFNα thanks to an improved pharmacokinetic profile and higher rates of viral clearance, or SVR. However, the rates of SVR in the most common HCV genotype, genotype 1, were only 40–50%, and even lower for those of African-American ethnicity, those with cirrhosis, and those co-infected with HIV [3]. Combined with suboptimal rates of cure, the need for a long duration of treatment (48 weeks for genotype 1), and significant side effects that limit tolerability and adherence, there is an urgent need for a more efficacious and better tolerated treatment.

Antivirals that specifically target the virus were long elusive, in no small part due to the lack of a robust *in vitro* model for HCV. With the development of

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successful cell culture models of HCV [4, 5, 6], our understanding of the viral structure, entry, replication, and host factors required for the viral life cycle expanded rapidly. Further, these *in vitro* models permitted efficient testing of putative antiviral agents. Rapidly on the heels of these tools, a number of new classes of antivirals have been developed. Direct acting antivirals (DAAs) are specifically designed to inhibit viral targets, and host-targeted antivirals (HTAs) inhibit host factors that are necessary for the viral life cycle.

In May 2011, the first two DAAs for the treatment of HCV were approved for the treatment of genotype 1 HCV. These drugs, telaprevir and boceprevir, both targeted the HCV NS3/4A serine protease. Approval for these drugs came on the heels of several landmark phase III studies that demonstrated their efficacy in both treatment-naïve and treatment-experienced patients with chronic HCV, with observed SVR in up to 60–70% of treatment-naïve patients.

The introduction of protease inhibitor (PI)-based triple therapies for HCV it has fundamentally altered our approach to managing chronic HCV: not only has it expanded the population of patients who are eligible for therapy, but it has also permitted shortening of therapy in those persons with favorable responses. Indeed, with higher SVR rates, the population of individuals chronically infected with genotype 1 HCV to whom therapy is offered has greatly expanded, and these patients are being treated with higher expectations of cure. However, there remain several limitations to PI-based triple therapy. First, there remains a continued requirement for PEG-IFNα and RBV because of the rapid selection of resistant variants with HCV PI monotherapy. Patients who cannot tolerate PEG-IFNα, due to psychiatric disease, autoimmune illnesses, or hematologic disorders, for example, are not candidates for therapy. Further, both PIs potentiate the anemia already encountered with the combination of PEG-IFNα and RBV, sometimes profoundly. Other adverse effects, such as rash with telaprevir, can be significant, and patients require close clinical monitoring given the risk of significant anemia and severe rash. Some patient populations, particularly African Americans and patients with advanced fibrosis, have a less robust response. Finally, the risk of breakthrough and resistance requires close monitoring, and "stopping rules" have been implemented to minimize the development of resistance.

Indeed, while the PIs and most novel antivirals in development are potent inhibitors of viral replication, monotherapy with any one agent is nearly certain to result in viral resistance. The HCV virus replicates rapidly, producing 10^{12} virions each day in a chronically infected individual; further, the viral RNA-dependent RNA polymerase (RdRp) is error prone and has an error rate of one nucleotide per genome with every replication cycle. Mathematical modeling has suggested that every possible single and double amino acid substitution is generated each day in chronic infection [7] and that resistance is inevitable. Indeed, analysis of treatment-naïve patients with genotype 1 HCV indicated that 9% of the studied patient population carried drug-resistant mutations [8].

The future of treatment regimens for HCV, therefore, rests in the design of regimens with drugs that are highly potent, have different targets, and have a high barrier to resistance. Given the poor tolerability of PEG-IFNα, all-oral regimens, in particular, are of greatest interest. The first proof-of-concept trial demonstrating that such a regimen was possible was INFORM-1, in which an NS3/4a PI (danoprevir) was combined with a nucleotide NS5B RdRp inhibitor (mericitabine). Sixty-three percent of treatment-naïve patients had undetectable HCV RNA at the end of 2 weeks of therapy, but, more importantly, no viral breakthroughs were observed during the same period [9]. Since then, a large number of all-oral regimens for chronic HCV have undergone or are currently undergoing clinical testing, and SVR data are beginning to emerge. The first proof that SVR could be accomplished without IFN was published in 2012: patients with genotype 1 HCV, who had failed to demonstrate prior virologic response to PEG-IFNα and RBV (so-called null responders), were randomized to receive an NS5A inhibitor, daclatasvir, and an NS3 PI, asunaprevir, with or without PEG-IFNα and RBV. In the all-oral group, 36% of these prior null responders achieved SVR [10].

It is no longer difficult to envision a future in which the vast majority of patients are treated with a combination of oral, targeted medications for a finite duration with extremely high rates of cure. Such all-oral regimens would combine antivirals from different classes, thus attacking different steps in the viral life cycle and limiting cross-resistance between agents. The clinical utilization of these novel antiviral medications will require an understanding of the viral life cycle and the development of these drug targets, which will be reviewed in this chapter.

HCV viral life cycle

HCV, a small, enveloped, single-stranded RNA virus, has been labeled a *hepacivirus*, belonging to the *Flaviviridae* family. The virus circulates in the host as a unique highly lipidated lipoviral particle (LVP), which shares many similarities to very-low-density lipoproteins (VLDLs) [11]. The LVP is composed of a nucleocapsid containing viral RNA and the HCV core protein. This nucleocapsid is surrounded by a lipid envelope that contains the two envelope proteins, E1 and E2; it is rich in cholesterol esters and closely associated with lipopro-

teins. It is thought that its similarity to VLDLs may contribute to its tropism to hepatocytes.

Several critical cellular factors have been found to be required for effective viral entry into the hepatocyte, and these include scavenger receptor class B type 1 (SRB1), CD81, two receptor tyrosine kinases (epidermal growth factor receptor [EGFR] and ephrin receptor A2 [EphA2]), and two tight junction proteins (claudin [CLDN1] and occludin [OCLN]). Most recently, Niemann–Pick-C1-like 1 (NPC1L1), a protein involved in cholesterol absorption, has also been shown to be an important receptor in HCV entry. Interestingly, the role of the LDL receptor in viral attachment and entry remains poorly understood. While the exact sequence of events in viral entry is not completely defined, it is thought that the envelope proteins or apolipoproteins initially interact with heparan sulfate proteoglycans, followed by an interaction between E2 and CD81, which together promote internalization of the virion [12].

Once internalized, HCV uncoats, revealing the nucleocapsid and naked viral genome. The viral genome consists of a positive-sense, single-stranded RNA, 9.6 kb in size, with a single open reading frame. The open reading frame encodes the 10 viral proteins, and is flanked by 5′ and 3′ untranslated regions (UTRs). There are important functional elements in both the 5′ and 3′ UTRs. The 3′ UTR is necessary for effective replication and is thought to direct the synthesis of negative-strand RNA. The 5′ UTR contains a recognition site for the liver-abundant microRNA-122, which supports viral replication. Also in the 5′ UTR is the internal ribosome entry site (IRES), which allows the virus to proceed to translation in a "cap-independent" (the usual 5′ ribosomal recognition site is not required to initiate translation) manner. The IRES recruits the 40s subunit, which is followed by the remainder of the translation initiation complex [13].

The viral genome is translated by host translational machinery into a single precursor polypeptide approximately 3000 amino acids in length. This polypeptide is cleaved by a combination of host and viral peptidases to yield 10 mature viral proteins. The viral proteins include the structural proteins: core, E1, and E2. The HCV core protein, in addition to its structural role in the nucleocapsid, also appears to play an important role in replication and viral assembly. The remaining seven proteins are the nonstructural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (see Table 26.1). During processing of the polyprotein, host proteases cleave the structural proteins, and the viral NS2/NS3 autoprotease cleaves at the junction between NS2 and NS3. The NS3 protein possesses several functional activities, including serine protease, helicase, and NTPase activities. Importantly, it forms a heterodimer with NS4A, which enhances its protease activity. The NS3/4A protease is required for processing the remaining nonstructural proteins, which include NS5A and the NS5B RNA-dependent RNA polymerase. The NS5A protein is a non-enzymatic protein that is essential for both replication and assembly, and exists in two phosphorylation states (basal and hyperphosphorylated) [13, 14].

The nonstructural proteins contribute to the formation of the membrane-associated replication structure, the so-called membranous web. The formation of the membranous web is directed by the NS4B protein, and it is composed of ER-derived membrane vesicles closely abutting lipid droplets. This structure, along with the remaining nonstructural proteins, forms a replication complex, which initially generates a negative-strand RNA intermediate, followed by a positive-strand RNA, which meets one of three possible fates: it is translated into a new viral polypeptide, replicated into a new viral RNA, or perhaps packaged into a new infectious virion.

In addition to viral proteins, several host factors have been demonstrated to have important involvement in HCV viral replication. These include microRNA-122 (miR-122), phosphatidylinositol-4-kinase-IIIα, and cyclophilin A.

Assembly of new HCV virions is thought to involve an interaction between the lipid droplet, core protein, and, potentially, NS5A. It has been proposed that the NS5A may serve a role in bridging replication and assembly, and its state of phosphorylation may dictate its functional role. The small p7 protein is required for assembly and release, though its exact role has yet to be identified. Assembly appears to parallel, and may even subvert, the cellular machinery utilized in the creation of VLDLs; indeed, apolipoprotein B, apolipoprotein E, and the microsomal triglyceride transfer protein have all been implicated in HCV assembly [15]. Newly formed infectious HCV virions are subsequently thought to be secreted in a similar manner to newly formed VLDLs into the bloodstream.

In theory, every viral protein and identified host factor are viable targets for antiviral drug development, but as will be discussed in this chapter, the farthest along are direct -acting antivirals targeting the NS3/4A protease and NS5B polymerase (Figure 26.1).

Direct-acting antivirals

NS3/4A serine PIs

The NS3 protein has several functional domains, including an N-terminal serine protease, which initially is involved in *cis* cleavage at the NS3–NS4A junctions. Subsequently, NS3 forms a heterodimer with NS4A and participates in the cleavage of downstream nonstructural proteins. The structure of the NS3 protease was first delineated in 1996 [16, 17]. Despite knowledge of the structure, the development of effective

Table 26.1 Viral proteins, their function, and drug development.

Viral proteins

	Target	Structural and functional considerations	Function in viral life cycle	Drugs in clinical development?
Structural proteins	Core	3 functional domains: RNA binding domain, hydrophobic domain, and C-terminal domain	RNA binding and core–core interactions, nucleocapsid assembly, association with cytoplasmic lipid droplets, and replication; may play a role in steatosis	None
	E1	Glycosylated type I transmembrane protein	Envelope protein; facilitates entry and also may play a role in viral assembly	None
	E2	Glycosylated type I transmembrane protein	Envelope protein; facilitates entry	None
Nonstructural proteins	p7	Two transmembrane domains linked by cytoplasmic loop	Creates an ion channel (viroporin) across the ER membrane; important for viral assembly and release	None
	NS2	N-terminal membrane anchor domain and C-terminal Zn-dependent cysteine protease	Autoprotease; required for assembly and infectivity	None
	NS3	Serine-type protease, RNA helicase, and NTPase	Viral polyprotein processing (with NS4A), cleavage of proteins of innate immune response (with NS4A), RNA replication, and virion assembly	Yes
	NS4A	Forms heterodimer with NS3	Protease activity (with NS3); may play a role in viral persistence	
	NS4B	Integral membrane protein	Directs formation of the membranous web and interacts with viral RNA during replication	Yes*
	NS5A	3–4 distinct domains; 2 states of phosphorylation	Essential for replication and likely for assembly; may inhibit interferon signaling; function may depend on phosphorylation state	Yes
	NS5B	Right-handed polymerase structure	RNA-dependent RNA polymerase	Yes

*The only inhibitor in clinical trials, clemizole, has not progressed beyond phase Ib testing.
(Source: Schaefer EA, Chung RT. Virology and immunology of hepatitis viruses. [13] inPractice® Hepatology. 2011. Available inPractice.com).

Host targets

Host target	Cellular function	Viral interaction	Drugs in clinical development?
Cyclophilin	Peptidyl-prolyl *cis-trans* isomerase, molecular chaperone	Interacts with NS5A and supports replication	Yes**
MicroRNA-122 (miR-122)	Comprises over 70% of hepatic miRs; exact cellular functions unknown	Binds two sites in 5′ UTR of HCV genome and promotes replication	Yes
HMG-Co-A reductase	Rate-limiting enzyme in mevalonate pathway of cholesterol biosynthesis	Generates geranylgeraniol; host FBL2 protein requires geranyl-geranylation to support replication	No*
Phosphatidylinositol-4-kinase-IIIα	Kinase; converts phosphatidylinositol (PI) to PI 4-phosphate	Supports generation of the membranous web	No

*HMG-co-A reductase inhibitors are FDA approved for the treatment of hyperlipidemia, but doses to inhibit HCV exceed those used clinically – adjunctive use with PEG-IFNα and RBV is being studied. **Currently on clinical hold.

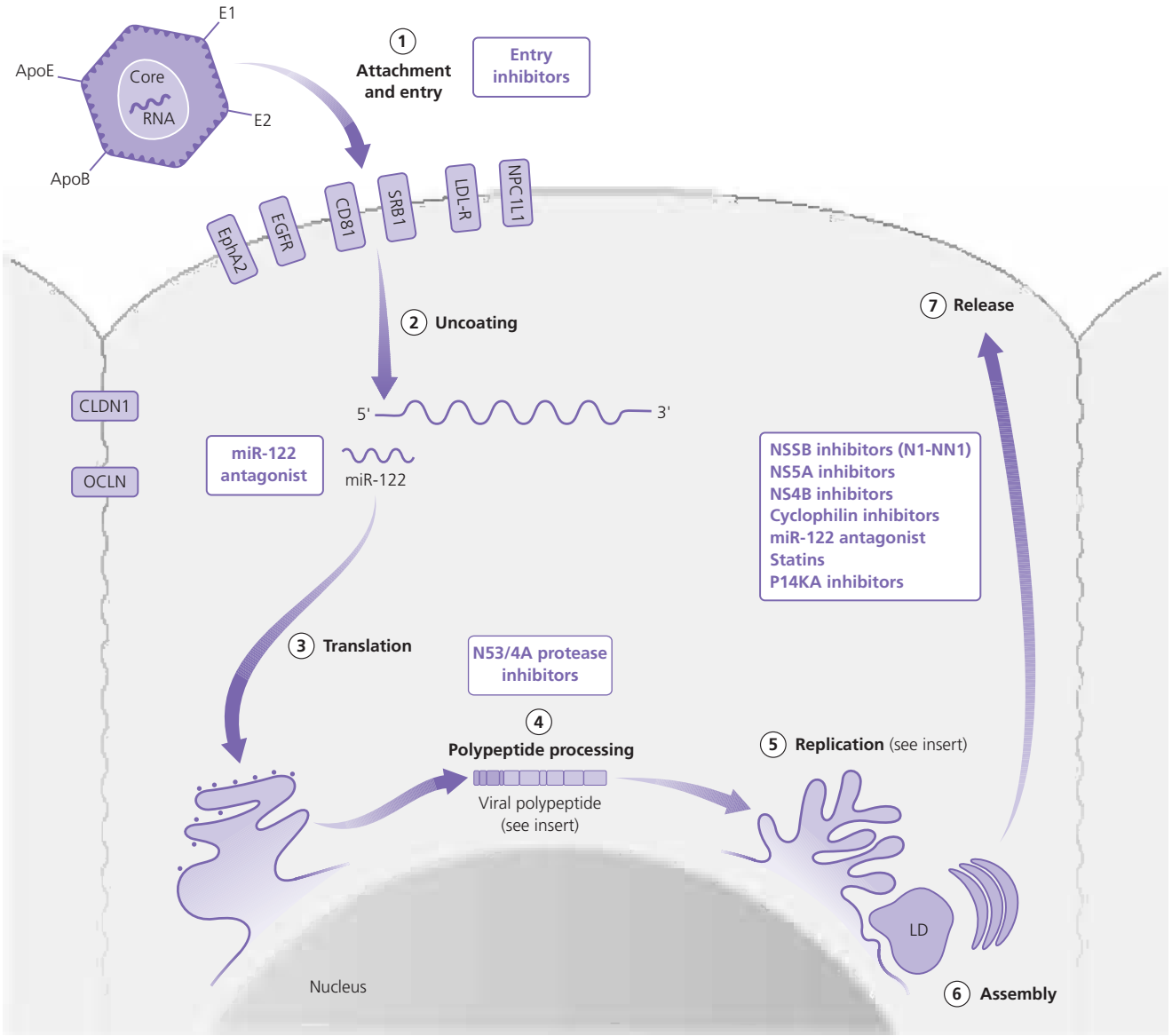

Figure 26.1 HCV viral life cycle and drug targets. The virus circulates as a highly lipidated lipoviral particle (LVP). The first step (1) is attachment and entry into the hepatocyte, which requires a number of host cellular proteins, including CD81, scavenger receptor class B 1 (SRB1), epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), claudin 1 (CLDN1), occludin (OCLN), and Niemann–Pick-C1-Like 1 (NPC1L1). The role of the LDL receptor (LDL-R) remains uncertain. Following entry, the virus uncoats (2), revealing the naked RNA viral genome. The genome interacts with microRNA-122 (miR-122), which is critical to support the viral life cycle. Translation (3) is initiated in a cap-independent fashion, and the genome is translated into a single polypeptide. The polypeptide is subsequently processed by a combination of host and viral proteases (4, insert A). Once the mature proteins are formed, replication (5) takes place on a specialized cellular structure, the membranous web (MW), which closely approximates a lipid droplet (LD). During viral replication, the nonstructural proteins NS4B and NS5B play critical roles. Cyclophilin A (CypA) interacts with the NS5A viral protein and supports replication (insert B). Following replication, the virus is assembled (6) into a new mature virion and released (7).

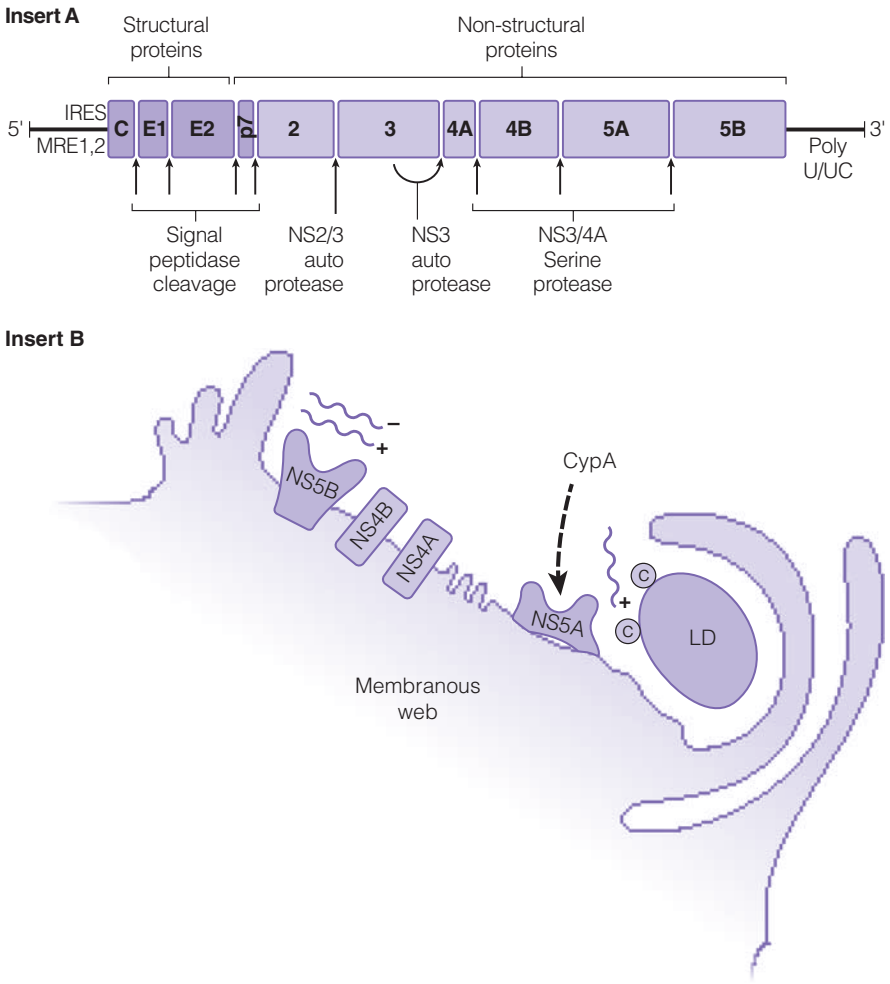

Figure 26.1 (*Continued*)

small-molecule inhibitors was limited by the shallow binding cleft. However, the first proof-of-concept study showing efficacy of a small-molecule inhibitor of NS3 in treating HCV was published in 2003, with the drug ciluprevir [18]. Further development of ciluprevir was subsequently halted due to significant cardiotoxicity observed in nonhuman primate models. However, a number of potent small-molecule inhibitors of NS3/4A have subsequently been developed. NS3/4A PIs can be chemically divided into two classes, the linear, tri- or tetrapeptide α ketoamide derivatives, and the macrocyclic inhibitors (Figure 26.2) [19]. The linear ketoamide derivatives interact at the catalytic site and result in reversible, covalent inhibition. The macrocyclic inhibitors bind noncovalently, and also bind to the catalytic site. All PIs developed to date were initially developed for and tested in genotype 1 infection, but several newer agents have been demonstrated to have activity against additional genotypes.

Telaprevir and boceprevir were the first two PIs to complete phase III testing, and have been approved by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of genotype 1 hepatitis C. The ADVANCE study was a phase III, double-blind randomized trial of telaprevir combined with PEG-IFNα and RBV in genotype 1 treatment-naïve patients [20]. The group receiving 12 weeks of telaprevir, PEG-IFNα, and RBV followed by an additional 12–36 weeks of PEG-IFNα and RBV depending on virologic response at weeks 4 and 12 had a 75% SVR, compared with 44% receiving PEG-IFNα and RBV alone. The REALIZE study was a phase III, double-blind randomized trial of telaprevir combined with PEG-IFNα and RBV in treatment-experienced patients [21], and it found that overall 64% of subjects receiving telaprevir, PEG-IFNα, and RBV for 12 weeks followed by an additional 36 weeks of PEG-IFNα and RBV achieved SVR. When stratified by prior response to therapy, prior relapsers fared best, with 83% achieving SVR, compared to 29% in prior null responders. The major adverse events in the phase III clinical trials were rash, anemia, pruritus, and anorectal complaints.

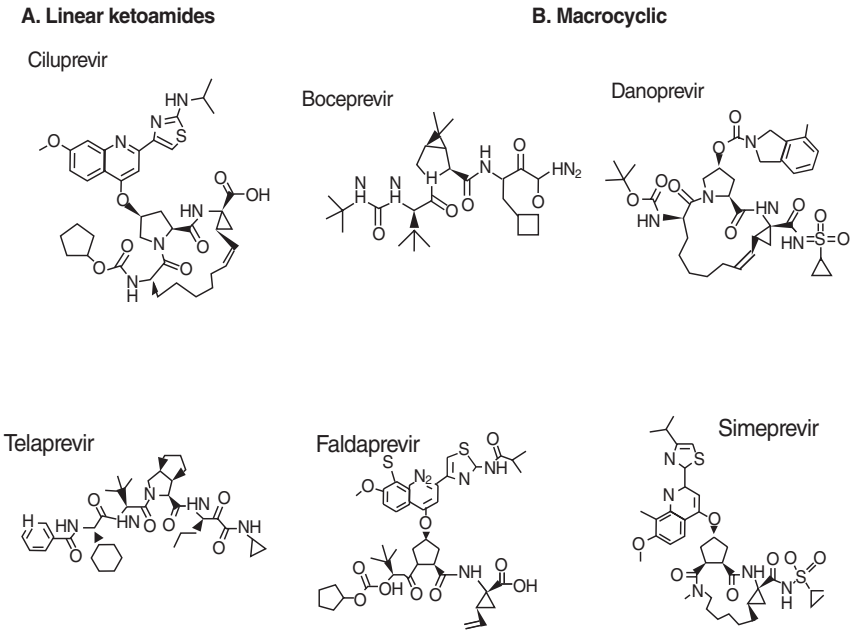

Figure 26.2 Examples of the structure of both linear ketoamide derivatives and macrocyclic inhibitors. (Source: Adapted from Kazmierski WM, Hamatake R, Duan M *et al.* J Med Chem 2012;55:3021–3026 [29]. Copyright (2012) American Chemical Society).

Boceprevir had similar phase III clinical data; one important difference is that the phase III boceprevir trials involved a 4-week "lead-in" period with PEG-IFNα and RBV prior to initiating triple therapy with boceprevir. In nonblack patients, those receiving triple therapy had SVR rates of 67–68%, compared with 38% receiving standard of care. Of note, the black cohort had lower rates of SVR of 42–53% in the groups receiving boceprevir, compared to 23% receiving standard of care [22]. The RESPOND-2 trial used boceprevir in previously treated patients (relapsers and partial responders), and found that boceprevir improved rates of SVR: 59–66% in the groups receiving boceprevir, compared to 21% receiving standard of care [23]. Anemia and dysgeusia were the major adverse events in the boceprevir-treated groups, and nearly one-half of the patients receiving boceprevir required erythropoietin support during therapy.

Both telaprevir and boceprevir require dosing every 8 hours, and telaprevir additionally requires fat intake at the time of dosing. Thus, while highly effective, the inconvenient dosing schedule and side effect profile leave the window open for newer PIs in development. The so-called second wave NS3/4A inhibitors hope to overcome these limitations, and they possess improved pharmokinetic profiles, fewer significant adverse effects, and, potentially, activity across a broader range of genotypes. An additional goal of newer NS3/4A PIs is to preserve activity against HCV variants that are resistant to telaprevir and boceprevir. Numerous second-wave NS3/4A PIs have advanced to phase II/III clinical testing (selected drugs are summarized in Table 26.2) [24].

Simeprevir is a potent macrocyclic inhibitor that has a 40-hour half-life permitting once-daily dosing. Combined with PEG-IFNα and RBV in a phase IIb trial, the reported rates of SVR were 75–85%; in this trial, a surprisingly high percentage of patients, 65%, receiving only PEG-IFNα and RBV also achieved SVR. Simeprevir has also demonstrated efficacy in treatment-experienced patients: in prior null responders, 51% achieved SVR on treatment with Simeprevir combined with PEG-IFNα and RBV, compared to 19% in those receiving PEG-IFNα and RBV alone [25]. The primary adverse effects observed in early clinical testing have been an asymptomatic rise in bilirubin and pruritus. Simeprevir appears to have efficacy against genotypes 1, 2, 5, and 6, but does not appear to have adequate activity against genotype 3.

Faldaprevir is a second-wave linear tripeptide PI. The structure of Faldaprevir was developed from a series of chemical modifications to the structure of ciluprevir, allowing for greater potency and specificity and improved pharmacokinetics [26]. In the SOUND-C1 trial, a phase I trial in which some patients received a combination of Faldaprevir, BI207127 (a nonnucleoside polymerase inhibitor), and ribavirin for 29 days, prior to receiving Faldaprevir plus PEG-IFNα and RBV for the remaining 20 weeks, 75–100% of patients achieved rapid virologic response (RVR) on triple, all-oral therapy

Table 26.2 Selected NS3 protease inhibitors with advanced clinical trial data.

Class	Drug	Dosing	Clinical data*	Adverse effects	Notes
Active site – linear	Telaprevir	750 mg three times daily	SVR: 69–75%	Rash, anemia, and pruritus	Requires fatty food with administration
	Boceprevir	800 mg three times daily	SVR: 63–66%	Anemia and dysgeusia	4 week "lead-in" with PEG-IFNα and RBV; rates of SVR lower in black patients
	Faldaprevir	120 mg once daily	SVR: 73–83%	Nausea, diarrhea, and jaundice	Included in all-oral regimen in SOUND-C trials
Active site – macrocyclic	Simeprevir	150 mg once daily	SVR: 83–84%	Elevated bilirubin, pruritus, and rash	SVR rates over 50% in prior null responders in a phase IIb study
	Danoprevir	100 mg twice daily, combined with low-dose ritonavir	SVR: 70%	ALT elevation and neutropenia	ALT elevation dose related, decreased with ritonavir boosting
Active site – other	Asunaprevir	200 mg twice daily	SVR: 83%**	Fatigue and diarrhea	ALT elevation observed in patients receiving high doses (600 mg BID)

*In treatment-naïve, noncirrhotic patients with genotype 1 HCV, combined with standard-of-care pegylated interferon (PEG-IFN) and ribavirin (RBV), unless otherwise indicated. **Phase IIa data: Subjects received triple therapy with asunaprevir and PEG-IFNα and RBV for a full 48 weeks.
Additional reference: [60].

[27]. Thus, Faldaprevir has already shown promise for interferon-sparing regimens.

Some of the newer PIs, most notably danoprevir, require concomitant administration of ritonavir for boosting of pharmacokinetic profiles. "Boosting" is borrowed from experience in PI treatment in HIV; ritonavir is a potent inhibitor of CYP3A4 at a steady state. Danoprevir in early clinical testing was found to have the adverse effect of aminotransferase elevation when given in high doses. Co-administration of danoprevir with ritonavir appeared to allow for therapeutic levels of danoprevir with less frequent dosing and less drug exposure, which appeared to limit hepatotoxicity [24]. However, the addition of ritonavir as an additional drug to what are likely to be complex regimens may limit adherence and tolerability.

Despite the enhanced potency of second-wave PIs, the NS3-4A inhibitors, as a class, have a relatively low barrier to resistance, and if PIs are administered as monotherapy, resistance will develop rapidly. While the term "second-wave" PIs has been used to denote protease inhibitors developed after telaprevir and boceprevir with improved pharmacokinetics and/or tolerability, "second-generation" PIs specifically have the important distinction of retaining antiviral activity against viral variants that have developed resistance mutations. Resistance occurs primarily due to amino acid changes in the NS3 protease catalytic site, leading to impaired binding of the small-molecule inhibitors, although there are additional resistance mutations associated with linear ketoamide inhibitors that reside outside the catalytic site. Importantly, two mutations appear to confer cross-resistance between linear ketoamide inhibitors and macrocyclic inhibitors, R155K/T and A156T, both of which impair binding at the catalytic site. The R155K substitution occurs more readily in HCV genotype 1a compared to 1b, because two nucleotide mutations are required for the amino acid change in genotype 1b but only one is required in genotype 1a [28]. This is thought to account at least in part for higher rates of SVR observed in genotype 1b when compared with 1a in clinical trials of PIs. Protease inhibitors that retain potent antiviral effects against these mutants are considered second generation (as compared with a second wave of first-generation inhibitors with more favorable pharmacokinetics and toxicities), and novel structures remain under active investigation [29].

NS5B RNA-dependent RNA polymerase inhibitors

The NS5B RNA-dependent RNA polymerase (RdRp) is a nonstructural protein that catalyzes the synthesis of new RNA viral strands during replication. The NS5B RdRp has a three-dimensional right-handed structure with a catalytic or active site surrounded by palm, finger, and thumb domains, all of which are targets for DAAs. There are two broad classes of HCV polymerase inhibitors: nucleos(t)ide inhibitors (NIs) and nonnucleoside inhibitors (NNIs).

Nucleos(t)ide inhibitors

NIs directly interact with and block the active site of the RdRp and cause termination of the elongating RNA

strand. The distinction between nucleoside and nucleotide inhibitors is subtle but important. Nucleoside inhibitors are analogs of natural nucleosides and, like the natural substrate, require phosphorylation by host kinases into nucleotide triphosphates before being incorporated into the viral genome. If the molecule is not recognized and phosphorylated by host kinases, it may be weakly phosphorylated and ineffective as a substrate. By contrast, nucleotide inhibitors are already phosphorylated and thus bypass host phosphorylation steps, particularly the first, rate-limiting phosphorylation step. The challenge with nucleotide inhibitors is achieving adequate concentrations in the liver; this has largely been overcome by the development of pro-drugs, which are effectively incorporated into the elongating viral genome at the active site, leading to chain termination [30]. The most attractive aspect of this class of compounds is its very high barrier to the development of resistance, making it unique among DAA classes. This feature is attributable to the inability of the highly conserved active site to tolerate mutation without incurring a major cost in replicative fitness.

Mericitabine is a nucleoside inhibitor pro-drug, which has efficacy against genotypes 1, 2, and 3 and has shown promise in a phase II trial, with an observed rate of SVR of close to 60% in patients with genotype 1 or 4 infection when combined with PEG-IFNα and RBV [31]. Sofosbuvir, formerly GS-7977, is a nucleotide pro-drug that possesses high activity against HCV both *in vitro* and *in vivo*. Combined with PEG-IFNα and RBV, Sofosbuvir has an SVR at 12 weeks (SVR12) of 88–91% in genotype 1 infection [32]. It also has shown immense promise as an agent for IFN-sparing regimens in genotype 2 or 3 infection. A study of patients with chronic genotype 2 or 3 HCV demonstrated that 12 weeks of Sofosbuvir combined only with ribavirin resulted in SVR12 in 100% of patients. These data suggest that nucleoside inhibitors may be highly effective components of interferon-free regimens [24].

The active site of the RdRp is highly conserved among genotypes, and mutations at the active site come at a significant cost to replicative fitness. Thus, few resistance variants have been identified *in vitro*, and none yet *in vivo*. The best characterized resistance mutation is an active site mutation, S282T, which reduces the affinity of the RdRp for the inhibitors, particularly in genotype 1a and 1b viral strains [33, 34]. Thus, nucleos(t)ide inhibitors are extremely promising as a class of DAAs with both high potency and a high barrier to resistance.

Nonnucleoside inhibitors

Nonnucleoside inhibitors (NNIs) bind NS5B away from the active site, at one of at least four allosteric sites. Binding at the allosteric sites impairs the enzymatic activity of the RdRp by causing conformational changes that prevent the initiation or elongation of the RNA strand. The four characterized allosteric sites are the "thumb 1" (benzimidazole) site, the "thumb 2" (thiophene and triazolpyridine) site, the "palm 1" (benzothiadiazene) site, and the "palm 2" (benzofuran) site [35, 36].

There are many more NNIs in advanced clinical development than NIs (see Table 26.3); however, NNIs have several limitations. First, the amino acid sequences at drug-binding sites are not conserved across viral genotypes, suggesting limited cross-genotype activity. Further, mutations do not necessarily come at a significant cost of viral fitness. Additionally, the nature of their interaction may lead to less potent inhibition of viral replication than the nucleoside inhibitors. Early clinical testing may reflect these limitations: for example, in phase IIb trials, filibuvir (thumb 2), tegobuvir (palm 2), ABT-333 (palm 1), and ABT-072 have SVR rates of 50–60% in genotype 1 infections, and filibuvir was associated with high rates of relapse [24, 37]. However, the potential contribution of these agents to all-oral regimens was demonstrated in a trial combining ABT-333 with ABT-450 (an NS3/4A PI) plus ritonavir and ribavirin, in which 90% of treatment-naïve patients with genotype 1 infection achieved SVR [38]. While NNIs will likely be important components in future oral anti-HCV regimens, they are limited by relatively low potency and low barrier to resistance.

NS5A inhibitors

The NS5A protein has several functions in the life cycle of HCV. It is critical for replication, is thought to bridge the steps of replication and assembly, and may additionally subvert interferon signaling and dampen the innate immune response. The protein has three functional domains: domain I, which is involved in membrane attachment and RNA bridging; domain II, which is thought to interact with cyclophilin A; and domain III, which is involved in viral assembly. As a non-enzymatic nonstructural protein, the discovery of the first-in-class NS5A inhibitors came by way of unbiased high-throughput small-molecule screening for compounds that inhibited HCV replication, using a chemical genetics approach and excluding molecules predicted to block the NS3/4a protease or NS5B RdRp [39]. From the screen emerged a compound that had weak, but highly specific, inhibition of HCV (BMS 858), and resistance to this compound was mapped to an amino acid residue on NS5A. The compound was subsequently chemically modified to a highly potent, highly specific inhibitor of HCV, daclatasvir (BMS 790052).

Daclatasvir was the first NS5A inhibitor to enter clinical testing. The compound appears to complement domain I of NS5A, and *in vitro* data suggest that it has

Table 26.3 Selected NS5B inhibitors in advanced clinical testing.

Class (site)		Drug	Dosing	Clinical data*	Adverse effects	Notes
Nucleos(t)ide inhibitors	Active site	Mericitabine	1000 mg twice daily	SVR: 59%	Well tolerated in phase IIb testing	
		Sofosbuvir	400 mg once daily	SVR12**: 91–98%	Anemia and neutropenia	In the ELECTRON study, 100% SVR in genotypes 2 and 3 with RBV only
Nonnucleoside inhibitors	Palm 1	ABT-333	Twice daily	SVR: 63%	Headache and nausea	
		ABT-072	Once daily	SVR: 52%	Headache and nausea	Combined with ABT-450 (PI) and RBV; 90% SVR observed
	Palm 2	Tegobuvir	30 mg daily	SVR: 56%	Headache and nausea	
	Thumb 1	BI 207127	Three times daily	SVR: 59–68%	Photosensitivity and rash	IFN-free regimen, combined with BI 201335 and RBV; higher response in genotype 1b
	Thumb 2	VX-222	Once daily	SVR12**: 83–90%	Headache, nausea, and diarrhea	Combined with telaprevir plus PEG-IFNα and RBV
		Filibuvir	Twice daily	SVR12**: 30–50%	Fatigue, nausea, and headache	High relapse rates observed

*In treatment-naïve patients with genotype 1 HCV, combined with standard of care pegylated interferon (PEG-IFN) and ribavirin (RBV), unless otherwise indicated. Duration of therapy varied based on trial stage and design and on virologic response. **SVR12: Undetectable HCV RNA at 12 weeks following completion of therapy.

efficacy against all six major viral genotypes. Like other DAAs, monotherapy with daclatasvir resulted in the emergence of drug-resistant variants, and similar to what has been observed with PIs, genotype 1a appears more prone to developing resistance than genotype 1b [24]. In a small, phase IIa, open label study, patients with genotype 1 chronic HCV (all 10 enrolled patients has genotype 1b) and a history of prior null response to PEG-IFNα and RBV were given dual therapy with daclatasvir plus asunaprevir (an NS3/4a PI). After 24 weeks of dual therapy, nine of the 10 patients enrolled achieved SVR12, but, as mentioned, all patients were infected with genotype 1b virus, which appears to be more responsive to combination DAA treatment than is genotype 1a [40]. Additional NS5A inhibitors are undergoing advanced clinical testing, including ABT 267 and GS 5885. As a class, NS5A inhibitors hold great promise in the treatment of HCV.

Other viral targets

While the NS3/4A PIs, NS5B inhibitors, and NS5A inhibitors are the furthest along in clinical development, other steps in the viral life cycle are being investigated as targets for DAA therapy.

NS4B, for example, is necessary for the formation of the membranous web, the ER-derived structure that contributes to the replication complex; it additionally has both nucleotide binding and NTPase activity, and is therefore thought to play an important role in the generation of positive-strand RNA [12]. The identification of a small-molecule inhibitor for NS4B utilized an unbiased approach, a small-molecule screen. First, the nature of the NS4B–RNA interaction was characterized, and then a library of compounds was tested for their ability to disrupt this interaction and to block HCV replication. Clemizole, an H1 histamine receptor antagonist not presently in clinical use, was validated as a compound that inhibited HCV replication by way of blocking NS4B–RNA interaction [41].

Viral entry is another potential target, and it has generated particular interest for its potential utility in the posttransplant setting – where blocking *de novo* infection of the newly transplanted liver would have tremendous clinical utility. As discussed in this chapter, there are numerous known mediators of HCV entry, which include the viral envelope glycoproteins E1 and E2 and host cellular proteins CD81, SRB1, EGFR, EphA2, CLDN1, OCLN, and NPC1L1. While neutralizing antibodies generated against the envelope glycoproteins may effectively disrupt the E2–CD81 interaction, no *in vivo* efficacy has yet been demonstrated [12]. ITX 5061 is a small-molecule inhibitor of SRB1, which has been shown to have *in vitro* antiviral effect against HCV, with synergistic effects when combined with IFNα and an NS3/4A PI [42]. While it retained potency in the setting

of resistance NS3/4A mutations, substitutions in E2 conferred high levels of resistance to ITX 5061. Phase I clinical testing of ITX 5061 is being pursued. Of interest, inhibitors of EGFR and NPC1L1, erlotinib (Tarceva) and ezetimibe (Zetia), are already FDA approved for other clinical indications. Ezetimibe is used in the treatment of hyperlipidemia, and erlotinib is presently used for the treatment of non-small-cell lung cancer (NSCLC). However, given its side effect profile, erlotinib may not be suitable for use in chronic liver disease.

Host-targeted antivirals (HTAs)

Cyclophilin inhibitors

Developing drugs against the host factors required for replication is an appealing strategy, as, at least in theory, such agents would possess a higher barrier to resistance than DAAs and would likely have pangenotypic effects, even if potency is not as robust as in some of the DAA classes. Cyclophilins were identified as potential therapeutic targets for HCV even prior to the characterization of HCV beyond non-A, non-B hepatitis virus. In 1988, it was observed that cyclosporine, an inhibitor of both cyclophilins and calcineurin (which confers the immunosuppressive effect for which it is commonly used), inhibited replication of non-A, non-B hepatitis. Due to its immunosuppressive effects, however, cyclosporine was not pursued as an antiviral therapy. Since that time, it has been clarified that cyclophilin A (cypA) appears to interact directly with HCV NS5A, and this interaction is important for viral replication. Thus, specific inhibitors that inhibit cypA, but not calcineurin, have been sought as drugs that may inhibit HCV without the attendant immunosuppression of calcineurin inhibition; these include alisporivir and SCY-635.

In phase II clinical trials, alisporivir has demonstrated antiviral efficacy against genotypes 1, 2, and 3. For the treatment of genotype 1 infection, alisporivir was tested in a phase IIb trial comparing PEG-IFNα and RBV to triple therapy with PEG-IFNα, RBV, and alisporivir. In this trial, 48 weeks of triple therapy resulted in an SVR rate of 76% compared with 55% in those receiving PEG and RBV. Interestingly, although resistant HCV variants did emerge, their appearance did not correspond to viral breakthrough [24]. Alisporivir has also been studied as a component of interferon-sparing regimens for the treatment of genotype 2 and 3 HCV infection. An open-label, phase IIb trial compared high-dose alisporivir alone, varying doses of alisporivir with RBV, alisporivir with PEG-IFNα, and PEG-IFNα plus RBV in treatment-naïve patients with genotype 2 or 3 infection. The trial found that SVR12 was achieved in 81% of those receiving high-dose monotherapy and 81–83% of those receiving alisporivir plus ribavirin [43]. The study highlights the promise of alisporivir in the treatment of HCV; however, concerns have emerged about the possibility of severe pancreatitis associated with alisporivir, including one death, and further development was placed on clinical hold by the FDA in April 2012.

SCY-635 is another CypA inhibitor also in phase II clinical testing. Phase I data suggest potent antiviral activity and hint at an additional antiviral mechanism, where cyclophilin inhibition may restore the host innate immune response. Individuals treated with the highest tested dose of SCY 635 (900 mg) showed restored markers of the IFNα-signaling pathway with therapy, and interleukin 28B (IL28B), a predictor of response to IFN-based therapy, also predicted response to SCY-635 [44]. These early clinical data of both alisporivir and SCY-635 support the concept that host-targeted antivirals may be valuable components in anti-HCV regimens, with strong potency, pleiotropic effect and high barriers to resistance.

miR-122 antagonists

MicroRNAs are small, noncoding, 22–23 nucleotide RNAs that act posttranscriptionally to regulate, and generally repress, gene expression by means of lowering target mRNA levels or by impairing translation [45]. MicroRNA-122 (miR-122) has been identified as a "liver-specific" microRNA, being found in abundant quantities in the liver, accounting for over 70% of all hepatic microRNAs, and not having significant expression in other tissues [46]. Interestingly, miR-122 has been found to be a host factor critical to HCV replication: unlike the classical association between microRNAs and their target RNA, miR-122 enhances cellular HCV RNA expression. An interaction between miR-122 and two seed sequences along the 5′ UTR of the viral genome appears to be principally responsible for this effect, although the exact mechanism has yet to be clearly identified. The microRNA recognition elements (MREs) are highly conserved across HCV genotypes. Inhibition of miR-122 poses an entirely novel paradigm for the treatment of HCV, whereby the host RNA environment is altered to block HCV.

Potent, durable, and specific inhibition of miR-122 was facilitated by a short, locked nucleic acid antagonist (LNA) that effectively sequesters miR-122 and blocks its interaction with host targets [47]. An LNA–anti-miR-122 has been demonstrated to be highly effective in suppressing HCV RNA in chimpanzees with durable antiviral effect [48]. This LNA–antagomir, miravirsen, has undergone phase II clinical testing, and these data have shown that once-weekly, subcutaneous administration of miravirsen (at doses of 5–7 mg/kg) results in significant sustained reductions in HCV viral load [24]. No

miravirsen-specific mutations were identified after 5 weeks of therapy and 18 weeks of follow-up, supporting the concept that this host-targeted antiviral indeed possesses a high barrier to resistance. However, further clinical data, and the evaluation of potential adverse effects from miR-122 sequestration, are still pending.

Other host targets: HMG Co-A reductase inhibitors and phosphatidylinositol-4-kinase (PI4KA)

The HCV life cycle is closely intertwined with host lipid metabolism, and the virus is thought to utilize products from the mevalonate pathway of cholesterol synthesis, the Bloch pathway of cholesterol synthesis, and fatty acids. Best characterized is the relationship between HMG-Co-A-reductase (HMGCR) and HCV: a downstream product of the mevalonate pathway, geranylgeraniol, is required for the interaction between host protein FBL2 and NS5A, and blocking HMGCR results in impaired viral replication [49]. Statins are HMG-Co-A reductase inhibitors in widespread clinical use for the treatment of hyperlipidemia and coronary artery disease. However, the doses required to inhibit HCV *in vitro* are far higher than the doses used in the clinical setting. Thus, while statins themselves do not appear to be a potent or promising class of HTAs, retrospective analyses have suggested that patients receiving PEG-IFNα and RBV for the treatment of genotype 1 infection had higher rates of SVR if they were concomitantly taking a statin [50].

Additional host targets have been actively sought, and high-throughput screening of host factors required for HCV replication have been a highly utilized tool to attempt to identify novel targets. Phosphatidylinositol-4-kinase (PI4KA) has been identified as a host dependency factor in several screens [51–53] and appears to be required for the arrangement and integrity of the membranous web [54]. A class of medications, the 4-anilino quinazolines, had been identified as inhibitors of HCV replication, and were initially thought, based on structural components, to work by perhaps blocking NS5A [55]. However, a recent analysis suggests that these may in fact function by interfering with PI4KA function, and members of this class may be targeted for further development as HCV antivirals.

Alternative interferons

Interferons are known to be critical effectors of the innate immune response, and type I interferons α and β have long been appreciated as important antiviral cytokines. The utilization of IFNα in HCV is based on its potent, but nonspecific, antiviral effect. IFNα functions by binding its receptor, the dimeric IFNα receptor (IFNαR), which leads to downstream activation of the JAK/STAT intracellular signaling pathway, and eventually increased transcription of genes involved in the antiviral response.

Type III interferons were first described in 2003, and they comprise three cytokines: IFNλ-1 (IL-29), IFNλ-2 (IL-28A), and IFNλ-3 (IL-28B). Like type I interferons, the type III interferons have been shown to have antiviral activity. Interestingly, the structure of IFNλ3 is much more similar to that of a cytokine (IL-10) than to type I interferons. The cellular receptor of IFNλ3 is a heterodimer composed of one IL10 receptor (IL10R) and one IFNλ receptor (IFNλR1). However, the signaling pathway of IFNλ3 and all type III interferons converges with type I interferon signaling [56] (Figure 26.3).

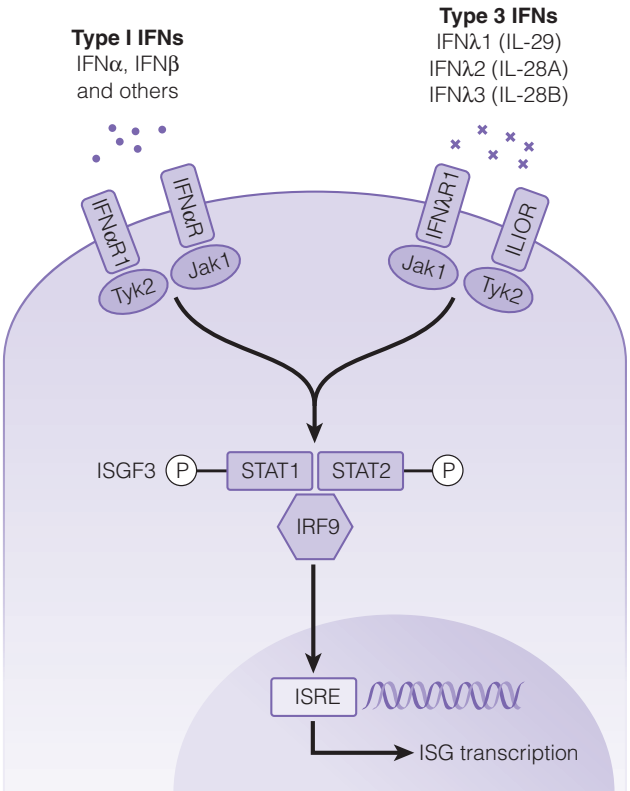

Figure 26.3 Type I and type II interferon signaling. Type III interferons (IFNλ1, IFNλ2, and IFNλ3) more closely resemble a small cytokine (IL10) than an interferon; however, they exert an antiviral effect mediated by the same signaling pathway as classic, type I IFNs. After receptor binding to a heterodimeric receptor of IL10 receptor and IFN-λ receptor 1, jak and tyk2 are activated and phosphorylate STAT1 and STAT 2, which in turn combine with IRF9 to create the heterotrimeric complex, the interferon-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus and activates transcription of interferon-stimulated genes (ISGs) by way of the interferon response element (IRE).

In a landmark genomewide association study (GWAS) in 2009, a series of polymorphisms surrounding the *IL28B* gene were identified as strong predictors of response to treatment of HCV with PEG-IFN and RBV [57], and several additional GWAS identified additional loci around *IL28B* shortly thereafter. While none of the loci were within the coding region of *IL28B*, these findings reinvigorated interest in the possible role of IFNλ in the treatment of HCV. Type III interferons are attractive therapeutic targets largely due to their tissue specificity – IFNλ1 receptors are expressed at high levels in hepatocytes, but not across all tissues [58]. Thus, IFNλ1 might exert a tissue-specific antiviral effect while sparing the significant systemic adverse effects observed with IFNα.

PEG-IFNλ1 has undergone clinical testing in the treatment of genotype 1 HCV. A phase IIb study compared escalating doses of PEG-IFNλ1 combined with ribavirin to PEG-IFNα plus ribavirin in treatment-naïve patients with genotype 2 or 3 infection. In this study, rates of SVR were similar between PEG-IFNλ1 and PEG-IFNα groups (57–83% compared to 40–67%, respectively). However, there were fewer adverse effects seen in the group receiving PEG-IFNλ1: for example, no dose reduction of RBV for anemia was required [59]. There was, however, no difference observed in neuropsychiatric side effects. While interferon-sparing regimens are widely regarded as the future standard of care for chronic HCV, there will certainly be subsets of patients who prove challenging to treat, and in whom resistance may be a significant issue. For these difficult-to-treat patients, interferon therapy may continue to play an important role, and PEG-IFNλ1 could be a better tolerated and highly effective interferon in such regimens.

Conclusion

Following the development of robust *in vitro* models of HCV, the field of drugs that are being developed to treat HCV has expanded at a dizzying pace. Strategies to identify inhibitors of HCV included targeted, chemical development based on known enzymatic structures and functions, and, in some cases, borrowed from prior experience with HIV. However, novel unbiased techniques, particularly high-throughput screening, have also been utilized to identify (1) small molecules that block the virus and (2) potentially targetable host factors that support viral infection. This has resulted in a large armamentarium of emerging weapons to combat HCV, which inhibit both viral targets (in the case of DAAs) and host proteins (host-targeted antivirals) and which selectively boost the host's innate immune response (alternate interferons). Thus far in clinical testing, different classes of drugs have demonstrated variable strengths. The PIs have high potency, but are limited by relative genotype specificity and a low barrier to resistance. The NS5B nucleos(t)ide inhibitors have great promise for pangenotypic activity and a high barrier to resistance. Nonnucleoside inhibitors have modest potency, limited genotypic coverage, and a very low barrier to resistance. NS5A inhibitors have high potency, increasing genotypic coverage, and a moderate barrier to resistance. Finally, HTAs, as a class, are expected to have a high barrier to resistance but may be less potent and must overcome host cytotoxicity concerns.

In the coming years, treatment regimens for chronic HCV will combine agents from these classes, and an understanding of the underlying biology and differences between classes will be critical for treating physicians. The fundamental tenets of designing effective regimens will be to combine agents with high potency with those possessing high barriers to resistance or, at a minimum, different resistance patterns. Further investigations are still required in special patient populations, particularly those who have undergone liver transplantation and those with HIV co-infection. However, it remains highly likely that a reality of interferon-free, oral, well-tolerated regimens of short duration will materialize for all but the most challenging treatment groups.

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Section V
Hepatitis D Virus

Chapter 27
Structure and molecular virology

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Summary

The hepatitis D virus (HDV) is a defective RNA infectious agent needing the presence of the hepatitis B virus (HBV) to complete its life cycle. HDV is present worldwide, but the distribution pattern is not uniform. Different viral strains are classified into eight genotypes found in specific geographical areas and often associated with severe disease outcome. The HDV particle is composed of an envelope, provided by the helper HBV, surrounding the 1.7 kB RNA genome and the HDV antigen. Replication occurs in the hepatocyte nucleus via a rolling-circle mechanism that leads to a full-length, complementary RNA as a replication intermediate. Since HDV does not possess the encoding capability for a RNA polymerase, replication is brought about by cellular polymerases.

Introduction

The HDV discovery dates back to 1977, when Rizzetto and coworkers reported the detection of a novel nuclear antigen – initially dubbed the delta antigen – in the hepatocytes of patients with a severe form of chronic hepatitis B [1,2]. Later experiments in chimpanzees showed that the hepatitis delta (or D) antigen (HDAg) was a structural component of a transmissible virus requiring the simultaneous presence of HBV to complete its life cycle. This premise is necessary to understand fully the unusual structure of HDV particles, which are composed of HBV envelope proteins surrounding a ribonucleoprotein inner structure, comprising the HDAg and the HDV RNA genome [3]. Initially known as "delta agent" or hepatitis "delta" virus, currently the term "hepatitis D virus" is preferred, even if "delta" is still frequently used.

HDV genome and classification

The HDV genome is a circular, covalently closed, negative-polarity, single-strand RNA composed of 1672 to 1694 nucleotides [4] (Dény P, personal communication). It is the smallest and only circular animal RNA virus, this structure being reminiscent of viroids and other small plant pathogens [5]. The degree of sequence similarity with viroids is, however, very low, and therefore the existence of a common ancestor is disputed. In addition, viroids do not code for any protein, which is at variance with HDV. A thorough and critical comparison of structural and replication cycle similarities between HDV, viroids, and other subviral pathogens (like virusoids) has been published recently [5].

Since the original report [6], several full-length HDV genomic sequences have been published and analyzed. Nucleotide number one has been arbitrary chosen at a unique *Hind* III restriction site [7]. Overall, HDV RNA has a high degree (~74%) of self-complementarity: as a consequence, it can fold on itself as an unbranched, double-stranded, rodlike structure [6, 7]. The genome contains a ribozyme domain spanning nucleotides 680 to 780 and a putative promoter site for a messenger RNA (mRNA) leading to the synthesis of its only antigen, HDAg [8]. Due to its unique structure and

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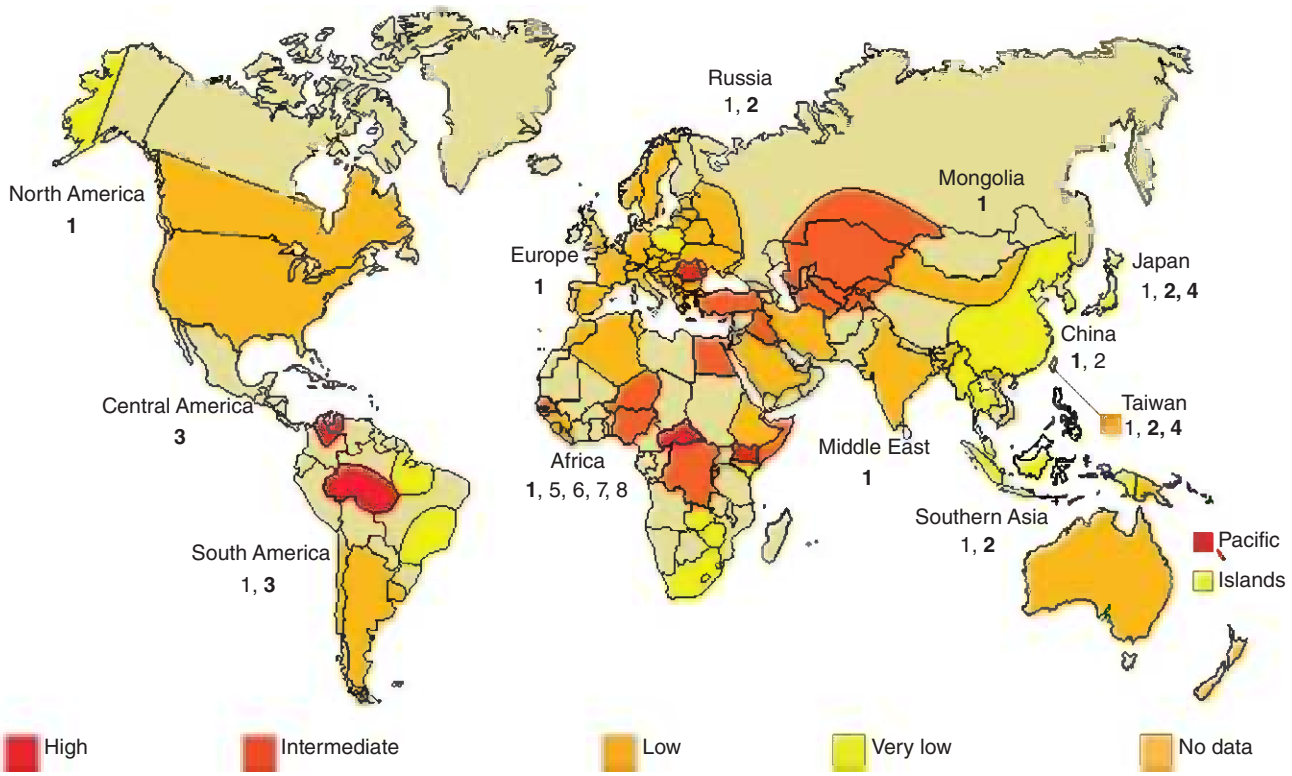

Figure 27.1 Schematic representation of the main areas of HDV distribution in the world. In bold, the predominant HDV genotypes for each geographic area (Source: Pascarella S, Negro F. Liver Intl 2011;31:7–21 [2]). (Color plate 27.1)

sequence, the HDV is classified as the single member of a novel genus, the *Deltavirus* [9].

The evolution rate of HDV RNA, as estimated by longitudinal studies of the viral quasi-species performed in hepatitis D patients, is quite high, as for most RNA viruses, and varies between 3.0 × 10E-2 (at the time of the acute or flare phase) and a less dynamic rate of 3.0 × 10E-3 base substitutions per nucleotide per year during the chronic phase of infection [10]. The high variability among different HDV isolates is believed to arise from the lack of proofreading activity of the RNA polymerase involved in replication, although recombination may also play a role [11]. Although highly conserved domains are found in the region containing the RNA autocatalytic cleavage sites and the RNA-binding domain of HDAg, HDV genomic RNA of different isolates may present up to 38% sequence heterogeneity. Initially grouped into three major genotypes, based on more recent analyses HDV isolates have been tentatively subdivided into up to eight major HDV clades [4]. With the exception of genotype 1, which is found worldwide, all other genotypes are found in specific geographic areas. Genotype 2 prevails in Japan, Taiwan, and Russia; genotype 3 in the Amazon basin; genotype 4 in Japan and Taiwan; and genotypes 5–8 in Africa (Figure 27.1) (for a review, see [4]). Infection with multiple genotypes may occur in individuals at high risk of repeated exposure. However, a single genotype dominates and only 10% of the viral population is represented by the minor strain [12]. The determination of HDV genotypes bears epidemiological interest only, at variance with other hepatitis viral infections, and there are no commercially available assays for HDV genotyping.

The hepatitis D antigen

HDV expresses a single protein called HDAg. A unique open reading frame (ORF), located on the RNA complementary to HDV genome, leads to the synthesis of two different forms of HDAg during the HDV life cycle. The small HDAg (S-HDAg) is 195 amino acids (aa) long, with a molecular weight of approximately 24 kDa, while the large HDAg (L-HDAg) shares the N-terminus with the S-HDAg but has an additional 19 aa at its C-terminus for a total length of 214 aa (27 kDa). The synthesis of the large HDAg requires the posttranscriptional editing of the antigenomic RNA brought about by a host enzyme called "adenosine deaminase acting on RNA-1" (ADAR-1) [13, 14]. The adenosine at base position 1015 of the

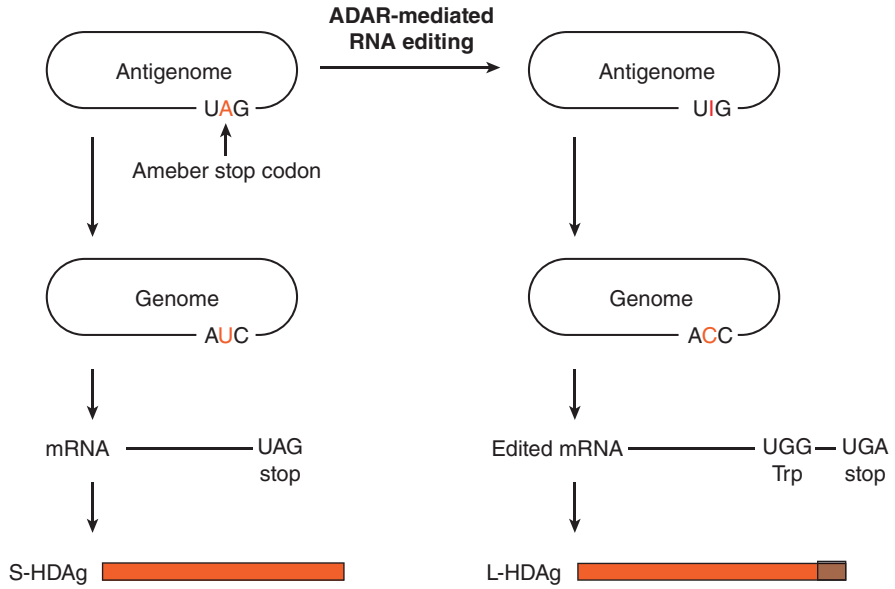

Figure 27.2 Editing of the HDV antigenomic RNA (Source: Pascarella S, Negro F. Liver Intl 2011;31:7–21 [2]). (Color plate 27.2)

antigenomic RNA is first deaminated into inosine. As a result, the amber stop codon (UAG) of the HDAg-encoding ORF is modified into UIG, with the inosine leading to the introduction of a cytidine in the genomic strand during the next replication round. When this modified genome is transcribed into HDAg mRNA, the amber stop codon is replaced by a UGG, which encodes for a tryptophan [13]. At the time of translation, this modified mRNA will direct the synthesis of HDAg with an additional 19 aa at its C-terminus (the L-HDAg), that is, until the next stop codon, located 19 codons downstream (Figure 27.2).

The S-HDAg is necessary for the initiation of genome replication [15], while the L-HDAg behaves as a dominant negative inhibitor [16]. As mentioned, the L-HDAg is necessary (and sufficient) for the assembly of the virion [17]. The HDAg binds to the viral genomic RNA, a property that may be facilitated by the rodlike structure of the latter [18]. HDAg may directly stimulate transcription elongation via the replacement of the host negative elongation factor, a transcription repressor bound to the RNA polymerase II [19].

In the absence of HBsAg, both L-HDAg and S-HDAg localize in the nuclei as they bear nuclear localization signals encompassing aa 35 to 88 [20]. L-HDAg is a nucleocytoplasmic shuttling protein, with a nuclear export signal at its C-terminus [21].

Several posttranslational modifications of HDAg have been reported. The L-HDAg contains a terminal CXXX box, which is a substrate for isoprenylation, a modification that increases its inhibitory effect on viral replication [22] and is necessary for viral particle formation. The prenylated L-HDAg is lipophilic and binds to the HBV envelope proteins [23]. Both HDAg forms are phosphorylated, with the major phosphorylation site being the serine 177: the S-HDAg is phosphorylated at serine and threonine residues, and the L-HDAg at serines only [24]. The phosphorylation of the S-HDAg (in particular of the serine 2) modulates HDV replication. The importance of HDAg phosphorylation in RNA replication is probably due to the fact that it mediates its RNA-binding activity. The S-HDAg is also methylated at the level of its RNA-binding domain. Mutations of this domain or use of a methylation inhibitor results in an inhibition of HDV RNA replication [25]. Also, acetylation of HDAg modulates HDV replication and regulates HDAg nuclear localization: a single substitution of lysine 72 by alanine decreases the accumulation of viral RNA, leading to an earlier expression of L-HDAg [26]. Finally, the HDAg undergoes sumoylation. The S-HDAg is a small ubiquitin-like modifier 1 (SUMO1) target protein through its multiple lysine residues. Fusion of S-HDAg with SUMO1 increases HDV genomic RNA and mRNA synthesis without affecting the level of the antigenomic RNA [27].

Virion structure

HDV particles are roughly spherical (36–43 nm) [3] and contain an internal ribonucleic structure surrounded by HBV envelope proteins and host lipids. The inner structure (19 nm) is composed of one single molecule of HDV RNA of genomic polarity complexed with about 70 molecules of both small and large HDAg forms [28]. HDAg

dimerizes through an antiparallel coiled coil. These dimers then interact to form octamers, which are arranged into a 50 Å ring resulting in a unique structure [29]. There is no evidence that the complementary, antigenomic RNA strand is packaged into virions. The HDV envelope is composed of about 100 copies of the three envelope proteins of HBV: the small HBV surface antigen (S-HBsAg), the middle (M-HBsAg), and the large (L-HBsAg). The relative proportion of these proteins is 95:5:1, resembling more the 22 nm particles of HBsAg than the complete virions [30]. The S-HBsAg is sufficient for HDV particle assembly, whereas L-HBsAg is necessary for infectivity and the M-HBsAg is not essential for assembly or infectivity [31]. The large HDAg (but not the small form) is necessary and sufficient to form virions, while the small HDAg form increases packaging efficiency.

The HDV life cycle

Virus attachment and entry

The early steps of HDV attachment involve the binding to the carbohydrate side chains of hepatocyte-associated heparan sulfate proteoglycans [32]. However, the true receptor of HDV and its detailed entry mechanism are unknown. HDV and HBV share the same envelope proteins. Thus, one would expect similar mechanisms of attachment and entry for both viruses. However, HBV is non-infectious when the virion is packaged with S-, M-, and L-HBsAg lacking the N-linked carbohydrates, at variance with HDV [33]. Some similarities exist: residues 5–20 of the pre-S1 domain in the L-HBsAg are needed for entry of both viruses. In fact, entry can be inhibited by synthetic peptides encompassing the 50 first amino acids of the pre-S1 domain [34]. Viral entry may implicate the clathrin-mediated endocytic pathway, as shown for other viruses that are endocytosed together with their receptor into endosomes.

The HDV replication

HDV replication occurs via RNA-directed RNA synthesis without DNA intermediates [5]. Three different RNAs accumulate during HDV replication: the genome (~300 000 copies per cell), the antigenome (~30 000 to ~100 000 copies), and the mRNA (~600 copies) (Figure 27.3). The antigenome is the exact complement of the

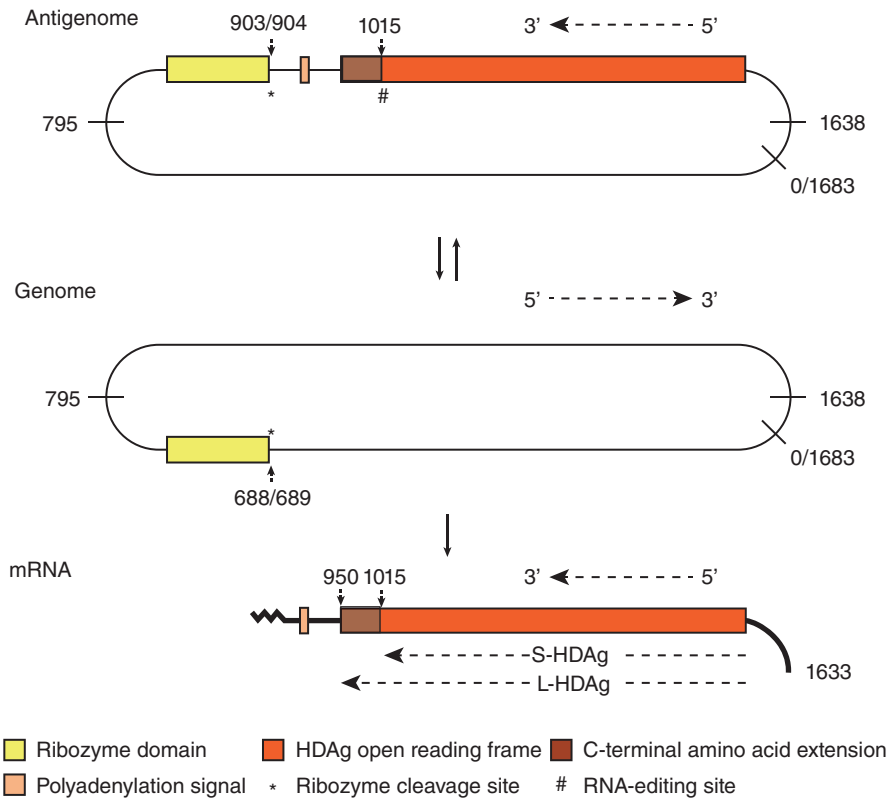

Figure 27.3 Schematic representation of the HDV antigenomic and genomic RNAs, as well as of the HDAg mRNA (Source: Pascarella S, Negro F. Liver Intl 2011;31:7–21 [2]). (Color plate 27.3)

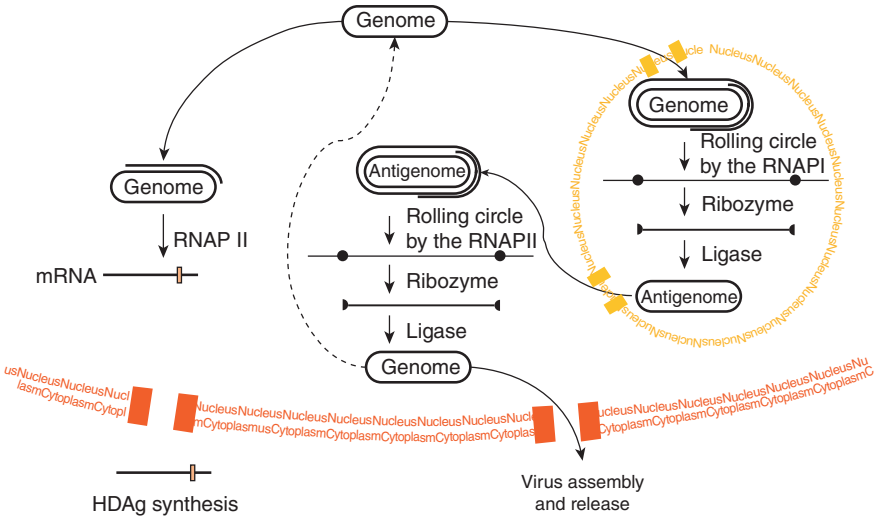

Figure 27.4 Double-rolling circle replication of HDV and localization of the different replication events (Source: Pascarella S, Negro F. Liver Intl 2011;31:7–21 [2]). (Color plate 27.4)

genome. It contains a unique ORF coding for the HDAg and, as the genome, a ribozyme of approximately 85 nucleotides in length [5, 35]. The 800-nucleotide linear mRNA has the same polarity as the antigenome, possesses a 5′ cap and a 3′-poly(A) tail, and directs the synthesis of HDAg [36].

Since HDV does not possess its own RNA polymerase, it exploits the human transcriptional machinery for its replication. The role of the host RNA polymerase II (RNAP II) is well established. RNAP II binds to the HDV RNA, and the HDV replication is sensitive to α-amanitin [37]. Resistance to α-amanitin, however, does not occur for the transcription of the antigenome, suggesting an involvement of RNAP I [38], and some data even suggest a role for RNAP III, which may bind to both genomic and antigenomic HDV RNA [39].

Replication occurs entirely in the hepatocyte nucleus, where HDV RNA is brought in by the HDAg, is activated by S-HDAg binding to HDV RNA, and occurs without any help coming from HBV and without DNA intermediates. Replication proceeds via a double-rolling circle mechanism, in analogy with plant viroids [5]. The transcription proceeds for more than one genome length, going twice through the ribozyme cleavage site. Then the antigenomic transcript undergoes an autocatalytic cleavage between nucleotides 688 and 689 via the antigenomic ribozyme, is folded into a rodlike structure, and is ligated by a host ligase [40] to form a circular template. The mechanism is the same to produce the genomic RNA from the antigenome: the genomic RNA will then either be incorporated into new viral particles or be used again as a template for antigenomic RNA synthesis. The ratio between genomic and antigenomic RNA is asymmetric, since the genome is several times more abundant than the antigenome. The mRNA, like the antigenome, is transcribed from the genome: however, the transcription of these two RNAs involves distinct start sites, uses different polymerases, and occurs in different subnuclear compartments, since the mRNA is transcribed in the nucleoplasm, like the genome, while the antigenome is transcribed in the nucleolus (Figure 27.4).

Virion assembly and release

The exact steps of HDV particles' assembly and release are unknown. HDV assembly is reduced after clathrin heavy-chain downregulation, whereas HBV is not. This suggests that even if the two viruses share the envelope proteins, their assembly and release mechanisms might be different. The assembly efficiency is, in addition, different between genotypes and correlates with the ability to interact with the clathrin heavy chain. Recent work has pointed out the implication of the amino acid at position 205 in virion release. Substitution of the proline by an arginine or an alanine in HDV genotype 1 significantly decreased the secretion of viral particles. The reverse substitution in genotypes 2 or 3 increased the assembly efficiency of HDAg [41].

The HDV dependence on the hepatitis B virus

As HBV is essential for HDV virion assembly and release, HDV infection is always associated with HBV infection. Two major patterns of infection can occur: co-infection and superinfection. A third pattern, the

so-called helper-independent latent infection, has been suggested to occur in the liver transplant setting, but its existence is disputed.

Co-infection is a simultaneous infection with both viruses that leads to an acute hepatitis B and D. Clinically, it is indistinguishable from a classical acute hepatitis B, and may lead to persistent HBV and HDV infection at a rate that is comparable to the progression to chronicity of the primary HBV infection in the adult (i.e., less than 5%). Superinfection is the HDV infection of an individual with predating HBV replication: in this case, progression to persistent HDV infection is virtually the rule [2].

Due to unknown interfering mechanisms, HBV replication is usually suppressed in hosts co-infected with HDV [2]. The molecular mechanisms leading to HDV interference on HBV replication are unknown. A specific profile of HBV RNA species has been reported in the liver of woodchucks infected with the woodchuck hepatitis virus and superinfected with HDV [42]. Recently, similar studies have been carried out in the livers of hepatitis D patients, where HBV genomes with large deletions in the basal core promoter–precore region were detected in HDV-positive patients but not in HDV-negative patients and were associated with lower HBV DNA levels in serum [43]. Whether these modifications of the helper nucleic acid species are related to the catalytic activity of HDV RNAs is a fascinating hypothesis that warrants further study. However, the most likely mechanism that may account for HDV inhibitory effects on HBV is viral interference.

Virus–host interactions

HDV replicates exclusively in hepatocytes. Even if initial chimpanzee studies and some *in vitro* experiments have suggested a direct cytopathic effect of HDV, the immune-mediated liver damage seems predominant, as suggested by the presence of inflammatory cells surrounding infected hepatocytes in patients with hepatitis D. Since HBV replication is usually suppressed by HDV, liver damage occurring in patients with chronic hepatitis D is believed to be mostly induced by HDV rather than by HBV.

HDV products have been shown to interact extensively with the host cell machinery. HDV replication is associated with increased histone H3 acetylation within the clusterin promoter, leading to an enhanced clusterin expression [44]. This modification is the same as that associated with the expression of factors of some oncogenic viruses. The clusterin protein overexpression, reported in several tumor cells, increases cell survival and thus may play a role in tumorigenesis, including in the development of hepatocellular carcinoma in chronic hepatitis D patients. However, the data concerning the effects of HDV on cell proliferation are not conclusive, since HDV seems to inhibit host cell proliferation [45].

HDV, like many other viruses capable of establishing a persistent infection, seems to have developed an anti-interferon alpha (anti-IFNα) strategy, which may account for the poor responsiveness to IFNα of chronic hepatitis D. HDV directly inhibits the IFNα signaling by interfering in the early steps of the Janus kinase (JAK)–signal transducers and activators of transcription (STAT) signal transduction pathway by inhibiting tyrosine kinase 2 (Tyk2), STAT1, and STAT2 phosphorylation [46].

Nuclear factor kappa light-chain enhancer of activated B cells (NFκB) activation is implicated in inflammation processes and in cancer. The L-HDAg (but not the S-HDAg) has been shown to sensitize to tumor necrosis factor alpha (TNFα)-induced NFκB signaling, probably through the direct association with TNF receptor-associated factor 2 (TRAF2), a protein implicated in the early signal transduction events [47]. This modulation of the NFκB signaling pathway may contribute to the pathogenesis of hepatitis D, amplifying the inflammatory liver damage.

Conclusions

After more than 30 years from its discovery, HDV is still a poorly characterized virus. Despite the advent of mass vaccination campaigns against HBV and the implementation of safer medical practices, HDV prevalence remains stable, especially in Western countries. Furthermore, severe outbreaks in resource-poor countries are still being reported, and no effective therapy is available. Thus, a continuous interest in HDV basic research is warranted: a better knowledge of the HDV life cycle and pathogenesis may help identify new approaches for treatment.

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Chapter 28
Epidemiology and natural history

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Summary

Hepatitis D virus (HDV) is the most severe form of viral hepatitis. Superinfection of hepatitis B surface antigen (HBsAg) carriers with HDV results in chronic evolution of the infection in the vast majority of cases. In contrast, co-infection of hepatitis B virus (HBV) and HDV frequently leads to severe acute hepatitis and may progress to acute liver failure. Anti-HDV-positive patients have an increased risk of experiencing liver-related morbidity and mortality. HDV patients may show complex and dynamic patterns of viral dominances. In most cases, HDV is dominant with high HDV RNA levels and suppressed HBV DNA. HDV is also dominant in triple-infected anti-HCV-positive patients where most subjects test HCV RNA negative. HDV is highly prevalent in Central Asia, eastern Turkey, some African countries, and the Amazon basin. In Western countries, HDV can frequently be detected in migrant populations and intravenous (IV) drug users. Vaccination against HBV needs to be applied to high-risk populations to prevent further spreading of the HDV infection.

Epidemiology of hepatitis D

It has been estimated that between 15 and 20 million individuals worldwide are infected with HDV [1, 2]. However, very limited data are available regarding the true frequency of HDV co-infections of HBsAg-positive patients. Population-based studies have rarely been performed, and most investigations of anti-HDV in hepatitis B patients were done in referral centers only – frequently including distinct special-risk groups. Moreover, the vast majority of studies on the epidemiology of hepatitis D were published 10 or even 20 years ago and only a few full papers on the prevalence of anti-HDV were reported in recent years. Finally, the diagnosis of hepatitis D was frequently based on serological markers. However, patients may become HDV RNA negative but remain anti-HDV positive, and thus liver disease may not be caused by HDV infection even in the presence of specific antibodies. HDV RNA testing is not well standardized, and there is still no World Health Organization (WHO) HDV standard available. Few sensitive quantitative HDV polymerase chain reaction (PCR) assays are available [3], but the performance of these assays across different HDV genotypes may be poor, which leads to further questioning of some study results. Thus, additional studies are needed to determine the true frequency of hepatitis D in HBsAg-positive individuals in most regions of the world.

Being linked to HBV, HDV is spread in the same way, mainly through parenteral exposure. Limited data are available on vertical transmission of HDV, but it is likely that this route of transmission is possible [1] .

HDV genotype 1 is the most frequent genotype and is distributed throughout the world. The vast majority of hepatitis D cases in Europe and North America are caused by HDV genotype 1. Genotypes 2 and 4 are

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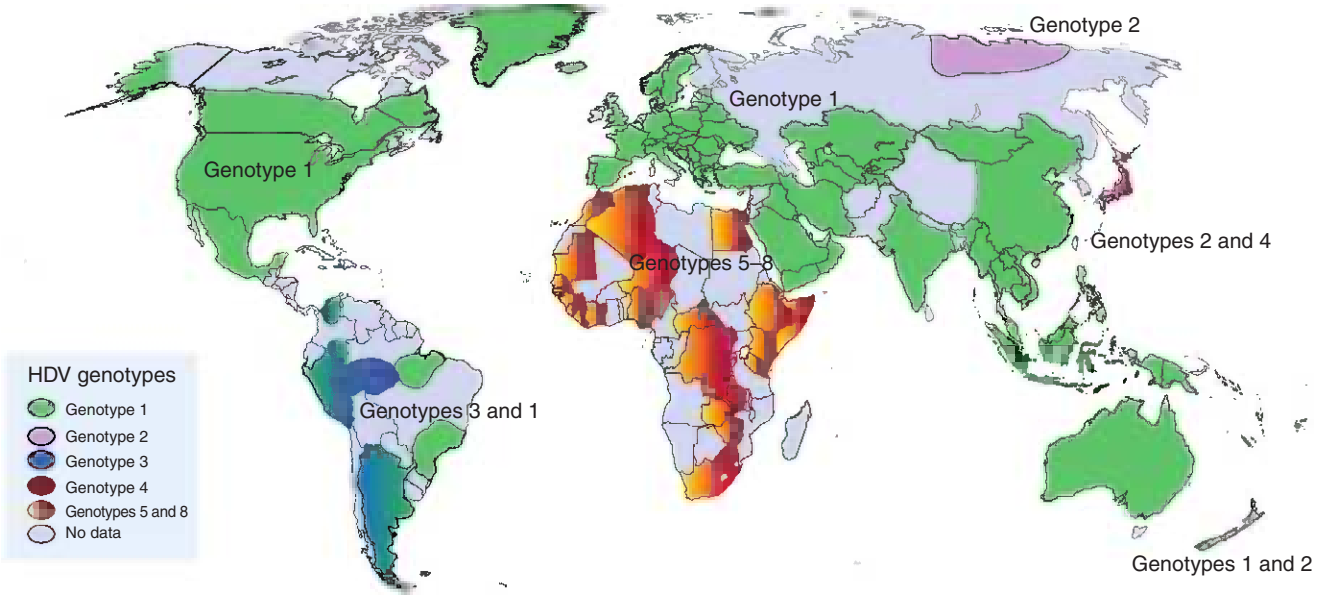

Figure 28.1 Geographic distribution of hepatitis delta genotypes. (Source: Adapted from Hughes SA, Wedemeyer H, Harrison PM. Lancet 2011;378:73–85 [1]). (Color plate 28.1)

mainly prevalent in East Asia and the Yakutia region of Russia, while genotype 3 is seen exclusively in the northern part of South America. HDV genotypes 5–8 have been described in sera of patients of African origin (Figure 28.1). There might be differences in the pathogenicity of different HDV genotypes. For example, HDV genotype 1 has been associated with more severe disease, whereas genotype 2 seems to cause a more benign course of liver disease [4]. HDV genotype 3 infection has also been linked with a particularly poor prognosis [5].

Prevalence of anti-HDV in Europe

The anti-HDV prevalence in HBsAg-positive patients declined in Italy and Germany during the late 1980s and the 1990s [6, 7]. In early Italian studies performed between 1980 and 1990, about 25% of hepatitis B patients had evidence for HDV co-infection [8]. After the introduction of vaccination programs against HBV, this frequency did sharply decline, and currently less than 10% of HBsAg-positive individuals test anti-HDV positive in Italy [8, 9]. A similar trend was observed in Turkey, where between <5% in western Turkey to >27% in southeastern Turkey of HBsAg-positive patients were HDV positive [10]. One German center also reported a decline in anti-HDV prevalence until 1997, while thereafter stable frequencies were detected [6]. This was mainly attributed to a high proportion of immigrants among the hepatitis D population. Less than 30% of anti-HDV-positive individuals in Germany were born in the country [11]. This experience is well in line with other reports from Germany [12], France [13], and London [14] also describing that the majority of patients testing anti-HDV-positive belonged to migrant populations (Table 28.1). The highest hepatitis D prevalence in Europe is currently reported in Romania, Moldavia, and Albania, followed by other southeastern countries and Russia [1].

IV drug use represents another risk group for HDV infections. About one-third of German hepatitis D patients report a history of drug abuse [11]. High anti-HDV prevalence was also described in HBsAg-positive individuals infected with HIV. An analysis from EuroSIDA, a prospective study involving more than 16000 patients infected with HIV-1 who were enrolled at 93 European centers, showed that overall 14.5% of HIV-positive and HBsAg-positive individuals tested anti-HDV positive [15]. Particularly high prevalence of more than 40% was detected in IV drug users, while only very few men who have sex with men were HDV infected. Moreover, considerable differences became evident between different European regions, with the highest anti-HDV prevalence in Eastern European patients.

The spectrum of HDV-associated liver disease also changed during the last three decades. While most anti-HDV-positive patients presented with acute hepatitis in the 1980s, anti-HDV is currently almost exclusively found in patients with chronic hepatitis. In Barcelona, the number of chronic hepatitis D cases only slightly declined between 1983 and 2007, while acute hepatitis D was very frequent before 1992 but almost absent after 2001 [21]. Thus, new infections with HDV became a rare event and the majority of cases currently presenting in

Table 28.1 Selection of recent studies on the prevalence of anti-HDV in HBsAg-positive individuals in Europe.

Reference	Country	HDV patients' country of residence	HDV prevalence in HBsAg-positive patients
[11]	Germany	Germany ~20% Turkey 27% Former Soviet Union 30%	11% (258/2363)
[12]	Germany	Germany ~13% Southern Europe 32.1% Eastern Europe 53.6% (1996–2002)	–/–
[14]	United Kingdom	Northern Europe 18.3% Southern and Central Europe 28.1% Sub-Saharan Africa 26.8% (2000–2006)	8.5% (82/962)
[15]	Europe	HIV-positive patients	14.5% (61/422)
[10]	Turkey	Different regions of Turkey	From 5% (west) to 27% (southeast)
[13]	France	Africa ~70% Western Europe ~15% Eastern Europe ~12%	–/–
[16]	Switzerland	Switzerland 39% Africa 21% Western Europe 15% Eastern Europe 11% Asia 14%	5.9% (101/1699)
[17]	Italy	Eastern Europe or Asia 7.4%	8.1% (95/1396)
[18]	Russia	Children 1 to 14 years, with or without liver disease (50%:50%)	4.7% (7/149)
[19]	Poland	Northern Poland	7.9% (5/63)
[20]	Italy and Albania	Albanian refugees in southern Italy	1.1%

liver clinics seem to be either immigrants or survivors of the HDV epidemic of the 1970s and 1980s.

Prevalence of anti-HDV in North America

Very limited data are available on the prevalence of HDV infection in North America. Some studies performed in the 1980s reported frequencies between 1.4% in blood donors and 30% in HBsAg carriers in Illinois [2]. Specific risk groups such as hemophiliacs or female prostitutes showed particularly high anti-HDV incidence rates. Recently, a surprisingly high prevalence of HDV antibodies was detected in IV drug users in Baltimore. Nineteen of 38 (~50%) chronically infected individuals were anti-HDV positive [22]. Still, more data are needed in order to take into consideration the different genetic backgrounds to determine the true health burden caused by HDV in North America.

Prevalence of anti-HDV in South America

HDV is known to be endemic in the Amazonia region, including the northwestern states of Brazil, eastern Peru, and some parts of Colombia and Venezuela. Patients are infected mainly with HDV genotype 3 in these regions in conjunction with HBV genotypes A or F [23]. HDV genotype 1 is also found but more frequently in other regions such as southern Brazil. Striking differences in HDV prevalence rates may occur even between different villages. It can happen that almost every hepatitis B patient in one village is co-infected with HDV, while HDV is basically absent in neighboring regions. Outbreaks of severe hepatitis among isolated populations have been described [24]. It is very likely that interfamilial spreading occurs during childhood, but details concerning the major routes of transmission are unknown. In contrast to western Amazonia, Amerindian populations of eastern Amazonia show very low HDV prevalence rates. The clinical picture of hepatitis D is different in Brazil because patients are much younger than European HDV-infected patients. Subsequently, more patients test HBeAg-positive in Brazil.

Prevalence of anti-HDV in Asia, Australia, and the Pacific regions

Dramatic differences in HDV prevalence rates can be observed in Asia. HDV is prevalent in Western Asia, such as in eastern Turkey, Syria, and Iran. Hepatitis D accounts for almost one-half of the cases of

hepatocellular carcinoma (HCC) in eastern Turkey [25]. In Iran, HDV is also considered a major health issue. Distinct risk groups such as hemophiliacs, HIV-positive individuals, and patients undergoing dialysis show a high frequency of anti-HDV, while co-infection rates are lower in urban settings such as Tehran [26]. High anti-HDV prevalence rates have also been reported for some Central Asian republics. A very high frequency of hepatitis D was reported in Mongolia, with up to one-third of chronic hepatitis cases being caused by HDV infection [27]. HDV seems to be highly prevalent in rural areas of Pakistan but also in liver clinics in Karachi and Jacobabad with co-infection rates exceeding 60% [26]. Limited data are available for India with rather diverse results of HDV prevalence among HBsAg-positive patients ranging between 2% and 19%. In contrast, HDV seems to be very rare in China, Japan, and most Southeast Asian countries. Similarly, HDV is infrequent in Australia. However, HDV can be detected in these regions in high-risk groups such as hemophiliacs, hemodialysis patients, prostitutes, and IV drug abusers. Still, even in high-risk groups the prevalence of HDV infection has declined in recent years. For example, the proportion of anti-HDV-positive prostitutes in Taiwan declined from more than 50% to less than 10% between 1988 and 2002. Similarly, more than three-quarters of IV drug users in Taiwan tested anti-HDV positive in 1985, while only 12/87 (14%) were anti-HDV positive in 2002 [28]. Still, HDV is prevalent in these risk groups and outbreaks may occur. It was interesting to note that HDV seems to have spread independently from HCV within a cohort of injecting drug users in Taiwan [29], suggesting different modes or dynamics of transmission between HCV and HDV.

Particularly high frequencies of HDV superinfection have been reported for distinct Pacific islands in studies published in the 1980s. Between 70% and 90% of individuals infected with HBV tested anti-HDV positive in some studies [26]. However, the current epidemiology of HDV infection in the Western Pacific region is not available and needs updating.

Natural history of hepatitis D

As mentioned, hepatitis D is considered the most severe form of viral hepatitis. Two scenarios need to be differentiated: simultaneous co-infection with HBV and HDV, and superinfection of an HBsAg-positive individual with HDV.

Simultaneous infection (co-infection) with HBV and HDV

Simultaneous HDV and HBV infection has been associated with more severe acute liver disease and even liver failure [30]. In the vast majority of cases, co-infection results in clearance of both infections. Similar to HBV mono-infection, children may be at a higher risk for chronicity. However, data on HDV infections in children are limited. Anti-HDV antibodies seem to persist in most patients for at least one decade after recovery even in immunocompromised individuals [31]. Recovery from HDV infection does not seem to induce complete protective long-term immunity. Reinfection of chimpanzees was possible after apparent recovery from acute hepatitis D [32]. Thus, individuals at risk might become re-infected with HDV, which could also explain the high prevalence rates in IV drug users or the intrafamilial spreading of HDV in certain high-endemic regions. Acute hepatitis D did become a rather rare event in Western countries in recent times [21], and only single cases of *de novo* infections are currently reported in European countries.

HDV superinfection

Superinfection of HBsAg-positive carriers can lead to acute hepatitis but frequently progresses to persistent infection. HDV superinfection may lead to transient or continued suppression of HBV replication. More than two-thirds of HDV-infected patients show very low levels of HBV DNA, and less than 20% of patients are HBeAg-positive [11, 33, 34]. In triple-infected HBsAg, anti-HDV, and anti-HCV positive patients, HCV replication is also suppressed in the vast majority of cases. Acute hepatitis D may even lead to clearance of HCV infection [35]. A molecular explanation of HBV suppression by HDV could be the suppressive effects of HDV p24 and p27 antigens on HBV enhancers [36]. Still, suppression of HBV and HCV replication is not associated with the level of HDV RNA in the blood [11], suggesting that the inhibitory effects of HDV are not directly linked to the extent of HDV replication. However, viral dominance may change over time. Different scenarios of changing patterns between HBV and HDV replication have been observed in one Spanish study following 37 hepatitis D patients from January 2006 to April 2007 [37]. The authors highlight the need for continued monitoring of HDV viremia by quantitative real-time reverse transcription PCR (rt-RT-PCR) assay as well as HBV DNA as hepatitis D is not stable but rather exhibits dynamic and possibly evolving viral profiles [38]. Moreover, HBV may replicate at significant levels possibly requiring antiviral therapy, according to current recommendations [39].

Chronic hepatitis D

Chronic hepatitis D frequently leads to end-stage liver disease, hepatic decompensation, and the development

of HCC [8]. Particularly severe courses of hepatitis D have been described in initial studies published after the discovery of HDV [40] during the 1980s and early 1990s [8]. Less severe disease activity but more frequent cases of cirrhosis in hepatitis D patients were observed in subsequent years [41]. Recent studies confirmed that HDV infection is indeed associated with a high frequency of liver cirrhosis, liver-related mortality, and overall mortality. In a cross-sectional study from Germany studying 258 anti-HDV-positive individuals, more than 50% of patients had biochemical or histological evidence of liver cirrhosis even though the mean age of this cohort was still younger than 40 years [11]. In contrast, one-quarter of patients had normal liver enzymes, but HDV replication was confirmed in only 64% of the subjects in that study. Liver cirrhosis developed in 62% of HDV-infected patients over a mean period of 233 months in Milan, with about one-half of them experiencing clinical complications including ascites, jaundice, and HCC. Liver decompensation was associated with female sex, alcohol abuse, and HDV replication [42]. Similarly, cirrhosis at presentation and lack of antiviral therapy predicted liver complications in another Italian long-term follow-up study. Of note, not female but male sex was independently associated with hepatic decompensation or HCC in this cohort of 188 patients [43].

HDV infection has been associated with a particularly high risk of developing liver cirrhosis in patients co-infected with HIV. In one Spanish study, 66% of patients infected with HIV/HBV/HCV/HDV but only 6% of subjects infected with HBV/HCV/HIV presented with liver cirrhosis [44]. Similarly, hepatitis D was associated with poorer overall survival in patients infected with HIV in Taiwan [45]. Thus, all HBsAg-positive patients infected with HIV should definitely be tested for anti-HDV as hepatitis D seems to take a particularly severe course in HIV-positive individuals.

Chronic HDV and HCC

Whether hepatitis D is also associated with an increased likelihood to develop HCC is controversial. Early studies did not find a strong association between HDV infection and HCC [46]. In contrast, a 3.2-fold increased risk to develop HCC was reported in a European multicenter study performed in the late 1990s [47]. However, this slightly increased HCC risk was based on only five out of 37 patients who developed an HCC in that study. Hepatic decompensations rather than HCCs were the most frequent clinical complications in hepatitis D patients in Turin [43], Düsseldorf [48], and Barcelona [21]. The annual HCC incidence was 2.8% in Italian patients with HDV co-infection [49] and 2.7% in one German cohort [48]. No increased HCC frequency was observed in another recent long-term follow-up study, where only 3% of the patients developed HCC during a median observation time of 13 years [37]. Similarly, our own experience suggests that HCC does not develop in a higher frequency in patients co-infected with HDV than in individuals mono-infected with HBV [50]. Overall, these data indicate that HDV itself has not any major additional oncogenic potential. Still, HCCs can develop, and thus regular state-of-the art HCC screening should be applied to HBV-infected patients as it is done for patients who are infected with HBV alone [39].

In summary, the severity of chronic hepatitis D has been confirmed throughout various studies performed in different countries. Fibrosis progression seems to be accelerated, as patients present with liver cirrhosis at a much younger age than individuals mono-infected with HBV. Still, factors associated with disease progression differ between cohorts, and more studies are needed to determine variables that are predictive of the clinical long-term outcome of hepatitis D. We recently suggested a baseline event anticipation (BEA) score for anti-HDV-positive patients. This easy-to-apply score included age (>40 years), sex (male), country of origin (Southeastern Mediterranean), bilirubin levels, international normalized ratio (INR) values, and platelet counts being associated with hepatic decompensation, HCC, liver transplantation, and death. The BEA score groups patients into low, medium, and high risk for developing liver-related clinical complications. The high performance of score was confirmed in two independent validation cohorts [50]. The BEA score needs to be evaluated in further studies (see www.hepatitis-delta.org).

HDV replication and lack of antiviral therapy have been associated with poorer outcomes in the Italian studies, indirectly suggesting that viral suppression or eradication by interferon alpha (IFNα)-based therapies will improve the long-term outcome of hepatitis D. However, pegylated IFNα is effective in only about 25% of patients [51], and thus future studies need to define the overall clinical benefit of antiviral therapies and also attempt to improve the overall treatment outcomes.

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Chapter 29
Treatment of hepatitis D

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Summary

Interferon is the only therapy with demonstrated efficacy for chronic hepatitis D. Nucleos(t)ide analogs against the hepatitis B virus (HBV) are not efficacious as they do not eradicate the underlying HBV that supports hepatitis D virus (HDV) infection.

The current therapeutic recommendation is pegylated interferon alpha (PEG-IFNα) given weekly for 12 to 18 months. Response is limited. Serum HDV RNA is undetectable 6 months post therapy in only about one-quarter of the patients; however, HDV may relapse in the patients as long as they remain HBsAg hepatitis B surface antigen (HBsAg)-positive. The only reliable endpoint of therapy is the clearance of HBsAg.

The current management of HDV patients is based on common practice rather than on evidence from clinical trials; therefore, it should be pragmatic and individualized. Therapy lasting longer than 12 months might be of benefit in selected patients with partial responses or in those with rapidly advancing disease.

Introduction

IFNα remains the only licensed therapy for hepatitis D more than 25 years after its empirical introduction into clinical practice [1]. While efficacious therapies are now available against hepatitis B and C, no specific therapy has been yet developed for chronic hepatitis D (CHD), which remains the most difficult to treat among chronic viral hepatitis.

Challenges in treatment of hepatitis D

The only requirement of HDV from hepatitis B virus (HBV) is the HBsAg, which is needed by the HDV to enter hepatocytes. HDV is not dependent on HBV replication, that is, it thrives independent of the level of HBV DNA in serum; usually HDV exerts an inhibitory effect on HBV replication, such that most patients with chronic hepatitis D have low or undetectable HBV DNA in serum [2].

After binding to a receptor on the liver cell via a domain in the N-terminal pre-S1 region of the large HBsAg [3], HDV RNA moves to the nucleus where it is replicated by redirected cellular RNA polymerases [4, 5]. Presumably the rodlike structure into which HDV RNA is driven by extensive intramolecular base pairing deceives host RNA polymerases into recognizing the viral molecule as a self-DNA.

Replication occurs through a rolling-circle mechanism; multimeric linear transcripts of the genome and antigenome elongate by rolling over the circular template of the other, are cleaved to a genome- or antigenome-sized monomer, and finally circularize into the infectious form by the HDV ribozyme [4].

Thus, HDV has no enzymatic outfit of its own to be targeted by antivirals, such as the proteases and polymerases of HCV and HBV; it diverts to its advantage the replicative machinery of the hepatocyte and uses as catalyst a structural part of its RNA rather than a protein catalyst.

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Potential targets for therapy could be the mechanisms contributing to disease, but the pathogenesis of hepatitis D remains unknown. Though expression of the HDV RNA and of the hepatitis D antigen (HD-Ag) in serum and liver is almost invariably associated with liver damage, there is no evidence that HDV is directly cytopathic, and the severity of hepatitis D does not correlate with the titer of HDV RNA in serum.

Hepatitis D may be an immune-mediated disease; the innate and adaptive immune response plays an important role in the clearance of the virus and of disease [6]. HDV subverts the adaptive immune response – CD4+ [7] and CD8+ T cell response is required for viral clearance, and cytotoxic CD4+ T cells were implicated in the pathogenesis of hepatitis D [8]. The interactions of the virus with IFN are unknown. The interference of HDV with IFNα signaling could represent a mechanism of viral persistence and of resistance to treatment; IFNα-induced intracellular signaling was impaired in HDV-transfected human hepatoma cells, the virus subverting the effect of IFNα by blocking Tyk2 activation, thereby resulting in selective impairment of activation and translocation to the nucleus of STAT1 and STAT2 [9]. Recent evidence suggests that interferon may block the spread of HDV by interfering with the initiation of productive infection of naïve hepatocytes [10].

Goals of treatment

In most viral infections, the primary goal of therapy is eradication of the virus with amelioration of liver disease.

Viral eradication remains the primary goal of therapy in chronic hepatitis D. However, this disease results from double HDV–HBV infection, and the definition and evaluation of a therapeutic response require the consideration of two viral infections. Therapy is considered successful if HDV remains undetectable 6 to 12 months after stopping IFN. This definition is extrapolated from the pattern of response in chronic hepatitis C, where a sustained viral response 6 months post therapy predicts eradication of hepatitis C virus (HCV) with almost 100% accuracy; however, this may not be the case for HDV, unless the concomitant HBV infection is also eradicated with clearance of HBsAg from serum.

Two points should be considered. Firstly, in the setting of an established HBV infection, the infectious potential of HDV is very high; titration of infectivity resulted in transmission of HDV to chimpanzees carrying the HBsAg with infectious serum diluted to 10^{-11} [11]; this is the highest infectious titer recorded for any hepatitis virus so far. Secondly, the most sensitive assays for the measurement of HDV RNA currently available have a lower detection limit of around 10 viral genome/ml, thus their sensitivity is by far lower than the natural infectivity threshold for the HBsAg carrier [12]. Furthermore, clearance of HDV RNA with treatment, even when determined with the most sensitive assays, may not mean that HDV is eradicated, and the residual undetectable HDV may be rescued post therapy, resulting in reactivation of HDV infection if the patient remains HBsAg-positive.

While the ultimate endpoint of therapy is the clearance of HBsAg, this goal is seldom reached. Therefore, the current therapeutic management of hepatitis D is pragmatic, aiming at achievable goals such as the control of HD viremia and the improvement of liver disease for as long as possible. Parameters of response to therapy are a negative HDV RNA test in serum, normal alanine aminotransferase (ALT), and histological amelioration in liver biopsy [13].

The detection of HDV RNA in serum remains problematic as no commercial test is available. Several homemade semiquantitative methods have been used to evaluate serum levels of HDV RNA, but they remain imprecise and difficult to handle in routine laboratory procedures, due to differences in sensitivity and to lack of standardization caused by the use of different sets of primers and a lack of reference standards. The development of an accurate and sensitive test for HDV RNA quantification in blood samples has several technical problems. Firstly, the extensive intramolecular complementarity (>70%) of the HDV RNA sequence impairs cDNA synthesis and therefore polymerase chain reaction (PCR) efficiency. Secondly, the genetic variability of the virus requires the design of primers and probes to target the most conserved regions of the genome; divergence between HDV genotypes is as high as 37% over the entire nucleotide sequence of the genome. As expected, a comparative analysis of HDV sequences of different genotypes indicated that the most conserved regions of the genome are located within the ribozymes; primers and probes targeted to the ribozyme segments reduce false negative results and optimize specificity for all genotypes. Another problem concerns the standardization of the assay and its comparison with other existing techniques. To date, there is no available international reference standard or control to calibrate a quantitative assay for HDV.

If HDV RNA testing is not available, a surrogate marker for the control of HDV replication and disease is the IgM type antibody to HDV (IgM anti-HD); this antibody is present at high titers in florid HDV disease but diminishes and disappears with spontaneous or IFN-induced remission of hepatitis D [14–16].

Therapy responders who become HBsAg-negative can be considered cured of HDV infection; those who lose serum HDV RNA and normalize liver enzymes *but* remain HBsAg-positive should be monitored with liver

Table 29.1 Controlled trials of treatment of chronic hepatitis delta virus (HDV) with standard interferon alpha (IFNα).

Reference	Number of patients	IFN schedule	End of treatment		End of follow-up	
			HDV RNA– negative	ALT normal	HDV RNA– negative	ALT normal
Rosina	12	Alpha 2b recombinant t.i.w. 5 MU/m² × 3 months	4 (33%)	4 (33%)	1 (8%)	1 (8%)
	12	Untreated	2 (17%)	0 (0%)	2 (17%)	0 (0%)
Rosina	31	Alpha 2b recombinant t.i.w. 5 MU/m² × 4 months 3 MU/m² × 8 months	14 (45%)	8 (25%)	14 (45%)	1 (3%)
	30	Untreated	8 (27%)	0 (0%)	IO (33%)	0 (0%)
Farci	14	Alpha 2a recombinant t.i.w. 9 MU × 12 months	10 (71%)	10 (71%)	0 (0%)	5 (36%)
	14	Alpha 2a recombinant t.i.w. 3 MU × 12 months	5 (36%)	4 (28%)	0 (0%)	0 (0%)
Gaudin	14	Untreated	1 (7%)	1 (7%)	0 (0%)	0 (0%)
	11	Alpha 2b recombinant t.ì.w, 5 MU/m² × 4 months 3 MU/m² × 8 months	7 (66%)	7 (66%)	1 (9%)	1 (9%)
	11	Untreated	4 (36%)	2 (18%)	NA	NA
Madejon	16	Alpha 2a recombinant t.i.w. 18 MU × 6 months 9 MU × I month 6 MU × I month 3 MU × 4 months	6 (50%)	4 (37%)	’0 (0%)	0 (0%)
	16	Alpha 2a recombinant daily 3 MU × 3 months 1.5 MU × 9 months	3 (19%)	1 (7%)	0 (0%)	0 (0%)
Borghesio	5	Lymphoblastoìd t.i.w. 10 MU up to normal ALT plus 12 months	NA	0 (0%)	NA	NA
	4	Lymphoblastoid daily 5 MU up to normal ALT plus 12 months	NA	2 (50%)	NA	NA

t.i.w.: three times a week; NA: unavailable data.
(Source: Niro GA, Rosina F, Rizzetto M. J Viral Hepat 2005;12:2–9 [1].)

chemistries and serum HDV RNA for possible reactivation of HDV.

Efficacy of therapy

Standard IFN

In studies in the 1980s and 1990s, standard IFNα given for 6 to 12 months resulted in normalization of liver enzymes in about 20–25% of the patients. Results were better with a high dose of IFN, 5 MU daily or 9 MU thrice weekly given for 12 instead of 6 months [1]. However, the rate of HDV clearance was lower than the rate of normalization of ALT, with virus persistence in many cases despite the temporary control of aminotransferases. These studies were heterogeneous, each included only a few patients, the design and therapy protocols were different, and the clearance of HDV RNA occurred at variable times from the beginning of therapy, sometimes after discontinuation of IFN. None of 10 Italian children improved clinically or histologically after one year of standard IFN [1], and treatment for the same duration was ineffective in Greek children [17]; however, in a recent report from Pakistan, 80% of 25 children with chronic hepatitis D responded to standard IFN [18]. Results of studies with standard IFN are summarized in Table 29.1.

Resolution of HDV infection and improvement of liver histology were reported by Lau in an anecdotal patient treated for 12 years [19], and resolution of cirrhosis was reported by Farci 12 years after therapy in four patients who were given 9 MU of standard IFN thrice weekly for 48 weeks [20]; however, it is difficult to reconcile the favorable clinical outcome with an IFN effect, as the patients were still HDV RNA–positive at the end of the original trial of therapy.

Type 3 liver–kidney microsomal antibodies (anti-LKM), which are occasionally present in florid HDV infection, did not impact response or adverse events; in different series, 10 anti-LKM-positive HDV patients completed IFN therapy without complications [1].

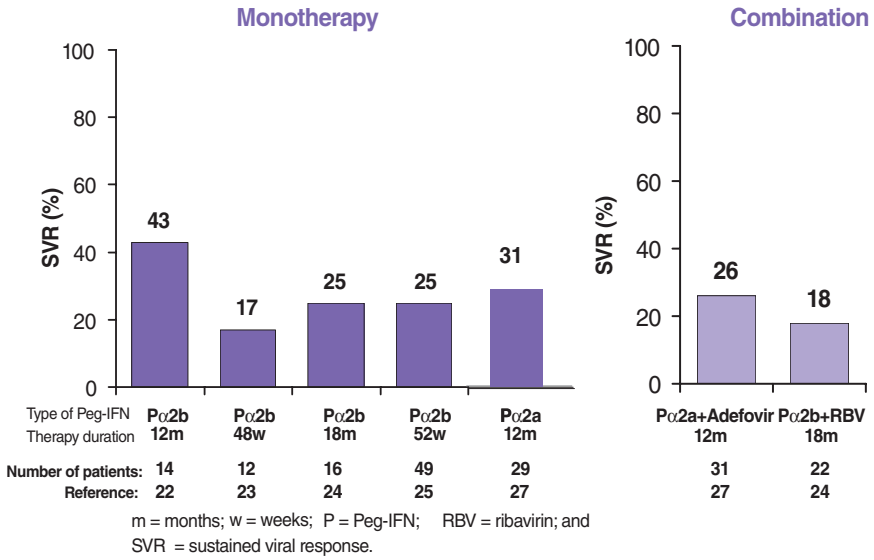

Figure 29.1 Results of the treatment of chronic hepatitis D treated with PEG-IFN as monotherapy or in combination with antivirals.

It was proposed that extension of therapy up to 24 months could give better results; however, prolonging standard IFN therapy to 2 years did not significantly increase the rate of sustained response compared to standard IFN treatment for 12 months [21].

Pegylated IFNs

The overall results have been better with PEG-IFN (Figure 29.1). Of 14 patients treated with PEG-IFNα-2b 1.5 μg/kg weekly for 12 months, 43% achieved a sustained clearance of serum HDV RNA [22]; in other series of patients treated with the same PEG-IFN, a sustained viral response was obtained in 18% to 25% of the patients [23–25]. PEG-IFN was efficacious also in patients with cirrhosis [26]. The largest study reported to date enrolled a total of 90 HDV patients in Germany and Turkey [27]. Thirty-one patients were assigned to receive 180 μg of PEG-IFNα-2a weekly plus 10 mg of adefovir daily, 29 received 180 μg of PEG-IFNα-2a weekly plus placebo, and 30 received 10 mg of adefovir alone daily; treatment was administered for 48 weeks, and the patients were followed up for 24 weeks post therapy. At the end of therapy, a virological response (HDV RNA undetectable in serum) was obtained in 23% of the patients given PEG-IFN with adefovir, in 24% of the patients given PEG-IFN with placebo, but in none of those given adefovir monotherapy. At the end of the post-treatment follow-up, HDV RNA was negative in 26% of the patients treated with PEG-IFN and adefovir, 31% of those treated with PEG-IFNα and placebo, and none of those given adefovir monotherapy; during the post-therapy follow-up, nine patients in the PEG-IFN groups become negative for HDV RNA, and two had a virologic relapse. There was no difference in efficacy between PEG-IFN and adefovir or PEG-IFN alone, with a cumulative sustained virologic response of 28% in both groups. These data suggest a marginal advantage of PEG-IFN versus standard IFN; however, the impact on liver disease was not consistent, more patients in the PEG-IFN groups had a worsening of histologic scores on biopsies performed at the end of treatment, and the ALT levels also normalized in patients who remained positive for HDV RNA during the follow-up.

Predictors of response include a low baseline HBsAg and HDV RNA titer and elevated ALT; HDV genotype 1 may be less responsive. The kinetics of HDV RNA during therapy may also be predictive; in the French study [22], HDV RNA was negative at 6 months of therapy in 75% of the patients who achieved an end-of-therapy response.

Antivirals against HBV

Though HBV synthesis is spontaneously inhibited in most patients with chronic hepatitis D, the advent of HBV antivirals led to the hypothesis that further pharmacological inhibition of HBV might repress the synthesis of HBsAg long enough for the infected hepatocytes to die, leading to eradication of HDV by preventing its spread to contiguous hepatocytes.

Famciclovir 500 mg three times a day given to 15 patients for 6 months or lamivudine 100 mg a day given for 12 or 24 months to a total of 36 patients had no effect

on markers of HDV infection or on the clinical or histologic features of hepatitis D [1]; only anecdotal cases of remission and resolution of chronic hepatitis D with lamivudine [28], or with tenofovir or emtricitabine associated with PEG-IFN [29], were reported. Adefovir monotherapy given at a dose of 10 mg daily for 48 weeks to 30 patients was not efficacious; none of the patients achieved a sustained viral response [27]. Although antivirals, as well as IFN, were able to control HBV replication in chronic hepatitis D accompanied by productive HBV infection, the clinical features of the liver disease did not ameliorate with control of HBV DNA synthesis.

Treatment of end-stage liver disease

Liver transplantation is lifesaving for end-stage liver disease [30]. The risk of graft reinfection and recurrent disease is distinctly lower for HDV than for HBV. Hepatitis B virus transmission is needed to support recurrent hepatitis D, but HBV replication is diminished in most HDV patients; therefore, clinically significant re-infection of the graft is often precluded by the low infectious HBV titer of the chronic hepatitis D patient undergoing transplantation.

Current prophylaxis with hepatitis B immune globulin (HBIG) and antivirals protect virtually every HDV transplant from recurrence of hepatitis D, with very good survival rates. A shortage of donors could tempt transplant centers to give an HBsAg-positive liver to a HDV patient; this should be resisted, as two such cases have been reported and in both HDV disease recurred [31, 32].

HDV latency

Liver transplantation has raised the issue of latency of HDV in humans.

When the first liver transplants for HDV were performed in Italy in 1987, an unexpected finding was the expression of the HD-Ag in biopsies of the grafted liver taken during surgery while intravenous HBIG was administered [33]. This finding can now be explained by an almost immediate reinfection with HDV, which gained access into the grafted liver through the HBsAg coat and replicated in the liver independently of HBV. This happens because even high-dose intravenous HBIG may not be sufficient to neutralize all circulating HBsAg-coated hepatitis D virions.

A controversial issue is how long HDV can maintain latency in human livers in the absence of HBV re-infection. Over a mean posttransplant period of 34 months, transient subclinical HDV re-infection (an HDV RNA blip in PCR) was observed in 5% of 117 HDV transplants performed in Torino [30]; serum HBsAg was negative in all the patients. Among 76 HDV transplants in Paris, 88% of those who remained HBsAg-negative had HDAg in liver or HDV RNA in serum during the first year post transplant, but only 5% remained HDV RNA–positive and HD-Ag-positive after the second year [34]. In a recent study from Hannover [35], HDAg remained detectable by immunohistochemical staining for up to 19 months post transplant in the grafted liver of six patients, in the absence of liver HBV DNA and cccDNA and of serum HBsAg and HDV RNA.

These data would indicate that HDV can remain viable in the hepatocytes for 2–3 years without HBsAg in serum; the issue of latent HDV infection seems to have limited clinical impact in the transplant setting, as HBV re-infection is required to rescue latent HDV and convert it to full-blown infection.

Future therapies?

Potential targets to therapy are the mechanism of HDV attachment to the liver cell and virion assembly (Figure 29.2). Virion assembly requires the integrity of critical regions of the HBsAg. The substitution of phenylalanine for tryptophan at positions 196, 199, or 201 of the small HBsAg [36] and conformational changes in the cysteines on the antigenic loop of HBsAg [37] block the entry of HDV into cells.

Acylated pre-S peptides derived from the large HBsAg envelope protein are potent inhibitors of the entry of HBV into hepatocytes, and promising results have been recently reported with Myrcludex-B, a myristoylated pre-S/2-48^{my2} peptide [38]; treatment with Myrcludex-B efficiently hindered the establishment of HDV infection *in vivo* and delayed the increase of HBV viremia and of intrahepatic cccDNA load.

Prenylation of the last four amino acids on the large HDAg (the so-called CXXX box) is critical to the interaction of this viral antigen with the HBsAg, and disruption of prenylation has been proposed as a therapeutic target in chronic hepatitis D [39]. Experiments *in vitro* and *in vivo* have shown that inhibition of prenylation prevented HD virion assembly and resulted in the clearance of HDV viremia; however, there is as yet no report that this strategy may be feasible and efficacious in humans.

Conclusions

The current recommendation for treatment of chronic hepatitis D is PEG-IFNα-2a given weekly for 48 weeks. Management is based on accepted common practice rather than on evidence from clinical trials; therefore, the approach should be pragmatic and individualized. Therapy protocols should be determined in each patient

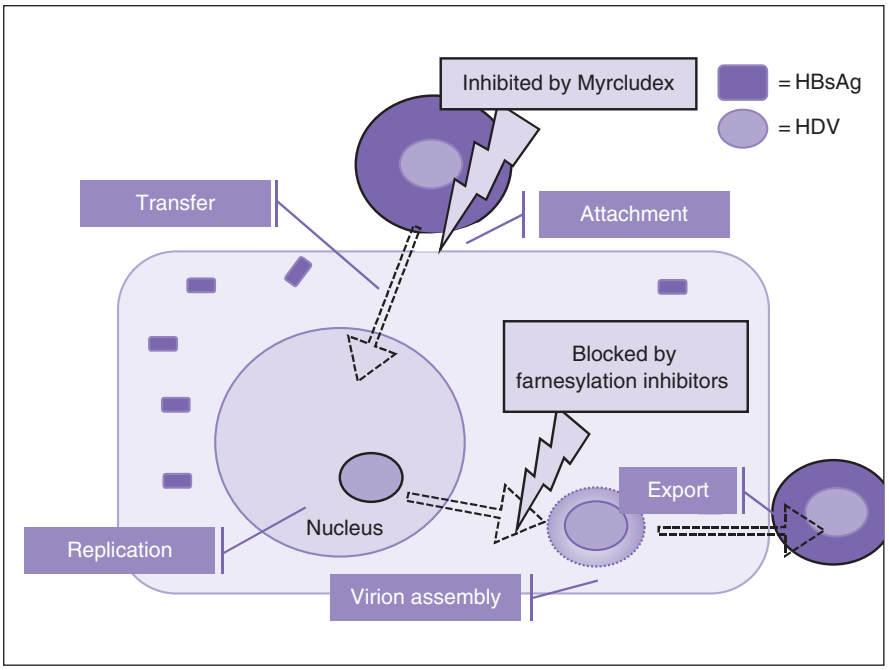

Figure 29.2 Targets for HDV therapy. Steps in the life cycle of the HDV are the attachment of the virion to the liver cell through an HBsAg receptor, transfer to nucleus, replication, virion assembly, and export. Potential therapeutic targets are the prevention of attachment of HDV to the liver cell membrane through modifications or disruptions of the HBsAg envelope and receptor, and of the process of HD virion assembly, which requires farnesylation (prenylation) of the large HD-Ag and the integrity of critical regions of the HBsAg. Myrcludex B has been proposed for the inhibition of HBV entry into hepatocytes, and prenylation inhibitors for the disruption of HD virion assembly. (Color plate 29.1)

according to the clinical evolution of disease and the pattern of HDV markers during therapy.

Treatment is indicated in patients with a chronic, active, and progressive hepatitis D. However, the limited potential of therapy should be weighed against the risk of adverse effects induced by IFN therapy, in particular in patients with advanced cirrhosis.

There is no rule for stopping therapy in patients who do not respond; however, sometimes the virologic response can be delayed, and it may be appropriate to continue therapy for at least one year before withdrawing. Prolonging therapy beyond 12 months might be of benefit in the occasional patient with partial virologic and biochemical response. In patients with rapidly advancing disease who respond to PEG-IFN but relapse upon withdrawal, extending therapy over several years may be considered.

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Section VI
Hepatitis E Virus

Chapter 30
Structure and molecular virology

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Summary

Hepatitis E virus (HEV), the causative agent of hepatitis E, belongs to the family *Hepeviridae*. At least four recognized and two putative genotypes of mammalian HEV have been reported. Besides humans, strains of HEV have also been genetically identified from swine, chickens, sika deer, mongoose, rabbits, and fish. The genome of HEV consists of three open reading frames (ORFs): ORF1 codes for nonstructural proteins involved in virus replication, ORF2 codes for a capsid protein that elicits neutralizing antibodies and is a target for vaccine development, and ORF3 codes for a small multifunctional protein involved in virion morphogenesis and viral pathogenesis. ORF2 and ORF3 are translated from a single bicistronic messenger RNA (mRNA) and overlap each other, but neither overlaps ORF1. The determination of the three-dimensional (3D) crystal structure of the HEV capsid protein should facilitate the development of vaccines and antivirals. HEV's taxonomy and genomic organization, its gene expression and functions, the 3D structure of the virions, and the life cycle of HEV replication are discussed in this chapter.

Introduction

HEV, the causative agent of human hepatitis E, is a public health concern in many developing countries of Asia and Africa, although the virus also causes sporadic cases of acute hepatitis E in many industrialized countries [1–3]. The mortality rate associated with HEV infection is generally low in the general population (less than 1%), although a relatively high mortality rate of up to 20–30% has been reported in infected pregnant women from developing countries [3]. Hepatitis E is now a recognized zoonotic disease, and pigs and likely other animal species are reservoirs for HEV [4–5]. HEV, a small, non-enveloped, single-strand, positive-sense RNA virus that is transmitted primarily via the fecal-oral route, is currently classified in the family of *Hepeviridae* [6]. With the identification of various animal strains of HEV from pigs, chickens, rabbits, deer, mongoose, and fish, the host range and genetic diversity of HEV are rapidly expanding [4–5]. Significant progress has been made in understanding the molecular biology of HEV, including the development of HEV reverse genetic systems [7] and determination of the 3D crystal structure of the HEV capsid protein [8].

Virion properties

The virions of genus *Hepevirus* are non-enveloped, icosahedral, spherical particles with a diameter of approximately 27–34 nm [2]. The avian HEV virions are similar in size and morphology to members of the genus *Hepevirus* [9–10] (Figure 30.1). The virion of HEV has a buoyant density of 1.35 to 1.40 g cm^{-3} in CsCl and 1.29 g cm^{-3} in glycerol and potassium tartrate gradients. Low-temperature storage (between –70 °C and +8 °C) and iodinated disinfectants can inactivate HEV virions [3, 5–6]. HEV is more heat labile than hepatitis A virus [11]. Virus infectivity remained after chloroform and ether

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treatment of avian HEV in liver suspensions; however, avian HEV infectivity was lost after incubating at 56 °C for 1 hour or 37 °C for 6 hours. Treatment of avian HEV in liver suspensions with 0.05% Tween-20, 0.1% NP40, and 0.05% formalin reduced virus infectivity by 1000-fold [3, 6, 10]. Incubation of homogenates of HEV-infected pig livers at 56 °C for 1 hour did not abolish virus infectivity, although the virus was completely inactivated after boiling or frying the HEV-infected liver for 5 minutes [4]. Whether there is a difference in thermolability among different species of HEV is unknown.

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Figure 30.1 An electron micrograph of an avian strain of hepatitis E virus particles (30–35 nm diameter) in a bile sample from a chicken with hepatitis–splenomegaly syndrome. Negative staining, bar = 100 nm. (Source: Haqshenas G, Shivaprasad HL, Woolcock PR *et al.* J Gen Virol 2001;82:2449–2462 [9] with permission from the Society for General Microbiology).

Taxonomy and classification

HEV was originally classified in the family *Caliciviridae*, largely due to its superficial similarity in morphology and genome organization to caliciviruses. Subsequent studies revealed that HEV does not share significant sequence homology with caliciviruses, and therefore HEV was subsequently declassified from the family *Caliciviridae*. Currently, HEV belongs to a new family, *Hepeviridae* [6].

Genotypes

There is a single genus, *Hepevirus*, within the family *Hepeviridae* [6]. The viruses within the genus *Hepevirus* all infect mammals, and strains of HEV in the genus have been genetically identified from human, pig, mongoose, deer, rat, and rabbit. HEV is the type species in the genus *Hepevirus*, which includes the four recognized genotypes with approximately 24–28% intergenotypic and 12–20% intragenotypic nucleotide sequence differences (Figure 30.2): genotype 1 consists of Burmese-like Asian strains of human HEV; genotype 2 is represented by a single Mexican strain and some African strains of human HEV; genotype 3 contains strains of HEV from humans in industrialized countries and from several

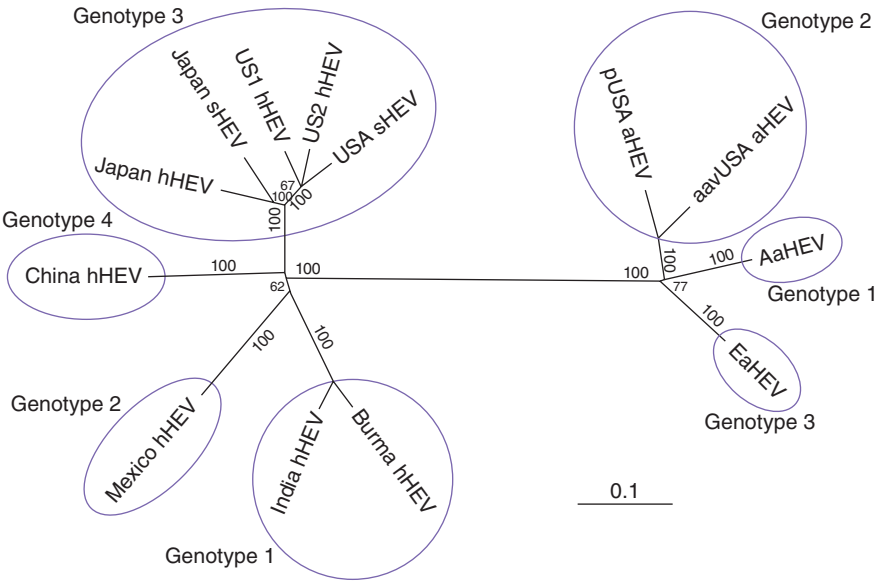

Figure 30.2 A phylogenetic tree of mammalian and avian strains of hepatitis E viruses. The four recognized genotypes of mammalian hepeviruses and the three recognized genotypes of avian hepatitis E virus are indicated. (Source: Bilic I, Jaskulska B, Basic A *et al.* J Gen Virol. 2009;90(pt 4):863–873 [17] with permission from the Society for General Microbiology).

animal species, such as pigs, deer, mongoose, and rabbit; and genotype 4 comprises strains of HEV from sporadic human cases in Asia and swine strains from pigs. The HEV strains recently identified from rabbits in China and the United States are distant members of genotype 3 [12–13]. There are also, as mentioned, two putative new genotypes of mammalian hepeviruses. The rat HEV strains detected from fecal samples of wild rats (*Rattus norvegicus*) in Germany [14] and the United States [15] appear to be a new genotype within the genus *Hepevirus*. More recently, a novel HEV strain detected from wild boars in Japan appears to represent another new genotype within the genus *Hepevirus* [16].

Currently, avian HEV is recognized as a floating species in the family *Hepeviridae* [6] (Figure 30.2). Phylogenetically, avian HEV forms a distinct clade separate from the mammalian hepeviruses. At least three genetically distinct genotypes of avian HEV have been identified from chickens worldwide: genotype 1 from chickens in Australia, genotype 2 from chickens in the United States, and genotype 3 from chickens in Europe and China [6, 17]. Since avian HEV shares only approximately 50% nucleotide sequence identity with the mammalian hepeviruses [10], avian HEV has been proposed as a separate genus, instead of a species, within the family [6]. Therefore, the current HEV classification will likely need to be revised soon.

More recently, a novel virus, designated cutthroat trout virus (CTV), was isolated from spawning adult trout in the United States, and the virus can be propagated in the Chinook salmon embryo (CHSE-214) cell line [18]. The genomic organization of CTV is similar to that of hepeviruses and avian HEV, and it shares significant (13–27%) amino acid (aa) sequence identity with hepeviruses and avian HEV. Therefore, the CTV in fish likely belongs to another new genus within the family *Hepeviridae*.

Inter- and intragenotypic recombination

Increasing evidence indicates that intra- and intergenotypic recombination events do occur among HEV strains. An analysis of 32 full-length HEV genomes identified two intragenotypic recombinants, strains China D and Nepal TK15; however, intergenotypic recombination was not evident in this study [19]. More recently, with the availability of additional full-length HEV genomic sequences, Wang *et al*. [20] analyzed 134 complete HEV genomes and identified both intragenotypic and intergenotypic recombination events. It appears that double recombination events for HEV can also occur, and that recombination between human and swine HEV strains is also possible [20]. This raises a potential concern for the emergence of new recombinant HEV strains in the case of co-infections of swine or humans with two different genotypes of HEV. An intergenotypic recombinant of HEV (DQ450072) was also identified and subsequently verified by phylogenetic analyses. Therefore, for accurate genotyping purposes, it is important to analyze complete HEV genomic sequences [21]. Under experimental conditions, viable and infectious intergenotypic chimeric HEVs have also been successfully produced by intergenotypic recombination [22]. Intergenotypic chimeric viruses containing the capsid gene either alone or in combination with its adjacent 5′ junction region and 3′ noncoding region from human HEV genotype 4 in the backbone of swine HEV genotype 3, but not in the backbone of human HEV genotype 1, are infectious in HepG2/C3A liver cells and in pigs, thus supporting the hypothesis that human HEV genotypes 3 and 4 are of swine origin [22].

Genomic organization

The genome of HEV is a single-strand, positive-sense RNA molecule of approximately 7.2 kb in size, with a 5′ m^7G cap structure and a 3′ poly(A) tail [6]. The HEV genome consists of a short 5′ noncoding region (NCR), three ORFs (1, 2, and 3), and a 3′ NCR (Figure 30.3). *In vitro* and *in vivo* mutagenesis studies revealed that the ORF2 overlaps ORF3, but neither overlaps with ORF1 [23]. The 26 bp 5′ NCR contains a cap structure in the 5′ end of the viral genome that plays a role in the initiation of HEV replication. It has been demonstrated that

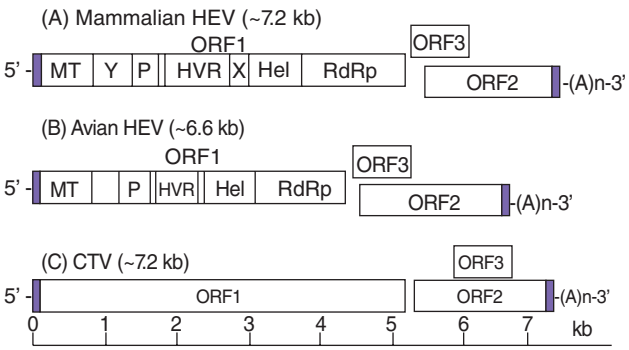

Figure 30.3 Schematic diagram of the comparative genomic organizations of hepatitis E virus (HEV) from mammalian ((A) mammalian HEV), chicken (B: avian HEV), and fish (C: cutthroat trout virus [CTV]) species: a short 5′ noncoding region (NCR), a 3′ NCR, and three open reading frames (ORFs). ORF2 and ORF3 overlap each other, but neither overlaps ORF1. ORF1 encodes nonstructural proteins, including putative functional domains; ORF2 encodes the capsid protein; and ORF3 encodes a small multifunctional phosphoprotein. NCR: nocoding region; MT: methytransferase; Y: "Y" domain; P; a papainlike cystein protease; HVR: a hypervariable region that is dispensable for virus infectivity; Hel: helicase; and RdRp: RNA-dependent RNA polymerase.

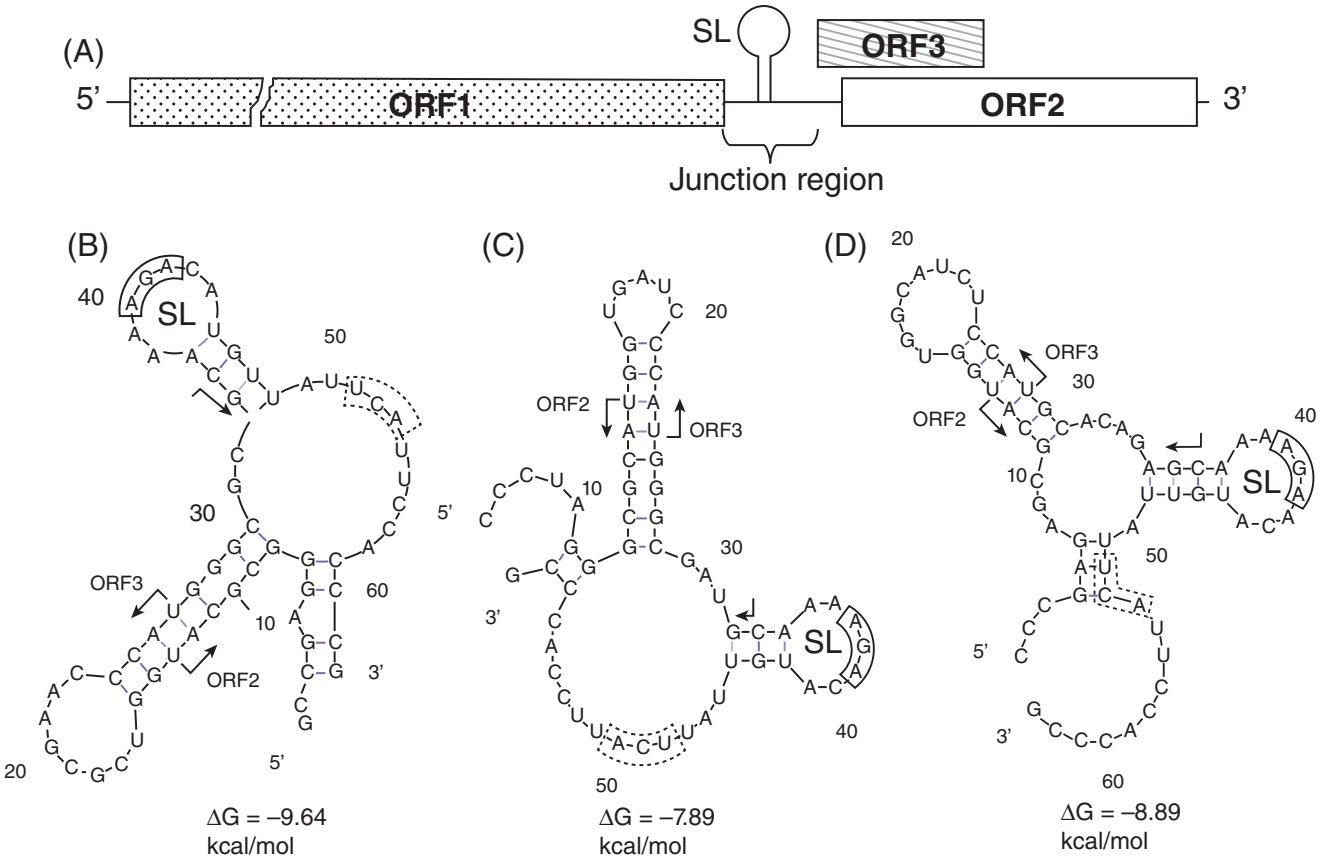

Figure 30.4 RNA stem loop (SL) structure in the junction region of the hepatitis E virus (HEV) genome. (A) Organization of the HEV genome. The position of the predicted SL structure is depicted. (B) Predicted secondary structure of the negative-polarity complement of the HEV genotype 1 (Sar55 strain) junction region. The sequence shown extends from nt 5096 to 5157. (C) Predicted secondary structure of the negative-polarity complement of the HEV genotype 3 (pSHEV-3 strain) junction region. The sequence shown extends from nt 5142 to 5200. (D) Predicted secondary structure of the negative-polarity complement of the HEV genotype 4 (T1 strain) junction region. The sequence shown extends from nt 5138 to 5200. The conserved AGA triplets in the subgenome promoters of alphavirus family members are boxed with solid lines. The HEV subgenome start site is indicated with arrows. The start sites of ORF2 and ORF3 are also indicated; the stop codon of ORF1 is boxed with dotted lines. (Source: Cao D, Huang YW, Meng XJ. J Virol 20;84:13040–4 [27] with permission from the American Society for Microbiology Press).

capped genomic RNA of HEV is infectious for chickens, pigs, rhesus monkeys, and chimpanzees [24–25]. A bicistronic subgenomic mRNA encoding both ORF2 and ORF3 proteins was identified *in vitro* [26]. At least two *cis*-reactive elements (CRE) were identified in the genome of HEV: one CRE located between the 3′ end of ORF2 and the 3′ NCR, and the other CRE in the intergenic region between the end of ORF1 and the start of ORF3 that may serve as the promoter for the 2.0 kb subgenomic mRNA [26]. A stem loop structure was identified in the junction region of HEV genome (Figure 30.4), and mutagenesis studies showed that both the sequence and structure of the stem loop in the junction region are important for HEV replication [27].

The avian HEV genome is approximately 600 bp shorter than that of mammalian hepeviruses [10, 28]. The genomic organization and putative functional domains are relatively conserved between avian HEV and mammalian hepeviruses [9], and common antigenic epitopes in the capsid protein between avian HEV and mammalian HEV have been identified [28]. The genome of CTV from fish is 7269 bp, and its genomic organization is also similar to those of the mammalian hepeviruses and avian HEV [18].

HEV proteins and their function

The ORF1, the largest ORF in the HEV genome, encodes the nonstructural proteins with putative functional domains analogous to those of the alphalike supergroup viruses. The major capsid protein encoded by ORF2 forms the virions. The ORF3 encodes a 13.5 kDa immu-

noreactive protein that is multifunctional and associated with virion morphogenesis and viral pathogenesis [6].

Nonstructural ORF1 proteins

The ORF1, located at the 5′ end of the HEV genome, encodes a 1693 aa nonstructural polyprotein that is important for virus replication. Similar to other single-strand positive-sense RNA viruses, a number of putative functional domains including methyltransferase, papainlike cysteine protease, helicase, and RNA-dependent RNA polymerase (RdRp) were identified in ORF1 [2, 6].

It has been reported that a purified 110 kDa protein encoded by aa 1 to 979 of ORF1 catalyzed the transfer of a methyl group from S-adenosylmethionine (AdoMet) to guanosine triphosphate (GTP) and guanosine diphosphate (GDP) to yield m(7)GTP or m(7)GDP. This 110 kDa ORF1 protein is identified as a capping enzyme with similar properties to the methyltransferase and guanylyltransferase of alphavirus nsP1. A monoclonal antibody recognizing 7-methylguanosine (m(7)G) can bind to HEV genomic RNA from two different HEV genotypes, and the cap analog inhibited the binding of HEV genomic RNAs, thus indicating that the genome of HEV is capped at its 5′ end [2, 7].

A purified recombinant protein encoding the helicase region (aa 960 to 1204 in ORF1) possesses nucleoside triphosphatase (NTPase) and 5′ to 3′ RNA duplex-unwinding activities [29]. The recombinant helicase protein hydrolyzed all rNTPs with moderate hydrolysis efficiency for dATP and dCTP, and less efficiency for dGTP and dTTP. Also, it was shown that the recombinant helicase protein unwinds RNA duplexes with 5′ overhangs showing a 5′-to-3′ polarity. A mutant helicase protein with a mutation in the nucleotide-binding motif I lost its ATPase activity by 70%. Another mutant helicase protein with mutations in the Mg(2+)-binding motif II had a 50% reduction in ATPase activity, and both helicase mutants lost the ability to unwind RNA duplexes [29]. In addition, the helicase domain also possesses RNA 5′-triphosphatase activity [30]. When incubated with 5′-[γ-^{32}P]RNA and 5′-[α-^{32}P]RNA, the purified recombinant helicase protein can release ^{32}P only from 5′-[γ-^{32}P]RNA, thus displaying specificity for the γ-β-triphosphate bond. Therefore, the helicase of HEV ORF1 may be involved in the first step of 5′ cap synthesis [30].

Two consensus LXGG cleavage sites were identified at positions 664 and 1205 in HEV ORF1 [31]. Since the LXGG sequence is generally recognized by viral and cellular deubiquitinating enzymes, a recombinant protein consisting of the methyltransferase and papainlike cysteine protease domains of HEV ORF1 was tested for deubiquitinating activity using fluorogenic substrates, and was found to cleave all four substrates. Another recombinant protein encompassing the helicase and RdRp domains spanning the LXGG site has no deubiquitination activity. Therefore, the methyltransferase and papainlike cysteine protease domains possess deubiquitination activity and may play a role in combating cellular antiviral pathways [31].

A hypervariable region (HVR) with extensive sequence variations was identified in HEV ORF1 [32]. By using human HEV genotypes 1 and 3 and avian HEV, it was demonstrated that deletions in the HVR do not abolish viral infectivity *in vitro* or *in vivo*, although evidence of virus attenuation was observed for HEV mutants with a larger or nearly complete HVR deletion [32]. To delineate the role of the HVR in HEV replication further, a panel of HEV mutants containing overlapping deletions in the HVR was constructed using human HEV genotype 1 and avian HEV infectious clone replicons to assess the effects of serial HVR deletions on viral RNA replication. The results showed that the replication levels of the HVR deletion mutants were markedly reduced in Huh7 cells and LMH cells, thus suggesting a role of HVR in the efficiency of viral replication [33]. Also, the HVR sequences between HEV genotypes were shown to be functionally exchangeable for viral replication and infectivity *in vitro*, although genotype-specific HVR differences in the efficiency of HEV replication were observed. Therefore, it appears that although HVR tolerates smaller deletions for virus infectivity, it may interact with viral and host factors to modulate the efficiency of HEV replication [33]. The HVR may also play a potential role in species specificity, as it is reported that the adaptation of HEV genotype 3 (strain Kernow-C1) from a chronically infected patient to propagate in cells of various animal species selected for a recombinant virus containing a 174 bp insertion of the human ribosomal protein gene in the HVR region of HEV ORF1 [34].

Capsid ORF2 protein

ORF2, located at the 3′ end of the genome, encodes the viral capsid protein of 660 aa. A signal peptide sequence in the N-terminus of the HEV capsid protein is believed to translocate the capsid protein into the endoplasmic reticulum (ER). A N-terminal truncated capsid protein (aa residues 112 to 660) can self-assemble into viruslike particles (VLPs) when expressed in baculovirus [7]. The asparagine residues 137, 310, and 562 are the three N-linked glycosylation sites on the capsid protein [35], and it has been shown that mutations within the glycosylation sites impair the formation of infectious virus particles, although the lethal effect is due to the altered protein structure instead of the elimination of glycosylation [36]. Three mutations in the HEV capsid protein

have been shown to contribute collectively to HEV attenuation: a F51L mutation in the capsid partially contributed to virus attenuation, whereas the T59A and S390L mutations in the capsid resulted in more drastic attenuation of HEV in a pig model [37]. The S390L mutation is localized within a putative sugar-binding sequence within the P1 linear domain (residues 314–453) of the C-terminus of the capsid protein that is strictly conserved among all four recognized HEV genotypes. This potential sialic acid–binding site forms a hidden pocket that may be responsible for binding to cell receptors [37].

The capsid protein of HEV elicits neutralizing antibodies and is the target for HEV vaccine development. Amino acid residues 578–607, 452–617, and 458–607 contain linear and/or conformational epitopes, and the conformational epitopes are believed to be critical for neutralizing activity [7–8, 38]. The aa residues Leu477 and Leu613 in the capsid protein are important in forming the neutralization epitope [39]. Schofield *et al*. [38] showed that two neutralizing monoclonal antibodies against the capsid protein partially recognize the same or overlapping epitopes located between aa residues 578 and 607. It has been reported that homodimers of the truncated HEV capsid proteins, E2 (aa 394–606) and p239 (aa 368–606), contain antigenic determinants and elicited protective immunity in rhesus monkeys. Mutational analysis mapped the dimeric interactions to five hydrophobic aa residues (Ala597, Val598, Ala599, Leu601, and Ala602) [7–8].

It is believed that, within the genus *Hepevirus*, there exists only a single serotype of HEV, and the capsid proteins among different genotypes of mammalian HEV in the genus *Hepevirus* are cross-reactive. In fact, even the nonmammalian avian HEV shares certain common antigenic epitopes in its capsid protein with mammalian hepeviruses [40]. Antigenic diversity, however, does exists as demonstrated by strain-specific and genotype-specific monoclonal antibodies against HEV. Although commercial vaccines against HEV are not yet available, experimental vaccines based on the recombinant HEV capsid protein are promising. In a large, randomized, double-blind, placebo-controlled phase III clinical trial in China, a recombinant HEV vaccine (HEV239) derived from bacteria-expressed capsid protein exhibited 100% vaccine efficacy after three doses of immunization [41]. The HEV239 vaccine has been approved under the brand name Hecolin by the China Food and Drug Administration, and became available in China in 2012. Another recombinant HEV vaccine derived from baculovirus-expressed capsid protein achieved 95.5% vaccine efficacy in a high-risk population in Nepal in a randomized, double-blind, placebo-controlled, phase II clinical trial [42].

Multifunctional ORF3 protein

ORF3, which overlaps ORF2, encodes a small cytoskeleton-associated phosphoprotein. The serine residue Ser-71 (previously predicted as Ser-80) is the phosphorylation site in ORF3 [7]. When phosphorylated, the ORF3 protein interacts with the ORF2 capsid protein, and the interaction is dependent on the phosphorylation of the ORF3 protein. The ORF2–ORF3 interacting domains have been mapped to a 25 aa region (residues 57–81) of the ORF3 protein, and such an interaction points to a possible regulatory role for ORF3 in HEV structural assembly. The ORF3 protein is known to bind to a number of cellular signal transduction pathway proteins, including the protein tyrosine kinases Src, Hck, and Fyn and the adaptor protein Grb2. A proline-rich region in the ORF3 protein binds to the src homology 3 (SH3) domains in the cellular proteins, thus modulating cell-signaling pathways [7].

The HEV ORF3 protein interacts with liver-specific alpha1-microglobulin and its precursor, alpha1-microglobulin–bikunin precursor (AMBP). A 41 aa residue region at the C-terminus of the ORF3 protein was shown to interact with bikunin, a kunitz-type serine protease inhibitor [7]. The AMBP is a liver-specific precursor protein encoding two different unrelated proteins, alpha(1)-microglobulin (alpha(1)m) and bikunin, and it specifically interacts with the HEV ORF3 protein. Therefore, the ORF3 protein may play a role in facilitating the export of alpha(1)m from the hepatocyte [43]. The interaction between the ORF3 protein and α_1-microglobulin–bikunin creates a potential immunosuppressive environment around the infected liver cells. The ORF3 protein has also been shown to interact with the fibrinogen, resulting in decreased fibrinogen secretion from Huh7 liver cells. Since fibrinogen is a hepatic acute-phase protein and plays an important role in maintaining host homeostasis and hemostasis during an acute-phase response, downregulation of fibrinogen expression is advantageous for HEV as the localized immune response around the virus-infected cells would be inhibited and delayed by decreasing the secretion of immunomodulatory fibrinogen [7].

In addition, the HEV ORF3 protein has been shown to regulate the expression of liver-specific genes by increasing hepatocyte nuclear factor 4 (HNF4) phosphorylation through the ERK and Akt kinases, leading to impaired nuclear translocation of HNF4 [7]. The ORF3 protein may also modulate the energy homeostasis in HEV-infected cells and thus contribute to pathogenesis [44]. The ORF3 protein interacts with microtubules and interferes with their dynamics, and thus the interaction of the ORF3 protein with microtubules may be necessary for the establishment of an HEV infection. The

ORF3 protein also interacts with hemopexin, which plays important roles in inflammation [7]. Taken together, the available data indicate that the small ORF3 protein of HEV is multifunctional and is believed to be involved in virus replication and pathogenesis.

Stages of HEV replication

As a fecal-orally transmitted pathogen, HEV is believed to enter the host through the gastrointestinal epithelial cells. After oral ingestion of the virus, evidence of HEV replication in the gastrointestinal tract has been documented [45]. After initial replication in the intestinal tract, HEV enters the bloodstream via viremia to reach its target organ, the liver. The life cycle of HEV remains largely unknown (Figure 30.5), largely due to the lack of a robust cell culture system for HEV propagation.

Attachment, entry, and uncoating

The entry site for HEV is believed to be at the gastrointestinal mucosa. It has been reported that a purified truncated HEV capsid protein p239 bound and penetrated cell lines that are susceptible to HEV replication, and caused inhibition of HEV infection in these susceptible cells. The binding between the capsid protein p239 and the cells was blocked by monoclonal antibodies specific against the p239 protein, suggesting that the capsid protein likely contains receptor-binding sites [7–8]. Kalia *et al*. [46] demonstrated that a 56 kDa recombinant capsid protein of HEV binds to cell surface heparan sulfate proteoglycans (HSPGs), specifically syndecans, in Huh7 human liver cells. Enzymatic or chemical removal of cell surface heparan sulfate significantly reduced the binding of the capsid protein to the Huh7 liver cells. Therefore, it appears that the HSPGs are the cellular attachment receptors for HEV, although the specific HEV cellular receptor(s) remain(s) unknown. It has been reported that the truncated HEV capsid protein P239 can bind to a chaperone, Grp78. Three-dimensional molecular modeling and docking suggested that Grp78 could serve as a putative receptor for HEV [8].

The uncoating and intracellular trafficking processes following HEV entry are largely unknown. It was shown that the HEV capsid protein is co-translationally, but not posttranslationally, translocated across the ER membrane, although glycosylation of the capsid protein is not required for ER localization [35]. Besides its presence in the membrane and surface of Huh7 cells, the capsid protein of HEV is also abundantly located in the cytoplasm [47]. It is believed that retro-translocation may be a pathway for the capsid protein to access to the cytoplasm. Surjit *et al*. [47] demonstrated that, when expressed in Huh7 cells, initially all capsid proteins are located in the ER, and subsequently a fraction of the capsid protein was retro-translocated to the cytoplasm. Certain cellular factors such as alpha-tubulin and HSP90 may involve the intracellular trafficking processes. The truncated capsid protein p239 binds to host cell proteins, Grp 78/Bip, alpha-tubulin, and HSP90 [48], and inhibition of HSP90 in the HepG2 liver cells blocked the intracellular transportation of the p239 capsid

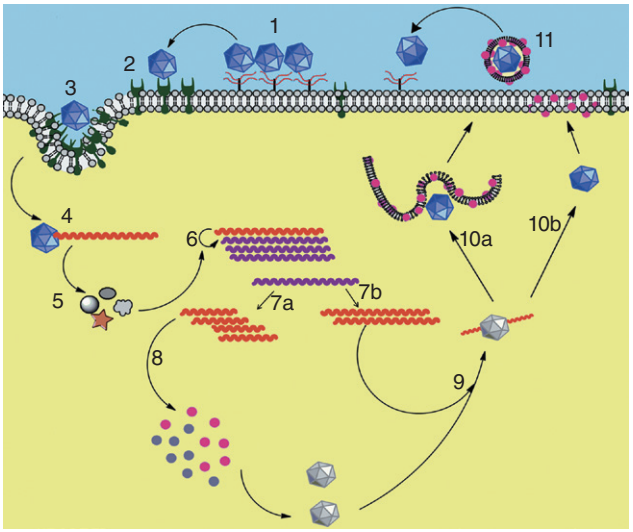

Figure 30.5 Proposed replication cycle of HEV. (1) HEV particles are concentrated on the surface of target cells through heparin sulfate proteoglycans (HSPGs) acting as attachment factors (red wavy lines). (2) HEV particles bind to an unknown cellular receptor. (3) The HVE particles are internalized by unknown mechanisms. (4) The virus uncoats to release genomic RNA (red noodles). (5) Positive-strand viral genomic RNA is translated in the cytoplasm into nonstructural proteins. (6) The nonstructural proteins, including the viral RNA-dependent RNA polymerase (RdRp), replicate the positive-strand genomic RNA into intermediate negative-sense RNA genomes (purple noodles). (7) The intermediate negative-sense RNA genomes then serve as the templates for the synthesis of 2.2 kb subgenomic RNAs (step 7a) as well as full-length positive-sense genomic RNAs (step 7b). (8) The positive-sense subgenomic viral RNA is translated into ORF2 (blue) and ORF3 (crimson) proteins. (9) The ORF2 capsid protein packages the viral genomic RNA to assemble new progeny virions, while the ORF3 protein may optimize the host cell environment for viral replication. (10) The ORF3 protein is associated with endomembranes (step 10a) or plasma membranes (step 10b) and may aid in viral egress. (11) Mature virions are associated with the ORF3 protein and lipids, which are subsequently removed by a unknown mechanism before the virus begins a new cycle of replication. (Source: Ahmad I, Holla RP, Jameel S. Virus Res 2011;161(1):47–58 [7] (the high-resolution drawing is courtesy of Dr Shahid Jameel, International Centre for Genetic Engineering and Biotechnology, New Delhi, India)). (Color plate 30.1)

protein, although the binding and entry of p239 to HepG2 cells were not affected, suggesting that HSP90 is involved in the intracellular transportation of the capsid protein [48].

It has been reported that the ORF3 protein localizes to early and recycling endosomes and delays the post-internalization trafficking of epidermal growth factor receptor (EGFR) to late endosomes and lysosomes [49]. Through its effects on EGFR trafficking, the ORF3 protein may prolong endomembrane growth factor signaling and promotes cell survival, thus facilitating viral replication [49]. The exact mechanism of HEV uncoating and viral RNA release remains unknown.

Transcription, translation, and replication of genomic RNA

After uncoating, viral genomic RNA is released to cytoplasm where transcription, translation, and virus replication occur. It is believed that the ORF1 polyprotein is directly translated from the positive-sense viral genome in the cytoplasm, although the exact mechanisms of translational and posttranslational processes of the ORF1 polyprotein are unclear. In fact, it remains unknown even whether the ORF1 polyprotein functions as a single protein with multiple functional domains or as individually cleaved smaller proteins. It has been reported that, when expressed in baculovirus, the ORF1 polyprotein can process into smaller proteins correlating with predicted domains. However, when expressed in a bacterial or mammalian expression system, the processing of the ORF1 polyprotein was not observed [50]. Nevertheless, a number of functional activities such as methyltransferase, guanylyltransferase, RNA 5′-triphosphatase, NTPase, and 5′-to-3′ RNA duplex-unwinding activities have been experimentally demonstrated for the ORF1 protein.

Like other single-strand positive-sense RNA viruses, HEV replication involves the formation of an intermediate negative-sense genomic RNA that subsequently serves as the template for the production of positive-sense progeny viral genomic RNA as well as subgenomic RNA for translation of ORF2 and ORF3 proteins. Negative-sense, replicative, viral RNA has been detected in the livers and gastrointestinal tissues of rhesus monkeys, pigs, and chickens experimentally infected with different strains of HEV [45]. In other positive-sense RNA viruses, the initiation of replication requires the interaction of the 3′ end of the viral genome with its RdRp and cellular factors to synthesize the negative-sense replicative RNA intermediate. It has been reported that recombinant HEV RdRp protein specifically binds to the 3′ end of the HEV RNA genome containing the poly(A) stretch, and thus the binding may direct the synthesis of complementary-strand RNA to initiate viral replication [7]. It has also been reported that the HEV replicase interacts with the 3′ end of the viral genome and localizes in the ER, which probably is the site of HEV replication [51].

Two subgenomic RNA species of approximately 3.7 and 2.0 kb were reportedly detected in the liver tissues of HEV-infected rhesus monkeys [2]. Also, in Huh7 human liver cells transfected with a HEV replicon, a bicistronic subgenomic mRNA encoding both the ORF2 and ORF3 proteins of HEV has been identified. In addition to the full-length HEV replicon RNA, a 5′-end capped subgenomic RNA was found to initiate at nucleotide 5122 downstream of the first two AUG codons in ORF3. This subgenomic RNA is bicistronic, as two separate AUG codons in different reading frames were found to initiate the ORF3 and ORF2 translation, respectively. Mutagenesis and *in vivo* animal study subsequently confirmed that the third in-frame AUG codon in the junction region is the authentic initiation site for ORF3 [23]. The ORF3 gene contains a *cis*-reactive element and encodes a protein required for HEV infection of rhesus monkeys [26]. A HEV mutant with a C-terminally truncated ORF3 was found to be non-infectious in pigs, indicating that an intact ORF3 is required for *in vivo* infectivity of HEV [23]. A conserved double stem loop RNA structure identified in the ORF1 and ORF2 junction region of the HEV genome is critical for virus replication, and both the sequence and the stem loop structure in the junction region play important roles in HEV replication [27].

Assembly and release

The mechanisms of HEV assembly and release remain largely unknown. The capsid protein is thought to package positive-sense viral genomic RNA into progeny virions. The HEV capsid protein has been shown to bind specifically to the 5′ end of the HEV RNA genome for viral packaging. A 76 nt region at the 5′ end of the HEV genome is responsible for binding to the capsid protein, and the interaction between the capsid protein and HEV RNA may play a role in viral encapsidation [7–8]. It has been shown that aa residues 126–601 in the capsid protein are the essential elements required for the assembly of VLPs [52].

The small ORF3 protein may play an important role in the morphogenesis and release of HEV virions. It has been shown that anti-ORF3 monoclonal antibodies can capture HEV particles in cell culture medium and in sera of infected patients, but not those in feces, indicating that the ORF3 protein is present on the surface of HEV virions released from infected cells [7]. An ORF3-deficient mutant virus replicated in PLC/PRF/5 and A549 cells as efficiently as a wild-type HEV, although when compared to the wild-type HEV-infected PLC/

PRF/5 cells, the ORF3-deficient mutant virus produced much lower numbers of progeny virions in the culture medium of infected cells [53]. Therefore, the HEV ORF3 is responsible for virion egress from infected cells and is present on the surface of released HEV particles that may be associated with lipids [53]. The PxxP motif in the ORF3 protein is a functional domain for HEV budding, and at least one of the two intact PxxP motifs in the ORF3 protein is required for the formation of membrane-associated HEV particles containing ORF3 protein on their surface [7]. The ORF3 protein interacts with tumor susceptibility gene 101 (Tsg101) through the PxxP motifs in the infected cells, and the Tsg101 and the enzymatic activities of dominant-negative mutants of Vps4 are involved in the release of HEV particles, thus suggesting that the multivesicular body pathway may be involved in HEV release [54].

Virion structure

The HEV virion is built with the ORF2 capsid protein, formed by capsomeres consisting of homodimers of a single capsid protein. When expressed in insect cells, the capsid protein of HEV was proteolytically cleaved into smaller polypeptides [45]. A truncated capsid protein containing aa residues 112–660, when expressed in a baculovirus system, self-assembles into VLPs. The aa residues 126–601 in the capsid protein are the essential elements for VLP formation [52]. It appears that RNA binding is required for the assembly of native HEV capsids [55]. Two types of VLPs with different diameters were observed [52, 55]: the small VLPs forming $T = 1$ structure are caused by deletion of the N-terminal basic domain, and the large VLPs forming $T = 3$ structure are the native virions [8, 55] (Figure 30.6). Both VLPs are formed with identical capsid subunits but different copy numbers: 180 subunits for $T = 3$, and 60 subunits for $T = 1$. In general, viruses with a $T = 3$ symmetry often also produce $T = 1$ small particles [55].

The 3D crystal structures of $T = 1$ VLPs for HEV genotypes 1, 3, and 4 are similar [55–57]: the VLPs contain 60 subunits of the truncated capsid proteins with icosahedral two-, three-, and fivefold axes in VLPs. The structure of a VLP containing a partial ORF3 protein (aa residues 70–123) fused to the N-terminal ORF2 protein (aa residues 112–608) contains two distinct domains: the shell domain and the protrusion domain [8]. The structure of the protruding domain E2s contains a neutralizing antibody recognition site and forms a tight homodimer important for interacting with host cells [58]. In a separate study, a 3.5 A structure of VLP reveals that each capsid protein contains three linear domains forming distinct structural elements: S (continuous capsid), P1 (threefold protrusions), and P2 (twofold spikes) [56] (Figure 30.6). A potential polysaccharide-binding site that may bind to a cellular receptor was identified in each domain, and sugar binding to the P1 domain at the capsid protein interface may lead to disassembly of the HEV capsid and cell entry [56].

Yamashita *et al*. [57] found that the Tyr-288 residue in the capsid protein is important for the assembly of VLPs. Mutational analyses indicated that the protruding

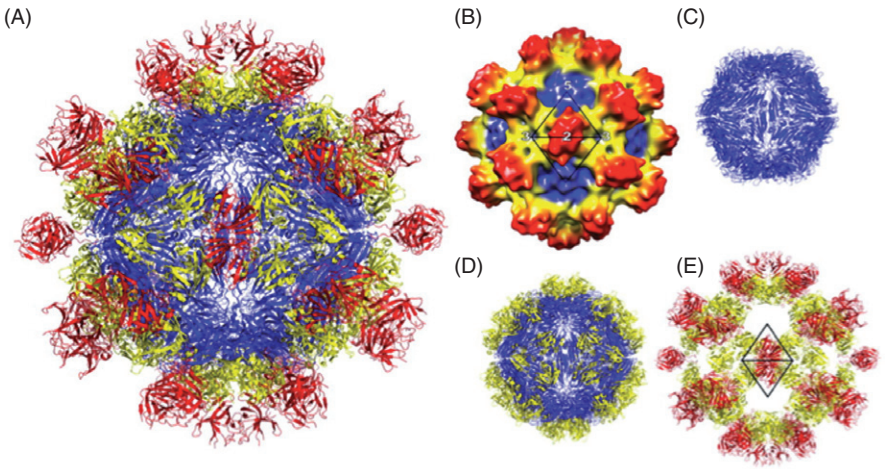

Figure 30.6 Structure of the hepatitis E virus (HEV)-like particle (viruslike particle [VLP]) ($T = 1$). (A) Crystal structure of an HEV VLP. The three domains, S, P1, and P2, are colored blue, yellow, and red, respectively. The VLP is positioned in a standard orientation with the three twofold icosahedral symmetry axes aligned along the vertical, horizontal, and viewing directions, respectively. (B) Cryo-EM reconstruction at 14 Å resolution. The surface is colored by radial depth cue from blue to yellow to red. (C) HEV VLP with only the S domain. (D) VLP with S and P1 domains. (E) HEV VLP with P1 and P2 domains. (Source: Guu TS, Liu Z, Ye Q *et al.* Proc Nati Acad Sci USA 2009;106:12992–7 [56] with permission from the National Academy of Sciences, USA). (Color plate 30.2)

domain contains neutralization epitopes and is involved in the binding of HEV virions to susceptible cells [57]. It has been demonstrated that dimerization of the E2s domain is essential for virus–host interaction, and the neutralizing antibody recognition site is located on the E2s domain [58]. Recently, a complex crystal structure of a monoclonal antibody (8C11 Fab) with an HEV genotype 1 E2s domain revealed that the Arg512 is a critical residue for the 8C11 Fab to neutralize HEV. The structures of HEV genotype 4 E2s and the E2s complex with 8C11 Fab revealed that the aa residue at position 497 of the Fab 8C11 epitope region of the E2s is distinct between HEV genotypes 1 and 4, and aa residue 497 appears to be important for HEV genotype recognition [59]. Molecular docking of the VLP crystal structure revealed that the Fab224 antibody covered three surface loops of a truncated capsid protein. Structural analyses of a chimeric HEV VLP containing an inserted B cell epitope tag of 11 aa that is fused to the C-terminus of the capsid protein revealed that the binding site of the Fab224 antibody is distinct from the location of the inserted B cell tag, thus suggesting that the $T = 1$ HEV VLP could be used to deliver foreign antigenic epitopes at the VLP surface [60].

The availability of the 3D virion structure of HEV will facilitate our understanding of the molecular mechanisms of HEV assembly and entry, and will also aid in the development of effective vaccines and antivirals against HEV in the future.

Conclusion and future prospects

Successful construction of HEV-infectious cDNA clones and replicons and identification of cell lines supporting a limited level of HEV replication provided the necessary tools to dissect the structural and functional relationship of HEV genes and to delineate the life cycle of HEV replication. The development of useful nonhuman primate, pig, and chicken animal model systems for HEV has afforded the opportunities to define the molecular basis of HEV replication and pathogenesis. The recent determination of the 3D crystal structure of the HEV capsid protein opens new avenues for structure-based HEV research in developing vaccines and antivirals and in understanding the molecular biology of HEV.

Despite the significant research progress made in the past decade, HEV is still an extremely understudied human pathogen. The life cycle of HEV remains largely unknown, and the mechanisms of HEV transcription, translation, and genome replication need to be delineated. Identification of a specific cellular receptor for HEV will help establish a more efficient cell culture system. Also, understanding the ecology and natural history of HEV will be important for effective prevention and control of this virus. There is also an urgent need to develop standardized serological and molecular diagnostic assays for HEV. Although the recently approved HEV vaccine in China and other experimental vaccines appear to be promising, it is important to evaluate the efficacy of the vaccines against the emerging genetically diversified strains of HEV, especially those animal strains with zoonotic potential.

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Chapter 31
Epidemiology, experimental models, and prevention: zoonotic aspects of hepatitis E

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Summary

Hepatitis E virus (HEV) is a positive-stranded RNA virus causing epidemic and sporadic acute hepatitis. It is now an established zoonotic agent with a global footprint. While the Old World HEV genotypes (genotypes 1 and 2) are associated with large waterborne anthroponotic epidemics, the more recent genotypes (3 and 4) are responsible for the zoonotic burden. HEV isolates from pigs, wild boars, and deer closely resemble autochthonous human strains of HEV and are found circulating rampantly all through the Western Hemisphere and parts of Asia, causing high seroepidemiological prevalence in their handlers and consumers. Animal experiments and cell culture systems have demonstrated the successful cross-species transmission of HEV. Chickens, rats, rabbits, mongoose, and cattle form an ever-increasing list of animals associated with HEV. With no available vaccine, changes in food habits, hygiene, and awareness and rigorous surveillance backed with reliable diagnosis comprise the only reprieve.

Introduction

HEV is a small, single, positive-stranded RNA virus that is classified in the *Hepeviridae* family and arguably the most frequent cause of acute viral hepatitis [1]. HEV remained for a large part of the twentieth century as an entity in the developing world, manifesting enteric non-A non-B hepatitis epidemics with an estimated disease incidence of 20.1 million cases per year caused by HEV genotypes 1 and 2 [2]. It is only with the emergence of genotype 3, isolated from autochthonous cases of hepatitis E in the United States [3] and simultaneously from domestic swine [4], that HEV showed signs of a global concern. HEV genotype 3 has ever since been recovered from humans sporadically and swine herds endemically throughout the Western Hemisphere and parts of Asia. An additional genotype, genotype 4, was recovered from humans in parts of Asia and subsequently from swine in the same regions [5]. The turn of the twenty-first century saw an increase in discoveries of animal strains of HEV from domestic pigs, wild boars, chickens, rabbits, rats, deer, mongoose, and possibly cattle and sheep and the existence of other animal species that are seropositive for HEV antibodies [6]. Seroepidemiological data show an ever-increasing global presence of HEV and a positive correlation between the seroreactivity of a population and its proximity to animals, raising public health concern for zoonotic infection by direct contacts with infected animals or through the consumption of contaminated animal meats. Several reports by now had demonstrated molecular associations between hepatitis E cases and eating raw or undercooked meat [7–8]. HEV is now a recognized zoonotic agent, and domestic pigs, wild boars, and likely other animal species are reservoirs of HEV. One prevailing view is that HEV may have evolved from being an animal pathogen to one causing zoonotic human infection [9]. Reports on experimental

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cross-species transmission in animal and cell culture models further establish the zoonotic potential of HEV [10–11]. Rabbits and chickens are proving to be the much wanted small-animal models for studying HEV pathogenesis [12–13]. All known genotypes of HEV share a common antigenic epitope resulting in a single serotype, which provides hope for the development of an effective vaccine. However, the complex epidemiology, disease pattern, and genetic and clinical diversity of HEV and the nonneutralizability of transfusion-associated HEV stand as challenges for any vaccine in the pipeline. Better diagnosis, surveillance, sanitary and food practices, and public awareness of the emerging trends in HEV remain to be the only preventive measures. This chapter is an effort to review the present complex epidemiological trends of zoonotic HEV and available experimental models to understand disease transmission and prevention.

General epidemiology and genetic heterogeneity

HEV genomes vary significantly and consist of at least four major mammalian genotypes (1–4) and one avian HEV. HEV epidemiology has a socioeconomic and genotypic basis, with the highest seroprevalence in countries affected with HEV genotypes 1 and 2 and poor water sanitation. Hyperendemic regions affected by genotype 1 include Southeast Asia, Central Asia, North Africa, and the Middle East with 20% to 45% of anti-HEV seroprevalence, while genotype 2 is restricted to Mexico and Nigeria. Genotype 1 and 2 strains are mainly associated with large waterborne anthraponotic epidemics and frequent sporadic acute hepatitis in these regions. Genotype 3 and 4 HEV strains are found in both human and other mammalian reservoirs (swine, wild boar, deer, and mongooses) and are mostly subclinical. Genotype 3 is found mainly in Europe, the United States, and Japan, and genotype 4 has been identified mainly in Asia (China, Japan, and India) with solitary cases reported in Germany and Spain. In Western countries, the seroprevalence within the general population is 1–3% in Europe and 2% in the United States. However, in these low-endemic areas, persons handling livestock and consumers of undercooked meat present with considerably higher seroprevalence than the general population. This is mainly due to the zoonotic transmission of HEV involving comestible animals in these regions infected with genotype 3 or 4. There are at least three genotypes of avian HEV worldwide represented by strains isolated from chickens in the United States, Australia, and Europe (Hungary and Spain) [14]. Avian HEV strains are very divergent with only 50–60% sequence identity with mammalian HEV strains, and therefore may be assigned a separate new genus distinct from the current genus *Hepevirus* [15]. The recently isolated rabbit HEV from China (Gansu and Beijing) and the United States (Virginia) most closely resembled HEV genotype 3 with approximately 78–82% sequence homology, suggesting that rabbit HEV may be a variant of genotype 3 [16–18]. Hepatitis E–like virus was identified in wild rats in Germany (Hamburg) and the United States (Los Angeles, California) with approximately 59.9% and 49.9% sequence identities, respectively, with known human and avian HEV, suggesting that rat HEV may represent an additional new HEV genotype within the *Hepevirus* genus [19–20]. Recently a novel strain of HEV was isolated and characterized from wild boar in Japan that was highly divergent from all known genotypes of HEV [21]. On the basis of nucleotide sequence analysis of 421 isolates, HEV has been further divided into 24 subtypes. Genotype 1 was more conserved and classified into five subtypes. The number of genotype 2 sequences was limited but can be classified into two subtypes. Genotypes 3 and 4 were extremely diverse and thus can be subdivided into 10 and seven subtypes, respectively, and designated as alphabetized subtypes (1a to 1e, 2a, 2b, 3a to 3j, and 4a to 4g) (Lu *et al.*, 2006).

Epidemiology: zoonotic aspect

Swine HEV

Zoonotic association of HEV was first reported when HEV-specific antibodies and RNA were detected in serum and stool samples of domestic swine in the Kathmandu valley of Nepal in 1995 [22]. However, the identity of the virus in the Nepalese pigs was not known as the virus was not sequenced. Following a serendipitous observation that the majority of adult pigs in the Midwestern United States were seropositive for HEV, a prospective study of swine herds from a commercial swine farm in Illinois helped in the identification of swine HEV in 1997, which was found to be closely related to, but distinct from, the human US HEV strains [4]. Swine HEV has now been detected in domestic pigs from nearly all major swine-producing countries of the world, both developing and industrialized, regardless of whether HEV is endemic in the respective human populations [15]. Apart from a single nonreproducible report claiming a genotype 1–like sequence isolated in a pig from Cambodia [23], at least two genotypes of swine HEV, genotypes 3 and 4, have been definitively identified and characterized from pigs worldwide (Figure 31.1). Although the mode of infection remains unclear, with high titers of the virus shed in the feces of infected pigs, it was primarily thought to be fecal-oral. However, experimental infection of naïve pigs with swine or human HEV via the oral route remains difficult [24]. Naïve pigs can become infected through direct

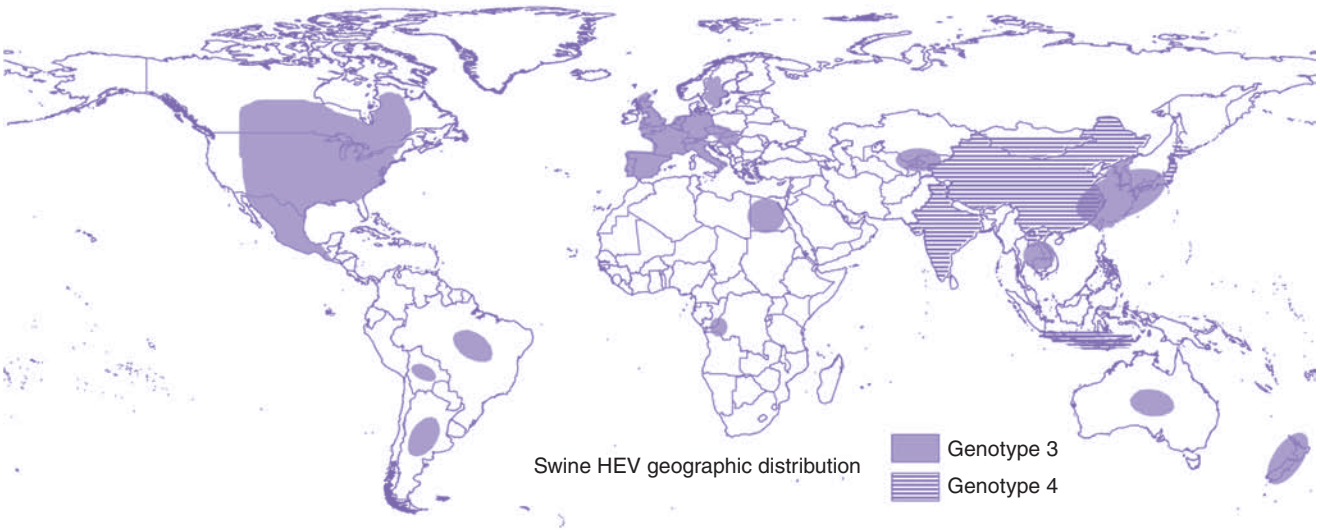

Figure 31.1 Distribution of HEV genotypes 3 and 4 in domestic swine (Source: Adapted, modified and updated from Purcell RH, Emerson SU. J Hepatol 2008; 48(3):494–503 [92]).

contact with infected ones [25]. Intravenous and intrahepatic modes of transmission are by far the most commonly used methods in experimental studies as they are more efficient than fecal-oral or contact exposure routes, with a limitation that they may not reflect the natural infection [26]. It was shown that the route of infection also influences the viral dissemination, when HEV was detected in urine after contact infection but not in intravenously inoculated pigs [25]. Swine HEV infection in pigs is subclinical as observed in both naturally infected pig herds and experimentally challenged pigs. No gross pathological lesions were detected in liver and other organs; some pigs, however, had mild to moderate multifocal and periportal lymphoplasmacytic hepatitis with mild focal hepatocellular necrosis [4, 27].

There is considerable information on the seroprevalence of HEV antibodies in serum derived from pigs, as detected by enzyme-linked immunosorbent assay (ELISA) and/or detection of viral RNA in serum, feces, slurry, or commercial liver from grocery store as identified by RT–PCR, in almost all major swine-producing countries, whether endemic or non endemic for HEV. Swine HEV has been majorly detected in China, Europe, the United States, Canada, Japan, Taiwan, Indonesia, Korea, South America, India, Australia, New Zealand, and Africa [6, 28]. All serological studies show a wide distribution of HEV in swine herds [28]. At the individual level, in market-age swine, the average seroprevalence varied greatly from study to study, from herd to herd, and from region to region: 56% of HEV-positive pigs in Japan, 23% in Argentina, 81% in Brazil, 51% in Laos, 68% in India (West India), 38% in Korea, 95% in the United States, 58% in Canada, 60% in China, and 100% in Spain [15, 28]. Anti-HEV prevalence in pigs is age dependent. Swine HEV generally infects pigs of 2–4 months of age. The infected pigs generally have a transient viremia lasting for 1–2 weeks, and fecal virus shedding for about 3–7 weeks [4, 29]. Most pigs younger than 2 months of age were seronegative, whereas the majority of pigs over 3 months of age were seropositive. Sows with high anti-HEV titers can passively transfer anti-HEV to their piglets, and thus some piglets are born seropositive. Maternal antibody usually waned by 8–9 weeks of age, at which point most piglets acquired infection. Swine HEV RNA is frequently detected in the sera and feces from pigs of 2–4 months of age, the period in which active swine HEV infections occur. Most adult pigs, although positive for IgG anti-HEV, are free of swine HEV RNA. Several reports describe the zoonotic potential of swine HEV, with detection of high seroprevalence among pig handlers and veterinarians and detection of HEV in retail pork, slaughterhouse wastes, and undercooked meat [28].

HEV from wild boars and deer

The discovery of swine HEV has led to the study of HEV in wild boars (*Sus scrofa*), which are indigenous in many countries and are hunted and consumed often raw or undercooked. HEV antibodies have been first detected in 15 out of 59 (25%) of wild-caught pigs in Australia [30]. However, the first reports confirming the role of wild boars in the zoonotic spread of HEV came from Japan. In one such study, patients hospitalized with

severe hepatitis were related to the ingestion of raw liver from wild boar [31]. In another study, a full-length genomic sequence of HEV amplified from a wild boar shared 99.7% nucleotide sequence identity with a virus isolated from a wild deer hunted in the same forest and with four human hepatitis E patients who consumed deer meats [8, 32]. These studies have established a clear foodborne zoonotic mode of transmission for sporadic hepatitis E in industrialized countries. The culinary habits in Japan of eating raw or undercooked wild boar and deer meat have lead to increased reports of zoonotic HEV associated with these animals. A high seroprevalence for HEV in Okinawa (Japan) wild boar hunters (25.3%) was shown as compared to the normal population in the same area (4.1–7.7%) [33]. The HEV seropositivity in wild boars varied from 4.5% to 25.5%, and the HEV RNA detection rate ranged from 1.1% to 3.6% in wild boars of different geographic regions in Japan. Several studies reported the presence of HEV in wild boars in European countries including Germany, Italy, Spain, the Netherlands, France, Sweden, and Hungary [34–37]. The HEV seropositivity in wild boars in these European countries varied from 12.0% to 42.7%, and the HEV RNA detection rate ranged from 2.5% to 25%. Most strains of HEV recovered from wild boars worldwide belong to genotype 3 and were genetically very closely related to human and swine strains described in the same geographic regions, thus suggesting possible interspecies transmission. However, recently, novel HEV sequences belonging to genotype 4 and to a new unrecognized HEV genotype [21] have also been detected from wild boars. IgG anti-HEV was detected in approximately 3% of the sika deer and 34.8% of Yezo deer samples in Japan [38–39] and 5% of red deer in the Netherlands [37]. The full-length genomic sequence of a strain of HEV was determined from a sika deer in Japan, and sequence analysis revealed that the deer HEV belongs to genotype 3 [32]. Genotype 3 strains of HEV were also identified genetically from roe deer in Hungary [40–41]. In these studies, reporting on divergent prevalence, sample sizes were generally small and of various origins such as the liver, sera, bile, and feces. Unlike swine HEV, there were limited data on the age of the animals tested, which makes it difficult to evaluate the real infection level of boar and deer HEV.

Avian HEV from chickens

HEV-like virus affecting chickens was first identified in Australia in 1999 as a big liver and spleen disease (BLS)–causing agent. Sequence analysis of a short 523 bp amplicon from infected chicken showed 62% identity to the helicase region of human HEV, and subsequently the virus was named BLS virus (BLSV) [42]. The BLS disease has been recognized in Australia since 1980, mainly affecting commercial broiler breeder flocks and causing decreased egg production and slight increase in mortality. Affected birds have hepatomegaly and splenomegaly, and histological lesions are characterized by a period of lymphoproliferation followed by a period of lymphoid destruction that coincides with clinical signs. A similar disease called hepatitis splenomegaly (HS) syndrome affecting broilers and layers has been observed across the United States and Canada since 1991. In 2001, the etiological agent causing HS syndrome was isolated and characterized from bile samples of affected chicken, and was named avian HEV due to its physical, genetic, and antigenic relatedness with human HEV [43]. In a follow-up field study, 11 additional avian HEV isolates from the United States were characterized and found to have 78–100% nucleotide sequence identities with each other, 79–88% identities with the prototype avian HEV, 76–80% identities with BLSV, and 56–61% identities with other known strains of human and swine HEV. In a later analysis, avian HEV was found to share only 50% nucleotide sequence identity over the complete genome with human and swine HEV strains [44]. The data from this study indicated that, like swine and human HEVs, avian HEV isolates are genetically heterogenic [45]. Avian HEV is detected in chicken farms of the United States, Canada, Spain, Hungary, Australia, and China. At present, three distinct avian HEV isolates are recognized, those from the United States, Europe, and Australia. The recent Chinese isolate shared 98% sequence identity with the European isolate. In the United States, avian HEV infection is enzootic in chicken flocks, and approximately 71% chicken flocks and 30% chickens were seropositive for avian HEV antibodies. It appears to be age dependent: approximately 17% of young chickens and 36% of adult chickens were positive for avian HEV antibodies [6]. In a study involving 29 flocks from Spain, 26 (89.7%) were found to have at least one seropositive case, and the proportion of positive animals ranged from 20 to 80% [46]. Similar age prevalence was shown in these flocks, with chicken above 40 weeks of age being more seroreactive. Avian HEV is mostly subclinical, and even healthy flocks were found to be seroreactive; in one prospective study, 14 seronegative chicken, monitored over 19 weeks, were found seropositive by 21 weeks without any signs of HS syndrome. It appears that avian HEV is readily transmitted within and between chicken flocks. Avian HEV infection has been experimentally reproduced via oronasal route of inoculation in chicken [47], and thus the route of transmission is presumably fecal-oral, although other routes of transmission cannot be ruled out. Chickens with HS syndrome display above-normal mortality in broiler breeder hens and laying hens of 30–72 weeks of age, with the highest incidence occurring between 40 and 50 weeks of age. In addition, in some cases, egg production

was reported to be dropped by 20%. Weekly mortality increases to approximately 0.3% and may sometimes exceed 1.0%. Similar to HS syndrome, the clinical signs for BLS in Australia also vary from mostly subclinical infection to egg drops that may reach 20%, with up to 1% mortality per week over a period of 3–4 weeks. Dead chickens associated with avian HEV infection usually have regressive ovaries, red fluid in the abdomen, and enlarged liver and spleen. Microscopically, liver lesions varied from multifocal hemorrhage to extensive areas of necrosis and hemorrhage and infiltration of heterophils and mononuclear inflammatory cells around the portal triad. In chickens experimentally infected by avian HEV, subcapsular hemorrhages and enlarged livers were observed in approximately one-fourth of the infected chickens [47]. The microscopic hepatitis lesions in experimentally infected chickens are characterized by lymphocytic periphlebitis and phlebitis in the livers.

Rat HEV

The first evidence of HEV in rodents came from a study of hepatitis E outbreak in Russia, where immuno electron microscope–positive HEV-like particles were detected in five of the 23 rodents caught from the affected area [48]. In the same study, seven white mice experimentally transmitted with human HEV seroconverted and shed virus in their feces 4 to 23 days post inoculation (dpi). In a prospective study from Nepal, experimental transmission of HEV was observed with three of the 27 challenged Wistar rats showing virus in feces 7 dpi, and in serum from 4 to 35 dpi [49]. Several seroepidemiological studies conducted on wild-caught rats in the United States showed high seroprevalence, with 90% rats from Hawaii, 73.5–77% rats from Maryland, 44% rats from Louisiana, and 80% rats from Los Angeles being seropositive for HEV [50]. Antibodies to HEV have been detected in various species of rats both commensal and noncommensal, including Norway (*Rattus norvegicus*), black (*Rattus rattus*), and cotton (*Sigmodon hispidus*) rats, and bandicoots, with the highest prevalence of antibodies found in the genus *Rattus*. Several other studies recorded the prevalence of anti-HEV antibodies including those from India showing 2.1 to 21.5% prevalence, Nepal showing 12% prevalence, Japan showing 31.5% prevalence among Norway rats and 13.3% prevalence among black rats, and Vietnam showing 20.9% prevalence [51–53]. In a study from Nepal, 9% of the 44 rats analyzed were shown to be viremic and sequence analysis of a 405 bp amplicon showed 96% homology with HEV genotype 1 sequences endemic in Nepal. However, no independent confirmation of this study exists, and efforts to establish genotype 1 or 2 infections in laboratory rats failed in the United States. By using a nested broad-spectrum RT-PCR, two authentic HEV sequences were amplified from fecal samples of wild Norway rats (*Rattus norvegicus*) from Hamburg, Germany. The rat HEV shared only approximately 60% and 50% sequence identity with human HEV and avian HEV, respectively. Subsequently, the complete genomic sequences of two strains of rat HEV have been determined, and sequence and phylogenetic analyses revealed that the rat HEV belongs to a previously unrecognized genotype [54]. In a recent study, two strains of HEV were isolated from urban rats of Los Angeles homologous to the rat HEV isolate from Germany [19]. Attempts to transmit the virus to rhesus monkeys failed; however, spotty transmission was observed in laboratory rats. In a recent study, the HEV genome was detected in 17.9% of the 56 Norway rats caught near a pig farm in Hokkaido, Japan [55]. A partially amplified ORF1 gene resulted in partially sequenced HEV belonging to genotype 3, which was genetically identical to the HEV prevalent in the swine from the source farm. The results indicate that these animals might be possible carriers of swine HEV in endemic regions. It remains to be determined if the rat HEV can cross the species barrier and infect humans or other animal species.

Rabbit HEV

Unique strains of HEV were recently identified from rabbits in Beijing, in Virginia (United States), and earlier from Gansu Province in China [16–18]. Full-length genomic sequences of these rabbit HEV were determined and found to cluster as a separate branch within HEV genotype 3. The first reports of rabbit HEV came from Gansu Province, in a study that reported 57% seroreactive serum samples from 335 rabbits and 7.5% were RT-PCR positive. Two representative full-length sequences were determined and were found to share 85% sequence identity with each other and 74%, 73%, 78–79%, 74–75%, and 46–47% nucleotide sequence identity to mammalian HEV genotypes 1, 2, 3, and 4 and avian HEV, respectively [17]. In a separate study from Beijing, approximately 55% (65/119) of the farmed rex rabbits were tested positive for anti-HEV antibody, with approximately 7% of fecal samples (8/115) also positive for HEV RNA. Sequence analysis of all eight amplicons showed 98.3–100% nucleotide homology with each other and had identities of 75.8–78.6%, 73.9–75.0%, 77.5–81.0%, 74.2–78.6%, and 54.8–57.6% with the corresponding regions of genotypes 1–4 and avian HEV, respectively [16]. HEV RNA and antibodies against HEV were detected in various breeds of rabbits from two farms in Virginia, United States [18]. The seroprevalence was higher in a farm where rabbits were caged in groups of 2–9 than in the other farm where rabbits were caged alone, reflecting differences in farming practices. The

overall seroprevalence of 36% among rabbits from the United States was lower than that among rabbits from China. Prevalence of HEV RNA in serum and fecal samples from rabbit farms in the United States (15 to 16% respectively) was higher than from farms in China. Sequence analysis showed the HEV isolate from the United States is different genetically from rabbit strains of HEV from China but is closely related and hence is grouped in genotype 3. Since the rabbit HEV belongs to genotype 3, it is possible that it may be zoonotic. In a prospective study, six of the nine rabbits inoculated with genotype 1 HEV and nine of the nine rabbits inoculated with genotype 4 HEV have seroconverted, but only two of the nine genotype 4–infected rabbits developed hepatitis, and none of the rabbits from the genotype 1 group developed hepatitis [56]. Sequence analysis identified 20 of the 25 determinants found in zoonotic strains in rabbit sequences, further suggesting a potential possibility of cross-species transmission of rabbit HEV. However, additional studies are warranted to determine the host range and species tropism of the rabbit HEV.

Mongoose HEV

Of the 184 mongoose specimens (Okinawa, Japan) analyzed in two separate studies, 8–21% were seropositive for IgG anti-HEV antibodies, much lower than that in pigs (58%), wild rats (44–94%), or wild boars (44%) in Japan [57–58]. Viremia was thought to be short-lived with the absence of HEV RNA even in IgM-positive mongoose. However, one animal was shown to be definitely positive for HEV RNA, and the full-length genomic sequence of this isolate was determined and shown to group with genotype 3 HEV, which was closely related to a genotype 3 swine HEV from Japan [58].

HEV prevalence in other animals

Antibodies to HEV have been detected from 4.4% to 6.9% of cattle in India [51], 4% to 30% of cattle and 9% to 12% of sheep in China [59–62], 2% of sheep in Spain, 3% of bison, and 15% of cattle in the United States [63]. Recently, a 189 bp ORF2 sequence of HEV was amplified from the fecal samples of eight cows and six sheep in China, and the eight HEV sequences from cows share 96–100% nucleotide sequence identity with each other, and 76–86%, 82–84%, 79–85%, an 84–96% nucleotide sequence identity with genotypes 1, 2, 3, and 4 of the mammalian strains of HEV, respectively. Similarly, six short HEV sequences from sheep are 99–100% identical to each other but share only 79–85%, 81–83%, 79–84%, and 85–95% nucleotide sequence identity to genotypes 1, 2, 3, and 4 of the mammalian HEV strains, respectively. Based on the available short sequence, it appears that the bovine and sheep HEV belongs to genotype 4 [64–65]. Anti-HEV antibodies were detected in 13% of the 200 studied work horses from Cairo, and 4% of the animals aged below a year were viremic. Sequence analysis showed 78% to 100% identity with human and animal strains of Egypt [66]. Serological evidence of HEV infection has also been reported in a number of other animal species including dogs, cats, goats, and rhesus monkeys [6]. However, HEV could not be recovered or sequenced from these animal species.

Experimental models

Animal models

Until recently, due to lack of an *in vitro* cell culture for HEV, experimental animals were preferred as alternate models for the study of HEV replication and pathogenesis. Initial nonhuman primate models, including tamarins (*Sanguinus mystax*) and cynomolgus monkeys (*Macaca fascicularis*) [67], rhesus monkeys (*Macaca mulata*) [68], and chimpanzees (*Pan troglodytes*) [69], helped in the discovery and characterization of HEV. The prospect of possible zoonosis for HEV came from an experimental infection of domestic pigs [70]. The discovery of swine HEV immediately followed with experimental cross-species transmissions of swine HEV in nonhuman primate models (rhesus macaques and chimpanzees) and human HEV in swine models [10]. Productive infections resulted in both cases, with similar biochemical, immunological, and histopathological changes including slight elevation of serum liver enzymes, fecal excretion, and seroconversion were observed, validating the genetic relatedness of swine and human HEV genotypes 3 and 4 [10]. However, human HEV genotypes 1 and 2 failed to establish any infection in the swine model [26]. The above models proved limited clinical interest for viral pathogenesis studies; as was the case with earlier human HEV animal models, they remained asymptomatic. A very moderate increase in liver enzyme activities and minor hepatic-pathological lesions were occasionally observed. Experimental infection of pregnant gilts with swine HEV genotype 3 did not lead to any fulminant hepatitis, abortion, or vertical transmission [71]. However, these models helped in demonstrating cross-species infections and extrahepatic sites of HEV replication in swine colon, lymph nodes (mesenteric and hepatic), and spleen [72]. The discovery of avian HEV led to the use of specific-pathogen-free chickens to study the avian strains of HEV and the associated HS syndrome. And it has been demonstrated that chickens can serve as a good model for the study of avian HEV replication and pathogenesis [47]. Unlike swine and nonhuman primate models, chickens can be readily infected with avian HEV via the natural oral route [47]. Avian HEV detected

in chicken egg white was found to be infectious, although no virus was detected in samples collected from the hatched chicks [73]. The host range of avian HEV appears to be limited. Although the avian HEV can infect turkeys [13], attempts to infect pigs and rhesus monkeys with avian HEV were unsuccessful1 [44]. Attempts to develop small-animal models for HEV met with limited success. Wistar rats experimentally inoculated with a strain of human HEV became infected [49]. It was also reported that BALB/c nude mice are susceptible to experimental infection with a genotype 4 strain of swine HEV [74]. More recently, it has been reported that rabbits can be experimentally infected with genotype 4 HEV [12]. However, transmission in small-animal models was not robust and was often spotty and nonreproducible.

In vitro cell culture

Early studies reported the propagation of hepatitis E virus (HEV) in either primary hepatocytes or several established cell lines, but replication was inefficient [75]. However, PLC/PRF/5, A549, HepG2/C3A cells, and swine cell lines like IBRS-2 were recently shown to be useful for propagating HEV. Successful culture using A549, PLC/PRF/5, and IBRS-2 cell lines were established for swine and boar HEV [11, 76]. Similar to human HEV cultures in these cell lines, establishment of culture depended on the load of the inoculum, with a minimum titer of not less than 1.8×10^4 copies per well [11]. In a recent study to identify human, pig, and deer cell lines permissive for genotype 1 and 3 HEV, it was found that genotype 3 virus infected swine cells more efficiently than human cells [77]. These culture models would be useful for expanding research on cross-species transmissions and as a tool for analyzing infectious titers of HEV in various biological and environmental samples.

Prevention

Except for the few clinical studies showing the use of ribavirin for the treatment of chronic cases and solid-organ recipients infected with HEV, there is no specific clinical regimen or immunoglobulin preparations for altering the course of acute hepatitis E infection [78–79], and the treatment currently is supportive. The use of other antivirals such as ribozymes and small hairpin RNA (shRNA) remained experimental both *in vitro* and in piglets [80–82].

Prevention remains the most effective approach against the disease. The development of serum antibodies in natural infection and the presence of a single serotype for all known genotypes of HEV are some favorable facts that suggest the feasibility of a HEV vaccine. The capsid protein encoded by ORF2 is highly conserved, highly immunogenic, and broadly neutralizing. Furthermore, it is a major target of cell-mediated immunity [83]. Thus the capsid antigen is the preferred protein for vaccine development. A 32 kDa recombinant ORF2 capsid protein of avian HEV expressed in *Escherichia coli* was found to confer immunity in the tested chickens [84]. Recent studies using swine and human HEV showed cross-genotype and cross-host-species protection in experimental pigs and rhesus monkeys, providing further hope for a broadly specific HEV vaccine [85–86].

Thus far, two HEV genotype 1 pORF2-based vaccines, the baculovirus-expressed 56 kDa protein and the *E. coli*–expressed protein designated HEV 239, have progressed to phases II and III of clinical trials, respectively, and were shown to be effective in preventing clinical disease caused by infection with HEV [87]. However, the exclusion of children, pregnant women, and chronic liver patients in these trials leads to questions regarding the safety and efficacy of the vaccines in these HEV-vulnerable groups. These vaccines should also have been tested for their efficacy in animals like pigs. Other important questions include the efficacy of these vaccines when used in the immediate post-exposure situation in preventing and controlling transmission during an outbreak, the length of vaccine-induced immunity, and when to vaccinate. Reports about the nonneutralizability of bloodborne HEV also question the efficacy of ORF2-based vaccines [88].

In the absence of any available vaccine, the most significant influence on HEV prevention is to control the source of infection. As a fecal-orally transmitted disease, contaminated water or water supplies are the main sources of HEV infections [89]. Many outbreaks in epidemic-hit countries have occurred due to leakage of sewage pipes into the municipal water supply. A barrier between these two supplies is essential for long-term prevention. Chlorination and filtration systems are generally inadequate if the source water is heavily contaminated [90]. Travelers to regions of endemic infection must maintain vigilance about the risks of contaminated water, ice, and food. Only boiled or bottled water should be consumed. This is especially true for individuals who have been vaccinated against hepatitis A and B and may have a false sense of security with respect to all forms of hepatitis. Particular information and surveillance must be taken with populations at higher risk of fulminant hepatitis such as pregnant women and people with underlying liver conditions. Women should avoid unnecessary travel to areas of endemic infection during pregnancy.

In industrialized countries, where zoonotic transmission may be the principal route of hepatitis E infection, studies measuring viral load and frequency of occurrence of HEV in pork at slaughterhouses and retail

outlets are needed. A critical load profile for transmission of HEV by various routes using laboratory primate models should be established. These findings would improve understanding of seropositivity to HEV in various exposed and unexposed groups, and the minimum viral load that is necessary for seroconversion or clinical disease [28]. Long-term studies, investigating the association between exposure to animals and anti-HEV IgG seroprevalence and clinical disease as outcomes, including genotyping of a larger number of isolates recovered from HEV IgM–positive individuals, are recommended [28]. Additionally, a critical review of the performance of ELISA tests, as a first step to developing assays with improved performance in assessing human exposure to HEV, is required to establish the specificity of the association between human seropositivity and exposure to swine or pork [28]. Case–control studies are recommended to further define risk factors such as age, gender, occupation, and level of pork consumption and to formulate relevant hypotheses for additional field and experimental research. The resulting information would allow the implementation of a formal risk assessment model with the main aims of quantifying the risk to public health posed by zoonotic HEV via human exposure to swine or pork, and evaluating potential control options [28].

Until these research data are available for formulating health policies, public awareness remains the most important strategy for preventing foodborne diseases like HEV. Governments should develop effective risk communication and knowledge transfer messages for the public, specifically for populations at risk. The key message is to avoid eating raw or inadequately cooked pork, particularly raw pork liver. HEV was shown to be viable even after heating at 56 °C for 1 hour [91]. Populations at risk such as pregnant women, transplant recipients, and individuals with underlying liver conditions should be informed on the possible risk of consuming raw pork, deer, or wild boar meat or having contact with infected pigs. Proper cooking of pork containing HEV would render it safe to eat. Information regarding safe food habits and cooking procedures for pork, poultry, and seafoods is provided by agencies like the US Food and Drug Administration (FDA) and the World Health Organization (WHO); however, the same information must be made available on the product at the retail level. Along the same line, pig handlers and veterinarians who are exposed to HEV must take hygienic measures after handling the animals. In swine, since HEV seems very contagious between animals, further investigation must be performed to determine if farming procedures would limit HEV dissemination. Commercial importation of swine is a very common practice, and a better surveillance strategy should be developed. Recently, a genotype 4 HEV was isolated in swine for the first time in Europe. Surveillance of swine, with wild boar and deer reservoirs, should be performed until all the routes of human exposure to HEV are identified. The widespread prevalence of HEV infection in different populations worldwide warrants an expansion of epidemiologic and intervention studies, and a robust, broadly specific hepatitis E vaccine is the need of the hour.

Final note: Regarding bat hepevirus, a recent study that analyzed 3869 bat specimens from 85 different species and five continents has detected HEV in African, Central American, and European bats [93]. Sequence analysis shows that bat hepevirus constitutes a distinct genus within the family *Hepeviridae*.

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Chapter 32
Clinical and pathological features, and diagnosis

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Summary

Hepatitis E virus (HEV) is a common infection worldwide resulting in significant morbidity. It typically results in a self-limiting illness characterized by the development of acute hepatitis, and the treatment is usually conservative. Overall, mortality due to HEV infection is low and arises from occasional cases of acute liver failure or in cases with preexisting liver disease. In pregnant women in developing countries, however, it is a serious infection with a high mortality. Recent evidence has suggested that chronicity can occur in the immunosuppressed and that this may lead to progressive liver injury and possible cirrhosis. Extrahepatic manifestations have also been described. There is evidence that treatment may be of benefit in the immunosuppressed and those with extrahepatic manifestations, although further research is required. HEV is increasingly being recognized in developed countries, and it should be considered as a cause in anyone presenting with hepatitis.

Introduction

HEV is a small, positive-sense, single-stranded, icosahedral, non-enveloped RNA virus member of the genus *Hepevirus* in the *Hepeviridae* family. Clinically and epidemiologically, HEV is similar to hepatitis A, and before diagnostic tests were developed it was termed "enterically transmitted non-A, non-B hepatitis" (ET-NANB). HEV was first recognized as a distinct entity in the 1980s, when sera from persons affected during large waterborne epidemics of acute hepatitis in Delhi and Kashmir, India, from the 1950s tested negative for serological markers of acute hepatitis A and B [1–3].

The hepatitis E genome was cloned in the 1990s [4], and reliable diagnostic tests have subsequently become much more widely available [5]. It is predominantly enterically transmitted via the fecal-oral route. Zoonotic transfer between animals (pigs, deer, and wild boar) and humans has also been shown [6, 7]. Hepatitis E is endemic in many tropical and subtropical countries (including India, China, Southeast and Central Asia, and Northern and Western Africa) and has resulted in multiple large epidemics of hepatitis E infection. Contaminated drinking water has been identified as the source of hepatitis E infection in the majority of these outbreaks, and hepatitis E infection may occur in both epidemic and sporadic forms in these endemic areas [8]. It has also been described in sporadic form in non-endemic countries, where hepatitis E infection is secondary to travel to endemic areas, or through zoonotic transfer. Typical hepatitis E infection results in a self-limiting illness of varying severity, with infected patients developing an illness that can vary from a severe hepatitis with jaundice to an asymptomatic anicteric illness.

Recently, evidence has started to emerge of extrahepatic manifestations of hepatitis E and also chronic infection with persistent viremia, usually in the context of immunosuppression. Treatment for patients with

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Table 32.1 Common symptoms associated with acute HEV infection.

Symptoms and signs	Frequency
Jaundice	90–100%
Nausea and vomiting	30–100%
Fever and sweats	25–95%
Abdominal pain	40–80%
Diarrhea	30–60%
Reduced appetite and weight loss	50%
Myalgia and arthralgia	30%
Pruritis	20%

viral persistence and also those with acute infection is being explored, and there is ongoing work with regard to developing an effective vaccine.

Clinical features

Acute hepatitis E

The acute illness caused by HEV infection is similar to that of other hepatotropic viruses [9] with a wide variety of possible symptoms, the most common of which are listed in Table 32.1.

In both endemic and non-endemic areas, acute HEV infection is associated with an illness characterized by malaise, significant liver enzyme elevation, and jaundice. It usually follows a self-limiting course and rarely results in acute liver failure. The severity of liver injury is variable, with anicteric hepatitis (biochemical abnormality only) and asymptomatic infection both described. The exact frequency of these milder presentations is not known, but is likely to exceed that of acute icteric illness. Those with preexisting liver disease are at high risk of severe illness, and hepatic decompensation is well described as a result of HEV infection [10]. While resulting in a similar clinical picture, different patterns of illness are seen between endemic and non-endemic areas. Outbreaks in endemic areas are associated with contamination of water sources, are more commonly associated with genotype 1 infection, infect those who are younger (10–40 years) [1, 2, 11], and there is a predilection for severe disease in pregnant women. Acute HEV arising in non-endemic areas is mainly due to travel to an endemic area or from zoonotic transfer; it is typically caused by genotype 3 or 4, affects older males (50–65 years) [12, 13] has a higher frequency of symptomatic presentation in those with preexisting liver disease [10, 14], and there is no predilection for pregnant women. In both endemic and non-endemic areas, men are more likely to be affected than women [8, 10, 14–18].

The incubation period of hepatitis E infection has ranged from 2 to 10 weeks in waterborne outbreaks following a short, well-defined period of water contamination [19, 20], Incubation is followed by a prodromal phase (1–27 days) with nonspecific malaise. The prodromal phase usually starts with nonspecific symptoms, including a flulike illness with nausea, vomiting, fever, abdominal pain, diarrhea or constipation, dysgeusia, and anorexia. This prodrome is then followed by an icteric phase, which is characterized by the development of jaundice, settling of fever, and return of appetite.

Hepatomegaly during this phase is common, with splenomegaly and transient lymphadenopathy described less frequently. Cholestatic symptoms with pale stools and pruritis can also be present in approximately 20% of cases [1, 10]. Laboratory abnormalities include a predominantly conjugated hyperbilirubinemia and an acute rise in both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (often up to 50 times the upper limit of normal) [21]. There may also be biochemical evidence of cholestasis with a raised alkaline phosphatase (ALP). These abnormalities gradually settle back to normal, typically over a 6-week period of resolution [10]. In a few cases, prolonged cholestasis has been described with an ongoing raised ALP, bilirubin, and pruritis. This usually follows a benign course and spontaneously settles back to normal over several months [10].

Mortality from patients hospitalized with hepatitis E infection ranges between 0.4% and 4.0% in endemic areas. [22] Data collected from population surveys during outbreaks of hepatitis E suggest much lower mortality, with rates in the region of 0.07–0.60%. The reported mortality rates appear to be higher in non-endemic areas, where mortality is up to 8–11% [23], possibly as a result of HEV infection occurring in those with preexisting liver disease [14]. This figure may well be an overestimate, though, due to underdiagnosis in milder cases in areas where HEV is less common and not tested for routinely. Mortality in pregnant women can be up to 20–25% in the third trimester [24].

Acute liver failure secondary to acute HEV

Acute liver failure secondary to massive liver necrosis occurs in a small subset of patients. HEV infection is the most common cause of acute liver failure in India, Pakistan, China, and Southeast Asia [25, 26], but it is rarely seen in non-endemic countries [27]. Patients with acute liver failure present with jaundice and progress to encephalopathy and coagulopathy, and mortality in this group is high [28]. Acute liver failure secondary to HEV infection typically results in a hyperacute pattern of

liver failure, although a sub-acute course has also been described [29, 30].

Acute HEV in pregnancy

Acute viral hepatitis in pregnancy has characteristically been associated with hepatitis E infection in endemic areas, where, during outbreaks, there is a higher incidence and severity of infection. In several studies of hepatitis E infection in pregnant women, no statistically significant difference was found between the rate of infection in the first, second, and third trimesters [1, 22, 31]. Mortality, however, rises during the second trimester and is greatest in the third trimester (20–25%) [24], when the risk of acute liver failure is highest [32]. Acute liver failure from HEV in pregnancy has a very short pre-encephalopathic period, with cerebral edema and disseminated intravascular coagulation (DIC) occurring more often. This may represent a severe manifestation of a Schwartzmann-like reaction [31].

The outcome of acute liver failure appears to be little different between pregnant women with hepatitis E infection and those with other causes of severe liver injury [33].

In a study of 220 consecutive pregnant women with acute hepatitis in a hospital in New Delhi, Patra *et al.* [30] found that acute liver failure was more common and that maternal mortality was increased (relative risk 2.7 and 6.0, respectively) in HEV-infected women compared with those who had hepatitis due to another etiology.

Vertical transmission from mother to fetus occurs readily and results in significant disease in the newborn. [32] Hepatitis E infection in pregnant women is also associated with premature delivery, low birth weight, and increased perinatal mortality (relative risk 1.2 for premature delivery, 1.9 for intra-uterine fetal death, and 1.8 for stillbirth). There is also a higher risk of antepartum hemorrhage (relative risk 4.1) [30].

Differences in the mortality rate secondary to HEV infection in pregnancy have been reported within endemic areas (e.g., northern India compared to southern India and Egypt). This may be explained by differences in viral genotype subtypes [34].

Studies from non-endemic areas show that acute viral hepatitis has no special predilection for pregnant women and follows a very similar course to that of nonpregnant women [35, 36].

The reason for severe liver injury in pregnancy secondary to hepatitis E infection remains unclear. Some recent evidence suggests that downregulation of the p65 component of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) in pregnancy may lead to more severe liver damage [37]. Other possible explanations include dysregulation of T cell responses in pregnancy [38–41] or associated malnutrition and folate deficiency [42].

Acute HEV in those with pre-existing liver disease

Patients with underlying chronic liver disease from any cause are at greater risk of developing severe decompensation as a result of infectious agents that would usually cause self-limiting illness in healthy individuals. HEV infection has been shown to be responsible for severe decompensation of chronic liver disease in this group with increased morbidity and mortality [12, 25, 43–45].

Acute HEV in cases with possible drug-induced liver injury

Drug-induced liver injury is common and accounts for approximately 3% of all hospital admissions due to jaundice [46, 47]. The diagnosis of drug-induced liver injury is based on the temporal relationship between the onset of drug therapy and the development of biochemical evidence of liver injury, improvement of liver injury on removal of the drug, worsening of biochemical liver injury on re-introduction of the drug, and the exclusion of an alternative diagnosis [48]. In two cohorts of British and American patients with suspected drug-induced liver injury, a retrospective analysis of samples revealed that a small but significant (21–22% in a British cohort and 16% anti-HEV IgG-positive and 3% anti-HEV IgM-positive in an American cohort) proportion of people diagnosed with drug-induced liver injury actually had HEV infection as the primary cause of their liver injury. Serologic testing for HEV should therefore be performed in patients with suspected drug-induced liver injury, especially if the clinical features are consistent with acute viral hepatitis [48, 49].

Chronic HEV infection

Until recently, HEV infection was thought to always follow a self-limiting course with spontaneous clearance. Chronic HEV infection with persistence of HEV RNA for over 6 months has now been described in the context of immunosuppression from solid-organ transplantation [50], chemotherapy, hematological malignancy, or HIV infection [51, 52]. Most reports of chronic HEV have been in solid-organ transplant recipients.

The description of chronic HEV infection was based on persistently elevated aminotransferase levels, histological features consistent with chronic viral hepatitis, detectable HEV RNA in serum, and the absence of other viral causes [50]. Although the biochemical and histological features in chronic HEV infection are typically

mild [53], progressive liver injury is possible, and a case of chronic HEV infection progressing to cirrhosis in a renal transplant recipient has been described [54]. The clinical and pathological course of HEV persistence in liver transplant recipients also suggests that these patients are at risk of progressive fibrosis and ultimately the development of cirrhosis if HEV is not cleared [54, 55].

To date, all cases where chronic HEV infection has been described have been due to genotype 3.

Extrahepatic manifestations

Several extrahepatic or nonhepatic features of hepatitis E infection have been described. These include acute pancreatitis and neurological, hematological, and autoimmune manifestations.

Acute pancreatitis

There have been several case reports of acute pancreatitis secondary to hepatitis E infection. All cases described thus far have been reported as genotype 1. The majority of cases described have been from the Indian subcontinent, and almost all cases were male. Acute pancreatitis occurred in the second or third week after the onset of jaundice and was characterized by typical symptoms of abdominal pain and serum amylase rise. In most cases, pancreatitis resolved spontaneously with conservative management. The underlying pathophysiology remains unclear at present, and the prognosis is linked to the severity of the liver disease [56–59].

Neurological manifestations

Case reports have described several neurological manifestations in association with hepatitis E infection. These manifestations are rare and include conditions as varied as Guillain–Barré syndrome, meningo-encephalitis, acute transverse myelitis, pseudo-tumor cerebri, seizures, and neuralgic amyotrophy [60–64]. A review by Kamar *et al.* [65] reviewed 126 patients with acute and chronic HEV infection and found evidence of neurological involvement in seven of these patients. These patients, collected from two different centers in the United Kingdom and France, had a wide spectrum of neurological involvement (inflammatory polyradiculopathy = 3, Guillain–Barré syndrome = 1, bilateral brachial neuritis = 1, encephalitis = 1, and ataxia or proximal myopathy = 1). All patients had genotype 3 infection, and HEV RNA was isolated from the cerebrospinal fluid (CSF) of four of the seven patients (all with chronic HEV). Neurological symptoms improved or resolved on clearance of HEV viremia. Neurological involvement has also been described in association with genotype 1 infection in patients mainly from the Indian subcontinent. The majority of patients with neurological involvement will recover fully over a period of weeks to months, although some may display residual symptoms.

Autoimmune and hematological manifestations

Some manifestations with an immunological basis have been described in a few patients with hepatitis E infection. These are uncommon and have mainly been described in case reports. The hematological manifestations associated with acute HEV infection are thought to have an immunological basis and include thrombocytopenia and hemolysis.

Thrombocytopenia has been reported in a few isolated cases [66, 67]. The platelet count improved spontaneously in most patients, although the use of intravenous immunoglobulin and corticosteroids is described. Thrombocytopenia has also been reported in other viral infections (e.g., hepatitis A) and is thought to be due to an immune mechanism [66].

Hemolysis with a positive Coomb's test (suggesting that hemolysis is immune mediated) appearing a few days to weeks after the onset of liver disease has been described [68]. Hemolysis has in some cases also been related to glucose-6-phosphate dehydrogenase (G6PD) deficiency [69, 70]. These patients developed anemia and severe hyperbilirubinemia, and may develop renal failure.

Treatment of HEV

Hepatitis E is a self-limiting illness in immunocompetent individuals, and as such there is usually no need for specific treatment. The treatment centers mainly on supportive management of fever, muscle aches, vomiting, and diarrhea.

Both T and B cell responses have been shown to be important for HEV clearance, and in one report 30% of solid-organ transplant patients with chronic hepatitis E cleared virus with a reduction in their immunosuppressive therapy [71, 72]. There have been several case reports of the use of both pegylated interferon (alpha-2a as well as alpha-2b) as well as ribavirin in patients with chronic hepatitis E infection [73–76]. These patients all had genotype 3 infection. The majority of cases treated with interferon for between 3 and 12 months achieved sustained viral clearance, although relapse has been described [28, 73–75]. Ribavirin has also been used on its own and resulted in a rapid fall in HEV RNA within one month of treatment, with subsequent viral clearance. The use of pegylated interferon and ribavirin has also been shown to be effective in patients co-infected with HIV [77].

Ribavirin use in acute hepatitis E infection has also been reported. A rapid fall in serum HEV RNA with improvement in laboratory values (ALT, bilirubin, and Prothrombin Index) was observed [78]. Further studies are needed to examine the role of ribavirin in acute severe hepatitis E infection, such as in HEV arising amid the background of existing liver disease. The activity of ribavirin against genotypes 1, 2, and 4 is not known.

Pathological features

The histological appearance of the liver in acute HEV hepatitis is characterized by a portal inflammatory component containing a significant number of polymorphs with a characteristic distribution toward the periphery of the portal tract. Lymphocytes and plasma cells are also present and are concentrated within expanded portal tracts with evidence of a mixed cholangiolitis. Kupffer cell aggregates may also be present [79].

The morphological features of HEV may be divided into classic and cholestatic types of acute viral hepatitis. Focal necrosis, ballooned hepatocytes, and acidophilic degeneration of hepatocytes are typical in the classic presentation, whereas cholestatic forms are characterized by bile stasis in canaliculi and glandlike transformation of hepatocytes. Submassive as well as massive hepatic necrosis may be present in fatal cases. [1, 80, 81].

The histological features of chronic hepatitis E are those of a chronic inflammatory response. There is evidence of portal inflammation and interface hepatitis with a lymphocytic infiltrate and variable degrees of piecemeal necrosis. These changes are not as florid as those found in acute HEV infection, but can result in progressive fibrosis or cirrhosis [50, 54, 55, 82].

Diagnostic tests and caveats in interpretation

Diagnostic tests for hepatitis E have become much more widely available in the last few years. There are several commercially available enzyme immunoassays for both IgM and IgG responses to HEV as well as reliable reverse transcriptase polymerase chain reaction (RT-PCR) assays for the molecular detection of HEV RNA in serum, feces, or bile.

Enzyme immunoassays for HEV antibodies

The majority of serological assays used to diagnose HEV infection use recombinant proteins derived from open reading frames 2 or 3 (ORF2 or ORF3) of the HEV genome. A positive IgM anti-HEV confirms acute hepatitis E, although rarely false-positive results for IgM anti-HEV have been described in autoimmune disease and rheumatoid arthritis [83]. A very high or rising titer of IgG anti-HEV supports the diagnosis of HEV infection. IgM anti-HEV appears just before or at the onset of symptoms, peaks during the symptomatic phase, and then declines over a 2–6-month period, after which it disappears. IgG anti-HEV appears shortly after IgM anti-HEV becomes detectable, increases through the acute phase, and may remain positive for years following infection. It remains unclear whether IgG anti-HEV confers immunity against re-infection [83].

The sensitivity and specificity of commercially available enzyme immunoassay kits for HEV have been evaluated. The IgM anti-HEV assays have a sensitivity ranging between 82% and 90% with a very good specificity of 99.5–100%. These results have been achieved across genotypes 1, 2, and 3, despite the use of recombinant proteins from genotypes 1 and 2 only [84]. The IgG anti-HEV assays range in sensitivity from 56% to 98%, but with very good specificity of 97–99.6% [85]. The enzyme immunoassays for HEV antibodies should be done as soon as possible after the onset of symptoms to avoid false-negative IgM results and/or to detect a rising IgG titer to distinguish recent from previous infection.

HEV RNA

The detection of HEV RNA allows for the diagnosis of active infection, and the use of HEV RNA testing led to the description of persistent infection in some immunosuppressed cases. Typically, HEV RNA becomes detectable in the serum just before the onset of symptoms and remains positive in the serum for approximately 1–2 weeks in the majority of cases, but may persist for up to 3–4 weeks [86, 87].

HEV RNA is detected through either conventional RT-PCR or real-time RT-PCR. Peptide sequences that are conserved across genotypes from ORF2 and ORF3 are used in both these tests so that HEV RNA from all genotypes will be detected. These assays have a very high specificity for HEV RNA, but vary in sensitivity [88]. Real-time RT-PCR has been shown to be more sensitive than conventional RT-PCR [89], but there is up to a 100–1000-fold difference in the sensitivity between different laboratories [88]. Further work with regard to standardization of these assays is required.

HEV RNA becomes detectable in the feces during the incubation period and continues to be shed in the feces for up to 3–4 weeks after the onset of symptoms [86, 90]. There have been isolated reports of prolonged fecal shedding of virus up to 121 days after the onset of hepatitis. The reason for this prolonged fecal shedding is not clear, but it has also been observed in pigs infected with HEV [90].

HEV RNA has been demonstrated in tissues other than the liver, and a transmission route other than the

fecal-oral route has also been suggested. In pigs, HEV RNA has been demonstrated in urine, suggesting an alternative route of transmission between pigs and humans [91]. HEV RNA has also been demonstrated in the CSF of patients with neurological manifestations [65].

Immunohistochemistry

The use of monoclonal antibodies against ORF2 and ORF3 proteins for the detection of HEV antigens in formalin-fixed, paraffin-embedded liver tissue has recently been described. These monoclonal antibodies successfully detected HEV antigens in fresh as well as decade-old liver biopsy specimens and may be useful in evaluating the epidemiology of HEV from preserved pathological specimens as well as adding a further confirmatory test in those who undergo liver biopsy where the diagnosis is not clear [92].

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Section VII
Clinical Aspects of Viral Liver Disease

Chapter 33
Clinical and diagnostic aspects of viral hepatitis

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Summary

Infections with viral hepatitis A through E have variable clinical presentation ranging from asymptomatic or mild flulike symptoms to jaundice and fulminant liver failure. Hepatitis A and E are fecal-orally transmitted, resulting in acute infection that is mostly self-limited. In contrast, hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV) are acquired via sexual, vertical, and parenteral transmission and can result in both acute and chronic infection. Testing for hepatitis A through E is based on serological and molecular tests.

Introduction

As mentioned, infections with viral hepatitis A through E can vary in their clinical presentation from mild flulike symptoms to jaundice and fulminant liver failure. While clinical manifestation alone cannot be used to differentiate the types of viral hepatitis, there are some differences in their natural history. Hepatitis A and E are fecal-orally transmitted, resulting in acute infection that is mostly self-limited. In contrast, HBV, HCV, and HDV are acquired via sexual, vertical, and parenteral transmission and can result in both acute and chronic infection. The severity of clinical symptoms depends on the interplay of the host immune response to the virus and can result in both hepatic and extrahepatic manifestations that can have clinical consequences. In addition, patients with chronic hepatitis who are co-infected with HIV or have a comorbidity such as heavy alcohol use are at increased risk for development of end-stage liver disease. Testing for hepatitis A through E is based on serological and molecular tests. This is important not only for diagnosis but also for making treatment decisions. The clinical features and diagnosis will be discussed in this chapter.

Hepatitis A

Hepatitis A virus (HAV), a member of the *Picornaviridae* family, is the most common cause of acute viral hepatitis worldwide. HAV is transmitted primarily by the fecal-oral route through contaminated water and food. As a result, HAV is endemic in countries of low socioeconomic conditions due to poor sanitation and lack of clean water resources. Less common modes of transmission include via sexual contact, injection drug use, and blood transfusion, though these are rare events. Vertical transmission of HAV is also unusual and has been identified in only a few instances.

In endemic regions, HAV commonly infects young children. The disease is typically asymptomatic for those younger than 6 years; only 30% develop nonspecific, anicteric, flulike symptoms. Infected children can

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transmit HAV, but high serological prevalence among individuals living in endemic regions limits widespread outbreaks.

In contrast, the incidence of HAV in developed countries is much lower due to higher living standards and the availability of a vaccine. However, the decreased disease incidence among children has resulted in reduced natural immunity. Thus, HAV infections in developed countries are now more commonly found in adults, usually as a result of international travel or sporadic outbreaks due to contaminated food.

Clinical features

The majority of HAV-infected children are asymptomatic. However, older children and adults are more likely to experience symptoms. Approximately 70% of adults develop jaundice accompanied by elevated serum aminotransaminase (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) levels. The incubation period ranges from 15 to 50 days. Individuals can experience prodromal symptoms before the onset of jaundice including anorexia, nausea, vomiting, malaise, fever, headache, and abdominal pain. Less common symptoms include chills, myalgias and arthralgias, cough and upper respiratory symptoms, constipation, diarrhea, pruritus, and urticaria. There is no prodromal phase for 15% of infected persons. The onset of the icteric phase is characterized by pale stools and dark urine due to an increase in bilirubin levels. This phase can last from 4 to 30 days, and younger individuals typically recover quickly. Pregnant women acutely infected with HAV are at risk for complications including premature contractions, placental separation, vaginal bleeding, and preterm birth. However, mothers typically have a complete recovery from HAV infection [1]. Other high-risk groups for complications include the elderly and immunocompromised persons.

Atypical clinical manifestations of hepatitis A

Relapsing hepatitis

HAV is a self-limiting disease and does not result in chronic infection. However, 3–20% of individuals may experience a relapse characterized by milder or similar symptoms to those of the initial infection. The relapse typically occurs 30–90 days from the initial infection, when levels of IgM antibody against HAV (anti-HAV) are still high. During this time, patients experience a second peak in serum ALT and AST levels. The most definitive method to diagnose a relapse is the analysis of stools for the presence of HAV RNA and proteins. Relapse can also occur after liver transplantation for HAV-related acute liver failure, which could be misinterpreted as an acute graft dysfunction. The mechanisms of relapse are unclear, but studies indicate that IgA anti-HAV expressed during primary infection may assist viral re-entry into hepatocytes.

Cholestatic hepatitis

Cholestatic hepatitis is characterized by prolonged jaundice that lasts at least 12 weeks. Older individuals infected with HAV may have prolonged prothrombin time and high bilirubin levels with severe cholestasis. Other symptoms include pruritus, fever, diarrhea, and weight loss [2]. During this time, bilirubin levels can be higher than 10 mg/dL. After an initial increase, ALT levels eventually return to normal. Since cholestasis spontaneously resolves with no sequelae, the prognosis of patients is typically favorable.

Fulminant hepatitis or acute liver failure

Acute liver failure (ALF) due to HAV infection is very uncommon, more frequently occurring in older and immunocompromised individuals [3]. Persons with preexisting liver infections such as hepatitis B (HBV) or hepatitis C (HCV) are also more susceptible to this complication. Comparison of viral load among patients of varying severity of infection indicates that a higher viral replication may cause a more vigorous immune response contributing to liver injury. As a result, although uncommon, young, healthy individuals may be at risk for severe complications due to HAV and HBV or HCV co-infection.

HAV and autoimmune hepatitis

Rare cases of autoimmune hepatitis type 1 (AH1) have been identified in individuals infected with HAV. HAV-infected persons developed AH1 typically within 5 months of infection. Individuals with a genetic background of asialoglycoprotein receptor (ASGPR)-specific T cell defects are predisposed to autoimmune hepatitis after viral infection. HAV infection triggers T cell or B cell reactivity to ASGPR, a hepatocyte–glycoprotein-specific receptor, causing cytotoxicity of normal hepatocytes.

Extrahepatic manifestations

Renal

Acute renal failure (ARF) is common in persons with fulminant hepatitis, but can also rarely occur in cases of nonfulminant hepatitis. Individuals can experience anuria, oliguria, hematuria, and proteinuria. The mechanisms of ARF associated with acute HAV infection are unclear, but glomerulonephritis, acute tubular necrosis,

and hepatorenal syndrome could contribute to the pathogenesis of ARF. An increase in bilirubin levels may also cause renal vasoconstriction and have toxic effects on renal tubules.

Neurological

Neuropathy and mononeuropathy due to HAV infection is very uncommon. Rare cases of encephalitis, meningoencephalitis, meningitis, acute transverse myelitis, and Guillain–Barré syndrome have been identified in HAV-infected patients. Neuropathies usually occur during the pre-icteric or convalescent phase and can spontaneously resolve. For protracted illness, treatment with intravenous steroids has been successful. In general, since HAV is neither neurotropic nor myotropic, a direct causal relationship between HAV and various neuromuscular diseases has yet to be identified.

Hematological

Hematological disorders have been associated with HAV infection, including cryoglobulinemia, thrombocytopenia, agranulocytosis, and aplastic anemia. These conditions are rare in acute infections and are more often associated with fulminant hepatitis.

Diagnosis

Serological tests for HAV

Currently, enzyme-linked immunosorbent assays (ELISAs) are clinically used for the diagnosis of HAV by the detection of IgM anti-HAV. Generally, IgM anti-HAV is detectable within 5–10 days post infection, and IgG anti-HAV appears soon afterward. IgM is present in the serum for up to 6 months, while IgG may remain present for the lifetime of the individual.

IgM anti-HAV positivity in the absence of symptoms of hepatitis may indicate an asymptomatic or a recently resolved infection of HAV. However, false positives in the absence of symptoms and false negatives may occur. IgG anti-HAV positivity in the case of IgM anti-HAV negativity is an indication of previous infection and immunity to HAV. For persons who have relapsed, IgM anti-HAV levels may rise after an initial disappearance.

Liver enzymes and bilirubin levels

Initial signs of liver damage include elevated levels of ALT and bilirubin. ALT levels are generally in the range of 100–1000 U/L but can be higher in patients with fulminant liver failure. Bilirubin levels are usually mildly elevated except in individuals with severe disease where values can reach higher than 10 mg/dL. Prothrombin time is usually well preserved in most cases but may be prolonged in fulminant disease. Serological testing revealing the presence of IgM anti-HAV confirms HAV infection.

Molecular tests for HAV

Since serological testing is a convenient and effective diagnostic tool, molecular tests are used for nonroutine diagnosis. HAV RNA can be extracted from stool and body fluids and detected by PCR technology.

Studies have indicated a correlation between differences in the HAV genome and disease progression. Determination of molecular genotype can be achieved by real-time (RT)-PCR and nucleotide sequencing. Comparison of the HAV genome from individuals with fulminant disease versus nonfulminant infection identified variances in amino acid substitutions in the 5′ nontranslated region and in the nonstructural protein 2B region [4]. In addition, analysis of HAV genotypes 1A and 3A revealed that longer hospitalization, higher ALT levels, and fulminant hepatitis were likely to be more associated with genotype 3A. Globally, genotype 1A is more common, while genotype 3A is highly prevalent in Southeast Asia. Korea has recently experienced a surge in prevalence of genotype 3A, which could reflect an emerging global trend.

Hepatitis B

Hepatitis B is a global epidemic with an estimated 350 million carriers worldwide. The prevalence of HBV carriers varies from 0.1% to 2% in Western developed countries such as the United States, Canada, New Zealand, and the countries of Western Europe. In high-endemic areas such as Southeast Asia, China, and Sub-Saharan Africa, the carrier rates can be as high as 10–20%. A high prevalence of HBV infection can also be found among Native Alaskans, Aborigines of Australia, and foreign-born immigrants from endemic countries. The wide variability in HBV carrier rates relates to the mode of transmission of the virus and age of exposure. In developed countries, the primary mode of transmission is via sexual contact, whereas in endemic countries vertical transmission from mother to child is the primary mode of HBV infection. Persons infected with HBV during adulthood typically clear the virus spontaneously, although about 2–5% of these individuals develop chronic hepatitis. On the other hand, 90% of individuals infected perinatally or during childhood develop chronic infections [5], thus serving as a reservoir for future HBV transmission. Chronic HBV infection is carcinogenic and can lead to serious consequences such as hepatocellular

carcinoma (HCC). This virus can also cause progression to cirrhosis, liver failure, and death.

Clinical features

Acute HBV

The majority of adult patients with acute HBV have a self-limited course. The incubation period ranges from 1 to 5 months and appears to correlate with the dose of inoculum at the time of exposure. During this time, individuals may experience general malaise, anorexia, nausea, vomiting, headache, right-upper-quadrant pain, jaundice, and, rarely, fulminant liver failure. In addition, about 5–10% of individuals develop a serum sickness–like syndrome characterized by skin rash, polyarthralgia, arthritis, and urticaria due to vascular deposition of circulating immune complexes of hepatitis B surface antigen (HBsAg), antibodies to HBV surface antigen (anti-HBs), and complements. Many patients give no history of acute hepatitis as anicteric hepatitis occurs more commonly than icteric hepatitis. However, persons with anicteric hepatitis are more likely to continue to progress to chronic hepatitis B infection than individuals with icteric hepatitis during the course of their illness.

Chronic HBV

It is estimated that 1.25 million people in the United States have chronic HBV infection, accounting for about 5–10% of US cases of chronic liver disease and cirrhosis. In addition, about 15–40% of these individuals will die prematurely from complications of chronic HBV such as cirrhosis or HCC [6]. The majority of patients with chronic HBV report no history of acute hepatitis, and the infection may come to attention without any symptoms except for abnormal liver function test with elevated ALT and AST levels. When symptoms are present, they include fatigue with occasional right-upper-quadrant discomfort. Chronic HBV is defined by the presence of HBsAg in the blood of an infected person for more than 6 months. The course of chronic HBV is mediated by the interplay of viral replication and host immune response. In individuals who acquired chronic HBV during adulthood, this typically consists of an early replicative phase with active liver disease and, later, a nonreplicative phase with remission of liver disease. In contrast, persons with perinatally acquired chronic HBV infection have an additional immune tolerance phase whereby viral replication is not associated with active liver injury.

During the initial phase of active viral replication with immune tolerance in patients with perinatally acquired HBV, a high level of HBV DNA and the presence of hepatitis B e antigen (HBeAg) can be detected without associated signs and symptoms of liver disease. These individuals have normal liver function tests and show minimal changes on liver biopsy due to the development of host immune tolerance to the HBV antigens. This phase may last for several decades and is associated with a low rate of spontaneous HBeAg seroconversion.

Transitioning from the immune tolerance phase to the immune clearance phase, the immune system attacks infected hepatocytes with active immune clearance of HBV. This phase is marked by high viral replication with associated liver injury as manifested by elevated ALT and chronic hepatitis on liver biopsy. Individuals may present with symptoms of acute hepatitis, with elevated IgM and IgG antibodies to hepatitis B core antigen (anti-HBc) leading to misdiagnosis of acute HBV infection and increased serum levels of alpha fetoprotein (AFP) from hepatic decompensation and necrosis. These exacerbations are often, though not always, associated with HBeAg seroconversion.

In the nonreplicative phase, patients are in the "inactive carrier state." During this phase, patient may achieve HBsAg clearance with development of antibody to hepatitis E antigen (anti-HBe). Individuals are asymptomatic with normal ALT, and have resolution of hepatic necrosis on biopsy and with undetectable HBV DNA [7]. However, some patients continue to have active viral replication despite negative HBeAg, which is termed "HBeAg-negative chronic hepatitis." These individuals have a mutant HBV variant such that HBeAg is no longer produced. They tend to be older and have more advanced liver disease.

Spontaneous HBsAg clearance occurs at an annual rate of about 0.1–0.8% [8]. In those without cirrhosis, prognosis is thought to be good after HBsAg seroconversion; however, clearance of HBsAg does not preclude the development of cirrhosis or HCC, particularly in those who seroconverted late in their life.

Fulminant hepatitis

Fulminant hepatitis is an infrequent but potentially fatal complication of HBV. In the United States, it accounts for 7% of transplant referrals for acute liver failure [9], but rates as high as 44% have been reported in Japan. The mechanism for fulminant hepatitis is not understood but is likely due to the host immune response to HBV infection resulting in hepatic necrosis and severe liver injury. Fulminant hepatitis can occur with acute hepatitis B infection; acute-on-chronic hepatitis B infection, with reactivation of HBV; or due to superinfection with hepatitis D virus (HDV) or other agents of viral hepatitis in patients with chronic HBV infection. Reactivation of HBV can occur spontaneously, due to immu-

nosuppression or after withdrawal of antiviral or interferon therapy. It is unclear why some people develop spontaneous reactivation of chronic hepatitis B infection and others do not. Viral mutations and differences in genotypes have been implicated in playing a major role in fulminant hepatitis, but further studies are still needed.

Extrahepatic manifestations

Renal

Several forms of renal diseases are associated with HBV infection. They include focal segmental glomerulosclerosis, membranoproliferative glomerulonephritis, membranous nephropathy, cryoglobulinemic glomerulonephritis, mesangial proliferative glomerulonephritis, minimal-change nephropathy, and IgA nephropathy. Individuals typically present with proteinuria and nephritic syndrome with mild liver disease. The underlying pathogenesis is immune complex mediated, with antibody–antigen complexes involving HBsAg, HBeAg, anti-HBc, and anti-HBe being found in glomerular and papillary basement membrane. Treatment with antivirals and interferon alpha (IFNα) may lead to remission and decrease proteinuria; however, corticosteroid treatment is not effective and may lead to increased viral replication [10].

Neurological

Guillain–Barré syndrome has been reported in persons with HBV infection, after HBV vaccination and during HBV treatment with IFNα therapy. In addition, acute transverse myelitis with acute HBV and recurrent demyelinating transverse myelitis in patients with chronic HBV have also been described. Direct measurements of HBsAg or immune complexes containing HBsAg have been found in the cerebrospinal fluid of some of these individuals. In others, circulating immune complexes containing HBsAg were shown to disappear after treatment, suggesting that perhaps autoimmunity may play a role in the pathogenesis of the neurological sequelae of HBV infection.

Rheumatological

Polyarteritis nodosa (PAN) is a well-described complication associated with HBV infection. While this is a rare complication of HBV infection, about 30% of patients with PAN are found to have HBsAg positivity. Patients often present with GI tract involvement, pulmonary symptoms, vascular nephropathy, malignant hypertension, and orchitis. Patient outcome is largely dictated by the severity of the vasculitis, underlying liver disease, and response to treatment, which relies on a combination of immunosuppressives, plasma exchange, and HBV suppression [11]. As with other forms of extrahepatic involvement, immune complexes containing HBsAg, immunoglobulins, and complements have been demonstrated in vascular lesions.

Hematological

Aplastic anemia is a rare but well-documented complication of HBV infection with high mortality. HBV can be found in extrahepatic cells such as circulating mononuclear cells, bone marrow cells, and the spleen. *In vitro* suppression of hematopoietic stem cells can be achieved in a dose-dependent fashion with HBV infection. Furthermore, several studies have postulated the role of HBV infection in hematologic malignancies in long-term survivors of aplastic anemia, such as the development of acute myeloid leukemia and myelodysplastic syndrome.

Other

The extrahepatic manifestations of HBV are diverse. While the renal, neurological, and hematological complications are well established, some less common manifestations also include cryoglobulinemia, myocarditis, and pancreatitis.

Special considerations

Occult hepatitis B infection

Occult hepatitis B infection (OBI) is defined serologically as the presence of HBV DNA in the absence of detectable HBsAg [12]. It may occur in the presence of anti-HBc, anti-HBs, or both [13]. OBI may exist rarely without serologic markers of past HBV infection. Therefore, OBI is detectable only by highly sensitive molecular techniques such as quantitative RT-PCR (qRT-PCR) and is important for diagnosis of acute and chronic hepatitis, cirrhosis, and HCC of unknown cause.

The molecular pathogenesis of OBI is complex and poorly understood. It was initially discovered with the availability of more sensitive assays for the detection of HBV proteins and DNA [12, 14]. However, OBI may develop due to molecular changes in the virus resulting in mutations that change the conformation of the HBsAg protein, rendering the protein undetectable by anti-HBs antibodies present in commercial kits [15]. Another reason for undetectable HBsAg levels in blood may be the formation of immune complexes during the resolution phase of acute HBV infection, whereas HBV DNA is still detectable [15]. OBI often occurs in the setting of co-infection with HIV, HCV, or both, due to presumed

viral interference effects on HBV replication; indeed, when HIV and HCV were cleared by antiviral therapy, HBsAg became detectable [16]. Therefore, there are multiple molecular mechanisms that contribute to the development of OBI.

The presence of OBI has substantial clinical significance, making it possible to clarify the etiology of idiopathic liver disease including cirrhosis and HCC [14, 17]. It is noteworthy that the prevalence of OBI is directly related to the carrier rate of HBV infection in various regions of the world. In this regard, OBI has been found in 30% (and, when combined with analysis of liver biopsy material, in 65%) of patients with idiopathic liver disease and cirrhosis [14]. Likewise, 75% of individuals with apparently unidentifiable causes of HCC were accurately diagnosed as having OBI. There is evidence of an increased risk for HCC with OBI in endemic HBV areas of the world [17]. Although the incidence of OBI in blood donors is reportedly low, when present it is infectious and can transmit acute infection to recipients.

Immunosuppressive therapy can have a profound influence on HBV replication leading to reactivation [18]. It is a particularly important diagnosis to establish for certain patient groups since they may not be routinely screened by ultrasensitive qRT-PCR-based assays for the presence of OBI. Lack of diagnosis has led to reactivation of HBV infection during chemotherapy for myeloproliferative disorders [18], and has resulted in the development of fulminant hepatitis and death [19]. Therefore, OBI screening prior to the initiation of chemotherapy needs to be performed. However, most if not all forms of immunosuppression can be associated with reactivation of OBI. Reactivation of HBV infection can occur in individuals who have detectable HBsAg or OBI with the presence or absence of anti-HBs and anti-HBc antibodies. Reactivation may occur with immunosuppression following renal or hepatic transplantation, with anti–tumor necrosis factor (TNF) therapy, or with immunosuppression induced by anti-tumor agents [19]. Reactivation may also occur rarely in persons who are HBV DNA negative but have antibodies to anti-HBc and anti-HBs in the blood, indicating past infection and clinical recovery; in this context, there may be very small amounts of residual HBV in the liver that will replicate under immunosuppressive conditions.

Therefore, it is mandatory that OBI be searched for in any patient undergoing immunosuppressive therapy. There are well-described algorithms for HBV prophylaxis in patients undergoing chemotherapy [20]. Most oncologists and hepatologists believe that prophylaxis with nucleoside analogs is far better than waiting until HBV reactivation occurs since the liver disease may occur suddenly, be quite severe, and not respond well to antiviral therapy. The general paradigm is to first identify OBI and then place individuals prophylactically on a nucleoside analog (e.g., tenofovir, lamivudine, and entecavir), all of which are suitable for this type of therapy. Substantial clinical experience has been obtained with lamivudine, and it has been found to be highly effective in preventing reactivation of HBV and subsequent development of severe or fulminant liver injury induced by chemotherapy [20, 21].

Hepatitis B infection and pregnancy

Acute and chronic HBV infection pose unique management challenges in pregnancy since there is a paucity of data to guide physicians on management of these individuals. While most women with chronic HBV do well throughout pregnancy, this is an immunosuppressive state. HBV exacerbation resulting in hepatic decompensation and hepatic failure has been reported during pregnancy and postpartum, particularly in women with advanced liver disease. Indeed, it is common to see a rise in ALT level in the postpartum period, and in one study from Japan about 43% of women in the postpartum period developed overt liver dysfunction with jaundice [22]. It is therefore imperative that women with HBV infection be monitored closely both during pregnancy and postpartum and to consider antiviral therapy if there is evidence of hepatic decompensation for the benefit of both the mother and the fetus [23].

While it is still controversial, HBV infection has been suggested to be associated with poorer pregnancy outcomes, resulting in higher rates of gestational diabetes, antenatal hemorrhage, and preterm labor due to the inflammatory response it provokes. In addition, it is evident now that high maternal HBV viremia ($>10^8$ copies/mL) is associated with higher risk of perinatal HBV transmission despite appropriate infant immunoprophylaxis [24] with hepatitis B immunoglobulins (HBIG) and hepatitis B vaccine, with rates as high as 25% [25]. Multiple studies have demonstrated the effectiveness of using HBIG alone or with lamivudine in the latter stages of pregnancy with successful reduction in vertical transmission. However, given the previous lack of safety data, the high rates of resistance to lamivudine, and potential infectious disease transmission from HBIG, this practice was not advocated. However, recent data from the Antiretroviral Pregnancy Registry showed no increased risk of birth defects with maternal use of lamivudine or tenofovir compared to the general population. In addition, a large prospective randomized trial has demonstrated the safety and effectiveness of telbivudine in preventing vertical transmission [26]. Given the long-term morbidity and mortality of chronic hepatitis B with vertical transmission, antiviral therapy should now be considered in all pregnant women with high viral load in reducing perinatal transmission [27].

HBV genotypes

Genomic sequencing has yielded 10 different HBV genotypes designated as genotypes A–J. These genotypes have allowed for epidemiological studies and enabled the geographical mapping of HBV. Genotype A is predominantly found in Northern Europe and the United States [28]; genotypes A and D are found in Africa and the Mediterranean; genotypes B and C are found in Asia; and genotypes E and H are found in West Africa and Central America, respectively. While the clinical relevance of the genotypes is still not well defined, it is suggested that these differences in viral characteristics may play a role in progression of liver disease, development of cirrhosis and HCC, and response to therapy [29]. For example, genotype B is associated with a better response to interferon therapy and earlier HBeAg seroconversion. In comparison, genotype C is associated with more severe liver disease and the development of cirrhosis and HCC. In the future, genotyping may help prognosticate patient outcome and therefore identify those in need of antiviral therapy.

HBV and HDV infection

HDV is a unique RNA virus whose infectivity and replication cycle rely on coexisting infection with HBV. It is estimated that about 5% of HBV carriers are infected with HDV [30]. In order for HDV to replicate, it uses the envelope protein produced by HBV. In this way, HDV infection and replication can result in the suppression of HBV replication. HDV infection is found worldwide, with the highest prevalence in South America, Southern Europe, the Mediterranean basin, the Middle East, south India, and parts of Africa. While the overall prevalence in Northern Europe and North America is low, given that it is primarily parenterally transmitted, it remains an important problem among injection drug users [31].

Infection with HDV can occur simultaneously with HBV (co-infection) or as a superinfection when HDV infection is acquired in individuals with existing chronic HBV. Co-infection of HBV with HDV is usually self-limited as HDV infection cannot persist after the resolution of HBV given its dependency on HBV for replication. Severe icteric hepatitis and fulminant hepatitis can occur but are rare.

In contrast, superinfection with HDV in a patient with pre-existing chronic HBV can result in severe hepatitis and acute liver failure. Individuals may have a high level of HDV viremia with suppression of HBV replication. The majority of patients result in chronic HDV infection with persistent HDV viremia. The natural history of chronic HDV infection is still controversial. In some studies, it has been linked to more rapid clinical progression to end-stage liver disease and HCC, particularly in injection drug users, but in other studies the presence of HDV infection did not seem to cause significant liver damage [32].

Diagnosis

Serological tests for HBV

The clinical diagnosis of HBV infection relies on the detection of three antigens produced by the hepatitis B virus and three antibodies produced by the host immune system in response to these antigens (Table 33.1). These antigens are HBsAg and anti-HBs, HBeAg and anti-HBe, and anti-HBc. In addition, direct measurement of HBV DNA by molecular hybridization and PCR methods can be performed with high sensitivities in cases of equivocal serologic testing or when making treatment decisions.

HBsAg can be detected within 6 days to 2 weeks after acute exposure along with HBV DNA before the onset of clinical symptoms (Figure 33.1A). Soon after the appearance of HBsAg, HBeAg is produced and abnormal liver function tests with elevated ALT can be seen. In self-limited cases of acute HBV infection, anti-HBs and anti-HBe are produced, resulting in the gradual disappearance of HBsAg and HBeAg in the serum. The period during the early stages of HBsAg clearance is known as the "serological window," in which there is undetectable HBsAg with only detectable IgM anti-HBc.

Table 33.1 Hepatitis B serology interpretation.

HBsAg	Anti-HBs	Anti-HBc IgM	Anti-HBc IgG	Interpretation
Neg	Pos	Neg	Neg	Vaccination
Neg	Pos	Neg	Pos	Prior exposure
Pos	Neg	Pos	Neg	Acute HBV infection
Pos	Neg	Neg	Pos	Chronic infection
Neg	Neg	Neg	Pos	1. False-positive lab test 2. Prior exposure with undetectable anti-HBs 3. Occult HBV infection with undetectable HBsAg 4. Resolving acute HBV infection

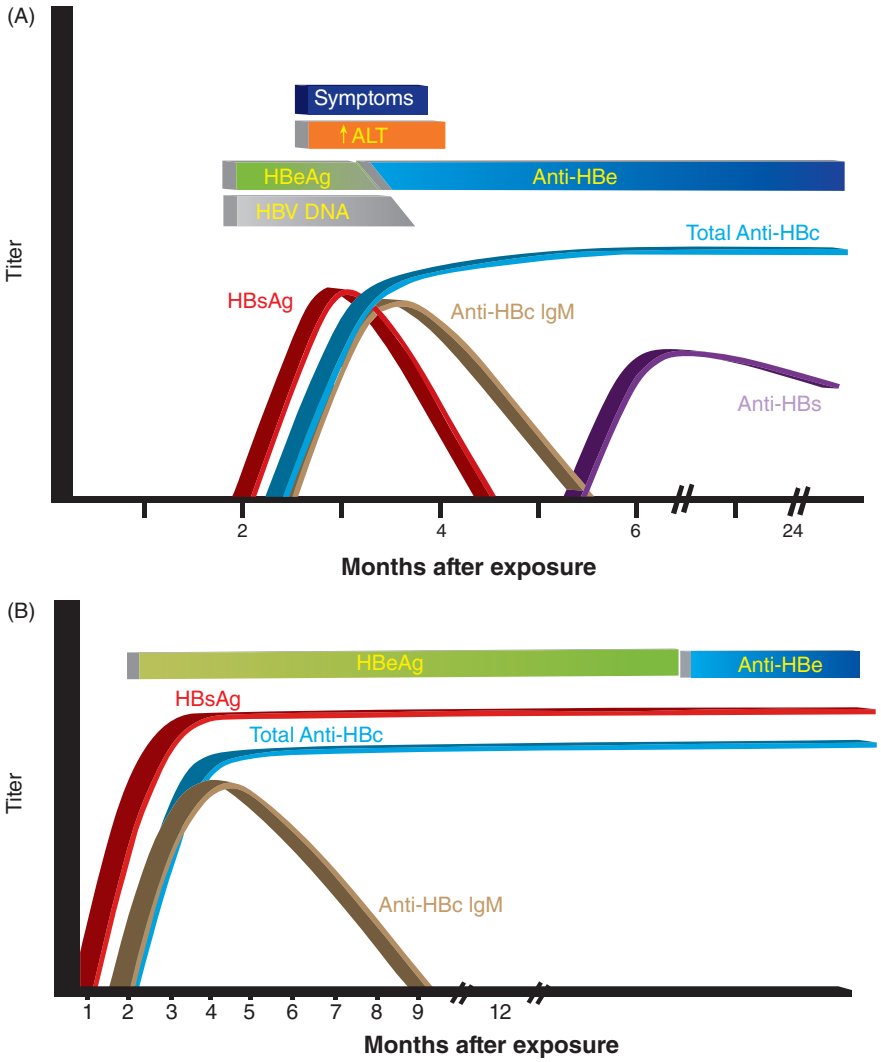

Figure 33.1 (A) Serological profile of acute hepatitis B infection with recovery. (B) Progression to chronic HBV. (Source: Adapted from Centers for Disease Control and Prevention. MMWR 2008;57(RR08):1–20 [51]). (Color plate 33.1)

Therefore, IgM anti-HBc is a marker of acute infection in contrast to IgG anti-HBc, which reflects current or previous infection. An anti-HBc test is commonly used to screen HBsAg-negative blood donors. Individuals with a high titer of anti-HBc in the absence of anti-HBs may be capable of transmitting HBV due to OBI [33]. During the acute infections, a high level of HBV DNA is found in the serum with decreasing viral levels as the infection resolves. Patients with detectable HBsAg 6 months after acute HBV progress to chronic HBV infection (Figure 33.1B).

Isolated anti-HBc can occur (1) during acute infection, with low or undetectable levels of HBsAg such as during the serological window described in this section; (2) with OBI, whereby HBsAg is low due to a low level of replication or undetectable due to mutations in the gene encoding for this envelope protein; (3) after resolution of prior infection with undetectable anti-HBs; and, lastly, (4) as a false-positive result. In these instances, measurement of HBV DNA is necessary as in the absence of HBV DNA, isolated anti-HBc likely represents a false-positive result or remote HBV infection.

HBeAg is produced soon after HBsAg appearance. HBeAg is a marker of high viral replication, infectivity, and liver injury. In acute infections, it disappears after several weeks; however, persistent HBeAg detection would suggest progression to chronic HBV infection. HBeAg disappearance or seroconversion occurs at about 10% annually in persons with chronic HBV. The disappearance of HBeAg generally coincides with a dramatic decline in viral replication to low or undetectable levels and normalization of liver function tests. In some individuals, however, active chronic HBV continues despite loss of HBeAg due to a pre-core mutation resulting in

the inability of the virus to produce HBeAg. These mutations are found largely in Asia and the Mediterranean, and patients typically have lower levels of HBV viremia but intermittent rather than sustained periods of liver inflammation and necrosis.

Molecular tests for HBV

Detection and measurement of HBV DNA levels are important for diagnosis of OBI and in following response to antiviral therapy. Both qualitative and quantitative molecular assays are commercially available with varying sensitivities for the low limit of HBV DNA detection. Molecular tests available for this purpose include (1) molecular hybridization and signal amplification techniques, (2) target amplification techniques, and (3) qRT-PCR technology. Given their sensitivity and accuracy, qRT-PCR techniques to detect HBV DNA have become more valuable than the other, older methods for HBV DNA detection.

Molecular hybridization requires the use of an oligonucleotide or RNA probe that hybridizes with the HBV DNA, resulting in a probe–DNA complex. This complex can be detected with multiple antibodies conjugated to alkaline phosphatase and, when treated with a chemiluminescent substrate, the complex can be measured by a light spectrometer. While this test is highly specific, its lower limit of detection is only 357 IU/mL. Target amplification techniques rely on the use of a specific primer that attaches to a strand of targeted DNA of the HBV virus. Using PCR amplification, this strand of DNA is then multiplied many times, resulting in HBV DNA detection with a lower limit of detection of 5 IU/mL (~25 copies/mL). qRT-PCR has a greater dynamic range and is faster than conventional target amplification PCR techniques. It can detect HBV DNA levels as low as 10 copies/mL.

Serological tests for HDV

In clinical practice, the diagnosis of HDV infection generally relies on the detection of anti-HDV. The production of anti-HDV is variable; therefore, repeated testing may be necessary. HDV infection is usually self-limited during co-infection with HBV, and anti-HDV disappears quickly after resolution of infection, leaving no evidence of prior infection. In contrast, in patients with superinfection of HDV resulting in chronic HDV infection, IgM and IgG anti-HDV can be found. It is important to note that IgM anti-HDV can be found in both acute and chronic infection; therefore, it cannot be used to distinguish the two.

The diagnosis of acute co-infection of HBV and HDV relies on the detection of anti-HDV with evidence of acute HBV infection (i.e., IgM anti-HBc). Similarly, the diagnosis of superinfection with HDV relies on the detection of anti-HDV in patients with evidence of chronic HBV infection (i.e., detection of IgG anti-HBc and HBsAg). However, given that HDV competes with HBV for viral replication, superinfection with HDV can lead to suppression of HBsAg production; therefore, in some individuals, HBsAg may be undetectable. Chronic HDV infection is suggested by persistent high titers of anti-HDV for more than 6 months.

In addition to anti-HDV, HDV antigen (HDAg) and HDV RNA can be measured to diagnose HDV infection; however, these assays are not widely available. They are limited to use in a few specialized laboratories.

Hepatitis C

Hepatitis C infection is found in about 170 million people worldwide, with an estimated 3.5 million (1.6%) of the US population with chronic infection. It is the leading cause of liver transplantation in the United States since chronic HCV is associated with high morbidity and mortality. Since the screening of HCV for blood products started in 1992, there has been a dramatic reduction in the number of HCV transmissions through blood transfusion. Injection drug use remains a major risk factor for transmission [34]. In addition, use of unsterilized needles, needlestick injuries, tattoos, body piercing, intranasal cocaine, and hemodialysis are other known risk factors for transmission. Vertical transmission can occur, with the risk of transmission relating to maternal viral load as well as co-infection with HIV. The risk of sexual transmission is low (<1%), which is in contrast to HBV (~25%).

Clinical features

Acute HCV

Acute HCV infection is generally asymptomatic or presents with nonspecific symptoms such as fever, malaise, nausea, anorexia, arthralgia, and vague abdominal discomfort. It accounts for 20% of acute hepatitis in the United States [35]. Fulminant hepatitis does not occur unless there is concurrent co-infection with another viral hepatitis such as HBV. The incubation period ranges from 5 to 26 weeks, with a detectable serum HCV RNA level in 1–2 weeks after exposure. The clinical course of acute HCV is illustrated in Figure 33.2. There are no specific useful markers that can differentiate between acute HCV and chronic HCV infection, as in both anti-HCV and HCV RNA will be detectable. In most patients, acute HCV will lead to chronic HCV infection, though about 14% of patients will spontaneously clear the acute infection [36].

Chronic HCV

Most patients with chronic HCV infection give no history of an acute illness and come to medical attention only when elevations in serum ALT and AST levels are found, usually during a routine physical examination. The evolution from acute to chronic HCV infection is displayed in Figure 33.3. These individuals may have had the infection for 1–2 decades and may already have significant inflammation and liver fibrosis at the time of diagnosis. About 20% of these patients may have cirrhosis, and progression can be hastened with concurrent alcohol abuse, non-alcoholic fatty liver disease, or co-infection with HIV. Complications due to cirrhosis and portal hypertension such as ascites, gastrointestinal bleeding from varices, hepatic encephalopathy, and HCC can result in high morbidity and mortality needing liver transplantation. The clinical outcome following HCV exposure is shown in Figure 33.4. In this regard, most individuals (80–85%) exposed to HCV develop chronic HCV infection.

Special considerations

HCV and alcohol

Although alcohol and HCV are both independent risk factors for causing liver damage, with different histo-

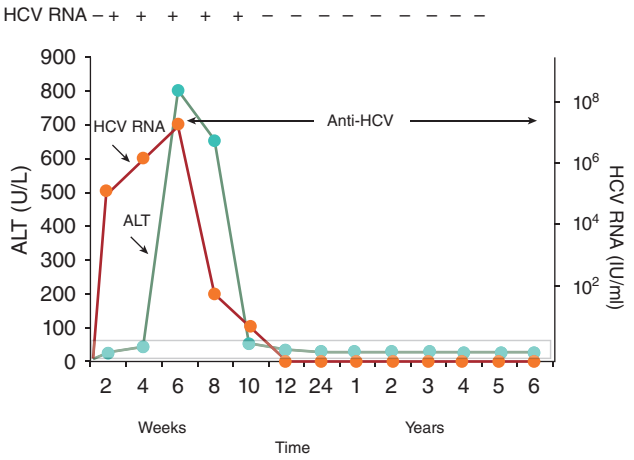

Figure 33.2 The clinical course of acute HCV. (Source: Hoofnagle, JH. 2002. Hepatology 36:s21–s29 [52]). (Color plate 33.2)

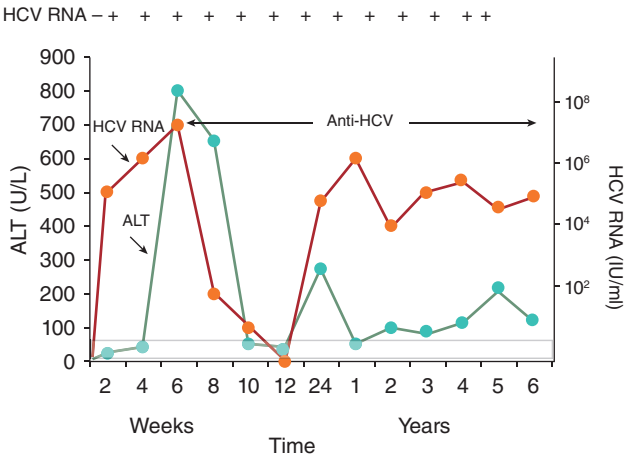

Figure 33.3 Illustration of the evolution from acute to chronic HCV infection. (Source: Hoofnagle, JH. 2002. Hepatology 36:s21-s29 [52]). (Color plate 33.3)

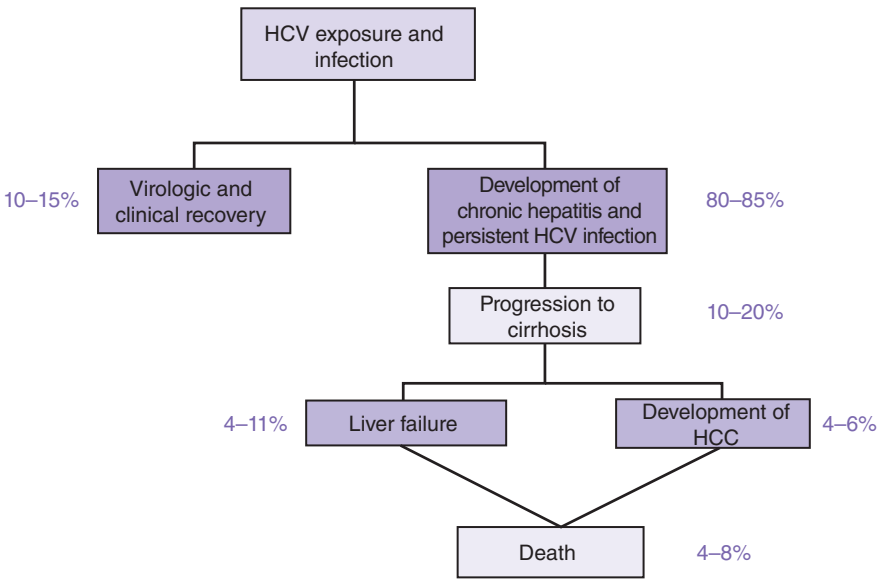

Figure 33.4 Clinical patterns of HCV exposure and infection. (Source: Di Bisceglie AM. Hepatology. 2000 Apr;31(4):1014–8 [53]).

logic and gene expression patterns, there is substantial evidence demonstrating the combined negative synergistic effects of both on the liver. Heavy drinkers (210 to 560 g/wk) with HCV have been observed to have an increased risk of advanced fibrosis, cirrhosis, and decompensated cirrhosis compared with nondrinkers [37]. In addition, the rate of fibrosis progression is doubled among drinkers compared to nondrinkers [38]. The synergistic or additive effects and mechanisms by which alcohol worsens HCV-related liver disease have not been clarified, but impairment of immune responses, enhanced viral replication, increased oxidative stress, and hepatocyte cytotoxicity have been postulated as major factors in liver disease progression [39].

HCV and HIV co-infection

Co-infection with HCV and HIV is common given the similar modes of transmission of these infections. While the high mortality from HIV infection has been largely related to the complications of AIDS from lack of effective treatment against HIV, this is no longer the case with the availability of highly active antiretroviral therapy (HAART). Instead, mortality in patients with HCV and HIV is increasingly due to complications of chronic liver disease. In co-infected individuals, serum HCV RNA viral load is typically much higher than in mono-infected HCV patients. In persons with HIV infection, exposure to HCV almost always results in chronic HCV, with rapid progression to cirrhosis, hepatic failure, and HCC.

Extrahepatic manifestations

Numerous extrahepatic manifestations of HCV have been reported. Some of these associations are well established, while others are not as well defined. These manifestations include mixed cryoglobulinemia, glomerulonephritis, leukocytoclastic vasculitis, neuropathy, large B cell lymphoma, autoimmune disease, and others.

Rheumatological

The clinical manifestations of mixed cryoglobulinemia can involve multiple organ systems, including the kidneys, skin, joints, and nerves. This is caused by vascular deposition of the cryoproteins containing HCV RNA, low-density lipoprotein, polyclonal IgG, and monoclonal IgM. Low serum levels of C3, C4, and CH50 are commonly seen in these patients, reflecting the activation of the complement system that mediates the inflammatory response. Rheumatoid factors (RF) are frequently positive, and in some laboratories RF may be a more sensitive test than cryoglobulin measurement given its dependency on appropriate collection and transport of the specimen to the testing laboratory. It is important to note that there are many asymptomatic patients with detectable cryoglobulins but no clinical manifestation of mixed cryoglobulinemia; therefore, the presence of cryoglobulins does not equate to having the clinical syndrome [40].

Renal

Membranoproliferative glomerulonephritis (MPGN) is the most common renal manifestation of chronic HCV infection, and the majority of individuals with MPGN have cryoglobulinemia. Patients present with nephrotic syndrome characterized by proteinuria, hypoalbuminemia, and edema. Decreased complement levels with immune complex glomerular deposits consisting mostly of IgG, IgM, and C3 are typically seen [41]. Other renal manifestations include proliferative and membranous glomerulonephritis.

Dermatological

Cutaneous manifestations of mixed cryoglobulinemia include findings of leukocytoclastic vasculitis with palpable purpura of the lower extremities, urticaria, livedo reticularis, and, in severe cases, painful ulcerations. Other implicated dermatologic manifestations of HCV include lichen planus (LP) and porphyria cutanea tarda (PCT) [42]. Individuals with PCT present with vesicles, bullae, and skin ulcers in sun-exposed areas.

Neurological

Peripheral neuropathy is the most common neurological manifestation of HCV infection, with most cases associated with cryoglobulinemia. It usually causes a sensory polyneuropathy, resulting in pain and, in some cases, motor deficits years later. Other rare neurological manifestations include optic neuropathy and encephalopathy.

Oncological

Chronic HCV infection and mixed cryoglobulinemia have been associated with the development of B cell non-Hodgkin lymphoma [43]. The most common forms of lymphoma found in these patients are follicular lymphoma, chronic lymphocytic lymphoma, lymphoplasmacytic lymphoma, and marginal-zone lymphoma. Patients typically have HCV infection for many years prior to the development of lymphoma, and in some case antiviral therapy may cause regression of the lymphoproliferative disease.

Others

Chronic HCV infection has also been associated with the development of adult-onset diabetes, thyroid disease, and autoimmune diseases such as Sicca syndrome, vitiligo, idiopathic thrombocytopenia, and myasthenia gravis.

HCV genotypes

There are currently six major HCV genotypes and more than 70 subtypes distributed geographically. In the United States, HCV genotype 1a accounts for about 57% of HCV infection, followed by genotypes 1b, 2, and 3. In Europe, the most prevalent genotype is 1b, followed by 1a, 2, and 3 [44]. Genotype 4 is largely found in Egypt, the Middle East, and Central Africa. Genotype 5 was originally found in South Africa but can also be found in specific regions of France, Belgium, and Spain. Genotype 6 is mainly found in Asia. While HCV genotypes do not influence the severity of liver disease, they have significant clinical importance in determining response to therapy.

Diagnosis

Currently, there is no definitive test to differentiate between acute and chronic HCV infection. Acute infection is suggested if there is a recent negative anti-HCV test or if the patient presents with new symptoms with negative anti-HCV but a detectable HCV RNA level.

Serological tests for HCV

The diagnosis of HCV infection relies on the detection of antibody to HCV (anti-HCV) and detection of viremia by measuring HCV RNA [45]. Current ELISA tests can detect anti-HCV as early as 7–8 weeks after HCV exposure; however, the specificity of the various assays does not discriminate between current active infection and past exposure with recovery. In addition, false-positive tests can occur. Therefore, in low-prevalent populations, confirmatory testing with a more specific recombinant immunoblot assay (RIBA) or direct measurement of HCV RNA levels should be performed.

Molecular tests for HCV

Both qualitative and quantitative molecular assays are available for measuring HCV RNA levels. This is important for several reasons: (1) for the diagnosis of acute HCV infection whereby anti-HCV has not been produced but active viremia is present, (2) to confirm active chronic infection, and (3) for monitoring the response to HCV antiviral therapy.

Qualitative assays consist of transcription-mediated amplification (TMA) or RT-PCR techniques whereby the reverse transcription of the HCV RNA produces a double-stranded DNA (dsDNA) template. This dsDNA template is then used for new transcriptions of HCV RNA and, with repeated cycles, allows viral RNA to be amplified. The qualitative assays are more sensitive than the quantitative assays and can detect viral loads at the lower limit of 50 /mL. Quantitative HCV is mainly used in monitoring response to HCV antiviral therapy, and commercially available qRT-PCR assays can detect to a lower limit of 615 /mL.

Hepatitis E

Clinical features

Hepatitis E virus (HEV), an RNA virus of the *Hepeviridae* family, is a global disease that differs in frequency, mode of transmission, and disease characteristics depending on geographical location. HEV is endemic to tropical and subtropical countries in Asia, Africa, and Central America [46]. In areas of poor sanitation, HEV is transmitted via the fecal-oral route, primarily by drinking contaminated water and, less commonly, by ingesting contaminated food. Transmission via parenteral and maternal-fetal routes has been documented, but is quite rare. HEV genotypes 1 and 2, which exclusively infect humans, are primarily associated with endemic regions [46]. In general, genotypes 1 and 2 cause a more virulent disease and infect individuals 15 to 35 years of age [47].

HEV was considered to be limited to endemic regions, and cases in developed countries were mainly due to travel to these areas. However, cases of sporadic HEV, primarily of genotypes 3 and 4, have been identified in the United States and Europe. In contrast to genotypes 1 and 2, genotypes 3 and 4 are not limited to humans, and are endemic to the swine and wild boar herds in Europe and Asia. Genotypes 3 and 4 more frequently affect elderly and immunocompromised individuals, including HIV-infected patients and organ transplant recipients. Possible modes of transmission are contact with infected swine, rats, or deer. In addition, consumption of undercooked venison or pork could lead to HEV infection since the virus is viable at temperatures below 56 °C. However, the direct cause of the sporadic outbreaks is unknown. The availability of testing resources and general awareness may be related to an increase in reported cases.

The incubation period of HEV spans 21–45 days. The infection is commonly asymptomatic, but some patients experience mild hepatitis. Common symptoms include jaundice, dark urine, pale stools, anorexia, enlarged liver, abdominal pain, nausea, vomiting, and fever. Ful-

Table 33.2 Comparison of hepatitis A and E serologic tests and clinical features.

	Hepatitis A	Hepatitis E
Family	*Picornaviridae*	*Hepeviridae*
Genome	RNA	RNA
Genome structure	Single-stranded Linear 7.5 kb	Single-stranded Linear 7.2 kb
Transmission	Fecal-oral Person-to-person 50–75%	Fecal–oral Person-to-person 0.7–2.2%
Incubation period (days)	15–50	15–64
Symptoms	Typically asymptomatic, mild hepatitis	Typically asymptomatic, mild hepatitis but can present with cholestasis resulting in severe pruritis
Chronicity	No	No
Age-specific seroprevalence	Acquired early in life (ages 10 and younger) in endemic regions	Acquired in young adulthood and adulthood (ages 15–35)
Mortality rate (general population)	0.5%	1–2%
Mortality rate (pregnant women)	Not well documented in pregnancy, but can cause gestational complications	Over 20%, especially during the third trimester

minant hepatitis due to HEV infection is rare, but elderly individuals and patients with underlying liver disease are susceptible to liver failure. Acute HEV is a self-limiting disease that usually does not lead to chronicity. However, chronic HEV has been identified in patients who have undergone liver transplantation. Chronic infection is characterized by persistently high ALT and AST levels and the presence of HEV RNA in the serum and stool for an average of 15 months after acute infection [48]. Organ transplant patients with acute HEV infections are also susceptible to developing chronic HEV infection due to immunosuppression. However, a reduction in the dose of immunosuppression therapy was associated with viral clearance [49].

The clinical features of HEV are rather similar to those of HAV, since both infections are enterically transmitted and present similar symptoms (Table 33.2). Thus, it may be difficult to clearly distinguish between HEV and HAV without the use of serological and molecular tests. However, HEV and HAV have some key differences. In endemic regions, HEV and HAV differ in age-specific seroprevalence. The anti-HAV seroprevalence rate is around 95% in children up to the age of 10 years. In contrast, anti-HEV is not commonly found in children. HEV mainly infects young adults and adults, and anti-HEV seroprevalence rates range from 15% to 60%. In addition, more cases of acute viral hepatitis during pregnancy are documented as HEV than HAV. Although the mortality rate of HEV is quite low, it dramatically increases to 15–25% in pregnant women, especially during the third trimester [50]. HEV-infected pregnant women are more susceptible to preterm deliveries, fetal death, perinatal mortality, and fulminant hepatitis.

Diagnosis

Serological tests

Serological tests are available to assess IgM, IgG, and IgA antibodies to HEV (anti-HEV) and HEV antigen levels in the serum. Since all genotypes of HEV confer a single serotype, these assays are applicable to all HEV infections. These serological tests for HEV are commercially available worldwide except in the United States. The antibody response to HEV is characterized by the appearance of IgM anti-HEV at the onset of symptoms, followed by IgG anti-HEV, which can remain in the serum for many years. Generally, individuals who are icteric and test positive for IgM anti-HEV with a rising titer of IgG anti-HEV have contracted acute HEV infection. However, some cases of HEV infection have low or absent antibody production. Thus, confirmation using molecular tests is necessary.

Molecular tests

Testing for the presence of HEV RNA in serum, bile, or stool samples is the most reliable method to diagnose HEV infection. qRT-PCR assays are more sensitive than serological tests and allow for earlier diagnosis and the determination of genotype. The window of viremia is usually 6–40 days post infection, before the increase in ALT levels and appearance of anti-HEV.

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Chapter 34
Treatment of acute hepatitis, severe acute hepatitis, and acute liver failure

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Summary

All hepatitis viruses can cause acute liver failure. For mild acute hepatitis, treatment is indicated only for acute hepatitis C virus (HCV), due to its high rate of chronicity if untreated, though in patients predicted to have a high chance of spontaneous clearance, 12-week observation seems reasonable.

Antiviral therapy is recommended for patients with severe acute hepatitis or acute liver failure due to hepatitis B virus (HBV). Causal treatment is not available for many types of acute liver failure. Patients with severe acute hepatitis and early signs of acute liver failure should be referred to liver transplant centers as early as possible.

Introduction

Medicine aims at identifying the cause of a disease and then eliminating its cause and/or ameliorating the insult's consequences. This is highly important in acute virus-induced liver injury, where the time for identification and elimination or amelioration of the injury might be short before a fatal outcome.

Definition of and discrimination between acute hepatitis, severe acute hepatitis, and fulminant hepatitis (or acute liver failure)

Acute hepatitis can be graded according to severity, as "acute hepatitis," "severe acute hepatitis," and, in its most severe form, "acute liver failure" or "fulminant hepatitis." The term "fulminant hepatitis" is controversial, as some have suggested fulminant hepatitis to be any acute hepatitis with severe coagulopathy, independent of the presence of hepatic encephalopathy (HE).

Thus, for practical purposes, it is simpler to divide acute hepatitis into three categories:

1. Acute hepatitis: hepatitis of less than 26 weeks' duration.

2. Severe acute hepatitis: acute hepatitis and the presence of coagulopathy with INR increased to >1.5.

3. Acute liver failure or fulminant hepatitis: acute hepatitis and the presence of coagulopathy and HE [1].

Exclusion of chronic liver disease is important because three principal differences in the management of acute liver failure and acute-on-chronic liver failure exist:

1. A different diagnostic approach: acute decompensation of chronic liver disease can be triggered by other factors (e.g., spontaneous bacterial peritonitis).

2. A different monitoring algorithm: patients with acute liver failure develop certain complications more frequently than those with acute-on-chronic liver failure (e.g., cerebral edema).

Viral Hepatitis, Fourth Edition. Edited by Howard C. Thomas, Anna S.F. Lok, Stephen A. Locarnini, and Arie J. Zuckerman.

3. A different therapeutic approach: only patients with acute liver failure are eligible for emergency liver transplantation in most situations.

Acute liver failure *may* be graded according to the time interval between onset of jaundice and HE as follows: hyperacute (<7 days), acute (7–28 days), and subacute (<26 weeks but >4 weeks). In general, a shorter interval is associated with a better prognosis for the patient. However, some now consider the acuteness to be more related to etiology (acetaminophen for the most part) and believe that the classification according to acuteness should be omitted, while others still believe that more acute presentation is associated with better prognosis in general [2].

Etiology of acute hepatitis and acute liver failure, and etiology-specific treatment options

Different etiologies of acute hepatitis and acute liver failure and potential treatment options are listed in Table 34.1, and described in this chapter. Table 34.2 itemizes suggested testing for patients with acute liver failure to help identify etiology, monitor disease progression, and predict prognosis.

In general, it is important to act as quickly as possible, ideally prior to the onset of HE. For several diseases, specific interventions are likely to be effective, but mostly before the onset of HE grades III or IV. The

Table 34.1 Causes of acute liver failure.

Etiology group	Etiology		Main characteristics	Causal treatment
Toxic (dose dependent)	Acetaminophen (US) Paracetamol (UK)		High transaminases and renal impairment	N-acetyl cysteine
	Amanita toxin (mushroom poisoning)		Severe nausea following a mushroom meal	Silibinin or penicillin G
	Carbon tetrachloride		Due to accidental inhalation	
Toxic (idiosyncratic)	Large list of different medications		Variable with different drugs or herbals	
Viral	Hepatitis A		More frequent with older age	
	Hepatitis B		Still the most frequent viral cause of acute liver failure	LAM, ETV, LdT, and TDF
	Hepatitis C		Rare: more frequent in Asia than in Europe and North America	IFN?
	Hepatitis D		Requires HBV co-infection, whether acute or chronic	IFN? LAM, ETV, LdT, and TDF for suppression of HBV replication
	Hepatitis E		Rare in Western world, but especially severe in pregnancy	Ribavirin in nonpregnant patients?
	Herpesviridae	HSV	High fever and leukopenia; poor prognosis, even with institution of acyclovir therapy	Acyclovir
		EBV	Rarely fulminant, but occasionally severe and acute	Ganciclovir
		CMV		Ganciclovir
		VZV	Might have no specific sign, and IgM anti-VZV might not be reliable; VZV-PCR might be required	No causal treatment known
	Coxsackievirus		Rare: mostly in neonates	No causal treatment known
	Adenovirus		Rare: mostly in immunosuppressed patients	No causal treatment known
	Parvovirus B19		Might be associated with aplastic anemia	No causal treatment known
	Hemorrhagic fever viruses		Rare	No causal treatment known

(*Continued*)

Table 34.1 (*Continued*)

Etiology group	Etiology	Main characteristics	Causal treatment
Immunologic	Autoimmune hepatitis	Hepatitis-like presentation, but with high IgG levels and positive autoantibodies; often needs histologic confirmation	Steroids +/– azathioprine
	GvHD	History of transplantation	Steroids
Metabolic	Wilson's disease	Typically young age, high bilirubin, low alkaline phosphatase, and hemolysis,	d-penicillamine or tetra-thiomolybdate
Pregnancy related	Fatty liver of pregnancy	Pregnancy, usually third trimester	Immediate delivery; no other causal treatment known
	HELLP-syndrome ("hemolysis" [H], "elevated liver tests" [EL], and "low platelet count" [LP])	Pregnancy or recent delivery	Delivery if still pregnant No other causal treatment known
Ischemic or vascular disease	Cardiovascular shock	AST >> ALT, hypotension due to heart failure or sepsis	Circulatory support and correct underlying cause
	Budd–Chiari syndrome	Imaging shows occlusion of hepatic veins	Anticoagulation with heparin followed by warfarin, angioplasty, or stenting of hepatic vein; TIPS
	Sinusoidal obstruction syndrome (SOS) (formerly known as veno-occlusive disease [VOD])	Following bone marrow transplantation and some other chemotherapeutics	Tissue plasminogen activator (tPA); defibrotide under FDA review (slight improvement in mortality: 62% vs. 75%)
Miscellaneous	Reye's syndrome	Mostly children, mostly following an acute illness, potentially related to salicylates	No causal treatment known
	Hyperthermic injury from heat shock	History of strenuous exercise in warm climate	Symptomatic, particularly hydration
	Malignant infiltration of the liver	Evidence for weight loss, health deterioration, and liver biopsy might be only way to identify	Chemotherapy of underlying disease if at all feasible
	Malaria	History of exposure and positive blood test	Antimalaria medication according to geography
	Leptospirosis	Rare: malnutrition might be required as cofactor for liver failure	No specific therapy established
	Dengue fever	Rare: malnutrition might be required as cofactor for liver failure	No specific therapy established; ribavirin?
	Typhoid fever	Rare: malnutrition might be required as cofactor for liver failure	Antibiotics
	Hemophagocytosis	Rare: part of hemophagocytic lymphohistiocytosis (HLH), also known as hemophagocytic syndrome	Cyclosporine, etoposide, and dexamethasone (and alemtuzumab)
	Tuberculosis	Rare: malnutrition might be required as cofactor for liver failure	Antituberculosis drugs (these drugs can also cause acute liver failure)
	Amoebic liver abscess	Rare: malnutrition might be required as cofactor for liver failure	Metronidazole and potentially drainage

Table 34.2 Recommended testing in patients with acute hepatitis progressing to acute liver failure.

Test concerning etiology	Tests to be ordered for assessment of severity and prognosis	Test to enable transplant listing
Anti-HAV IgM	Bilirubin	Anti-HIV
Anti-HBc IgM	Arterial pH	AB0 blood group typing
HBsAg and HBV DNA	INR	Drug screen
Anti-HCV	Factor V	
HCV RNA	Creatinine	
	AST	
Anti-HEV	ALT	
Anti-HSV IgM HSV PCR	Alkaline phosphatase	
Anti-HDV (if HBsAg or anti-HBc IgM is +)	gGT	
Anti-CMV IgM CMV PCR		
VZV PCR		
IgG levels		
ANA	Conjugated bilirubin	
AMA	Lactate	
Toxicology screen	Ammonia levels	
Acetaminophen–paracetamol level	Lipase	
Ceruloplasmin	Alpha fetoprotein (as marker of cell regeneration)	
Urine copper excretion or Kayser Fleischer ring		
Pregnancy test	CBC	
Adenovirus DNA	Urea	
Parvo B19 RNA	Sodium	
Coxsackievirus RNA	Potassium	
Tyrosine levels	Chloride	
Galactose	Bicarbonate	
	Calcium	
Questions	Magnesium	
Recent medications	Phosphate	
Recent toxin and mushroom exposure	Glucose	

likelihood of survival without liver transplantation is limited once HE is present, especially after HE stage III or IV is evident.

It is vital that all patients with acute hepatitis and HE should be evaluated for transplant benefits and ideally be transferred to a transplant center. Moreover, transfer should occur prior to HE [1].

Viral hepatitis, acute hepatitis, and acute liver failure

All hepatitis viruses are capable of inducing a form of acute liver failure, especially hepatitis viruses A, B, D, and E. There have been rare reports of acute liver failure in relation to HCV infection.

In recent times, nonviral etiologies have become more frequent, while the incidence of viral-induced acute liver failure has decreased as the result of more widely available vaccinations and other preemptive approaches to prevent transmission via sexual exposure or injection drug use and as the result of universal precaution in healthcare settings.

Hepatitis viruses: HAV to HGV

Hepatitis A virus

Hepatitis A virus (HAV) infection causes only acute infection, and no chronic case has yet been described. HAV infection is mostly a benign infection when acquired in childhood. Morbidity and mortality increase with the patient's age (leading to death in approximately 2.5% of patients older than 49 years in one study [3]; and 1.8% in those older than 60 years compared to 0.7% between ages 40 and 59, 0.5% between ages 15 and 39, and 0% younger than 15 years in another study [4]. Underlying chronic hepatitis B was found to be a risk factor for a deadly course of acute hepatitis A, with 0.05% (15/27346) mortality among hepatitis B surface antigen (HBsAg)-positive individuals versus 0.009% (25/283400) among HBsAg-negative individuals during a Shanghai outbreak in 1988 [5]. Of note in less developed countries, HAV might carry a higher risk of mortality due to malnutrition. Given the safety of HAV vaccines, universal HAV vaccination should be considered.

Risk factors for a severe course of hepatitis A

While acute hepatitis A mostly runs a mild course, it can progress to severe hepatitis. The most widely recognized risk for more serious disease in acute hepatitis A is older age. This is consistently found in several studies.

In addition, underlying liver disease seems to be a risk factor, especially with viral hepatitis C or B. One study reported in 1999 suggested a higher rate of fulminant cases of HAV infection in the background of underlying chronic HCV infection [6], but this was not universally confirmed. Still, vaccination against HAV is recommended for patients with chronic liver disease. Underlying chronic hepatitis B has been found to be a risk factor in several studies, including the Shanghai outbreak mentioned earlier.

High HAV viral load has been associated with more severe disease [7], but it is important to note that patients with fulminant hepatitis A often have undetectable HAV RNA in their serum at presentation, possibly reflecting an aggressive immune response [8]. There are no data supporting a benefit of antiviral therapy in patients with acute liver failure due to HAV.

There could be a genetic risk factor, suggested by case reports of familial clustering of fulminant hepatitis A, such as a mother and son in the US Acute Liver Failure (ALF) study, three siblings of Iranian origin in a study from Israel, and two siblings in a study from Turkey. Whether clustering of acute liver failure in these cases is due to host genetic factors or more virulent HAV genotypes or viral sequences is not clear. Active vaccination against HAV could prevent both infection and disease, and combined passive and active prophylaxis can prevent outbreaks when given before clinical onset.

Specific treatment for HAV

There is no established treatment for acute hepatitis A, and treatment is usually not necessary because it is usually a relatively mild and self-limiting illness. Once there is evidence of coagulopathy or encephalopathy, liver transplant evaluation should be considered. No antiviral has been proven effective in humans. *In vitro* studies found antiviral activity for several compounds – amantadine, ribavirin, glycyrrhizin, and pyrazofurin [9, 10] – but none has been confirmed to have antiviral activity or to improve outcomes in humans.

When acute hepatitis A progresses to acute liver failure, transplantation represents the only therapeutic option. Several reports have described persistence of HAV after liver transplantation, which can lead eventually to recurrent liver failure [11]. How often this occurs is unclear. It is also unknown whether passive prophylaxis with immunoglobulin can prevent such recurrence.

In summary, no treatment for acute HAV is needed in most cases, and no treatment is established.

Hepatitis B virus

Infection with HBV is preventable by active immunization, and universal vaccination of newborns should be available. However, active vaccination programs vary geographically (see Chapter 14). In most countries, including but not limited to the United States, Taiwan, and most if not all European countries, incidence of acute HBV infection is on the decline, thanks to widening vaccine coverage.

In the absence of vaccination, HBV is highly contagious. It is estimated that 3 to 200 copies of HBV, depending on the virus strain, are sufficient to cause infection [12, 13]. The progression to chronicity in immunocompetent adults varies between 0.2% and 13% in various studies [14], and is somewhat higher in immunocompromised patients. The low rate of chronicity in immunocompetent patients makes prevention of chronicity not a likely indication of antiviral therapy.

After an incubation period of 2 weeks to 6 months, jaundice develops in about 14% to 30% of infected individuals [15, 16]. The likelihood of symptomatic disease following HBV infection is likely influenced by both the infectious dose within an inoculum and the sequences of the infecting strain. In a study evaluating patients who received a serum-stabilized yellow-fever vaccine, jaundice developed in 5% to 40% of patients who received different vaccine lots [17], with jaundice occurring more frequently among older individuals. Increases in attack rate and severity of acute HBV infection with age were also suggested by a report from an outbreak in North Carolina. In a nursing home with 40 residents, eight of 15 considered susceptible to an HBV infection due to lack of immunity developed acute hepatitis B, and six of these eight (75%) elderly patients died from complications of acute HBV [18]. In 2005, about 1% (24/2452) of patients reported to the US Centers for Disease Control and Prevention with acute HBV infection and with information on their survival died from their hepatitis B infection [19]. In addition, a few patients were transplanted for fulminant hepatitis B. HBV remains an important cause of acute liver failure [20].

Risk factors for more severe form of acute hepatitis B are less well established compared to hepatitis A. Age has been reported to be relevant [21], as well as the viral sequence, where viruses harboring basal core promoter and/or precore mutations (precore [G1896A and G1899A] and core promoter [T1753A/C, T1754C/G, and A1762T/G1764A]) were associated with more severe acute hepatitis B, in addition to seronegativity for

hepatitis B e antigen (HBeAg) [22]. Co-infection of HBV with HCV or hepatitis D virus (HDV) is also associated with a higher risk of severe acute hepatitis and acute liver failure.

Specific treatment for acute HBV infection and liver injury

Treatment of acute hepatitis B

While most patients with acute hepatitis B will not require any treatment due to low chronicity rate and low rate of severe disease, a minority of patients with more severe disease may benefit from intervention. For acute hepatitis in the absence of coagulopathy, antiviral therapy is not necessary [23].

Treatment of severe acute hepatitis B

There have been several cases series that indicate efficacy of antiviral therapy. In addition, there are two randomized studies in patients with severe acute hepatitis B. One underpowered study from India reported no benefit from lamivudine in acute hepatitis B, and this included patients with severe acute hepatitis B defined as fulfilling two of the following three criteria: HE, serum bilirubin >10.0 mg/dL, and international normalized ratio (INR) >1.6. That study suggested faster recovery in 31 lamivudine-treated patients compared to 40 patients who received placebo, but the difference was not significant, possibly due to the small number of patients [24]. In contrast, a study of 80 patients from China with severe acute hepatitis B, bilirubin about nine times the upper limit of normal (171 μmol/l), and an INR between 1.4 and 1.6 found higher mortality in patients in the control group (10/40; 25%) versus the lamivudine-treated group (3/40; 7.5%; p = 0.034) [25].

Treatment of acute liver failure due to hepatitis B

No placebo-controlled trial has been published, but cases series have suggested lower mortality in patients with acute liver failure, when treated with nucleosides before more advanced HE has emerged [3]. In a study recruiting only patients with established liver failure, no benefit of nucleosides on survival was observed [26], but the authors concluded that nucleos(t)ide analogs are indicated in these patients to reduce viral load prior to transplant.

Which patient with acute hepatitis B should be treated with antiviral therapy?

For patients with acute hepatitis B without impairment of coagulation parameters, antiviral treatment is not required. However, for patients with severe acute hepatitis defined by the presence of impaired coagulation parameters and for patients with acute liver failure, antiviral therapy should be initiated. Any treatment intervention is more likely to be effective when initiated early. According to the American Association for the Study of Liver Diseases (AASLD) guidelines, antiviral therapy in acute hepatitis B should be initiated once INR increases over 1.5.

How to treat?

There is good evidence for an overwhelming immune response in the pathogenesis of HBV-related acute liver failure. Therefore, an immune-stimulant such as interferon alpha (IFNα) could be dangerous. Interferon had been explored as an option for acute hepatitis B in the past, without deleterious effects but also without evidence of benefit [27].

Oral nucleos(t)ide analogs inhibit HBV replication with an immediate decline of serum HBV DNA. Lamivudine was first reported to prevent death in a few case reports of patients who developed acute reactivation of hepatitis B. Likewise, lamivudine was observed to be effective in ameliorating HBV reactivation in a series of renal transplant recipients. These studies showed that early start of lamivudine therapy appears to be essential.

The same appears to hold true for severe acute hepatitis B infection in immunocompetent patients: several case series have reported lower mortality in patients treated with lamivudine for severe acute hepatitis or acute liver failure due to HBV [28]. In addition, two case series showed significantly better survival in lamivudine-treated patients compared to historic controls (Table 34.3). Two studies reported a multivariate analysis, where in both studies, age over 45 and the presence of SIRS (systemic inflammatory response syndrome) were detrimental, while lamivudine therapy was associated with improved survival [29, 30].

Given the efficacy and safety of nucleosides reported so far, they would be preferred in the setting of severe acute or acute liver failure, and interferon should not be used because of the risk of infection and worsening of liver failure.

Which antiviral to use in acute hepatitis B?

Most studies have used lamivudine; however, it is likely that other nucleos(t)ide analogs such as telbivudine, entecavir, or tenofovir may be equally effective. Adefovir has lower antiviral activity and potential nephrotoxicity, and is therefore not preferred. Tenofovir also has potential nephrotoxicity. Telbivudine is associated with a high rate of drug resistance, but this may not be a

Table 34.3 Lamivudine treatment in patients with severe acute hepatitis B: patient survival in two case series with historic controls and two series with concurrent controls*.

Studies with historic controls		
Control (no lamivudine)	Lamivudine treated	Reference
4/20 (20%)	14/17 (82.4%) $p < 0.001$	[29]
6/23 (26.1%)	7/10 (70%) $p = 0.026$	[30]
	7/14 (50%) $p = 0.17$**	
Studies with concomitant controls		
Control (no lamivudine)	Lamivudine treated	Reference
1/3 (33.3%) non-MHH	15/20 (75%) $p = 0.2$, non MHH	[29]
6/39 (15.4%)	14/38 (36.8%) $p = 0.032$	[31]
6/40 (15%)***	14/40 (35%) $p = 0.069$***	
Total controls	Total lamivudine treated	
17/86 (19.7%)	50/85 (58.8%), $p < 0.001$	
17/87 (19.5%)***	50/91 (54.9%)**, *** $p < 0.001$	

*At a center where lamivudine was not yet implemented in treatment of fulminant hepatitis B.
**Four patients reported to have been excluded for receiving a liver transplant.
***Including three patients who received a liver transplant.
MHH stands for Medical Hochschule Hannover, where the single center's experience was reported together with experience from five non-MHH sites.

major concern in patients with acute hepatitis B in whom the duration of treatment is expected to be short.

The AASLD practice guidelines for HBV note the lack of sufficient data and recommend lamivudine, telbivudine, or entecavir as options; lamivudine and telbivudine could be considered if the anticipated duration of treatment is short [1, 4]. The 2012 European Association for the Study of the Liver (EASL) clinical practice guidelines on hepatitis B recommend entecavir or tenofovir [31].

Acute-on-chronic hepatitis B

In the absence of a history of chronic hepatitis B, it is difficult to discriminate "acute hepatitis B" from "acute-on-chronic hepatitis B." Both can present with a similar clinical picture, and hepatitis B core antibody (anti-HBc) IgM can be positive in both acute hepatitis B and acute-on-chronic liver failure due to HBV. Anti-HBc IgM titers are usually higher in acute hepatitis B compared to exacerbations of chronic hepatitis B [32].

If there is suspicion of a preexisting chronic hepatitis B or known chronic hepatitis B, antiviral treatment should be initiated promptly and nucleos(t)ide analogs with high barriers to resistance should be used given the need for a long duration of treatment.

There are currently two different definitions for acute-on-chronic liver failure (ACLF):

1. The Asian Pacific Association for the Study of the Liver (APASL) defined ACLF [33] as acute hepatic insult manifesting as jaundice (bilirubin ≥5 mg/dl [85 μmol]) and coagulopathy (INR ≥1.5 or prothrombin activity of <40%), complicated within 4 weeks by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease.

2. The second definition was suggested by an EASL–AASLD single-topic conference: "Acute deterioration of pre-existing, chronic liver disease, usually related to a precipitating event and associated with increased mortality at 3 months due to multi-system organ failure" [34].

In a small study from India, 14 patients with reactivation of HBV and ACLF were randomized to tenofovir, while 13 patients received placebo. The 3-month mortality was 6/14 (42.8%) and 11/13 (84.6%, p=0.03) respectively, and survival was related to fast reduction of HBV DNA within 2 weeks [35].

While the ethics of placebo control in such populations is questionable, the results of that study were clear: early treatment intervention has the potential to reverse the course of an otherwise very grim disease. Therefore, the 2009 APASL guidelines recommended antiviral therapy in all patients with ACLF due to hepatitis B [7]. Even in the absence of clinical signs of liver failure, patients with severe exacerbations likely benefit from antiviral treatment.

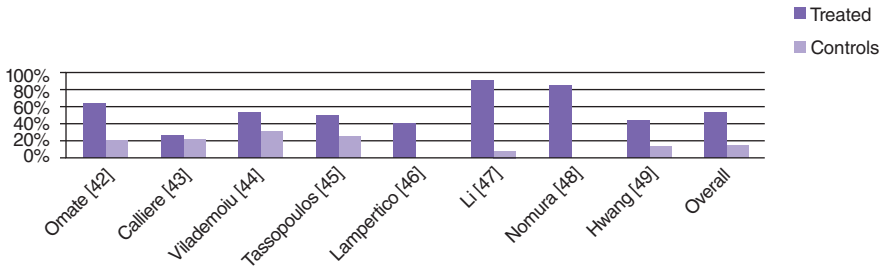

Figure 34.1 HCV clearance in prospective randomized trials comparing interferon regimens with spontaneous course [42–49].

Hepatitis C virus

For the most part, acute HCV infection appears to run a milder clinical course than acute HBV, with transaminases above 1000 IU/mL being infrequent and icterus not common [36]. However, chronicity evolves in more than 50% of patients with acute HCV infection, though the chronicity rate is somewhat lower in symptomatic patients than is the case in asymptomatic patients. Severe acute hepatitis or death due to liver failure is rare in acute HCV infection but has been reported. Cases have been reported more frequently in the 1990s from Japan [9] but very rarely from Western countries. Also in Japan, the proportion of acute liver failure due to HCV has declined [37].

Specific therapy for acute HCV

Most cases of HCV infection go unnoticed because of its mild clinical course. However, for those clinically evident cases of acute hepatitis C, there is currently some consensus that interferon is helpful in preventing chronicity among a substantial number of patients [38, 39]. In different meta-analyses, the effect size differed substantially, but in each analysis clearance was higher among patients receiving interferon, even though up to 55% of patients cleared infection spontaneously [40, 41]. The most recent meta-analysis suggested that early intervention with interferon leads to higher clearance rates than delayed treatment [58], with 55% spontaneous clearance rate, 62.5% clearance rate if treatment is initiated more than 24 weeks after diagnosis of acute hepatitis C, 66.9% when treatment is initiated between 12 and 24 weeks, and 82.5% when treatment is initiated within 12 weeks. Patients were not evaluated in relation to symptoms in this analysis, but in general, symptomatic patients have a higher likelihood of spontaneous clearance.

Very few randomized trials have been performed in acute HCV infection; however, those trials suggest an even greater effect of early therapy, which is largely related to a very low rate of spontaneous HCV clearance in these studies (see Figure 34.1) [42, 43, 44, 45, 46, 47, 48, 49].

Can IL28B change the approach to early versus delayed treatment intervention?

Similar to *IL28B*'s role in predicting response to PEG-IFN plus ribavirin therapy for chronic hepatitis C, several studies evaluated its role in predicting spontaneous clearance. These studies found a >50% spontaneous clearance rate in patients with a favorable *IL28B* genotype. This high spontaneous clearance rate suggests that *IL28B* can be helpful in individualizing treatment for patients with clinically evident acute hepatitis C [50]. Patients with an *IL28B* genotype (i.e., rs12979860 CC) that is more responsive to interferon, especially in the presence of jaundice, have a more than 50% chance of spontaneous viral clearance and can defer treatment as they are likely to respond to interferon-based therapy even if treatment is initiated at a later time point. In contrast, patients with less favorable *IL28B* genotypes are unlikely to clear HCV spontaneously and are also less likely to respond to interferon, particularly if treatment is initiated late. Testing for HCV genotype and *IL28B* genotype would guide individualized care. Patients with "favorable" *IL28B* genotype and/or patients with HCV genotype 2 or 3 infection can be observed for 12 weeks, and treatment can be initiated if spontaneous clearance does not occur.

When and how to treat acute hepatitis C

The current recommendation for treatment of acute hepatitis C is PEG-IFNα monotherapy (PEG-IFNα-2a, 180/week, or PEG-IFNα-2b, 1.5 μg/kg/week, for 24 weeks). Although there is not sufficient evidence that PEG-IFN is superior to standard interferon in acute hepatitis C, once-weekly administration is likely to help with compliance and facilitates direct observed therapy if necessary, such as for drug-abusing patients. Such treatment is expected to achieve viral eradication in >80% of patients compliant with the medication, when

initiated early [11]. Interestingly, ribavirin appears not to be required in cases of acute hepatitis C, though most experts would consider adding ribavirin at week 4 if HCV RNA remains detectable after 4 weeks of PEG-IFN monotherapy.

HCV-related acute liver failure

Due to the rarity of acute liver failure due to HCV, no treatment has been established. However, interferon may be an option. While IFNα treatment may cause a flare in patients with hepatitis B, this is rarely seen in patients with hepatitis C. IFNα might be safer in HCV-related acute liver failure than in HBV-related acute liver failure, but risk of bacterial and fungal infection might be increased. In future, direct-acting antiviral agents that are more potent and have less side effects might be considered.

Hepatitis D virus

Incidence of acute HDV infection is on the decline since the introduction of HBV vaccination. HDV infection requires ongoing HBV infection. Co-infection of HBV and HDV is more frequently associated with acute liver failure than acute HBV mono-infection, while HDV superinfection in a person with underlying chronic HBV infection may present as exacerbation of chronic hepatitis B or acute hepatitis in patients with previously undiagnosed chronic HBV infection [51]. In the latter case, patients may present with acute-on-chronic liver failure. In general, co-infection is more likely to lead to severe acute presentation but rarely leads to chronicity, while superinfection of HDV usually leads to chronicity [52].

In an Italian study demonstrating a recent decline in incidence of acute HDV, two of 218 patients co-infected with HDV, but none of the 128 HDV-superinfected patients, died [53].

Specific therapy for acute HDV infection

The only treatment option for chronic hepatitis D is interferon, which has limited efficacy (see Chapter 29). At present, it appears that combining interferon with a nucleos(t)ide analog does not confer any overall benefit [54]. In a study by Wedemeyer *et al.* [54], the primary endpoint of sustained virological response 24 weeks after a 48-week therapy was not significantly different between PEG-IFN plus adefovir versus PEG-IFN alone; however, a decline in HBsAg levels of more than 1 log(10) IU per milliliter from baseline to week 48 was observed in 10 of 31 (32.3%) patients receiving the combination in comparison with two of 29 (6.9%; p=0.01) receiving PEG-IFN alone. There are no data on the use of IFN with or without nucleos(t)ide analog in patients with acute liver failure due to HBV/HDV co-infection or acute-on-chronic liver failure due to HDV superinfection. These patients should be evaluated for liver transplantation and receive prophylaxis to prevent HBV re-infection as that would also prevent HDV re-infection. A majority of the patients with HBV–HDV co-infection will have a self-limiting course and do not require antiviral therapy.

Hepatitis E virus

HEV is the most common cause of enterically transmitted non-A, non-B hepatitis [55]. There seems to be a difference in the clinical course between infected patients in highly endemic regions and those infected in less endemic regions. In highly endemic regions, most infected individuals are in the 10–40-year age range, while in low-endemic areas, patients with acute hepatitis E are generally older [56]. In both instances, men are more likely to be affected than women [57]. Among affected women, the disease has a more severe clinical course if they are pregnant. In some of the endemic regions, HEV has become the most frequent cause of sporadic acute viral hepatitis. Some data suggest that HEV viral load correlates with disease severity.

One particular problem is the high rate of acute liver failure among pregnant patients acutely infected with HEV. A large Pakistani single-center experience, including 1015 patients with fulminant hepatitis aged between 15 and 40, found that HEV-induced acute liver failure was more frequent among females than males (30.4% vs. 23.1%; p=0.03), but even more frequent among pregnant compared with nonpregnant women (59.4% vs. 30.4%; p<0.0001). A similar trend was observed for mortality from HEV-associated fulminant hepatic failure: 37.1% for males, 46.0% for nonpregnant females, and 51% for pregnant females [58]. One study explored the relation of viral load and pregnancy status in acute hepatitis E versus fulminant hepatitis E, and found that in addition to a higher prevalence of HEV RNA in serum among pregnant women, the viral load was highest in pregnant women with fulminant hepatitis E (5870 ± 1.5E5 IU/mL), followed by pregnant women with mere acute hepatitis E (343.3 ± 216.44 IU/mL), nonpregnant women with fulminant hepatitis E (199 ± 225 IU/mL), and nonpregnant women with acute hepatitis E (13.8 ± 7.8 IU/mL). All women with detectable HEV RNA were infected with HEV genotype 1 [59]. A subsequent study at the same institution confirmed higher HEV viral load in pregnant women with fulminant hepatitis E compared to those with mere acute hepatitis E (139 994.0 ± 103 104.17 versus 768.92 ± 1105.40 copies/ml; p=0.046) [60]. In addition, the authors found IL10 and IL12 to be elevated in pregnant women with fulminant hepatitis, while both progesterone receptor expression and progesterone-

induced blocking factor expression in the placenta were reduced in women with fulminant hepatitis E [61].

HEV infection is uncommon in the Western world, and HEV-induced acute liver failure is even rarer. The reasons for the different clinical course in the Western world compared to high-endemicity areas is not well understood at present, but might be related to different HEV genotypes and different nutrition status. However, HEV always has to be kept in mind in the differential diagnosis of hepatitis, especially when tests for hepatitis A–D are negative. In a British study, 6/50 (12%; 95% CI: 5.7–23.9%) patients with suspected drug-induced liver injury (DILI) were found to have acute HEV infection [61]. In the Western world, there appears to be an association between male gender and older age, which was also seen in a US study of 318 patients with presumed drug-induced liver injury; in this study, 9/318 (2.8%; 95% CI: 1.5–5.3%) were positive for HEV IgM, and these nine patients were significantly older (mean age 67.2 vs. 47.0, $p<0.001$) and were mostly men (8/9 [89%] versus 104/268 [34%] of anti-HEV IgG and IgM-negative patients, $p=0.003$) [62]. Most HEV cases in low-endemic areas appear to be the result of genotype 3 infection, without history of travel to areas where HEV is endemic. The source of HEV infection seems to be zoonotic transmission from contact with live animals, particularly swine, or from eating undercooked pork. In high-endemic areas, genotypes 1 and 2 dominate. In general, acute HEV infection is self-limiting, but in immunosuppressed patients chronic HEV infection has been observed.

Specific therapy for acute hepatitis E

Two fairly large phase II studies demonstrated the efficacy of vaccination [63, 64], but no vaccine is currently available and licensed for use globally, though China has licensed a vaccine according to WHO's Hepatitis E Fact sheet N°280, from July 2012.

For nonsevere cases, no treatment is required; however, in severe cases with coagulopathy, treatment might be considered on an individual basis. As viral load might be responsible for dictating disease severity [13], antiviral therapy could be effective. In a few cases of chronic HEV, interferon and/or ribavirin have been reported to have antiviral activity, resulting in sustained clearance of HEV. At least one case report demonstrated the feasibility of ribavirin therapy in the setting of severe acute hepatitis E [65]. Importantly, emergency delivery has not been shown to modify the outcome for pregnant women. Both interferon and ribavirin are contraindicated during pregnancy. Liver transplantation should be considered in those with acute liver failure, but this option is not available in most countries where HEV is endemic.

Other viral infections that can cause acute hepatitis and acute liver failure

Herpesviridae

Herpesviridae can cause hepatitis and, in rare cases, acute liver failure. Herpesvirus simplex I and II viruses (HSV-1 and HSV-2), Epstein–Barr virus (EBV), varicella zoster virus (VZV), and the human herpes virus 6 (HHV-6) have been reported to cause acute liver failure. Additionally, adenovirus infections have been attributed to cause fulminant hepatitis, especially in immunosuppressed or pediatric patients. In neonates, coxsackievirus B infection may present as fulminant hepatitis. The role of Parvo B19 virus, previously reported in relation to fulminant hepatitis, has recently been questioned. Finally, hemorrhagic fever viruses such as dengue, lassa, ebola, and yellow fever may also induce fulminant hepatitis.

There is not sufficient evidence to conclude whether acute liver failure can be a result of cytomegalovirus (CMV) infection, even though it is a well-known hepatotropic virus.

GB virus C (GBV-C), alias hepatitis G virus (HGV), and other presumed "hepatitis viruses"

Two initial reports in 1996 suggested an association between GB virus C or hepatitis G virus (HGV) and acute liver failure, but this association was subsequently not confirmed.

Another virus, transfusion-transmissible virus (TTV), was also not confirmed to cause acute liver failure. Neither HGV nor the TTV is considered to be associated with fulminant hepatitis or with hepatitis at all [66].

Nonviral causes of acute liver failure

Among nonviral factors contributing to acute liver failure, the most common in developed countries is acetaminophen (in the United States; paracetamol in the United Kingdom) intoxication. Overdose leading to poisoning might be intentional in a suicide attempt or non-intentional due to chronic use of pain medications. Other causes of acute liver failure include Wilson's disease, intoxication by *Amanita phalloides* as a result of mushroom poisoning, acute fatty liver of pregnancy, severe forms of HELLP (**h**emolysis, **e**levated **l**iver enzymes, and **l**ow **p**latelets) syndrome, acute Budd–Chiari syndrome, ischemic hepatitis, autoimmune hepatitis, and a variety of drugs such as halothane and isoniazid. Finally, sepsis, hyperthermia, malignant infiltration of the liver, and Reye's syndrome may, in rare

cases, cause acute liver failure (for a complete list, see Table 34.1).

Prognosis and indication for liver transplantation

Acute or severe acute hepatitis is not an indication for liver transplantation. However, these patients may progress to fulminant hepatitis or acute liver failure. Thus, patients must be monitored for signs of recovery versus deterioration.

Basically, prognosis of fulminant hepatitis depends on halting further cell damage and on the ability of hepatocytes to replicate, thereby resulting in liver regeneration. Regeneration is likely to decrease with age and history of prior damage to liver cells. This explains why a higher risk of death is observed in older patients with acute hepatitis and in those with preexisting underlying liver disease. Prognosis deteriorates dramatically once HE sets in.

The most widely used prognostic scoring system, the King's College criteria [67], was established more than three decades ago but still appears to be the best and most widely used scoring system. It is a relatively simple score that requires limited information that is readily available: cause of liver failure due to acetaminophen-paracetamol or other causes, serum pH, prothrombin time, creatinine, HE grade, age, bilirubin, and interval between onset of jaundice and onset of encephalopathy (see Table 34.4).

Table 34.4 Prognostic scores for acute liver failure.

Scoring developed with cutoffs for indication for liver transplantation

Criteria of King's College London [67]	
Indication for transplantation is met when the criteria listed below are met	
Paracetamol intoxication	Other etiologies
Arterial pH <7,3	PTT >100 sec (INR >6,7)
Or all of the following:	Or three of the four following criteria:
PTT >100 sec	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
	Unfavorable etiology (hepatitis of unknown origin, drug-induced liver injury)
French criteria (Clichy) [68]	
Indication for transplantation is met when the criteria listed below are met	
Factor V <20 % and patient age <30 years	
Or	
Factor V <30 % and hepatic encephalopathy grade 3–4°	
BiLE score (bilirubin, lactate, and etiology) [70]	
BiLE score >6.9	
BiLE score = bilirubin ([μmol/L]/100) + baseline lactate (mmol/L) + 4 (for indeterminate ALF, Budd–Chiari syndrome, or phenprocoumon toxicity) – 2 (for acetaminophen toxicity) + 0 (in case of any other ALF etiology)	

PTT: prothrombin time; INR: international normalized ratio.

Additional scoring systems that might be helpful in predicting mortality but have no clear established cutoff point for indication of liver transplantation

MELD (model of end-stage liver disease) score (9.57 × $\log_e$ creatinine (mg/dL) + 3.78 × $\log_e$ bilirubin (mg/dL) + 11.20 × $\log_e$ INR + 6.43)
SOFA Score (http://clincalc.com/IcuMortality/SOFA.aspx) Requires PaO_2 and FiO_2, platelets, bilirubin, Glasgow Coma Score, mean arterial pressure, and creatinine
APACHE II Score Requires body temperature, mean arterial pressure, heart rate, respiratory rate, PaO_2 and FiO_2, arterial pH, sodium, potassium, creatinine, hematocrit, white cell count, Glasgow coma score, and bicarbonate
Clinical prognostic indicators Any three of the following: age ≥ 50, interval between jaundice and hepatic encephalopathy ≥7 days, grade III or grade IV hepatic encephalopathy, presence of cerebral edema, prothrombin time ≥35 seconds, and creatinine ≥1.5mg/dl
Liver Injury Unit (LIU) score LIU = 3.584 × peak total bilirubin (mg/dl) + 1.809 × peak PT (seconds) + 0.307 × peak ammonia (μmol/l) Substituting INR for PT, the formula was LIU =3.507 × peak total bilirubin + 45.51 × peak INR + 0.254 × peak ammonia.

Other factors that have been found to have prognostic value include lactate, factor V, and AFP [68, 69], but no score, including the SOFA (Sequential Organ Failure Assessment) and APACHE (Acute Physiology and Chronic Health Evaluation) scores, has proven to be superior to the King's College criteria. Most studies showed that the King's College criteria have high specificity but low sensitivity; therefore, there is a need for an alternative scoring system with improved sensitivity so patients who might benefit from liver transplantation can be identified early [1].

General treatment and prophylaxis recommended for patients with fulminant hepatitis and acute liver failure

While acute and severe acute hepatitis do not require any treatment other than supportive care and disease-specific intervention, as outlined in this chapter and in Table 34.1, patients with acute liver failure may benefit from N-acetyl cysteine (NAC) administration regardless of underlying etiology [70]. Importantly, the earlier in the course NAC is given, the greater the benefit that was observed [70]. This is well in line with observations for antiviral therapy in fulminant hepatitis B, where death despite antiviral therapy has been mostly associated with delayed treatment initiation.

Complications of acute liver failure and their treatment

Acute liver failure leads to impairment and potential failure of several other organs. These extrahepatic complications frequently determine the eventual outcome of patients with fulminant hepatitis. Host defenses are compromised in patients with liver failure, leading to increased risk of bacteria as well as fungal infection [34].

Hepatic encephalopathy and cerebral edema

The frequency of severe HE, subsequent cerebral edema, and increased intracranial pressure (ICP) appears greatest in patients with acute onset compared to subacute onset of HE after development of jaundice. The pathophysiological basis is multifactorial and includes various circulating toxins, in particular ammonia, that impair neuronal function and cause damage to the blood–brain barrier.

Lactulose or retention enemata are commonly used to treat HE in patients with chronic liver disease, and more recently rifaxamin was approved for that indication. Whether they are helpful in the setting of acute liver failure is questionable. L-ornithine-L-aspartate (LOLA) can decrease circulating ammonia levels and has been shown to be beneficial in chronic liver disease, but a randomized study in India failed to demonstrate improvements in ammonia levels, HE, or survival for ornithine aspartate compared with placebo in patients with acute liver failure [71].

In acute liver failure, increased ammonia levels induce astrocyte swelling. Over time, cerebral perfusion is impaired by a loss of its autoregulation and by progressive cerebral edema with increasing intracranial hypertension. Finally, brain stem herniation and death of the patient may follow.

Cerebral edema is the main cause of death in acute liver failure. To exclude cerebral bleeding, a cranial computed tomography (CT) scan may be necessary, but CT scans are insensitive in detecting early cerebral edema and the utility of this procedure should be balanced against a potential increase in ICP due to moving the patient.

Direct quantification of ICP can be best performed by epidural ICP-monitoring catheters [72]. However, their use is associated with specific risks like bacterial infection or intracranial bleeding. To date, no controlled clinical trials have been published demonstrating a beneficial effect of epidural catheters. Therefore, it is recommended only for patients waiting for liver transplantation with HE of at least grade III. In addition, cerebral oxygen supply can be estimated by the jugular bulb oxygen saturation measurement [73].

Cerebral perfusion pressure (CPP) should be kept above 50–60 and is calculated by subtracting ICP from mean arterial pressure (MAP). Basic measures to minimize increased ICP include establishing a quiet environment with minimal interventions, elevating the patient's head [1], and sedation, when required.

The osmotic diuretic mannitol is the first-line treatment when there is evidence of increased ICP. In patients with renal failure, mannitol should be used with caution and may require hemodialysis or hemofiltration.

Therapeutic hyperventilation leads to cerebral vasoconstriction in ventilated patients. It can be used in incipient cerebral edema if the cerebral perfusion pressure is maintained at sufficient levels. In advanced cerebral edema, hyperventilation improves acute intracranial hypertension for only a short period of time. Prolonged hyperventilation can aggravate cerebral hypoxia and should be avoided.

Mild hypothermia (body temperature of 32–33 °C, or 89.6–91.4 °F) has been suggested, especially as a bridge to transplantation. There are conflicting data concerning impairment of liver regeneration due to hypothermia. Other therapeutic measures without sufficient evidence include thiopental, which reduces cerebral metabolism, and flumazenil, which antagonizes endogenous GABA-like toxins. If seizure activity is present, therapy with phenytoin is indicated.

Coagulopathy and bleeding complications

Coagulopathy as a consequence of disturbed hepatic protein biosynthesis is a hallmark of severe acute hepatitis and acute liver failure. Factor V and prothrombin time–INR (international normalized ratio based on prothrombin) are used to assess the severity of liver injury and hepatic function of viable hepatocytes. Factor V is used in the Clichy criteria for transplant listing, but one study suggested that although factor V was superior to INR, factor VII was superior to factor V [1]. Factor VII has the advantage of the shortest half-life, providing more accurate indication of changes in liver function.

Spontaneous bleeding is rare; therefore, transfusion of platelets and/or fresh frozen plasma (FFP) is indicated only when there is evidence of bleeding or prior to interventions. To avoid disequilibrium of clotting factors, FFP is preferred over factor concentrates. Antithrombin III (AT III) is selectively administered in cases of disseminated intravascular coagulation. If an intervention is required and FFP does not succeed in correcting coagulopathy, activated factor VII might be considered in very select cases. A general use of activated factor VII is not recommended due to its risk for arterial thromboembolic complications [74].

For prevention of upper gastrointestinal tract bleeding, acid suppression therapy is recommended in the form of H2 blocker or proton pump inhibitor (PPI); however, no studies to prove or disprove this approach exist.

Infections

Bacterial and fungal infections contribute highly to mortality in acute liver failure. These patients have increased infection rates as a consequence of various pathophysiological changes that impair cell-mediated and humoral immunity. Because of the high incidence of infections in acute liver failure, different prophylactic antibiotic combinations have been evaluated in the past, but none has proven superior to monitoring. The specific regimen should be chosen according to the local microbiological spectrum and resistance patterns. Prophylactic antimicrobial therapy is used on all patients in some centers, but there is no evidence of survival benefit and it cannot be recommended routinely. Vigilant monitoring for infection is of utmost importance to detect infection such that antimicrobial therapy can be promptly initiated at the earliest sign of infection.

Renal failure

Acute renal failure is a frequent complication of acute liver failure [67], and those with acetaminophen-paracetamol intoxication are especially at risk. The principal causes of acute renal failure are relative hypovolemia, parenchymal ischemia, and acute tubular necrosis by direct nephrotoxicity. Volume status should be controlled by continuous monitoring with central-venous catheters to avoid any increase in intracranial pressure due to volume overload. Electrolyte levels have to be carefully controlled and corrected where necessary. In cases of incipient renal failure, early renal replacement therapy is indicated. Continuous veno-venous hemofiltration (CVVH) seems to be better tolerated and should therefore be preferably used. Anticoagulation with heparin should be administered with caution because of the increased risk of bleeding.

Circulatory failure

Splanchnic vasodilatation is characteristic of acute liver failure. Septicemia is mimicked by increased cardiac output, reduced systemic blood pressure, reduced systemic vascular resistance, and reduced vascular filling pressures. Relative hypovolemia plays an important pathophysiological role in the etiology of other complications like renal failure. However, intracranial hypertension can cause an increase in systemic blood pressure.

Monitoring of central venous pressure is mandatory to manage fluid balance.

Respiratory failure

In patients with acute failure, respiratory failure is often part of multi-organ failure. Usually, it occurs only after the patient is already intubated for advanced HE. Respiratory failure is associated with a very high subsequent mortality risk [72]. Arterial hypoxemia can be caused by intrapulmonary shunting, adult respiratory distress syndrome (ARDS), pneumonia, aspiration, intrapulmonary hemorrhage, and pulmonary edema.

Metabolic derangements

Reduced gluconeogenesis and depletion of glycogen stores can result in hypoglycemia; therefore, close monitoring of blood glucose is mandatory.

Liver transplantation

Conventional orthotopic liver transplantation (OLT) is the only proven therapy for patients with acute liver failure. However, correct timing of liver transplantation is crucial. On the one hand, transplantation should be avoided in patients with a realistic possibility of hepatic regeneration and eventual survival. On the other hand, transplantation has to take place before irreversible complications like brain stem herniation develop, in

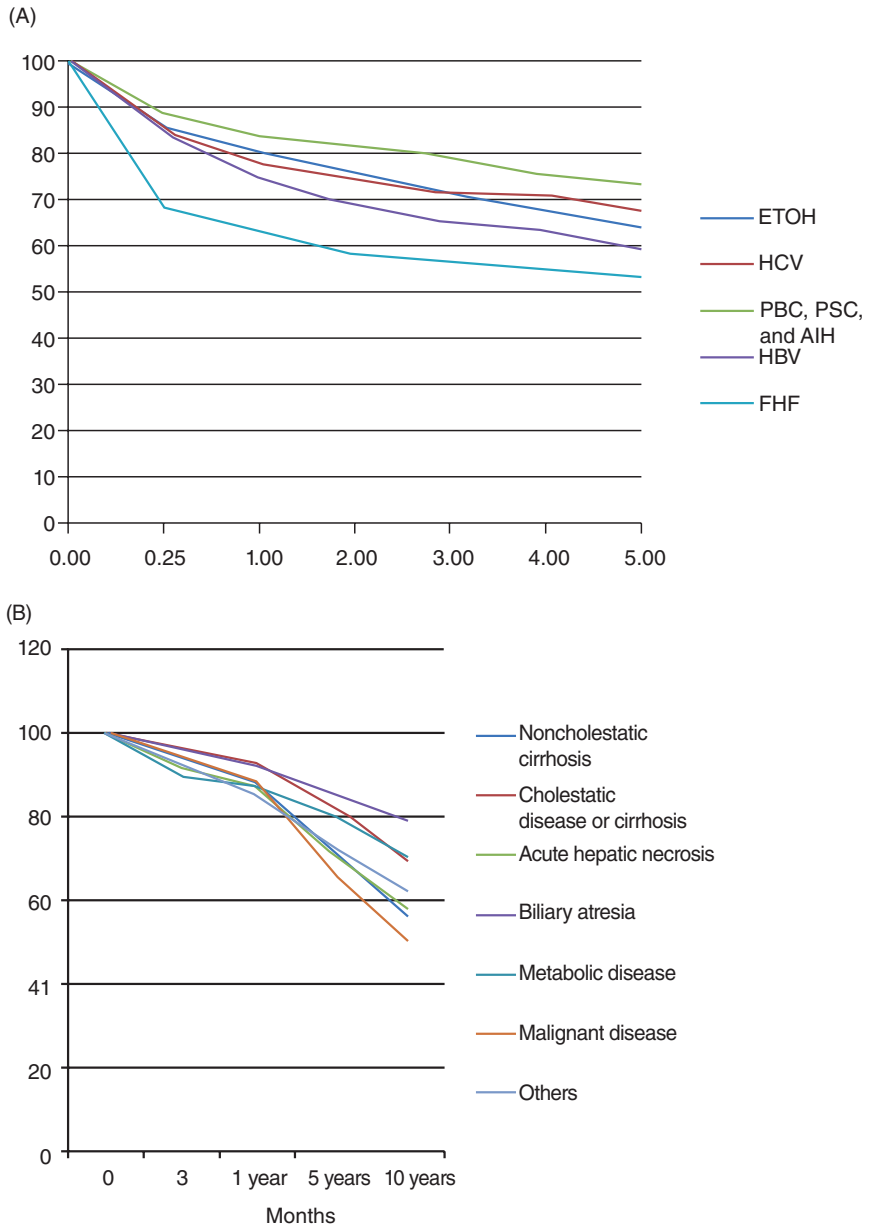

Figure 34.2 (A) Five-year survival according to etiology based on Pittsburghs first 4,000 transplants prior to the year [77]. (B) Survival according to etiology based on an 2009 OPTN/SRTR report for patients transplanted in the US [78]. (Color plate 34.1)

which case the intervention would be futile. To prevent unnecessary loss of time, every patient with acute liver failure should be referred to a specialized center for evaluation for liver transplantation. Transplantation is indicated in patients with an estimated mortality above 80%. Contraindications are irreversible brain damage, uncontrolled sepsis, AIDS, advanced comorbidities, or a malignant disease. Patients with acute liver failure 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 was fair for patients with acute liver failure (see Figure 34.2A) but seems to have improved with lower early mortality in more recent years (see Figure 34.2B), likely due to improved patient management and patient selection. If the patient survives the first few months after OLT, long-term prognosis is comparable to or even better than in patients transplanted for chronic liver disease.

Whole-organ transplantation is limited by the shortage of suitable donor organs. As a result, other options have been used, including splitting a liver from a

deceased donor for two recipients or transplanting part of a liver from a living donor. The logistics of finding and evaluating a suitable living donor in the short period of time before severe complications develop are complex, and there is also an ethical concern regarding whether the potential donor can fully grasp the risks involved.

In fulminant hepatitis, the liver has the potential for partial or full recovery. Therefore, technical variants of liver transplantation were introduced that allow the patient's own liver to remain in place, named "auxiliary liver transplantation." There are two potentials with this procedure. Firstly, if the native liver regenerates, immunosuppression can eventually be weaned. Secondly, in situations of small-for-size graft (i.e., in settings of living donor transplantation), an auxiliary graft that on its own might be too small to sustain survival works together with the remaining native liver to sustain survival.

The future

Hepatocyte transplantation

The transplantation of hepatocytes is still an experimental procedure, and it is not foreseeable when it may become a reality. The necessary amount of hepatocytes seems to be much higher in patients with fulminant hepatitis than in those with hereditary metabolic diseases. Thus, it is unlikely that hepatocyte transplantation will evolve as a tenable solution for fulminant hepatitis until it has become established for inborn metabolic disorders.

Xenotransplantation

After the initial euphoria about transplantation of xenogenous organs, it currently appears further away than it seemed about a decade ago. Thus, it is not expected to become a reality in the foreseeable future. A major reason for this drawback is that transplantation of organs from nonhuman species lead to hyperacute antibody-mediated rejection in addition to cellular rejection. While rejection may be overcome with genetically engineered humanized animals such as pigs, there are other obstacles such as risk of zoonotic infections and immunogenicity of secreted proteins made by livers from other animal species [75].

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.

"Extracorporeal liver perfusion" has been performed using pigs, baboons, humans, calves and cows, and combinations thereof, but none of these attempts in a total of 198 patients has suggested substantial benefit over regular intensive unit care [76]. "Artificial support systems" are basically modified hemodialysis or hemofiltration approaches employing sorbent-based systems using charcoal or albumin; the two most studied devices are "fractionated plasma separation and adsorption" (FPSA) and "molecular adsorbent recirculating system" (MARS). Finally, the "bioartificial liver support system" would include, in addition, cartridges containing human or other mammalian hepatocytes.

None of these systems has proven efficacy; therefore, they have no place outside clinical trials at present.

Hepatectomy as the *ultima ratio*

Hepatectomy as the *ultima ratio* in desperate situations for patients awaiting liver transplantation for fulminant hepatitis might be considered. Removal of the "necrotic liver" can improve cerebral edema and circulatory parameters. This approach has, however, been performed only very rarely because of the difficulty in ensuring the availability of a liver before the patient succumbs.

Conclusion

Acute hepatitis ranges from asymptomatic liver injury identified only by elevated liver transaminases to severe acute hepatitis and finally liver failure. Acute viral hepatitis usually does not require specific treatment, with the exception of hepatitis C. For severe acute hepatitis, treatment appears reasonable when specific intervention is feasible (e.g., nucleos(t)ide analogs for severe acute hepatitis B). Management of acute liver failure requires specific therapies directed at the etiology, management of its complications, close monitoring, and evaluation for liver transplantation if indicated.

The main viral agents are the classic hepatitis viruses (A, B, D, and E, but rarely C). Common extrahepatic complications include cerebral edema, coagulopathy with risk of bleeding, bacterial and fungal infections, changes in metabolism, and multi-organ failure (especially renal failure). Because these complications dictate survival, therapy should be aggressive and begin early. However, despite significant improvements in intensive care medicine, mortality remains high. The prognosis of patients with acute liver failure can be predicted by clinical scores, but these scores have a low sensitivity. In patients with an estimated high probability of a fatal

course, liver transplantation represents the treatment of choice. Therefore, early referral to a transplantation center is mandatory.

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Chapter 35
Hepatitis and hemophilia

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Summary

Patients with hemophilia A and B are deficient of coagulation factors VIII and IX respectively and suffer from spontaneous and traumatic bleeds as a result. Treatment is with intravenous replacement of these factors, which until recently were prepared from thousands of plasma donations. Many patients treated prior to the introduction of viral inactivation in 1985 were infected with hepatitis B, hepatitis C, and HIV. Virtually 100% of individuals exposed to nonvirally inactivated clotting factors have been infected with hepatitis C, of whom 80–85% are chronically infected. Response to treatment and progression to end-stage liver disease (ESLD) in patients with hemophilia are similar to those of HCV-positive individuals in the general population infected for a similar length of time.

Introduction

Hemophilia is an inherited bleeding disorder caused by a deficiency of either factor VIII or IX in hemophilia A and B, respectively. The incidence of hemophilia A is 1 in 10 000 and hemophilia B 1 in 50 000 male births. Severely affected individuals have <1% of factor VIII/IX in their plasma and classically develop hemarthroses leading to hemophilic arthropathy by early adulthood [1]. Additionally, persons with hemophilia (PWH) can suffer bleeding into muscles as well as bleeding after injury and surgical or invasive procedures. The bleeding can be treated with the intravenous administration of the appropriate clotting factor, which is prepared immediately before use by reconstituting a lyophilized concentrate. Although hemophilia A and B are the best known, there are a number of other inherited bleeding disorders treated with concentrates, including von Willebrand disease and deficiencies of fibrinogen and factors II, VII, X, XI, and XIII.

The use of clotting factor replacement has transformed the lives of PWH in terms of both morbidity and mortality. In the early 1900s, when replacement therapy was unavailable, the mean life expectancy was 12 years [2], whilst in the 1990s it was close to the normal population in HIV-negative individuals [3]. Up to the Second World War, the only replacement therapy available was transfusion of whole blood, whereas after this fresh frozen plasma became available. A major advance in the mid-1960s was the introduction of the method to produce cryoprecipitate, which made it possible, for the first time, to administer concentrated (but still relatively dilute) clotting factor. Clotting factor concentrates were introduced in the early 1970s.

Clotting factor concentrates

Clotting factor concentrates are prepared from pools of plasma containing up to 30 000 donations. Prior to 1985, concentrates were prepared and infused into recipients

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Table 35.1 Prevalence of viral infections in 138 patients at the Sheffield Haemophilia and Thrombosis Centre treated with nonvirally inactivated concentrates prior to 1985.

Viral infection	% positive
Previous hepatitis A	37
Current hepatitis B	2
Previous hepatitis B (anti-HBs or anti-HBc)	48
Hepatitis C	100
HIV	26

(Source: Makris, M *et al.* Br J Haematol, 1996. 94(4):p.746–52 [4]).

without any viral inactivation, resulting in any infections present in the donated plasma being transferred to and usually infecting the recipient. Table 35.1 shows the prevalence of concentrate-transmitted infections in PWH in Sheffield in individuals treated with nonvirally inactivated concentrates prior to 1985 [4]. As a result of the start of the HIV epidemic and the realization that there was an excess of this infection in PWH, it was decided to introduce viral inactivation of concentrates to try to eliminate any possible transmission of HIV. This proved to be highly successful in eliminating not only HIV but also hepatitis C infection by concentrates. A number of viral inactivation methods were used by different manufacturers, including dry heat treatment at 80°C for 72 hours, pasteurization, vapor heat treatment under pressure, solvent detergent treatment, as well as nanofiltration [1]. These methods were highly effective, with the exception of an outbreak of hepatitis A in 1992 due to failure of the solvent detergent treatment to inactivate this virus, which lacks a lipid envelope and is thus not susceptible to viral inactivation by this method [5]. Since the mid-1990s, regulatory authorities have required that all clotting factor concentrates undergo two different viral inactivation steps before release.

Viral inactivation is one of several measures introduced to reduce the risk of viral transmission by concentrates, and other measures include donor selection, donor and minipool nucleic acid testing (NAT), as well as plasma hold, where donations are frozen but not used until a donor is shown to be negative 6 months after the donation. The latter measure reduces the chance of transmission during very early infection before the development of IgG antibody (seroconversion).

Rates of infection

PWH exposed to nonvirally inactivated concentrates prior to 1985 had an almost 100% chance of being infected with non-A, non-B hepatitis with their first exposure to concentrate. This was shown in two studies from the United Kingdom that measured alanine aminotransferase (ALT) and aspartate aminotransferase (AST) every 2 weeks after first exposure to concentrate [6, 7]. The identification of hepatitis C in 1989 led to a large number of reports of the prevalence of hepatitis C in hemophilic cohorts. Initial reports underestimated the prevalence at 59% due to the low pickup rate with the first-generation enzyme-linked immunosorbent assay (ELISA) for hepatitis C antibody (anti-HCV) [8], but the subsequent second-generation ELISA tests with recombinant immunoblot assay (RIBA) confirmation showed the true figure to be close to 100% in individuals exposed to nonvirally inactivated concentrates [4]. Franchini reviewed the data of hepatitis C virus (HCV) genotypes in publications from seven large cohorts and found genotype 1 in 45–74%, genotype 2 in 6–19%, genotype 3 in 10–42%, and genotypes 4 and 5 in <4% of patients [9].

Hepatitis B infection occurred in up to 50% of PWH treated with nonvirally inactivated concentrates, but the majority cleared the virus spontaneously with only 2% having chronic infection [4]. HBV infection was previously a major cause of transfusion-transmitted disease [10]. Thus, a cohort effect is observed: the majority of intensively treated older patients with hemophilia have serological evidence of previous HBV infection. Similarly, a high prevalence of hepatitis D among hepatitis B surface antigen (HBsAg)-positive PHW was reported [10–12].

Many PWH treated prior to 1985 also have evidence of past infection with hepatitis A, but it is not clear how many of these infections were community acquired or concentrate related. A relatively small number of patients were infected due to hepatitis A transmission by solvent detergent–treated concentrates in 1992 [5].

Hepatitis A viremia in donors is short, and this together with the current viral inactivation methods have meant that this is no longer a major issue. Despite the safety of the current plasma-derived concentrates, because of the theoretical infection risk and the safety of the hepatitis vaccines, there is a recommendation that all PWH likely to be treated with plasma-derived concentrates should be vaccinated against hepatitis A and B [13]. No concentrate-related hepatitis E transmissions have been described.

Investigation of liver disease

With the exception of liver biopsy, the investigation of chronic liver disease in hemophilia is the same as in nonhemophilic individuals. These patients are frequently evaluated at hemophilia centers, but it is important that their care is reviewed by a hepatitis specialist [14].

Liver biopsy

Liver biopsies have been carried out since the late 1970s and have provided considerable information on the

severity of the liver disease [15]. Whilst centers performing liver biopsies were reporting that a high proportion of patients have advanced liver disease, in the form of advanced fibrosis and cirrhosis [15], other centers not performing biopsies were questioning whether the raised transaminases seen in these patients were of any significance and felt this was a small price to pay for the availability of concentrates [16].

There has been a reluctance to perform liver biopsies because of the perception that their performance was dangerous. The first large series reported was based on a questionnaire sent to centers in the United States and Europe by Aledort, who asked centers to report their experience of liver biopsies in hemophilia up to 1981 [17]. Centers reported 126 liver biopsies, a 12.5% incidence of "bleeding," and also two deaths due to bleeding. This study, however, did not require documentation of bleeding, and the bleeding group included patients who needed more FVIII or stayed in hospital for longer than anticipated [17]. More recently, Theodore and colleagues reviewed the data of 13 further reports of series of liver biopsies in patients with hemophilia covering 387 biopsies and found no excess bleeding other than a single case of hemobilia; no deaths were reported [18].

Originally, all biopsies were performed percutaneously on inpatients, but more recently reports of transjugular liver biopsies have predominated [19], and at least one center actually performed liver biopsies in PWH as an outpatient procedure [20]. Liver biopsies are undoubtedly expensive because they have to be covered with clotting factor concentrate; one center reported that they routinely do biopsies in patients who are having other unrelated major surgery because the patients are already receiving sufficient FVIII/IX to have their hemostatic system normalized [21]. Although liver biopsy in hemophilia is safer than once considered, it must be performed only if the information it provides cannot be obtained by a safer and cheaper way and if knowledge of the result will influence the patient management. Liver biopsies are no longer required before treatment with antiviral therapy. A number of protocols in terms of clotting factor cover for the performance of liver biopsy in hemophilia exist; the one used in our centers is shown in Table 35.2.

Table 35.2 Protocol for clotting factor replacement to cover liver biopsy in hemophilia.

Prior to biopsy – exclude inhibitor (antibody to FVIII/IX)
Immediately pre-biopsy: treat to 1.00/ml (i.e., 100 iu/dl or 100%)
12 h post biopsy: measure level and treat to 1.00 iu/ml
24 h post biopsy: measure level and treat to 1.00 iu/ml
48 h post biopsy: measure level and treat to 0.50 iu/ml

Non-invasive monitoring

In view of the obvious limitations of liver biopsy in hemophilia, alternative approaches for monitoring disease progression with methods such as the Fibrotest and Fibroscan have been attempted. Initial reports involved the use of biomarkers and included the Fibrotest, the AST-to-Platelet Ratio Index (APRI), the Forns Index, and hyaluronic acid measurement. The Fibrotest computes a score based on the patient's age, sex, and results of the serum haptoglobin, alpha2-macrogblobulin, apolipoprotein A1, gamma glutamyl transpeptidase (GGT), and bilirubin levels. Maor and colleagues used the Fibrotest and found that 22 of 132 patients tested had F4 fibrosis. They found discordance among the different biomarkers but overall felt that the use of the Fibrotest was able to identify patients with significant fibrosis [22]. The non-invasive tests appear to be performing as well in hemophilic as in nonhemophilic individuals, including those co-infected with HIV.

An alternative non-invasive method is liver stiffness measurement using transient elastography (TE) with the Fibroscan instrument. This technique uses ultrasound to measure elasticity (stiffness) of the liver, which is directly related to the degree of fibrosis present. In nonhemophilic patients, TE has been correlated with liver fibrosis as detected by liver biopsy. Several groups have reported their experience of TE in cohorts of hemophilic individuals. In the Utrecht cohort of 124 patients, severe fibrosis was found in 18% and cirrhosis in 17% [23], whilst in Denmark the corresponding figures among 141 patients were 17% and 2.9% respectively [24]. Maor and colleagues compared the Fibrotest and Fibroscan in 57 PWH who had both tests and found a reasonable but not perfect correlation [25]. These non-invasive methods are more accurate in predicting very mild fibrosis or cirrhosis, and may help avert liver biopsy for patients whose liver histology may be at the extremes of the spectrum of fibrosis [26].

In some centers, the Fibroscan is used to form the basis for the strength of recommendation for antiviral therapy (consider treatment if <7.5 kPa, and recommend it if ≥7.5 kPa), and 6-monthly ultrasound monitoring for hepatocellular carcinoma (HCC) detection is offered only to those with a TE score of ≥10 kPa [27].

The natural history of hepatitis C in hemophilia

Clotting factor concentrates for use in hemophilia were introduced in the early 1970s, and almost all PWH who were infected with HCV had been infected by 1985, which was the date of the introduction of highly effective viral elimination techniques during the manufacturing process. At the time of writing this chapter in 2012, most

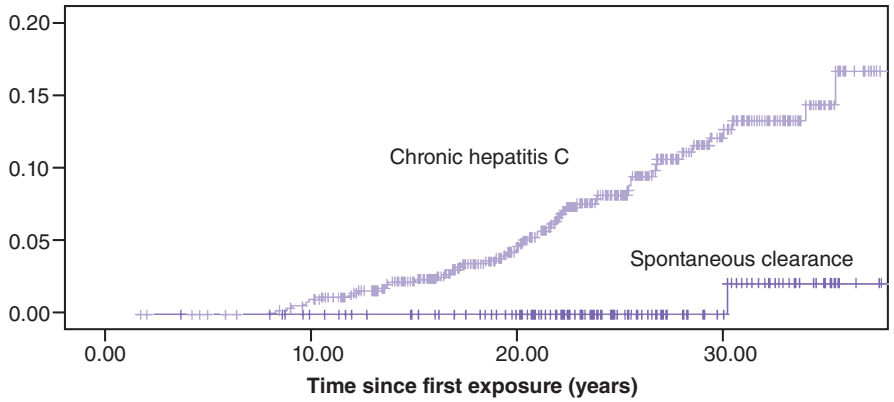

Figure 35.1 Cumulative incidence of end-stage liver disease in 687 chronic HCV-infected PWH and 160 PWH who cleared the HCV spontaneously (Source: This research was originally published in Blood. Posthouwer, D *et al.* Blood, 2007. 109(9):p.3667–71 [28] © the American Society of Hematology).

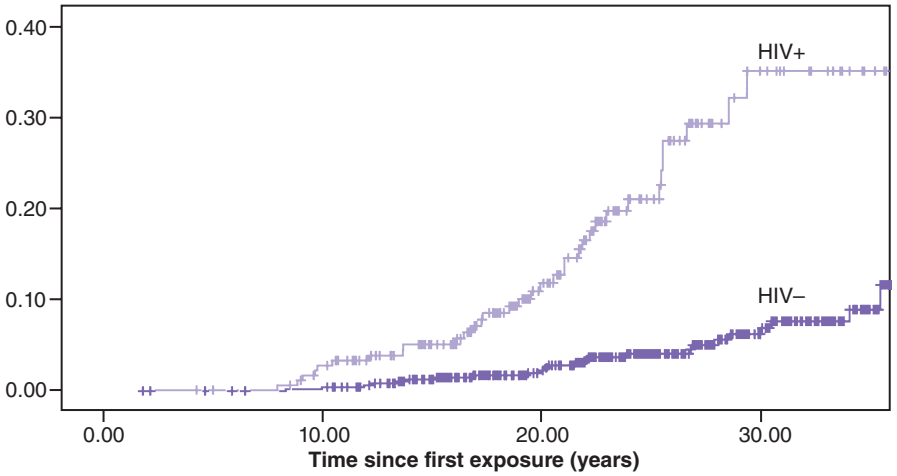

Figure 35.2 Cumulative incidence of end-stage liver disease in 190 HIV-infected PWH and 497 HIV-negative PWH. All patients were anti-HCV-positive. (Source: This research was originally published in Blood. Posthouwer, D *et al.* Blood, 2007. 109(9):p.3667–71 [28] © the American Society of Hematology).

PWH will have been infected with HCV for 27–42 years. A number of cohorts of hemophilic patients have been extensively studied, including those from Royal Hallamshire Hospital in Sheffield, Royal Free Hospital in London, and van Creveld Clinic in Utrecht, who reported their data together in 2007 [28]. Of 847 HCV antibody–positive PWH, 160 (19%) cleared the virus spontaneously whilst the rest developed chronic HCV. Figure 35.1 shows the development of ESLD (defined as the occurrence of liver failure with at least one complication: ascites, bleeding esophageal varices or hepatic encephalopathy, HCC, or liver-related death) in patients who cleared the virus spontaneously and those with chronic HCV. After 35 years of infection, ESLD developed in 11.5% of the HIV-negative individuals and in 35.1% of individuals co-infected with HIV and HCV (Figure 35.2). Independent risk factors for ESLD included HIV co-infection, older age, alcohol abuse, and infection with HCV genotype 1 [28]. Death from liver failure in HCV-positive individuals is among the commonest causes of death in patients with inherited bleeding disorders. In a report on the whole UK cohort of 4865 PWH, deaths due to liver disease were 16.7 times higher than in the general population and 5.6 times higher for liver cancer [29]. In the national Italian cohort report on 443 PWH who died between 1980 and 2007, 13% of all deaths were due to liver disease, and this was second only to deaths from HIV, which accounted for 45% of the total [30].

Hepatocellular carcinoma

In patients with HCV-related cirrhosis, the risk of HCC is 3–6% per year, whilst it is approximately one-half this in individuals with advanced fibrosis [31]. In a prospective European study where malignancies are reported, HCC is the commonest malignancy seen in

PWH (European Haemophilia Safety Surveillance [EUHASS], unpublished data). In view of this high rate of HCC, patients with advanced liver disease should undergo surveillance for early detection of HCC. In hemophilia, however, where liver biopsies are not carried out, it is difficult to identify the group of cirrhotic individuals who require HCC surveillance. In a prospective study of 385 unselected HIV-positive Italian PWH who underwent annual ultrasound screening for HCC, six new cases were identified over a 4-year period of follow-up, or 0.4% per year. Unfortunately, in all cases the tumors were multicentric and not eligible for curative treatment [32]. In a follow-up study by the same group, 6-monthly liver ultrasound surveillance identified more HCC than a 12-monthly approach, but the difference was not significant [33]. If a PWH is known to have cirrhosis or advanced fibrosis (on the basis of liver histology or non-invasive testing), they should undergo 6-monthly liver ultrasound screening [34]. It is important to appreciate that patients with cirrhosis who have had a sustained virologic response (SVR) to anti-HCV therapy will also need to continue to undergo HCC surveillance with ultrasound [34]. The management of PWH and HCC has recently been reviewed and should essentially follow the same pathway as in persons without hemophilia [35].

Antiviral therapy

With the exception of unavailability of liver histology, the management of chronic hepatitis C in hemophilia is similar to that for the nonhemophilic population. The first randomized trial of interferon monotherapy in hemophilia reported efficacy of this treatment in 1991 [36]. Although the initial results were poor, they progressively improved with the introduction of ribavirin (RBV) and pegylated interferon (PEG-IFN). In a review of all studies of antiviral therapy for chronic hepatitis C carried out in hemophilia, interferon monotherapy resulted in SVR in 95/434 (22%) patients, standard interferon plus RBV in 136/318 (43%), and PEG-IFN and RBV in 96/168 (57%) [37]. In a large multicenter cohort study from the United Kingdom and Netherlands that included all HCV genotypes and involved 295 PWH with chronic HCV, the SVR rates for interferon monotherapy were 26%, for standard interferon and RBV 45%, and for PEG-IFN and RBV 59% [38]. Response to antiviral therapy in terms of SVR was associated with a marked difference in the development of ESLD during long-term follow-up as shown in Figure 35.3 [38]. In the largest single-center study of 367 chronic hepatitis C mono-infected PWH from Iran treated with PEG-IFN and RBV, the overall SVR was 61%, with factors favoring a higher SVR being younger age, lower Body Mass Index, lower HCV viremia, and genotype non-1 infection. The SVR was 51% for genotype 1 and 71% for non-1 individuals [39].

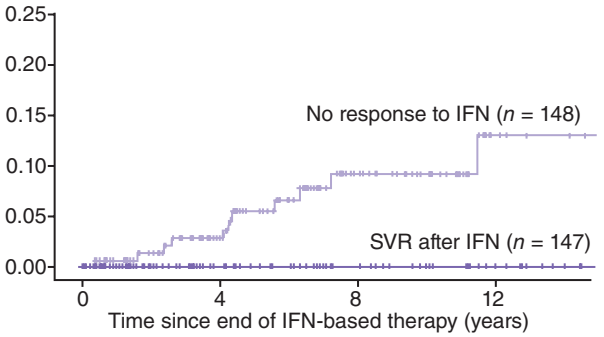

Figure 35.3 Risk of end-stage liver disease in hemophilic patients with and without a long-term response to anti-HCV therapy (Source: This research was originally published in Blood. Posthouwer, D *et al.* Blood 2007. 109(9):p.3667–71 [28] © the American Society of Hematology).

Interferon alpha (IFNα) is difficult to apply in decompensated cirrhosis (Child–Pugh class C) and may precipitate deterioration [40]. Patients with signs of decompensated cirrhosis, such as jaundice, marked coagulopathy, spontaneous bacterial peritonitis, variceal bleeding, and/or hepatic encephalopathy, meeting Child–Pugh class C criteria should be considered for liver transplantation.

A single-nucleotide polymorphism (SNP) upstream of the interleukin 28B (IL28B) gene, which encodes the type III IFN, IFN lambda-3 (IFNλ-3), is associated strongly with response to IFN treatment. The location of these SNPs on chromosome 19 suggests an important role of IFNλ signaling for HCV infection [41]. In addition to IL28B polymorphism status, the presence of advanced fibrosis has been shown to reduce SVR rates by up to 40% [42]. Increasing age, obesity, insulin resistance, and male gender have also been shown to be associated with lower SVR rates [43].

Higher rates of co-infection with HIV and HCV have occurred in hemophiliacs. HCV, with or without HIV, increases the risk of insulin resistance and diabetes [44].

Co-infection with HIV affects the natural history of HCV infection. Lower clearance rates (5% to 10%) are seen in HIV-seropositive individuals with acute HCV. HIV accelerates HCV disease progression. Cirrhosis is more prevalent among HIV-positive than HIV-negative patients and is emerging as a major cause of morbidity and mortality in patients co-infected with HIV–HCV [29, 45, 46].

Chronic immune activation is a distinguishing feature of liver and PBMC gene signatures from patients co-infected with HCV–HIV, and it may contribute to hepatic fibrogenesis. Co-infection may complicate HCV antiviral therapy. HCV is an independent risk factor for highly active antiretroviral therapy (HAART)-

associated hepatotoxicity. This effect is generally outweighed by a reduction in the overall risk of liver-related mortality when HAART is administered.

Antiretroviral agents for co-infected patients should be selected carefully, since some are more likely to induce hepatotoxicity. The indications for treatment of hepatitis C are generally based on a combination of virologic findings and a full hepatic assessment. Most guidelines indicate that all HIV-positive patients with chronic HCV should be considered candidates for treatment. Treatment is strongly recommended for patients with elevated serum aminotransferases, CD4 counts of greater than 350 cells per μL, HIV RNA lower than 1000 copies per mL, and no alcohol intake, although the degree of fibrosis needs to be taken into account. As with HCV mono-infection, the primary goal of HCV treatment for co-infected patients is SVR.

A full discussion of antiviral therapy of hepatitis B, C, and D is beyond the scope of this chapter. PWH with chronic hepatitis B or D receive the same treatment as other patients for chronic hepatitis B; the indications and goals of treatment are similar and discussed elsewhere.

Unfortunately, current treatment of hepatitis C with PEG-IFN and RBV has severe limitations, although a substantial proportion of patients can achieve viral eradication. Important progress is being made in the development of new treatments, particularly in new specific inhibitors of hepatitis C.

Several potential viral targets have been identified. Inhibitors of the NS3/4A protease with activity against HCV genotype 1 infection are currently the most advanced, with several drugs licensed or at phase III trial evaluation. Two protease inhibitors (telaprevir and boceprevir) were approved for the treatment of HCV genotype 1 in 2011. The initial development paradigm has been to add a single DAA (predominantly a protease inhibitor) to an IFN and RBV backbone. For treatment-naïve patients with chronic hepatitis C genotype 1 infection and prior treatment failure, the addition of a protease inhibitor to a backbone of PEG-IFN and RBV has resulted in a significant increase in SVR, although the response rate in prior null responders, particularly those with cirrhosis, remains very low. In general, patients with hemophilia are candidates for treatment with the first-generation protease inhibitors [47, 48]. Patients with hemophilia and HIV–HCV co-infection are likewise candidates for treatment with new direct-acting antivirals. Provisional data suggest that response rates are improved in co-infected patients (to 70%), but larger studies are required to confirm these findings.

Potential drug–drug interactions in patients co-infected with HCV–HIV receiving antiretroviral agents (ARVs) require careful selection of agents. Boceprevir and telaprevir are both substrates and inhibitors of cytochrome P-450 3A4. Telaprevir is a substrate of P-glycoprotein. Telaprevir may increase levels of cyclosporine, tacrolimus, atorvastatin, and amlodipine. Co-administration of abacavir with PEG-IFN and RBV has been associated with an increased risk of nonresponse to hepatitis C therapy, and an interaction between abacavir and RBV has been suggested. Both drugs are guanosine analogs. Inhibitory competition for phosphorylation is the likely mechanism. Combinations of zidovudine with RBV and PEG-IFN can lead to increased risk of anemia. The use of didanosine alongside RBV is associated with an increased risk of mitochondrial toxicity, which is attributable to the active metabolite of didanosine, dideoxy adenosine 5-triphosphate.

Interactions between the first-generation protease inhibitors and ARVs are expected, particularly with HIV protease inhibitors and nonnucleoside reverse transcriptase inhibitors. To date, however, relatively few studies have examined drug–drug interactions between HCV protease inhibitors and ARVs. Pharmacokinetic studies have shown that co-administration of efavirenz with either of the HCV protease inhibitors lowers levels of the HCV protease inhibitors. Levels of boceprevir decreased in patients taking efavirenz. Co-administration with efavirenz could potentially compromise telaprevir levels. The dosage of telaprevir should be increased when given with efavirenz. As there are little data to guide dosage adjustments of boceprevir when given with efavirenz, co-administration of these two drugs is not recommended. Co-administration of ritonavir-boosted HIV protease inhibitors with HCV protease inhibitors has produced more heterogeneous results: atazanavir–ritonavir appears to have a modest impact on HCV protease levels. The integrase strand inhibitor raltegrevir does not appear to affect telaprevir levels significantly and is not anticipated to affect boceprevir. Boceprevir substantially reduced the trough levels of the studied ritonavir-boosted HIV protease inhibitors (atazanavir, lopinavir, and darunavir). Effects with tenofovir are not expected to be clinically significant.

Combination clinical trial DAA regimens are now increasingly more commonplace as other classes of DAA have reached more advanced stages of clinical evaluation. The next phase of therapy for hepatitis C will evolve to interferon-free regimens for many, although several regimens may be invoked depending upon the genotype, subtype, and prior response. Unfortunately, there is limited experience of these agents in clinical trials in PWH, and there is an urgent need to include PWH cohorts in the next wave of trials.

Liver transplantation

The first report of liver transplantation in hemophilia was in 1969 in the hemophilic dog model, which demonstrated that the procedure was also associated with

normalization of the FVIII level [49]. The first orthotopic liver transplant in a PWH was reported in 1985 in a 15-year-old boy whose transplant was associated with less than average blood loss, and exogenous FVIII was no longer required 18 hours post transplant [50]. Over 100 liver transplants have been carried out in PWH worldwide. FVIII/IX concentrate is administered immediately before the surgery by either bolus injection or continuous infusion and for the immediate postoperative period for 12–48 hours, after which no further concentrate is required [51]. Co-infection with HIV–HCV is not a contraindication to liver transplantation in hemophilia, provided patients are controlled on HAART and not severely immunosuppressed [52], but caution must be exercised for drug interactions. The indications for liver transplantation in humans with hemophilia are the same as for nonhemophilic individuals, but the procedure has the major advantage of producing a phenotypic cure of the hemophilia as a result of FVIII production by the transplanted liver.

A particularly challenging situation is liver transplantation in PWH and antibodies to FVIII (inhibitors). Two potential problems arise: firstly, the immediate perioperative management; and, secondly, the long-term outcome. The acute treatment can be with high-dose FVIII (if the inhibitor is not very high) or recombinant factor VIIa, whilst in the long term the response in the reported cases has been variable and included elimination of the inhibitor (due to presumed tolerance from the continuously produced FVIII) as well as recurrence both rapid and delayed [53].

Prevention

All patients with HCV should also be tested for hepatitis A virus (HAV), HBV, hepatitis D if HBsAg-positive, and HIV. If negative, patients should be vaccinated against both HBV and HAV. The immune response to HAV vaccine is good, even in relatively immune-suppressed individuals, while successful HBV vaccination relies on several factors, including high CD4 counts (>500) in those with HIV co-infection.

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Chapter 36
Hepatitis in persons infected with HIV

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Summary

With shared routes of transmission, viral hepatitis is very common in patients with HIV infection. The increase in liver-related mortality is causing concern. It is important, therefore, to screen individuals infected with HIV for viral hepatitis and to consider antiviral therapy. For patients with HIV and chronic hepatitis B virus (HBV) infection, an antiretroviral regimen with activity against HBV is recommended. Pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy has reduced efficacy in HIV-positive patients with chronic hepatitis C virus (HCV) infection, and it is hoped that the newer directly antiviral agents, despite potentially challenging drug interactions, will improve the success rate in this group.

There are many important epidemiological and clinical interactions between HIV and the hepatotropic viruses. The pattern of co-infection varies according to the risk group. In men who have sex with men (MSM), for example, hepatitis A virus (HAV) and HBV are common, whereas the injecting drug user (IDU) or recipient of contaminated blood products is more likely to be exposed to hepatitis C virus (HCV) infection. Recent reports, however, describe outbreaks of HCV infection in HIV-positive MSM. There are also reports of chronic hepatitis E virus (HEV) infection in this setting.

It is recognized that HIV infection can modify the clinical course of viral hepatitides whether these occur before the onset of HIV infection, simultaneously, or subsequently. The clinical importance of this has increased with the improved survival of the HIV-infected individual.

Hepatitis A virus infection

HAV infection is common in MSMs and IVDUs, but the acute illness does not seem to be more severe or prolonged in the HIV-positive patient. HAV vaccination is recommended for these high-risk individuals. There are reports, however, of reduced antibody response in patients infected with HIV [1].

Hepatitis B virus infection

With the reduction in AIDS-related deaths following widespread use of antiretroviral (ARV) therapy, liver disease has emerged as a significant cause of morbidity and mortality in patients co-infected with HIV and HBV [2, 3]. All HIV-positive patients should therefore be screened for markers of HBV and HCV exposure and infection, and those with absent HBV markers should be offered HBV vaccination and infection protection advice. All HBV-infected patients should be offered HIV testing.

Epidemiology

There are approximately 350 million hepatitis B carriers and 40 million persons infected with HIV worldwide

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and, with shared routes of transmission, an estimated 4 million patients co-infected with HIV–HBV. However, prevalence and routes of transmission vary by geographic region. In a study of participating Northern European HIV centers (EuroSIDA), HBsAg co-infection prevalence was 9%, with most HIV-positive MSM having serological markers of past HBV infection [3].

Rates of HIV–HBV co-infection are slightly lower among IDUs and are generally lower in those infected through heterosexual contact.

In endemic regions of Asia and Africa, HBV is often transmitted perinatally from mother to child or before the age of 5 years through contaminated injections, scarification practices, or close household contact. Heterosexual transmission is a major concern, particularly among Sub-Saharan HIV-positive patients, and HBV co-infection was seen in 5% of South African attendees of urban HIV clinical services [4]. With immigration and global population movements, greater numbers of people co-infected with HIV–HBV may be found in heterosexual populations of countries with historically lower HBV prevalence.

Natural history

Cellular interaction

It is unclear whether HIV per se can cause hepatitis or other types of liver disease. Hepatomegaly, steatosis, and abnormal transaminase values are seen in both ARV-naïve and treatment-experienced patients.

Direct viral interaction at the cellular level would require that both HIV and HBV infect and replicate within the same cell lines. In an analysis of infected lymphoid cells from patients with chronic HBV infection, HBV DNA was present in peripheral blood mononuclear cells (PBMCs) in all patients with HIV and HBV infection, but in only 50% of those who were HIV-negative [5].

Experimental models suggest that HIV can infect and replicate within CD4-expressing sinusoidal endothelial cells and Kupffer cells. Resultant functional changes have also been described, leading to reduced synthesis of von Willebrand factor and reduced secretion of endothelin-1. Human hepatoma cell lines, which are not CD4 expressing, can also be infected with HIV. Direct interactions at the molecular level are thought to occur. The long terminal repeat (LTR) of the HIV genome has several sequences in which cellular transcription factors can bind and upregulate expression of viral genes. NFκB, a transcription factor, is generally bound to an inhibitor (IκB) within the cytoplasm of the cell and on activation is separated from IκB, passes to the nucleus, and binds to the LTR. Cytokines such as tumor necrosis factor alpha (TNFα) can induce NFκB [6], and it is thought that the X gene product of HBV can act in a similar way [7]. Studies with hepatocellular carcinoma (HCC) 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 [8].

Table 36.1 Effect of HIV on HBV infection.

Effects
Acute infection
Increased carriage following acute infection
Milder acute disease
More severe acute disease with prolonged transaminitis
Chronic infection
Higher HBV DNA load
Reduced hepatocyte necrosis
Reduced response to treatment
Increased risk of HBV re-activation or re-infection
Increased progression to cirrhosis
Increased risk of HCC
Decreased response to interferon
Increased lamivudine mutations
Vaccine
Reduced response to vaccination

Clinical outcome

See Table 36.1 for descriptions of both acute and chronic HBV infection.

Acute HBV infection

Retrospective analysis of the US Centers for Disease Control multicenter HBV vaccine study has yielded valuable data on the interaction of HIV and HBV [9]. In unvaccinated individuals, the risk of chronic carriage of HBV was 21% in HIV-positive individuals versus 7% in the HIV-seronegative group. Patients co-infected with HIV and HBV and with higher CD4 counts were more likely to clear infection.

In this study, acute hepatitis B was more severe in the presence of HIV co-infection. Peak transaminase values were similar in both HBV mono-infected and HIV–HBV co-infected groups, but the risk of clinical illness was higher and the duration of elevated transaminase values (more than 200 IU/L) significantly prolonged in the HIV–HBV co-infected group.

Chronic infection

Effect of HIV on HBV disease

In the presence of HIV infection, immune control of HBV is reduced, leading to a reduction of HBsAg clearance and higher HBV DNA levels in untreated patients.

Studies of liver biopsies in HIV treatment–naïve, HBV-positive patients have revealed increased expression of hepatitis B e antigen (HBeAg) and HBV polymerase, but reduced necroinflammation in the HIV-positive group [10].

In untreated persons co-infected with HIV–HBV, more rapid progression to cirrhosis is reported when compared with HBV mono-infected persons. Moreover, HCC may develop at an earlier age and be more rapidly progressive in the HIV–HBV co-infected population. High levels of HBV replication may be associated with the development of fibrosing cholestatic hepatitis [11]. This phenomenon is mainly seen in liver transplant patients with high levels of HBV replication, and it is thought that at very high levels of HBV replication, the virus may have a direct cytopathic effect.

In an untreated patient with declining cellular immune deficiency, reactivation of HBV replication (despite sometimes previous HBs antigen seroconversion) may occur. The mechanism of HBV reactivation in HIV infection is not clearly understood and does not necessarily correlate with changes in the CD4 count or other quantifiable changes in immune function. HBV reactivation, or re-infection, should be considered in a patient with HIV and past HBV exposure who develops a flare in the transaminase levels.

In occult HBV infection, HBsAg may be negative, with seropositivity for anti-HBc, and the diagnosis may depend on the exclusion of other causes of liver disease, by the detection of HBV DNA levels or by appropriate immunohistochemical stains of liver biopsy material. The prevalence of occult HBV infection varies from 10% to 43%.

Treatment

The treatment aims in HBV-infected patients are to prevent hepatic damage by suppression of HBV replication, ideally with the loss of HBeAg and ultimately the loss of HBsAg, and conversion to anti-HBe and anti-HBs antibody, respectively. Limiting HBV viremia reduces the risk of developing cirrhosis and HCC, and loss of HBsAg is associated with best survival and lowest risk of HCC or liver-related death. There are also potential public health benefits with reduced risk of HBV transmission.

The need for treatment of HBV infection in a person infected with HIV requires an assessment of the clinical stage of both HBV and HIV disease. In assessing HBV infection, both HBV DNA and liver fibrosis assessment are required. Alanine aminotransferase (ALT) measurement alone is not sufficient, as patients may have significant liver fibrosis in the presence of normal transaminase values. Therefore, in patients with a HBV DNA greater than 2000 IU/mL, assessment of liver fibrosis by fibroscanning or liver biopsy should also be considered.

In cases of mild liver fibrosis (METAVIR stage F0/1), monitoring of HBV DNA and ALT accompanied by an ultrasound every 3–6 months is recommended. If more significant liver fibrosis is present (METAVIR F2 or above), hepatitis B treatment should be initiated.

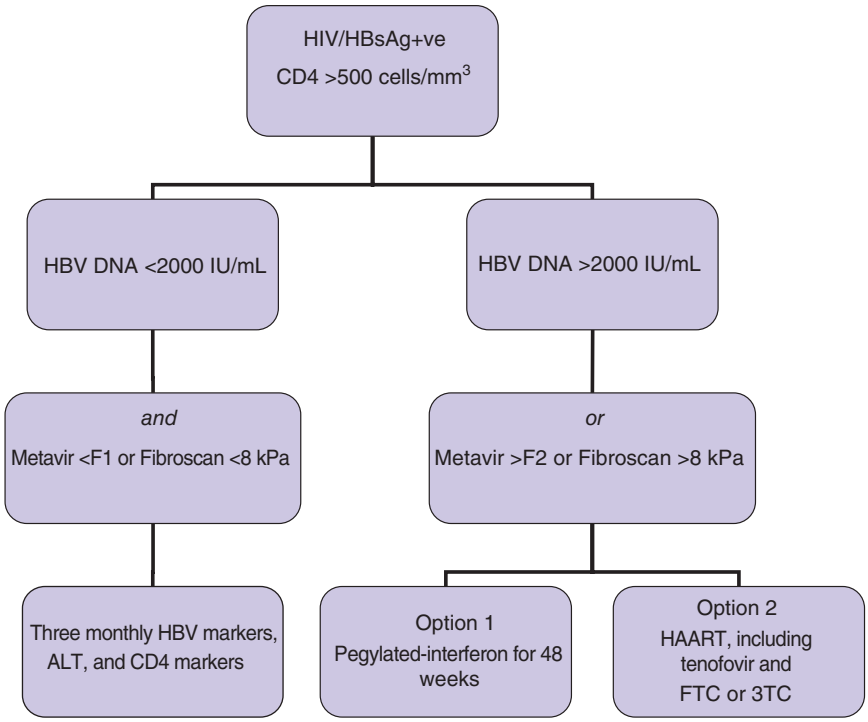
Patients with an HBV DNA of less than 2000 IU/mL tend to have minimal hepatic necroinflammatory activity, a benign course of fibrosis progression, and a low risk of HCC development. However, especially in patients infected with HBeAg-negative variants, fluctuations in HBV DNA and ALT can occur. Monitoring of the activity of the HBV DNA and ALT accompanied by an ultrasound every 6–12 months is recommended. In patients with HBV DNA less than 2000 IU/mL, but elevated transaminases and/or signs of advanced liver fibrosis, alternative causes of hepatitis and liver toxicity should be excluded and treatment for HBV considered.

In the Multicentre AIDS Cohort Study, an eightfold increased risk of liver-related mortality was seen among those co-infected with HIV–HBV compared to individuals mono-infected with HIV, particularly among subjects with low CD4 nadir counts [12]. Subsequent studies have shown a reduced risk of liver-related death in patients on an ARV regimen containing lamivudine [13].

Anti-HBV therapies

Interferon (IFN)-based therapies for patients co-infected with HIV–HBV avoid the potential of future viral resistance. (See Figure 36.1 for an HIV–HBV co-infection management algorithm.) However immunosuppression, whatever the underlying cause, is associated with lower treatment response rates. In patients with HIV infection, it is not known whether the poor response to IFN therapy results from reduced T cell function or the abnormal cytokine milieu in HIV infection, with high levels of acid-labile IFN, downregulation of IFNα receptors, or the presence of IFN antibodies. Standard IFN and PEG-IFNα therapies have generally low response rates in the patient co-infected with HIV–HBV. PEG IFNα therapy is also associated with greater adverse effects in patients co-infected with HIV–HBV, but is a potential therapeutic option, especially in patients with an elevated ALT, serum HBV DNA less than 2000 IU/mL, minimal fibrosis, and HBV genotype A. HBV genotype A predominates in Sub-Saharan Africa (genotype Aa) and Europe (genotype (Ae), and is the predominant genotype in persons co-infected with HIV–HBV in the United States and Europe.

If HBV DNA levels fail to decrease by at least 1 $\log_{10}$ by week 12 of treatment or remain higher than 2000 IU/mL by week 24, then treatment is unlikely to be



Hepatitis B virus (HBV) management if CD4 >500 cells/mm^3

Option 1: Consider if alanine aminotransferase (ALT) is greater than the upper limit of normal, and if there is a low level of

HBV DNA, minimal hepatic fibrosis, and HBV geno A.

Option 2: Consider in all patients FTC (emtricabine): 3TC (lamivudine).

Figure 36.1 Management of HIV–HBV co-infection.

successful and should be discontinued. Combination studies with PEG IFNα and adefovir, for example, have shown no added benefit [14]. PEG-IFNs should be used with caution in cirrhotic patients and avoided in patients with decompensated cirrhosis.

IFNα-based therapies are rarely used in this setting, and the main approach to therapy has been with the use of antiretroviral agents that also have activity against HBV infection. British HIV Association (BHIVA) treatment guidelines [15] recommend initiation of ARV earlier in those with HIV–HBV infection.

Entecavir monotherapy, which was not thought to have anti-HIV activity, was previously considered as an option for the patient with HIV–HBV infection and a high CD4 count who did not meet the usual criteria for initiation of ARV therapy but for whom there were clinical concerns regarding the HBV infection. Reports, however, have shown that exposure to entecavir therapy may encourage HIV resistance [16], and it is therefore no longer considered safe as HBV monotherapy in a patient with HIV–HBV infection. It may have a role, however, in a patient on effective ARV therapy (HIV <50 copies/mL) where the patient is unable to tolerate a tenofovir-based regimen and where additional agents are required to treat the HBV infection.

Treating HBV as part of antiretroviral therapy

The recommendation, therefore, is that the patient with HIV-HBV infection should be prescribed ARV with a tenofovir–emtricitabine or tenofovir–lamivudine "backbone" and a third agent such as a nonnucleoside reverse transcriptase inhibitor or protease inhibitor (PI). The baseline HBV viral load is often very high in this setting, so it may take many months, if not years, for the HBV DNA to become undetectable. Resistance to tenofovir is unusual in HBV infection but should be considered in a patient who develops viral breakthrough (≥1.0 log IU/ml increase from nadir). It is important, therefore, that the HIV–HBV infected patient has regular checks of HBV DNA levels and that if a decision is made to switch from a tenofovir-based regimen to another

regimen, an additional agent such as entecavir is prescribed for the HBV infection, as a sudden cessation of HBV therapy can lead to a flare in the transaminase values and risks of hepatic decompensation.

Immune restoration associated with effective antiretroviral therapy can lead to partial restoration of cellular immunity with an increase in the CD4 count, immune recognition, and destruction of infected hepatocytes, leading to an increase in the transaminase levels and clearing of markers for HBV infection [17]. This can be part of a more generalized immune reconstitution inflammatory syndrome (IRIS). Biochemical hepatitis several weeks after initiation of therapy may cause clinical concern as it is difficult to ascertain whether this represents hepatoxicity or a "healthy" immune response to the HBV infection that may lead to immune lysis of infected hepatocytes and resolution of the HBV infection.

In patients with cirrhosis, liver ultrasound and alpha feto-protein estimation should be performed, at least every 6 months, for early detection of HCC, and endoscopic variceal screening should also be offered.

Only a few specialist transplant centers can offer transplantation for the patient infected with HIV. Graft and patient survival rates are similar in HBV mono-infected patients versus patients infected with HIV-HBV when the transplantation is for cirrhosis-associated hepatic decompensation, but results are less favorable in the co-infected patients when the indication is HCC [18].

HBV prevention

Protective levels of antibody develop in 90–95% of immunocompetent adults after vaccination with hepatitis B vaccination. The response is reduced in those with chronic disease such as renal failure, and studies have shown that the response is also reduced in those with HIV infection. It has also been noted that the levels of both naturally acquired and postvaccination anti-HBs antibody levels decrease more rapidly in the patient with HIV. In one study [19], HBV vaccination was administered to 35 MSM, and only one of the 18 HIV-negative men failed to develop an antibody response, whereas eight of the 17 HIV-positive group failed to respond.

HIV patients with CD4 counts less than 500/mm^3 are less likely to respond to HBV vaccination (33%) compared to those with CD4 counts greater than 500/mm^3 (87.5%), and the persistence of protective antibody is reduced in the presence of HIV infection [20]. Patients infected with HIV should be offered a repeat course of HBV vaccine at double the conventional dose if they fail to respond to the initial vaccine course.

Hepatitis C virus infection

HCV infection is common in patients with HIV infection. HCV is mainly bloodborne, and therefore HIV–HCV co-infection is particularly seen in intravenous drug users and in those who received contaminated blood products prior to the introduction of donor screening or viral inactivation treatment of plasma-derived products.

Recently, however, there have been outbreaks of HCV infection in HIV-infected men who have sex with men (MSM), and this has raised questions regarding the possibility of sexual transmission in this group.

Epidemiology

When HCV antibody and polymerase chain reaction (PCR)-based tests were introduced in the 1990s, it was apparent that many patients with HIV infection were also co-infected with HCV. This had already been suspected clinically from various risk groups as many had deranged liver function tests with negative tests for hepatitis A and B and were deemed to have "parenterally transmitted non-A, non-B hepatitis." The introduction of increasingly reliable virological tests confirmed that the majority of these patients had HCV infection.

The prevalence of HCV in individuals infected with HIV varies considerably. It is uncommon in Sub-Saharan African patients, where heterosexual transmission is the main route of HIV infection, but is common among patients with a history of injecting drug use. Globally it has been estimated that there are 4 to 5 million people with HIV–HCV co-infection.

Patients co-infected with HIV–HCV tend to have much higher levels of HCV RNA, and this is reflected, for example, in the higher rates of mother-to-baby transmission of HCV in mothers with HIV–HCV co-infection.

Heterosexual transmission of HCV is rare in the HCV mono-infected patient, and studies of co-infected heterosexual patients with hemophilia and their sexual partners demonstrated significant sexual transmission of HIV infection, but lower rates of HCV transmission.

Therefore, until recently, HIV–HCV infection was mainly evident in patients who had received infected blood products or shared contaminated needles.

Studies in genito-urinary (GUM) clinics suggested that there was a slight increase in the HCV prevalence of MSM attending GUM clinics when compared with heterosexual clinic attendees, but often there were very little data on their detailed background of injecting drug use and the like.

It is only recently that there have been outbreaks of acute HCV identified in HIV-positive MSM [21]. Early reports came from European cities (London, Paris, Rotterdam, Amsterdam, and Berlin), and more recently

cases have also been described in the United States and Australia. This has raised several questions regarding HCV transmission in this setting.

Cases were often diagnosed when patients were found to have abnormal liver function tests as part of their regular clinic blood tests. The differential diagnoses in this setting include other viral infections and drug hepatotoxicity. It became apparent that many of these patients were anti-HCV antibody negative for some months and, in some cases, years and that the gold standard of diagnosis was the HCV RNA test [22].

Very few HIV-negative MSM have been diagnosed with acute HCV infection, and there are concerns that the condition may be underdiagnosed in this group as acute HCV rarely causes significant symptoms or signs such as jaundice. Seroprevalence studies are underway with HIV-negative MSM to evaluate this further.

Questionnaire studies of MSM infected with HIV who have become infected with HCV have revealed that those who become infected are often engaging in high-risk sexual behavior with multiple partners [23]. They have also revealed that drug use may be a factor, with injection of drugs such as methamphetamine and ketamine and snorting of cocaine. It is unclear, therefore, whether these outbreaks of HCV represent sexual transmission, with possibly the higher HCV RNA levels seen in co-infected patients enhancing transmission, or whether the other risk factors are more relevant.

Natural history

Acute hepatitis C virus infection

The outbreak of acute hepatitis C among MSM infected with HIV has enabled detailed studies of this group. Acute infection in this setting can occur as a co-infection where the individual acquires both viruses at the same encounter or, as is more often the case, as a superinfection where the individual infected with HIV subsequently acquires HCV infection. Acute HCV infection is usually asymptomatic, and this appears to also be the case in the co-infected or superinfected individual. Jaundice is rarely evident, but some patients require hospital admission for intravenous fluids and symptom control if significant vomiting occurs. Data suggest an increased risk of chronic HCV infection in patients infected with HIV.

A detailed prospective longitudinal cohort study was performed in our center [24]. 112 HIV-positive patients with acute hepatitis C were studied. Spontaneous clearance was observed in 15% of the patients, and 85% of the patients developed chronic infection. A subgroup of those who went on to develop chronic infection had fluctuating levels of viremia with an initial decrease and then a subsequent increase. Detailed analysis of these patients showed that many had "superinfection" with new strains of HCV infection and partially effective T cell responses. Spontaneous clearance was associated with a 2.2 $\log_{10}$ drop in the HCV viral load within 100 days of infection, hyperbilirubinemia (≥40 μmol/L), an elevated transaminase value (ALT ≥ 1000 IU/L), and a high baseline CD4 count (≥650 mm^3), and it was seen only in those with genotype 1 infection.

Chronic infection

Before the advent of effective ARV therapy, patients infected with HIV generally died because of immunosuppression-associated opportunistic infection (OI) or malignancy. Although it was recognized that many patients had HBV or HCV co-infection, their liver disease was rarely a major cause of morbidity or mortality as patients succumbed to their HIV infection before they developed the life-threatening complications of chronic viral hepatitis. It is only since the mid-1990s with the availability of more effective ARV therapy and improved survival that HIV–HCV co-infection has become of clinical relevance in these patients.

With an early diagnosis of HIV infection and effective ARV therapy, the mortality rate of individuals infected with HIV and the need for inpatient treatment of OIs have significantly declined. Now, however, many of the HIV patients co-infected with HCV require inpatient care as a result of their HCV-related liver disease.

A Spanish study described an increase in hospital admissions with decompensated liver disease from 9.1% in 1996 to 26% of HIV admissions in 2002 [25]. By 2004, this had decreased to 11%, which the authors attribute to the availability of more effective antiviral therapy for HBV and HCV and perhaps, also, to a secondary effect related to the improvement in immune function seen with more effective ARV.

The co-infected patient now, therefore, has a reasonable prognosis in terms of their HIV infection but may survive to face the potentially lethal complications of liver disease.

Postmortem studies in France have demonstrated the clinical importance of hepatitis co-infection [26]. In 2000, 822 patients were studied, and 29% were HCV infected. HBV infection was present in 8%, and 4% were both HBV and HCV infected. 110 patients overall died of end-stage liver disease, and the risk of HCC was 15%. This risk was highest in the HBV-infected group (50%), and HCC was identified in 10% of patients with HCV co-infection and 13% of those with both HBV and HCV infection.

A follow-up study in 2005 [27] found an increased incidence of HCC (25%) and found that HCV co-infection was the main risk in 25% of these cases. Although 50% of the HCV-infected patients had received antiviral

therapy for HCV, 98% of the group had HCV viremia when tested post mortem.

Other studies have shown that in patients with HIV and HBV or HCV infection, HCC development occurs at an earlier age than in the mono-infected patient and the patient infected with HIV has a more rapidly progressive course with reduced survival.

It had already been recognized that in patients with other causes of immunosuppression, such as long-term corticosteroid therapy, a more rapid progression of HCV associated liver disease occurred. Rates of liver fibrosis have been evaluated in patients with HIV–HCV infection, and several studies have shown more rapid disease progression.

In one early detailed study, patients co-infected with HIV–HCV were compared to those with HCV mono-infection [28]. The researchers matched the controls according to other risk factors that are known to cause more rapid disease progression – male sex, consumption of alcohol, and age at time of infection. More rapid progression of liver disease was apparent in the group infected with HIV.

Since the publication of that study, there have been significant improvements in ARV therapy, and this appears to have also had a beneficial effect on the rates of progression of HCV-associated liver disease with survival benefits. This is heartening for patients with HIV–HCV infection who have failed to respond to HCV treatment or who have been unable to tolerate it.

One study [29] examined liver-related and overall mortality in 285 patients with HIV–HCV from 1990 to 2002. The investigators compared the outcomes in patients who had had no ARV therapy to those who had been treated with nucleoside analogs only and to those who had been treated with more active ARV regimens available after 1995. Liver-related mortality rates were lowest in the group who received the more recent and more effective ARV regimens. This resulted in a significant survival benefit for the treated patients.

In the SLAM-C study of patients co-infected with HIV–HCV, the effects of long-term IFNα therapy were evaluated [30], and interestingly those patients in the control arm (no interferon therapy) but taking effective ARV therapy demonstrated very little progression of their liver disease.

Studies have also shown that ARV therapy can increase the cellular immune response to HCV antigens and inhibit viral replication. T cell responses were evaluated over time in 80 HIV–HCV patients in one study. The proportion of patients with T cell responses to HCV core peptides increased with the duration of ARV therapy and, presumably, the associated immune restoration [31]. These studies have informed guidelines and encouraged clinicians to commence ARV therapy at an earlier stage in patients co-infected with HIV–HCV.

The presence of other viral infections such as HBV and HDV can increase the rate of hepatic fibrosis, and it is also recognized that patients with HIV have high risks of nonviral liver diseases, which can also cause more rapid progression of their liver disease and possibly reduce the chance of them responding to antiviral therapy.

Several studies have reported high levels of alcohol consumption in patients with HIV infection. In one US study, for example, the rates of heavy drinking among HIV-positive patients were almost twice those of the general population. Those with HIV–HCV co-infection have particularly high levels of alcohol consumption, which is likely to increase the rate of progression of their liver disease and increase the risk of liver-related death.

Non-alcoholic fatty liver disease (NAFLD) is also common in patients with HIV infection. When AIDS first emerged, this was mainly recognized in patients with HIV-associated wasting or "slim disease." Now, it is commonly seen in well-nourished patients and is often associated with insulin resistance. It is unclear whether this relates to the metabolic effects of chronic viral infection or to ARV therapy.

The effects of HCV infection on HIV progression are less clear. The Swiss cohort study compared the response to ARV therapy in patients mono-infected with HIV versus patients co-infected with HIV–HCV. The group reported that although both groups had a similar HIV viral load response to treatment, the CD4 response was decreased in the HIV–HCV group [32]. More recent studies from this and other groups, however, have not confirmed these observations, and this may relate to the availability of more effective ARV.

Management of HCV infection

Acute infection

It is important to consider the diagnosis of acute HCV infection in a patient who develops an acute hepatitic illness, even if they do not appear to have the "usual" risk factors. In the setting of HIV infection, it can take many months, if not years, for the patient to develop an anti-HCV antibody response, and it is important therefore to check for the presence of HCV RNA [22].

In acute infection, treatment with PEG-IFN and RBV has resulted in sustained virological responses (SVRs) of approximately 60% in patients with HIV [33]. This is much lower than the SVR rates of more than 90% in the HIV-negative patient but higher than the SVR rates seen when treating chronic infection.

HCV genotype does not appear to influence the SVR rate in acute HCV infection.

There has been uncertainty as to when to initiate HCV therapy in patients with acute infection. Some of these

patients will clear the infection spontaneously, and there are concerns regarding the toxicity of PEG-IFN and RBV and the risks of superinfection. Detailed studies of patients infected with HIV and with acute HCV have helped identify those who are less likely to clear infection spontaneously so that they can receive HCV therapy at an early stage [24]. The optimal timing of treatment has not been determined, but it is thought that earlier treatment (e.g., after 3–6 months) is preferable if the patient has failed to clear the virus spontaneously.

Chronic infection

HIV guidelines recommend that patients are screened at the time of HIV diagnosis for HCV infection, with repeat tests annually if negative at baseline.

When a patient is found to have both HIV and HCV infection, it is important to stage both diseases. With HIV infection, this entails a detailed health check with blood tests, including HIV viral load and CD4 count. In assessing the stage of HCV infection, there may be clues regarding the chronicity from the history. There would be concerns, for example, that if the patient had a background of injecting drug use several years in the past, they have more risk of advanced liver disease.

The presence of splenomegaly is not unusual in a patient with HIV infection and therefore is not necessarily an indicator of portal hypertension as it would be in an HCV mono-infected patient. Similarly, thrombocytopenia may be associated with HIV infection per se and not necessarily an indicator of HCV-associated hypersplenism. Jaundice is seen in a subgroup of patients taking atazanavir, an HIV PI. Caution is therefore advised when considering the use of the APRI, Child–Pugh, and other scores that depend on hematological and biochemical parameters as reliable indicators of the severity of liver disease.

Patients should be screened for HBV and HAV immunity, and offered the appropriate vaccination if non-immune. A baseline liver ultrasound is recommended. The availability of fibroscanning has reduced the need for liver biopsy in these patients. Nevertheless, a liver biopsy is useful if the fibroscan is indeterminate, it suggests advanced disease, or there are clinical concerns regarding other coexistent liver diseases. Patients with a diagnosis of cirrhosis should be offered screening for HCC and esophageal varices.

Treating the HIV infection

In many patients, treating the HIV infection is the clinical priority. Current PEG-IFN and RBV therapy for HCV is rigorous and requires detailed monitoring. In the APRICOT study, patients [34] treated with PEG-IFN and RBV had a median CD4 cell decrease of 140 cells/mm^3. It is important therefore that, if the patient requires ARV therapy, they are stable on a suitable regimen with, ideally, a CD4 count of at least 350/mm^3.

We have already described the beneficial effects of effective ARV therapy on the progression of liver disease. The choice of ARV therapy is important in this setting. There are concerns that certain antiretroviral agents can interact with PEG-IFN and RBV. During the APRICOT trial, hepatic decompensation occurred in 14 patients [35], and a didanosine-containing ARV regimen appeared to be a particular risk. Six of the patients died. All of the patients with hepatic decompensation had cirrhosis, emphasizing the potential risk of PEG-IFN and RBV in patients with advanced liver disease. Stavudine was also associated with a high risk of serious adverse events. Didanosine and stavudine are rarely used now as first-line ARV agents, but for patients on regimens featuring these agents a switch to an alternative ARV regimen is recommended.

There have also been concerns regarding zidovudine-containing regimens and the increased risk of anemia when the patient is also prescribed PEG-IFN (risk of myelotoxicity) and RBV with risks of hemolysis and, more rarely, aplastic anemia.

Abacavir-containing regimens have been reported to be associated with lower SVR rates when treating the HCV infection [36]. This has been attributed to an interaction with ribavirin at the cellular and phosphorylation levels. Subsequent studies have not confirmed this finding.

A subgroup of patients treated with atazanavir develop hyperbilirubinemia. Generally, the other liver function tests remain normal, and this side effect is thought of as being mainly a cosmetic nuisance. In the setting of chronic liver disease, however, the jaundice may cause unnecessary concern.

There are many potential drug–drug interactions when treating co-infected patients [37]. Exposure to HIV PIs may be reduced by concomitant administration of the HCV PIs and vice versa.

Treating the HCV infection

The international APRICOT trial was the largest co-infection trial to date [34], and 868 patients were randomized to receive standard (thrice-weekly) IFN with RBV (400mg bd) versus PEG-IFN and placebo versus PEG-IFN RBV for 48 weeks. (See Figure 36.2 for an HIV–HCV co-infection management algorithm.)

Highest SVR rates were seen in the PEG-IFN and RBV treatment arms, with SVR rates of 62% in patients with genotypes 2 and 3 infection (versus 36% in those treated with PEG-IFN and placebo and 20% in those treated with standard IFN and RBV).

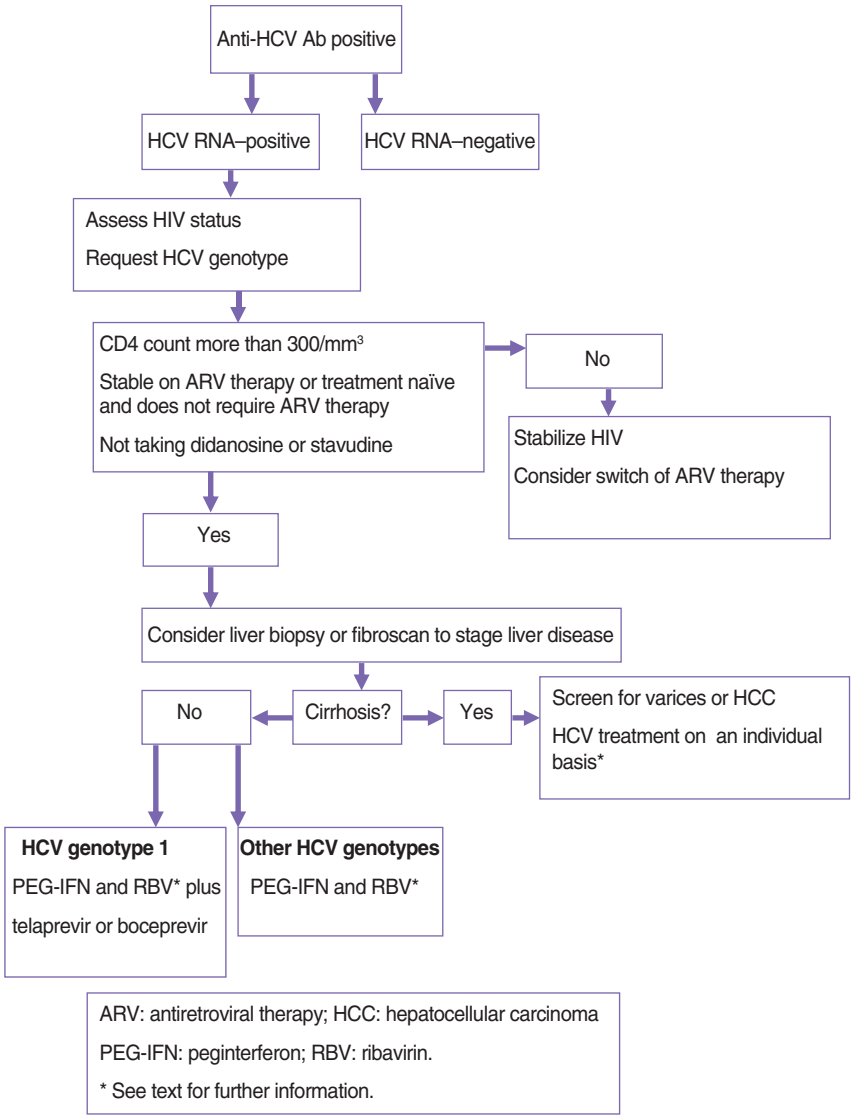


Figure 36.2 Management of HIV–HCV co-infection.

The response rates were much lower in the patients with genotype 1 infection. The highest SVR was in the PEG-IFN and RBV arm (29%), with an SVR rate of 14% for those treated with PEG-IFN and placebo and 7% for those who received the standard interferon–RBV arm.

The baseline HCV viral load was particularly important in the genotype 1–infected patients. In those with a baseline viral load of ≤ 800 000 IU/mL, the SVR rate was 61% versus 18% in those with higher baseline viral loads.

These SVR rates are obviously lower than when treating HCV mono-infected patients, and despite careful recruitment of "stable" patients there were high withdrawal rates (25–39%) because of drug toxicity and other reasons.

This large study was particularly useful in developing clinical practice guidelines recommending treatment for stable patients with genotype 2 and 3 infection, but encouraging patients with genotype 1 infection and mild liver disease to wait for more effective therapies.

Since this study, there have been several advances in HCV therapy. Shorter treatment courses are recommended for patients with genotype 2 and 3 infection, and response-guided therapy (RGT) has also evolved with cessation of ineffective therapy if there is very little initial response to antiviral therapy.

In the APRICOT study, low-dose RBV (800 mg/day) was administered to patients whatever the genotype. This reflected concerns regarding anemia when zidovudine-containing regimens were more commonly

prescribed. There have been subsequent concerns that 800 mg of ribavirin is not the optimal dose for patients infected with genotype 1 or 4.

Low-dose ribavirin was also prescribed in a French co-infection study (ANRS HCO2 RIBAVIC) [38] when 412 patients were randomized to receive 48 weeks of ribavirin, 400 mg twice daily. with weekly PEG-IFN or thrice-weekly standard IFN. Overall, the SVR rate was 27% in the PEG-IFN treatment arm and 20% in the standard IFN arm.

In this trial, the SVR rates for genotype 2 and 3 infection were similar for patients treated with PEG-IFN (44%) and patients treated with standard IFN (43%). For genotype 1 or 4 patients, the SVR rates were only 6% in the standard IFN arm and 17% in the PEG-IFN arm. Retrospective analysis showed that the virological response at 12 weeks helped predict a successful treatment outcome and supports the strategy of RGT. This trial also raised concerns about the use of didanosine-containing ARV regimens, with 11 reported cases of pancreatitis or "symptomatic hyperlactemia."

Weight-based dosing was tried in the PARADIGM study [39] and was not shown to be advantageous. 410 patients co-infected with HIV–HCV with genotype 1 infection were randomized to receive PEG-IFN with RBV 800 mg/day versus weight-based doses (1000–1200 mg/day) of ribavirin. SVR rates of 19% (low-dose RBV treatment arm) and 22% (weight-based RBV treatment arm) were recorded.

However, weight-based dosing of RBV was included in a Spanish trial [40], where 95 patients with HIV–HCV were randomized to receive standard IFN or PEG-IFN with daily RBV (800 mg for genotype 2 or 3, and weight-based dosing of 1000–1200 mg for genotype 1 infection). The duration of therapy was 24 weeks for patients with genotype 2 or 3 and a low (<800 000 IU/mL) HCV viral load, and 48 weeks for all other patients. The SVR rates were significantly higher in genotype 1 or 4 patients who received the PEG-IFN regimen (38% versus 7% in those who received standard IFN and RBV). The SVR rates when treating genotype 2 and 3 patients were similar in both treatment arms (53% for PEG-IFN and RBV versus 47% for standard interferon and RBV). The current standard of care would be to advise higher doses (1000 mg/day if less than 70 kg and 1200 mg/day if more than 70 kg).

The PRESCO study group [41] planned to evaluate the potential benefits of longer duration treatment courses in 389 patients with HIV–HCV infection. All patients received weight-based RBV dosing with PEG-IFN (a 48–72-week course for patients infected with genotype 1 or 4, and 24–48 weeks for genotype 2 or 3).

The SVR rates of 35% for genotype 1 or 4 and 72.4% for genotype 2 or 3 are among the highest seen in HIV–HCV infection trials, but a high dropout rate in the longer treatment arms precluded analysis of this as a treatment option.

Clinical trials of antiviral therapy for chronic HCV in individuals infected with HIV has, in common with the HCV mono-infected patient group, shown a lower response rate in African Americans and an influence of the host IL28-B genotype. In one of the largest studies to evaluate the effects of this [42], the virological outcome in 331 patients co-infected with HIV–HCV (159 genotype 1) was reviewed. As with HCV mono-infected patients, the SVR rates were higher in those with HCV genotype 1b (versus 1a), and the strongest influence of the IL28-B genotype was seen in the patients with HCV-1a.

Directly acting antivirals (DAAs)

Protease inhibitors (PIs)

A major advance for patients with genotype I HCV infection has been the introduction of the PIs telaprevir or boceprevir in combination with peginterferon and ribavirin. Results from trials in HCV mono-infected patients show increased SVR rates, with shorter duration therapy an option for many patients. There are limited data on the use of telaprevir and boceprevir in patients with HIV–HCV infection, and there are concerns regarding the potential for drug interactions, particularly if the patient is on a PI as part of their ARV (as discussed in this chapter).

In one study, 59 patients were randomized to receive 48 weeks of PEG-IFN and RBV with telaprevir or placebo for the first 12 weeks [43]. The SVR rate at 12 weeks (SVR 12) after cessation of therapy was 74% in the triple-therapy arm and 45% in the dual-therapy arm.

A co-infection trial with boceprevir is also ongoing, with 100 patients recruited [44]. Patients initially received a "run-in" of 4 weeks of PEG-IFN and RBV to assess their initial response, and then a further 44 weeks of PEG-IFN and RBV plus boceprevir or placebo. The SVR 12 rate after cessation of therapy was 61% in the triple-therapy arm and 27% in the dual-therapy arm.

Liver transplantation

Only a small number of transplant centers are able to offer liver transplantation as an option for patients co-infected with HIV–HCV who develop hepatic decompensation or HCC within transplant criteria. There are therefore limited data on the outcome following transplantation.

High rates of severe bacterial and fungal infection are reported in patients co-infected with HIV–HCV, and a

US study [45] found that both graft and patient survival were reduced when patients with HIV–HCV were compared to patients with HCV mono-infection.

Hepatitis D virus (HDV) infection

All patients with HBV infection should also be screened for HDV infection. Normally, HDV has an inhibitory effect on HBV replication, but this effect may not be evident in patients with HIV infection, and it is therefore not unusual to detect high levels of HDV RNA and HBV DNA. In the absence of effective ARV, more rapidly progressive liver disease is seen in patients with HIV–HBV and HDV infection. Treatment options are limited for patients with HDV infection. Staging of the liver disease should be offered and a trial of IFNα therapy considered should there be evidence of progressive disease.

Hepatitis E virus (HEV) infection

Chronic HEV infection has been recognized in the setting of severe immunosuppression, and there are case reports of chronic HEV infection in patients with HIV infection [46] and anecdotal accounts of successful antiviral therapy with RBV monotherapy or in combination with PEG-IFN. In a patient with unexplained abnormal liver function tests, it is recommended that the patient be screened for HEV infection.

Hepatitis GBV-C

There have been some interesting observations in patients with HIV and GBV-C co-infection. Patients infected with GBV–C infection tend to have higher CD4 counts, and some studies have also reported improved survival in co-infected patients [47]. It is unclear whether this relates to an interaction at the cellular level or to an enhanced T-helper-1 response.

Summary

In summary, advances in ARV therapy have improved the survival chances for the patient with HIV–HBV and HIV–HCV infection. Challenges remain, however, with reduced SVR rates when treating the HCV infection and complex drug interactions with the newer anti-HCV agents.

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Chapter 37
Migration, hepatitis B, and hepatitis C

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Summary

The prevalence of hepatitis B and C varies considerably between countries and even regions of the world. Some countries have far higher rates of HBV and HCV than others. Today at a time of accelerating migration, however, when people are moving in increasing numbers from high-prevalence to lower prevalence areas, migration is changing the global distribution and profile of HBV and HCV. In doing so, it is presenting new public health and clinical medicine challenges. Many of these challenges remain ill-defined and poorly responded to by health authorities and systems.

Introduction

Hepatitis B (HBV) and hepatitis C (HCV) are two of the main causes of liver disease and hepatocellular carcinoma worldwide [1]. In terms of the burden these infections represent for the individuals, their families, and the healthcare systems of the countries concerned, HBV and HCV have together become one of the most urgent public health challenges facing the world [2].

As with most other major infectious diseases, the social and geographic distribution of viral hepatitis varies between regions of the world and groups of people, and although 50% of the world's population lives in parts of the world that are characterized by a high prevalence of HBV [3], some countries have a higher prevalence of HBV and HCV than others, and within these countries some groups of people are more at risk than others.

From 1990 to 2005, the prevalence of chronic HBV infection decreased in most regions of the world, but the absolute number of HBsAg-positive people nevertheless increased from 223 million in 1990 to 240 million in 2005 [5]. As can be seen in Figure 37.1, high-HBV-prevalence areas include most of Southeast Asia and the Pacific Basin (excluding Japan, Australia, and New Zealand), Sub-Saharan Africa, the Amazon Basin, parts of the Middle East, the Central Asian Republics, and a number of countries in Eastern Europe. Within these areas, it is estimated that up to 90% of the population is exposed to HBV before the age of 40, and that 8–20% of them become HBV carriers [6]. In countries such as China, Senegal, and Thailand, high HBV infection rates are common among infants and young children. On the other hand, in Panama, New Guinea, the Solomon Islands, and Greenland, and in certain native populations such as Alaskan Indians, HBV infection rates among infants are relatively low, but then increase rapidly in older children [6].

Regions with a low HBV prevalence include most of North America, Western and Northern Europe, Australia, and parts of South America. In these areas, less than 20% of the population is estimated to be infected, and fewer than 2% are thought to be carriers [6, 7]. All other parts of the world tend to fall into an intermediate range in which between 2% and 8% of a given population is estimated to be carrying HBV.

Within these broad regional prevalence classifications, however, there is growing evidence that patterns of HBV and HCV may be changing, more rapidly than previously expected. Among the factors contributing to this changing epidemiology of viral hepatitis, the accelerating pace of migration both within and between countries calls for more attention than it has received to date.

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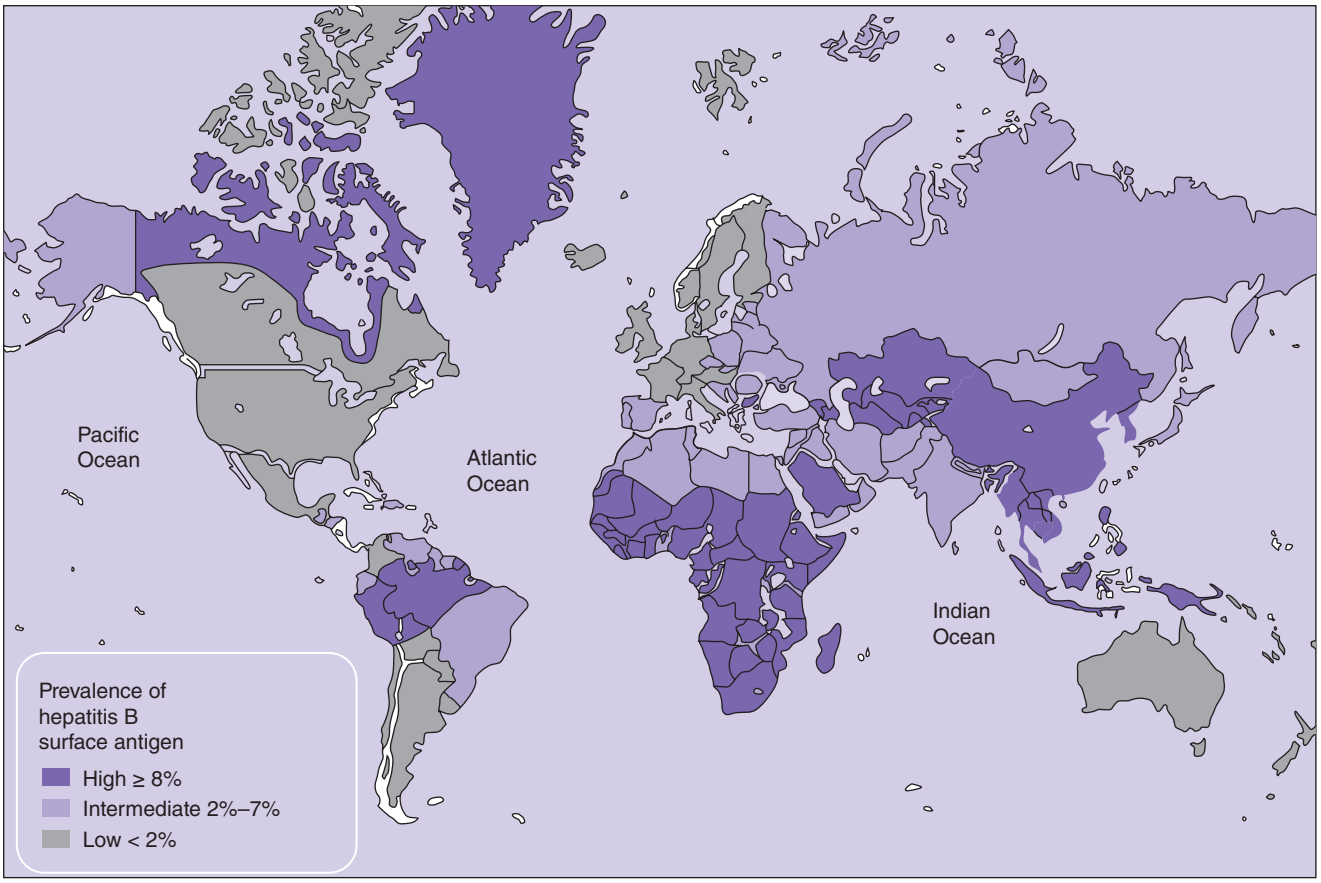

Figure 37.1 Map illustrating the global prevalence of hepatitis B surface antigen. (Source: Centers for Disease Control and Prevention [4]).

People on the move

The UN estimates that at least one in every 33 people in the world today is a migrant, and that together migrants have come to constitute a population equivalent to the fifth most populous country in the world [8]. Even these figures, however, could be an underestimation of the true number of people on the move. In China, 3.5 million people are reported to be moving from countryside to cities every month, and in India, Mexico, Nigeria, and Brazil, the massive growth in cities continues to be primarily due to a relentless pattern of rural-to-urban migration. Meanwhile, an estimated 30–40 million people move every year across highly porous borders of developing countries in search of work [9], and an additional growing phenomenon of trafficked people may account for another 12 million migrants [8, 10]. Globally, there are also around 50 million refugees and internally displaced people, and a group that is rarely taken into account, namely tourists, who in 2012 numbered over 1 billion. Finally, a group that is often forgotten is the estimated 40 million nomads and Roma people, who in some countries are constantly on the move and remain relatively unknown to local health authorities.

In Europe, where migration between European countries and into Europe is growing, there are a conservatively estimated 40 million migrants from all parts of the world. In some of the smaller countries of Europe, migrants now make up more than 30% of the total population, and in some European cities such as Geneva they make up as much as 50% of the population. The vast majority of migrants are people who have moved in search of work and a new life. Most of them arrive with work contracts, have visas, and are known to the national and local authorities. A small minority of migrants, however, does not apply for visas, and remains fairly anonymous and invisible to health registries and services. Often called "undocumented" or "irregular" migrants, their health profiles and needs are difficult to define.

Population movement and health

The interaction between population movement and health is a dynamic and complex one that can involve a number of stages, each of which has its own implications for exposure to disease and/or movement of disease [11]. Migrants are usually relatively young when

they move, and most have not yet developed the types of health problems that typically emerge with age. However, when people move, no matter what the reason for their movement, they nevertheless carry medical histories or "health prints." In some cases, migrants relocate to other countries unconsciously carrying a disease. This is the case with hepatitis B and C; most people do not know they have it until much later in life, when they develop symptoms of disease, and when it is often too late to do much about them. It is important to remember that this is true of all people who move, including tourists who travel briefly for pleasure and businesspeople who move frequently for short periods of time as part of their work.

In the first stage, migrants, travelers, or tourists move and take with them whatever health prints they have developed as a result of the social, economic, and physical environments in which they were brought up and lived. These health prints reflect the diseases that people have been exposed to, the outcomes of those disease episodes, and their level of satisfaction with whatever healthcare services were accessible to them at the time in those locations.

The health prints that people carry with them also include the beliefs and attitudes about health and disease they have learned from their parents or formed in response to their own personal experiences with disease incidents. Because poverty and conflict continue to be the main drivers of migration, many of the health prints that are being carried around the world tend to have been formed in the context of communicable diseases and poorly resourced health systems that typically lack essential equipment such as sterilization units and disposable needles and syringes [12].

In stage 2, the health prints people carry with them when they move are further modified by what happens to them during their migration. Many migrants are unable to travel directly to their final destinations and are compelled to take circuitous and difficult routes that involve stopping to live and work in locations along the way. Each time they do so, they risk being exposed to whatever are the dominant health problems of the locations they transit in, as well as whatever healthcare exists there and is accessible to them. Given that the transit settings in question are often as poor as the settings that migrants left in their countries of origin, the continued risk of exposure to diseases of poverty and poor healthcare practices remains high.

The third stage of movement is that in which people reach their final destinations, which are usually socioeconomically better situations than the ones that they left and/or transited through. The reality, however, is that in many of these settings migrants are economically and socially pressured to move into subcultural, ethnic minority environments in which traditional behaviors, health beliefs, and customs persist and can continue to expose migrants to diseases that, while unusual in the local host community, may remain highly prevalent in the ethnic community. In many of these settings, the healthcare experience of migrants can also be limited because of social, cultural, or administrative factors, even if and when universal access is otherwise guaranteed [13, 14]. The term "ethnic minority" is used to refer to people who arrive as migrants or refugees and who tend to move into communities made up of people from the same countries. They often self-identify as being ethnically different and stay in their own communities where they can retain their language, culture, and customs. Ethnic minorities may have different health profiles from the host population and may be maintaining customs and ways of life that contribute to the development of particular types of health problems.

A further complication for health in the context of population movement often comes in a final stage in which migrants periodically return home to see relatives they have left behind and take with them their children, who may have been born and brought up in relatively better off countries but are now abruptly exposed to diseases of poverty, including HBV and HCV. Even adults returning to communities of origin for short visits can be exposed to diseases that are uncommon in the countries in which they have resettled and are working [15]. See Figure 37.2.

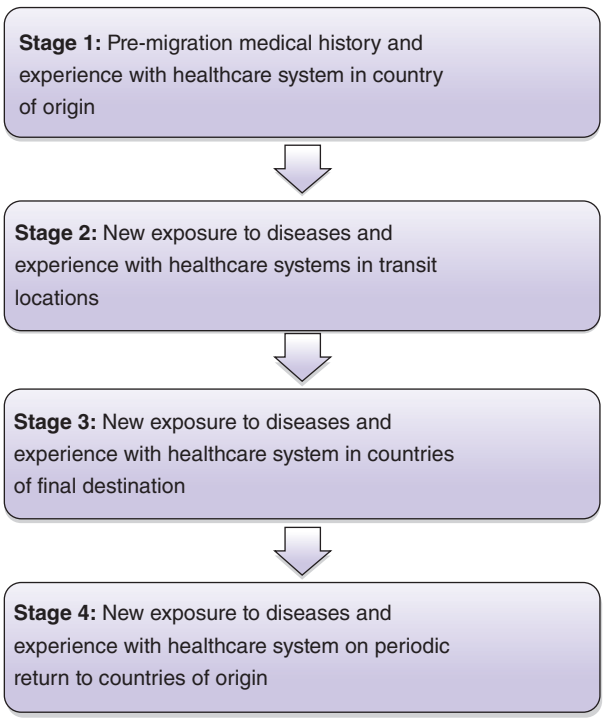

Figure 37.2 Flow chart of migration stages. (Source: Carballo M, Cody R, O'Reilly E *et al.* Migration, Hepatitis B and Hepatitis C: Report prepared for the Summit Conferance on Hepatitis B and C, Brussels 2010 [16]).

Population movement and viral hepatitis

Knowledge about the nexus between migration and health in general is improving, but in the specific case of viral hepatitis there has been little research, and the link between population movement and HBV or HCV remains poorly understood. There are a number of reasons for this, the most important one being that while biomedical research into viral hepatitis has been intense and has resulted in major progress in therapeutics, neither HBV nor HCV has received much attention from public health specialists who might have taken up the question of how population movement affects viral hepatitis patterns. This may in part be due to the natural history of viral hepatitis and the fact that because people, including migrants, living with HBV and HCV often remain asymptomatic for many years [17], they are unlikely to seek action from healthcare providers. The World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC), for example, estimate that of the approximately 350 million chronic HBV carriers in the world, most are unaware that they have been infected [18]. In Europe, as many as 40% of the people living with HCV are similarly thought to be unaware of their condition [19] and are unlikely to come to the attention of healthcare providers. In the countries from which most of today's migrants are moving to escape poverty, public awareness about viral hepatitis is likely to be even lower than it is in the United States or Europe.

Understanding the link between population movement and viral hepatitis has not been helped by the fact that the studies that have been done on the subject have often used small samples that have not necessarily been representative of larger migrant populations. Many of these studies have also used different data collection methods and, in some cases, have simply involved adding questions to larger surveys that were not initially designed to address viral hepatitis.

Emerging HBV and HCV patterns

Despite limitations in comparable data, the emerging picture is one in which migration may now be driving changes in the distribution of viral hepatitis in Europe. In Germany, where migrants make up only 13% of the population, people with a migrant background make up almost one-half (42%) of the 503 040 HBV carriers there [20]. A particularly high risk of chronic infection has been found among ethnic Germans returning from former Soviet Union countries, and in general migrants of all origins have been estimated to have a 4.3 times higher risk than native-born Germans of developing chronic HBV infection [20]. A more recent study has highlighted high rates of HBV- and HCV-related cancer and mortality in migrants from former Soviet Union countries [21]. In Ireland, although 88% of all the reported cases of acute HBV involve Irish-born people, over one-half (56%) of those with chronic HBV are likely to be from Sub-Saharan Africa, another 19% from Eastern Europe, 8.3% from East Asia and the Pacific region, and 6.3% from South and Southeast Asia [22]. Similarly, in the Netherlands, one study reported that 77% of all chronic HBV patients were foreign born, and nearly all came from intermediate- or high-endemic regions [23]. The intergenerational effect of this is that between 58% and 72% of all HBsAg carriers are estimated to be first-generation migrants. In the United Kingdom, where an estimated 3780 new HBV infections occur every year and about 269 people progress to chronic carrier status, the combination of growing intercontinental tourism by UK nationals and the widening scope of in-migration is expected to increase rates of infection in the near future [24]. The UK Health Protection Agency estimates a net annual in-migration of 6571 people living with chronic HBV [25], and the Hepatitis B Foundation estimates that 50% of the doubling of HBV cases in the past 5 years has been due to migrants from Africa, Asia, Russia, and new EU countries [26]. A more recent study concluded that 96% of chronic HBV infection in the United Kingdom is due to migrants from, and tourists going to, countries of high HBV endemicity, and that people from ethnic minorities are also more likely than others to be admitted for care for, and die from, HCV-related severe liver disease [27]. Because the prevalence of HCV is high in many of the countries that many migrants come from, the WHO has calculated that migrants may have accounted for around 10 000 cases of HCV in the United Kingdom [28]. Seroprevalence studies in the United Kingdom also suggest a rising rate of HCV in blood donors of South Asian and Polish origin [29]. In Spain, an early study of HBV in a cohort of recently arrived Sub-Saharan African migrants reported past HBV markers in 36.4% of adults and 33.3% of children; HCV markers were found in 8.6% of adults and 2.4% of children [30]. A recent study has reported hepatitis B surface antigen (HBsAg) in 10% of Sub-Saharan African patients and a prevalence of co-infection with HCV in 1.6% [31]. Similar findings have been reported by a study in Portugal that found a 7.3% HBV prevalence rate in migrants from Sub-Saharan Africa as well as elevated rates of sexually transmitted infections [32]. An HBV prevalence of 29% has also been reported for the Roma in Spain, a relatively neglected nomadic population about whom little is known [33]. An Italian study in Foggia reported a 10.7% HBV prevalence in over 550 migrants [34], and a 2008 study in Verona found 9.3% of irregular migrants from Sub-Saharan Africa to be HBsAg-positive [35]. In France, the impact

of periodic-return migration on children of migrants has also been highlighted, and it has been shown that children are often infected during their summer visits to relatives in North African countries with a high prevalence of viral hepatitis [36].

Studies of pregnant women have repeatedly reported higher rates of HBV among migrants than native-born women in France, where women of West Indian, Southeast Asian, and Sub-Saharan African origin were found to be especially at risk of carrying HBV [37] and HCV [38]. A Greek study similarly found a high prevalence (12%) of HBV in pregnant women of Albanian origin [39], and much the same has been reported from Germany, where studies of pregnant women have reported rates of 8% HBeAg-positivity [40] and 7.5% in women from Albania [41].

To what extent the presence of migrants is changing national profiles is nevertheless less clear. In Italy, the relatively massive migration from HBV-endemic countries into northern Italy does not appear to have significantly altered the prevalence of HBV [42], and a study of HBV prevalence in preschool children in Denmark found that even among children of migrants from high-endemic areas, the prevalence remained low [43]. Danish studies of HBsAg in migrants between 1998 and 2002 similarly concluded that the prevalence of HBV in migrants is not changing the overall profile of HBV in the general population [44].

Substance abuse and migrants

The role and reason for substance abuse in the context of migration have been largely neglected and call for more attention. The process of migration, including uprooting, which is often abrupt and difficult, involves leaving behind close relatives and moving under precarious conditions. It then means trying to resettle into social environments that are often unwelcoming and ill prepared to receive migrants. Coping with the stress of this takes many forms, and migrants and their children often turn to drugs and alcohol to deal with these difficulties and the loneliness and marginalization that come in the wake of migration. In the case of the children of migrants, there are also indications that they experiment with and use drugs in order to demonstrate rejection of the culture and authority of their parents and that of the host country as well [11]. Local drug dealers who are well aware of the dilemmas faced by young migrants and their children tend to target them within ethnic minority settings. An association between injecting drug use and HIV, which can be used as a surrogate indicator of the risk of viral hepatitis, in mobile populations has been highlighted by a number of studies [45, 46] and has highlighted the fact that injecting drug use in migrants often involves more sharing and poorer cleaning of equipment than in other groups of similar age and socioeconomic background [47, 48].

Migrants and sex work

To what extent the presence of HBV and HCV in migrants represents a danger to host groups is not clear because their social networks do not often overlap in ways that might constitute a transmission threat. This is equally true of other communicable diseases such as tuberculosis [11], but it is not to say there is no potential for outward spread. In Europe, as many as 47% of all female sex workers, 47% of all transgender sex workers, and 32% of male sex workers are migrants [49], reflecting the growing trafficking industry from Eastern Europe and Central Asia [14] and the fact that as the economic options for migrants, especially irregular migrants, become fewer, sex work can become an increasingly viable option and deserves urgent attention.

Prevention and management of viral hepatitis

The prevention and effective management of viral hepatitis depend not only on the availability of vaccines and therapeutics, both of which are becoming increasingly available and rapidly perfected, but also on how people perceive the disease and what they believe can be done at the individual and healthcare levels. The mix of factors involved in this complex process has been subsumed in a variety of behavioral models that build on a conceptual framework proposed by Rosenstock [50], and it includes five constructs: (1) perceived susceptibility, (2) perceived severity, (3) perceived benefits, (4) perceived barriers, and (5) self-efficacy.

1. Perceived susceptibility to viral hepatitis is difficult in all social groups, be they migrant or nonmigrant, because of the so-called silent or asymptomatic nature of the disease. The fact that so few people hear and know about this "silent" disease, or are aware of their personal infection status, means that few can be expected to worry about their susceptibility. Migrants tend to move from poor social and economic backgrounds where health information in general, and advice about viral hepatitis in particular, is poor, so this can be especially problematic. Even knowledge about the potential implications of unprotected sex and/or injection and piercing with contaminated equipment can be limited.

2. In cultures where viral hepatitis is very prevalent and treatment remains poorly developed, the disease may not be understood in ways that encourage people to take action to prevent transmission of the disease as well as promote seeking diagnosis and treatment. The concept of the perceived severity of viral hepatitis thus presents challenges. Viral hepatitis can have a long incubation before problems begin to be noted or diagnosed; many people have difficulty in projecting symptoms

that can be years away, and it can be difficult for laypeople to relate their original exposure to a virus they hear little about. It is also made difficult by the tendency for hepatitis-related morbidity and mortality to be referred to by symptoms rather than by original infection with HBV or HCV. Moreover, for migrants coming, as many do, from countries and cultures that have traditionally emphasized the past and present rather than the future, thinking about the severity of a disease that is ill defined and "silent" can be difficult.

3. Understanding the benefits of taking primary or secondary preventative action or even of seeking early diagnosis requires that people first of all understand the severity of the disease and are able to weigh up the pros and cons of action. In the case of migrants not used to being able to intervene to alter the course of diseases in general, this can be difficult, especially given that little if any attention has been given to providing public information on viral hepatitis to migrants and nonmigrants alike. Much also depends on the experiences a person has previously had with healthcare, including disease prevention. In the case of people migrating from poor-socioeconomic countries and communities, which is most often the case with migrants, they may not have had the positive experiences needed to encourage them to seek healthcare.

4. The concept of perceived barriers – that is to say, the assessment by people of what prevents them from acting on seeking information and care, and acting on advice given by health professionals – requires that people be able to distinguish between real and perceived barriers. In the case of migrants, this is often complicated by the fact that differences in language and cultural background can make it difficult for migrants to understand what healthcare services are available to them and how they can be accessed and used [51, 52, 53]. The administrative procedures surrounding access to healthcare can be off-putting to migrants, who are unused to bureaucratic procedures and who may also suspect them as ways of "tracking" them as migrants. Many migrants come from countries where people grow up suspicious of all officialdom and learn how to avoid it. There may also be fundamental differences in health and healthcare beliefs, and indeed attitudes toward healthcare personnel themselves. A further "reality" is that migrants often feel, rightly or wrongly, unwanted and unappreciated by both the public and healthcare personnel, and are hesitant about seeking attention from the health sector or indeed any other sector [14]. Moreover, migrants who do not have clearly defined legal and medical insurance status, which is a common problem, fear being rejected by healthcare personnel, a fact that few healthcare systems seek to correct.

5. As far as self-efficacy is concerned, it is important to note that the process of migration can be a psychologically debilitating process in which migrants are repeatedly confronted with reminders of the fact that they were not able to do well in their countries of origin, may not speak the language of the countries they have moved into, may lack professional skills or recognized diplomas in these new countries, and are typically relegated to low-status and difficult jobs that locals are not willing to do. None of this enhances feelings of self-efficacy, and in the case of other diseases such as type 2 diabetes, the lack of self-efficacy has been shown to detract from migrants' capacity to self-medicate and change eating behaviors in response to glucose indicators. In the case of viral hepatitis, the perceived lack of self-efficacy or "feelings of powerlessness" can be an important barrier to seeking diagnosis and/or then managing disease, including treatment adherence.

Real barriers to care and treatment

In addition to the range of perceived barriers outlined in the "Prevention and management" section, it is worth emphasizing that many of the barriers to better coverage by viral hepatitis care are real. Administrative rules and procedures are often complex and time-consuming, and they call for work and residence permits, health insurance papers, and permanent addresses that migrants, especially irregular migrants, cannot always provide. Incomplete or nonexistent medical insurance problems often arise because employers fail to fulfill obligations to provide insurance to migrants who do not understand the system or their right to coverage. For all these reasons, migrants use public healthcare facilities far less than the general public [54].

Poor health literacy has also emerged as an important impediment. Health illiteracy, including the inability of people to understand how a healthcare system works and find their way through it, is a major challenge for migrants. Proximity of healthcare services and the timing of service openings can also pose challenges to migrants who work unusually long hours, especially those who do not live near hospitals and are ineligible to access primary care physicians. The "right" of migrants to take time off for medical reasons is often not known to them and rarely explained by employers.

For many female migrants, cultural attitudes toward gender in their countries of origin and in the ethnic minority communities they may be living in can also restrict healthcare-seeking behavior [55], and this is further exacerbated if and when local healthcare providers insist that migrant women must accept being seen by male as well as female doctors and nurses.

There are also many situations in which the policies governing access to healthcare can be counterproductive. Forced isolation of asylum seekers, for example, is a growing tendency in Europe, and in Austria it has been shown to discourage migrants' voluntary

participation in HBV prevention programs. Some countries are also requiring that irregular migrants seeking healthcare do so through national immigration services that are then required to report their presence to the police, thus making the process highly unlikely to attract migrant involvement in viral hepatitis or other programs [56].

HBV vaccination

Since 1991, WHO has advocated universal vaccination against hepatitis, and at present some 177 countries have reported that they have national programs [57]. What level of coverage these programs have achieved, however, remains unclear. If childhood vaccine coverage is used as a surrogate marker, there is reason to believe that levels of coverage with respect to HBV vaccination probably remain low, especially in low-resource countries.

Even in EU countries, however, HBV immunization remains selective and far from universal. The United Kingdom, Ireland, the Netherlands, Denmark, Finland, Iceland, Norway, and Sweden have chosen HBV vaccination strategies that are group specific and focus on what are considered to be at-risk groups. These groups include healthcare workers, injecting drug users, those living with HBV-positive partners, men who have sex with men (MSM), sex workers, and people with hemophilia or others regularly receiving blood or blood products, and they have been the ones routinely vaccinated. Critics of this approach point out that since more than 30% of those living with acute HBV infection do not have identifiable risk factors, targeted population approaches may be missing a sizable proportion of those for whom vaccination would be justified [58]. This is certainly the case with migrants, who in some countries are eligible for routine healthcare only once they acquire residency status. Given that the time conditions required for residency differ considerably between countries, and that migrants tend to see emergency wards as their only viable source of healthcare [14], the opportunities for HBV vaccination may be few. Certainly in countries where irregular migrants have only the right to emergency care, HBV immunization is not covered [58].

In some countries, children of asylum seekers are eligible for HBV vaccination, and in others adult asylum seekers are also eligible [59], but policies vary considerably and are often so rigid on the legal requirements that the right to coverage is not even clear to healthcare providers, let alone potential beneficiaries [60]. In other situations, regulations concerning refugees as opposed to asylum seekers make it virtually impossible for the latter to benefit from viral hepatitis initiatives [61].

The need for outreach programs aimed at groups such as the Roma has been tackled by countries such as Bulgaria, where universal infant HBV immunization was implemented in 1991, but even there the national program remains heavily subsidized by the United Nations Development Program [62], and it is not clear if this initiative would receive sustainable support from national authorities if UN support were to be terminated.

Conclusions

The link between population movement and national profiles of viral hepatitis is becoming clearer, and certainly in many parts of Europe, migration appears to have become an important driver of the changing patterns of HBV and HCV. To what extent national policies on viral hepatitis, where they exist, are taking this into account is far less evident. Many countries simply do not have a national policy, and where they do, there is little evidence that the migration phenomenon has been taken into account. In part, this may be because there has been relatively little research on the possible impact of migration on the incidence and prevalence of viral hepatitis. There has certainly not been very much translational research on the subject.

There are many reasons why the issue of migration and its implications for the distribution of HBV and HCV calls for attention. First of all, the growing pace of migration within and into Europe and the fact that people are increasingly moving from countries and regions of the world with relatively higher prevalence rates of both these infections mean that the number of people infected with one or both of these diseases will inevitably rise. The second is that for a variety of reasons, migrants of all types often remain outside the mainstream of national health systems and hence are invisible to epidemiologists and health planners. A silent disease that is carried by people who then go on to potentially remain invisible and anonymous to health systems has all the makings of a serious epidemic. It also has all the makings of a major economic burden on national systems that could, if more were done to identify and respond to migrants with the problem, act proactively to bring them into a care and treatment framework that would massively reduce later costs associated with liver and other diseases.

Identifying and acting accordingly are not without their challenges, however. The experience that public health workers have gained in the field of HIV shows just how important it will be to always seek the voluntary and well-informed participation of migrants in screening and treatment. Migrants, at best, tend to remain on the margins of mainstream society. At worst, they remain hidden until their health problems are acute

and socially problematic. Reaching out to migrants in relevant languages and respecting cultural norms and needs will be essential parts of this. It will also be important to acknowledge the barriers that migrants face in accessing healthcare, and take steps to reduce if not eliminate these barriers.

Some researchers have suggested that the overall epidemiological profile of HBV and HCV is not necessarily being affected by migration, but there is considerable reason to believe that this is not the case and that policies and programs are needed to halt the spread of this disease through timely, human rights–based approaches that avoid stigma and discrimination, and offer the type of excellent preventative and therapeutic biomedical action that is increasingly available. The shortage of good data on the spread of viral hepatitis in the context of migration also suggests a need for far more public health research in this general area. It is also clear that current immunization programs need to be reassessed and, where necessary, reformed so as to be more inclusive and capable of reaching people based on real-time evidence.

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Chapter 38
Occupational aspects of hepatitis

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Summary

The most important occupational aspects of viral hepatitis relate to the potential transmission of the bloodborne members of the group – hepatitis B and C viruses (HBV and HCV) – from patients to healthcare workers (HCWs) and from HCWs to patients. Consideration of these issues therefore forms the bulk of this chapter. Hepatitis A, D, and E viruses are then considered briefly, and some discussion of compensation for occupationally acquired infection is presented at the end.

Hepatitis B and C viruses

Occupations where there is at least a theoretical risk of acquisition of HBV or HCV infection 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 largest and most obvious occupational group at risk is HCWs (including paramedics and emergency responders) and others such as police officers and firefighters. 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 healthcare setting, there has been an increasing realization that virus may travel in the opposite direction (i.e., 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 demonstrate convincingly an increased prevalence of markers of HBV infection in workers exposed to blood and bodily fluids. In the United States, 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 10 times for physicians and dentists, and more than 10-fold for surgeons and laboratory workers having frequent contact with blood samples [1]. Similar findings have been reported for emergency physicians and oral surgeons.

In 1990, the US Centers for Disease Control and Prevention (CDC) 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 [2].

Surveillance of acute hepatitis B infections in the United Kingdom 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 36 per 100 000 for HCWs. Comparative rates in the years 1980–1984 were 6 and 37 per 100 000,

Viral Hepatitis, Fourth Edition. Edited by Howard C. Thomas, Anna S.F. Lok, Stephen A. Locarnini, and Arie J. Zuckerman.

respectively [3]. Again, surgeons and laboratory workers were the subgroups with the highest rates of infection.

Occupations allied to healthcare may also carry a risk of HBV infection. Increased frequency of markers of HBV infection compared to either blood donors or the general population has been reported in embalmers (who commonly give a history of needlestick injuries at work), staff and specialized teachers in schools for those with learning disabilities, and emergency ambulance staff and paramedics [4]. Studies of police officers, prison officers, and firefighters on the whole have failed to find a significant increased risk. Among professionals unrelated to healthcare 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, and unapparent parenteral infection arising from dermatitis and small cuts from scissors has been suggested to account for an excess of HBV markers in hairdressers in Scotland.

Modes of occupational transmission of HBV to workers

The most obvious potential route of occupational transmission of HBV is via inoculation of infected blood by sharps injuries (needlestick injuries with blood-contaminated needles, or cuts with blood-contaminated scalpels 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 [5]. 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 may pose a risk for transmission of HBV, including amniotic, pericardial, peritoneal, pleural, synovial, and cerebrospinal fluids and semen and vaginal secretions. Exposures to non-blood-stained feces, nasal secretions, sputum, sweat, tears, urine, and vomitus are not thought to pose a risk of infection. Hepatitis B transmission has been reported following human bites, 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.

While percutaneous injuries are the most obvious route of exposure to HBV, in reality, many infections acquired by HCWs arise from direct or indirect blood or body fluid exposures that inoculate HBV into cutaneous scratches, abrasions, burns, or other lesions, or on mucosal surfaces [6]. HBV has been demonstrated to survive in dried blood at room temperature on environmental surfaces for at least 1 week.

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 sharps injuries, the risk of transmission per exposure (considered in this chapter), and the HBV immune status of the HCW population.

The prevalence of HBV carriers in the patient population will vary widely according to the population under study. The United Kingdom 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 – for example, in the United Kingdom, in hospitals for those with learning disabilities, prevalence rates between 1% and 12% have been reported.

Blood exposure incidents are, unfortunately, all too common in a hospital setting, the most obvious occurrences being during operative procedures. Risk factors include long procedures, high blood loss, major operations, and wound closure with staples. Other potential exposures include glove tears and perforations, and eye splashes with blood and other bodily fluids. Other HCWs, including nursing, laboratory, domestic, and portering staff, also suffer blood exposures. Widely differing rates are reported in different studies, and there may be considerable inaccuracies due to underreporting.

In an Italian national surveillance program of occupational exposures to bloodborne 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 [7]. No HBV seroconversions were observed, even in 158 known susceptible HCWs, 117 of whom received post-exposure prophylaxis (PEP). In a UK study of surveillance of significant (percutaneous and mucocutaneous exposures to an infected source) occupational exposures of HCWs to bloodborne viruses, percutaneous exposures (carrying the highest risk of transmission) to HBV accounted for only 7% of all reported exposures in a 10-year period. No HBV seroconversions were reported after 409 HBV exposure incidents in the period 1997–2010.

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% (discussed further in this chapter).

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. However, the United Kingdom currently operates a selective vaccination policy – targeting vaccine at those subgroups of the population deemed to be at significant risk of exposure to HBV. In occupational terms, the official UK guidance [8] explicitly identifies "Health-care workers including students and trainees," "laboratory staff who handle material that may contain virus," "staff and residents of residential accommodation for those with 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 gray area as to precisely which individuals within an organization do or do not require vaccination – and, given the excellent safety and efficacy record of the vaccine, policies should aim to be inclusive rather than exclusive.

Voluntary vaccination policies may be less effective than mandatory ones – there is evidence that a considerable proportion of all exposure-prone procedure-performing HCWs may not be vaccinated under a voluntary system. However, mandatory policies are difficult to implement, and therefore most countries rely on highly recommending HBV vaccination to HCWs, while reserving the right to determine the infection status of individuals who refuse vaccination.

What is a protective response to vaccination?

Protection acquired from vaccination is directly proportional to the level of anti-HBs achieved. Importantly, vaccinees who do not generate any detectable anti-HBs response are as susceptible to HBV infection as placebo recipients, and there is no evidence to suggest that such vaccinees may somehow be protected by nonhumoral mechanisms (e.g., T cell immunity) generated by the vaccine. However, the titer of anti-HBs that guarantees protection against infection is controversial. In many countries, including the United States, 10 IU/L is accepted as a cutoff level above which the individual is deemed to be protected [6]. Certainly, there are vanishingly few reports of acquisition of either symptomatic acute HBV infection or 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. In the United Kingdom, 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 of 10–100 IU/L are regarded as suboptimal. One concern that led to this approach was the specificity and 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 [9].

What is the need for booster doses of vaccine?

The anti-HBs level in vaccinees 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) will eventually become anti-HBs-negative in the absence of further antigenic stimulation. Initial vaccination policies therefore built in stipulations for booster doses following the initial vaccination course, mostly adopting a pragmatic approach of universal boosting at specified time intervals (e.g., 5 years). 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 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. The European Consensus Group on Hepatitis B Immunity has therefore argued that, in 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 [10]. This is now the official policy in the United States. In the United Kingdom, the extant guidance [8] still recommends a single booster dose 5 years after completion of the primary course.

Management of non- and hyporesponders to vaccination

In an occupational health setting, it is standard practice to check the anti-HBs response induced by the vaccine. Individuals have the right to know whether or not they have been protected, and knowledge of response to an initial course of vaccination is essential in managing any subsequent exposure incident. About 10% of otherwise healthy adults will mount no detectable anti-HBs response. These individuals should receive a complete second course of vaccination. If this fails to induce an anti-HBs response, then they will need hepatitis B immunoglobulin (HBIg) for protection if subsequently exposed to the virus. A further 10% make somewhat suboptimal responses in the range of 10–100 IU/L. These individuals should be given one stat additional dose of vaccine, and then the standard booster dose at 5 years. One possible explanation for a non- or suboptimal response to vaccination is, of course, that the vaccinee is already a carrier of HBV. Testing for markers of current or past infection in these individuals is therefore good clinical practice.

A variety of strategies have been tried to improve anti-HBs responses to current vaccines. Intradermal administration of vaccine may improve antibody responses, but current vaccines are not licensed for use in this way. The effect of a variety of immunostimulants (e.g., interleukin-2, erythropoeitin, interferon-alpha, and granulocyte macrophage colony-stimulating factor) administered at the same time as the vaccine has been studied, but none have been convincingly demonstrated to be effective. More immunogenic vaccines may be developed. Vaccines containing pre-S1 and pre-S2 regions of the HBsAg, in addition to the conventional S region, may circumvent genetic nonresponsiveness 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 [11]. However, production of that vaccine is technically difficult, and it has not 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 nonresponders (titer <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 titer of anti-HBs reached after the third dose was over a log higher with the AS04-adjuvated vaccine [12]. A novel DNA vaccine against HBV has been shown to induce protective levels of anti-HBs in nonresponders to conventional vaccine.

Infection control policies

Approaches to reduce hazardous exposures to bloodborne viruses (BBV) can be categorized as (1) taking special precautions with patients or specimens known or suspected to be infected, or (2) adopting 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, and goggles as appropriate), and minimal manipulation and safe disposal of sharp instruments. In recent years, there has been a major shift toward 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, while practicing universal precautions does reduce the frequency of blood exposures among trained HCWs.

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. Other infection control measures include double gloving, spectacles or visors to avoid eye splashes, impermeable gowns to prevent skin contamination, effective systems for collecting and disposing of used sharps, and safer needle and syringe combinations that reduce needle handling and automatically protect the needle after use.

Post-exposure prophylaxis policies

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 United Kingdom [13] define a significant exposure as one that is percutaneous (needlestick or sharps

Table 38.1 HBV post-exposure prophylaxis for reported incidents deemed to be of significant exposure.

	HBV status of donor		
	HBsAg-positive	Unknown	HBsAg-negative
HBV status of exposed person			
Not vaccinated or unknown anti-HBs response	Accelerated vaccine course HBIgx1	Accelerated vaccine course	Standard vaccine course
Known vaccine Responder	Booster	Consider booster Booster	Consider booster Booster
Known vaccine Nonresponder	HBIgx1 Consider booster	HBIgx1 Consider booster	No HBIg Consider booster

(Source: Adapted from UK Department of Health [8]). HBIg: hepatitis B immunoglobulin.

injury, or bite), mucocutaneous (non-intact skin, conjunctiva, or mucous membrane) if involving blood, or sexual. The HBV status of the source may be known, unknown but determinable, or unknown and not determinable. The HBV status of the person exposed may be a known responder to vaccine, a known nonresponder to vaccine, unknown, or a known HBsAg carrier. On the basis of these variables, algorithms can be constructed to indicate the appropriate post-exposure prophylaxis (PEP) needed in different circumstances; a simplified version of one of these is shown in Table 38.1. The options for action to be taken vary from nothing 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%. The most recent guidelines in the United States are provided by the CDC [6].

Transmission of HBV from infected workers

HBV transmission from infected workers via exposure-prone procedures

There are around 50 reports of HCW-to-patient transmission of HBV in the literature, with over 500 infected patients and transmission rates of 6–15%. In the United Kingdom, between 1975 and 1990, there were 12 outbreaks of HBV infection associated with infected HCWs, with 91 infections identified [14]. 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 were performed (i.e., a "look-back"), acute icteric HBV infection occurred in 1–2% of patients, with an overall transmission rate (including asymptomatic cases) of 4–9%. In 20 such outbreaks in the United States involving over 300 infected patients, all 17 surgeons who were tested were HBeAg-positive.

The risk of an infected HCW transmitting HBV is dependent on the type of work performed, with obstetric-gynecological and cardiothoracic surgeons being the two most frequently implicated in HCW-to-patient transmission. 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. The term "exposure-prone procedures" (EPPs) was coined in 1991 by the CDC 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.

Table 38.2 Risk of transmission of HBV by prevalence band of country of origin of healthcare worker (HCW).

Prevalence band	Typical prevalence	Risk per 500 000 exposure-prone procedures (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

Estimated transmission during EPP is 5%. (Source: Adapted from UK Department of Health [15]).

Magnitude of risk of transmission from infected workers

In the absence of any control measures, this will depend on the prevalence of HBV infection among 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 [14], 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 – see Table 38.2 [15].

Prevention of transmission of HBV from workers

The occurrence of HCW-to-patient transmission of HBV has driven the generation of policies designed to reduce this risk and thereby protect patients. In 1981 in the United Kingdom, 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. UK policies have since been modified at various intervals, with the trend toward the introduction of ever more restrictive guidelines.

A major revision occurred in 1993, with the explicit intention of removing HBeAg-positive HBV carriers from the pool of HCWs allowed to perform EPPs, based on the extensive evidence available at the 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; nonresponders to vaccine should, after appropriate counseling and consent, be tested for HBsAg; those found to be HBV carriers should then be tested for HBeAg/anti-HBe; and those found to be HBeAg-positive were banned from performing EPPs. No restrictions were placed upon infected HCWs who were HBeAg-negative.

For HBeAg-positive surgeons, this guidance had devastating effects, effectively ending their surgical careers. 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. There was also a significant effect on the healthcare service through the loss of surgical expertise.

Subsequent to the 1993 guidelines, there have been nine HCWs who have transmitted infection to at least 15 patients, three of whom died of fulminant infection (Table 38.3). This is considerably less than the 75 transmissions per year estimated to arise in the absence of any regulatory guidelines (see Table 38.2), but was nevertheless disappointing. In all instances, the guidelines had been correctly instituted. The first eight surgeons in Table 38.3 were known to be anti-HBe-positive HBV carriers (and therefore entitled to perform EPPs), while 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).

The failure of the 1993 guidelines was subsequently shown to be because all the anti-HBe-positive surgeons harbored precore mutants and had high levels of HBV DNA [16]. In the light of these unexpected incidents, further restrictions on HBsAg-positive HCWs were introduced in 2000 [17], stipulating that HBsAg-positive HCWs without HBeAg should be further tested for HBV DNA; HCWs with DNA levels below 10^3 copies per mL were allowed to continue to perform EPPs, subject to annual testing of their HBV DNA levels, but HCWs with DNA levels above this value were banned from EPPs. The cutoff 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 4×10^4 copies/mL [16]; (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; and (3) potential variability in viral load within individuals over time. The level of 10^3 copies per mL is felt to err on the side of caution. Other countries have opted for different cutoffs (e.g., the Netherlands use 10^5 copies/mL) [18].

Given the critical role of the HBV DNA level, procedures were put in place to ensure accurate testing, which was initially restricted to two designated laboratories. Two samples from each HCW were required, taken a few days apart. The assays were internally controlled using a WHO standard of 10^3 copies/ml. To date, around 500 anti-HBe-positive HCWs have been tested in this way, of whom around 60% have DNA levels above the cutoff. There have been difficulties in implementing this guidance, and a number of changes have recently been proposed, including extending testing to any specialist virology laboratory accredited by Clinical Pathology Accreditation UK Ltd; testing only one, rather than two, samples from the HCW; and reporting the viral load in IU/ml rather than copies/ml (which will render the cutoff at 200 IU/ml). These are intended to make it easier for HCWs and occupational health departments to comply with the guidance.

Table 38.3 HCW-to-patient transmissions of HBV in the United Kingdom since the 1993 guidelines.

Surgical specialty	Number infected (number died)	Exposed patients tested
1. General	1	—
2. Obstetrics and gynecology	3	92
3. Obstetrics and gynecology	1	111
4. General or urology	1	21
5. Orthopedic	1 (1)	188
6. Orthopedic	1 (1)	—
7. Cardiac	2	125
8. General	3 (1)	—
9. Surgical house officer	2	—

The availability of effective and safe antiviral agents capable of suppressing HBV replication has created a new dilemma for policy makers. Should anti-HBe-positive HCWs be allowed to perform EPPs if their HBV DNA levels are maintained below 10^3 copies per mL by taking antiviral therapy? One concern would be that maintenance of safe levels of HBV DNA would then be dependent on compliance with therapy. 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. The rapidity of emergence of drug resistance is known to be dependent on the initial HBV DNA level, and when resistant mutants do appear, HBV DNA levels rise gradually, usually to no higher than the pretreatment load. Thus, it may be safe 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 must be no greater than 10^5 copies/mL, and that viral load must be checked every 3 months. The residual risk of patients acquiring HBV infection under those circumstances must be extremely low. Such a policy condones the use of antivirals for the sole purpose of allowing a HCW to resume his or her operative career, an indication that is unlicensed. This policy was adopted in the United Kingdom in 2007 [19].

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 harbor low levels of anti-HBs [9]. Thus, if only nonresponders 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 of HBV-infected HCWs with detectable anti-HBs, with some even >100 IU/L. The importance of this loophole is demonstrated by surgeon 8 in Table 38.3. 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 titer 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 that allow known HBV-infected HCWs to continue to perform EPPs should emphasize that any needlestick or sharps injury to the HCW received while 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. 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

Occupational exposure to HCV infection is likely to occur through the same routes as for HBV (i.e., needlestick and sharps injuries, and mucosal splashes). There are well-documented examples of acquisition of HCV infection by workers through needlestick injury [20]. 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. However, most serosurveys report a low prevalence of anti-HCV in this group, and, with one or two exceptions, HCWs are no more likely to have markers of HCV infection than other population groups. Similar data have been reported for dental-care workers and, in the United States, for emergency responders (including firefighters, paramedics, and emergency medical technicians). Taken together, the data in Table 38.4 make it difficult to conclude that HCWs are more likely than the general population to have markers of HCV infection, in marked contrast to HBV infection (as also discussed in this chapter), at least before routine HBV vaccination was introduced.

Modes of occupational transmission of HCV to workers

As with HBV, the most obvious routes of occupational transmission of HCV are via sharps injuries, such as needlestick incidents. 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. 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 discussed in this chapter for hepatitis B virus.

Table 38.4 HCV seropositivity rates in occupational groups.

Country	Year	Number positive/ number tested	%	Comparison group (if any) and comments
A. Healthcare workers				
Germany	1992	6/1033	0.6	5/2113 (0.24%) blood donors
Italy	1992	45/495	4.8	39/3575 (1.1%) blood donors
				58/576 (10.1%) factory workers
	1995	61/3073	2.0	
	2003	39/1800	2.2	Higher than blood donors but lower than general population
	1996	12/472	2.5	8/285 (2.8%) healthy individuals
United States	1993	23/167	1.4	Higher if markers HBV infection, blood transfusion needlestick
	1993	7/943	0.7	0.4% blood donors
	1995	7/770	0.9	Higher if surgeon
United Kingdom	1994	3/1053	0.28	
	1997	4/1949	0.2	
	2001	30/10654	0.28	
France	1996	9/557	1.6	
	1996	2/328	0.7	0/112 (0.0%) other workers
Belgium	1994	5/120	4.1	Higher if hemodialysis nurses, and higher with years on dialysis ward
Sweden	1994	6/880	0.7	Same order as first-time blood donors
Hungary	2001	13/477	2.7	Higher with age
India	1994	0/90	0.0	
	2000	0/200	0.0	
Pakistan	1996	4/95	4.2	13/91 (14%) controls
Syria	2001	6/189	3.2	1% in general population
Lebanon	2001	2/502	0.4	Comparable to local blood donors
Turkey	2003	2/702	0.3	23/5670 (0.4%) blood donors
South Africa	2002	7/402	1.8	
Argentina	1994	7/439	1.6	
B. Dental-care workers				
United Kingdom	1997	2/167	1.2	0.5% estimate in general population
	1992	0/94	0.0	
United States	199?	7/343	2.0	Oral surgeons: Higher with age and years in practice
		2/305	0.7	
				Dentists: practice higher with age, years in practice
	1991	8/456	1.75	1/723 (0.44%) blood donors
Swiss	2001	1/1056	0.1	0.5–1.0 estimated blood donors
C. Emergency responders				
United States	2003		1.3–3.6	No difference from appropriate referent groups in general US population
	2002	5/406	1.2	Firefighters
		2/274	0.7	Corrections personnel
		0/29	0.0	Police officers
	1999	64/2136	3.0	Firefighters
	1991	9/437	2.1	Firefighters
	1992	5/382	1.3	Firefighters 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	8.0	0/50 local blood donors

1. The prevalence of HCV infection in the patient population. This will vary considerably. In a London teaching hospital, 8.5% of 200 needlestick 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 [21]. The HCV infection in one-quarter of the source patients was discovered only as a result of follow-up of the needlestick injury. None of 102 patients in a UK hospital for patients with learning disabilities was found to have antibodies to hepatitis C. The prevalence of HCV infection in patients in dialysis units is highly variable (e.g., from 0 to 39%). The clientele attending accident and emergency departments may have a high prevalence of

bloodborne virus infection – of 2523 patients attending an inner-city emergency department in the United States, 18% were positive for HCV, and 5% were positive for HBsAg.

2. The frequency of blood exposures among HCWs capable of allowing transmission. This was discussed in detail in this chapter. Published estimates of this frequency vary from annual rates of 0.1 to 5 per surgeon.

3. The risk of transmission following an exposure to an infected source. Henderson reviewed published data available in 2003 and identified 39 acquisitions of HCV infection in HCWs from 2506 (1.6%) needlestick exposures, although these figures may not be entirely accurate, as it is difficult to rule out double reporting of incidents or transmission events in studies by the same sets of investigators. The CDC guidelines quote an average incidence of anti-HCV seroconversion after accidental percutaneous exposure from an HCV-positive source of 1.8% [6]. In the United Kingdom, the Health Protection Agency surveillance of significant occupational exposures to bloodborne viruses in the period 1997–2010 received reports of 2153 HCWs exposed to an HCV-positive source from whom follow-up data were available. Percutaneous injuries accounted for 74% of exposures, nearly two-thirds of them from hollow-bore needles. There were 14 seroconversions, giving a crude transmission rate following percutaneous injury of just under 1%. Altogether, there are now 18 documented in the United Kingdom, including four cases from Scotland [22].

The Italian Surveillance scheme (SIROH), comprising data from 41 participating hospitals, reported HCV transmission in 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 [7]. In the case of cardiothoracic surgery, 987 percutaneous and 255 mucocutaneous exposures were reported, of which 257 source patients were anti-HCV-positive. No seroconversions were observed. 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.* [23] 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 20 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. Prevention strategies include ensuring adequate education of HCWs about phlebotomy and intravenous cannula insertion, ensuring the availability of suitable sharps-disposal containers, introducing safety cannulas, reducing the 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.

The post-exposure management of an HCV exposure incident in a HCW differs markedly from the situation with HBV, as there is no vaccine for either pre- or post-exposure active immunization. Also, passive protection with pooled gamma-globulin is not recommended, although there are data predating the characterization of HCV that immune serum globulin prophylaxis reduced the risk of posttransfusion non-A, non-B hepatitis [24]. However, in contrast to HBV infection, potentially curative treatment for HCV infection exists. Rates of virus clearance in acute HCV infection following early initiation of pegylated interferon alpha therapy are high, approaching 100% in otherwise immunocompetent individuals. This therefore allows construction of a rational approach to the management of HCV-related needlestick exposures.

The UK recommendations [25] state that a baseline serum sample from the needlestick recipients 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, comparison with the baseline sample will indicate whether the infection arose from the needlestick injury. Where patient-to-HCW transmission of HCV is suspected, the HCW should be referred to an appropriate specialist for consideration of early therapy and the associated high chance of viral clearance. There is some uncertainty surrounding precisely when therapy should be started. There is at least a 25% chance that acutely infected HCWs will spontaneously clear the infection, thus sparing themselves 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 and Australian authorities, 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, 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" [6]. 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 (discussed further in this chapter). 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, given the unpredictable nature of needlestick injuries and their unfortunate frequency in HCWs. Any other policy 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 be implemented only if the HCW concerned reports the incident to the appropriate authority. There is evidence that reporting of such incidents is suboptimal, even after high-risk exposures.

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. The United Kingdom has the largest number of reported incidents, with seven surgeons known to have transmitted infection to 20 patients. Lookback exercises resulted in 8560 at-risk patients being tested, giving a transmission rate of 0.23% (see Table 38.5, which also gives data on non-UK incidents).

The transmission rates for each surgeon are reasonably consistent, with all except the Spanish and American cardiothoracic surgeons being <0.5%. Taking all reports together, the transmission rate is 42/13403 (0.31%).

In addition to these well-documented surgeon-to-patient incidents, there have been eight reported instances of transmissions associated with anesthetists. For four of these, the precise mode of transmission has not been identified, but presumably must have arisen through breaches in infection control. For the other four, there is evidence that the anesthetist was sharing the patients' narcotics for self-injection, and thereby contaminating the needle or syringe used for patient injection. Over 300 patients are thought to have acquired HCV infection in this manner.

Modes of occupational transmission of HCV from workers

In retrospective investigations, it is often impossible to identify the precise mechanism of transmission of a bloodborne 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 anesthetists and, indeed, well-documented nosocomial outbreaks of HCV 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

Ross and colleagues [26] have constructed a model to determine the risk of occurrence of HCW-to-patient

Table 38.5 Transmission of HCV infection from HCW to patient.

Country and year	Surgeon	Probable cases	Patients tested	Transmission rate
United Kingdom, 1995	Cardiovascular	1	270	0.37
United Kingdom, 1999	Gynecologist	7	3628	0.19
United Kingdom, 2000	General	2	627	0.32
United Kingdom, 2000	General	4	1145	0.35
United Kingdom, 2001	Obstetrician	1	198	0.51
United Kingdom, 2002	Obstetrician/Gynecologist	2	1315	0.15
United Kingdom, 2004	Obstetrician/Gynecologist	3	1377	0.22
	Subtotal (i)	20	8560	0.23
Spain, 1996	Cardiothoracic	5	222	2.25
Germany, 2002	Orthopedic	1	207	0.48
Germany, 2002	Obstetrician	1	2285	0.04
United States	Cardiac	14	937	1.50
Germany, 2008	General	1	1192	0.08
	Subtotal (ii)	22	5843	0.38
	Total	42	14403	0.29

Subtotal (i) is the total number of probable cases and the number of patients tested in transmissions reported in the UK. Subtotal (ii) is the total number of probble cases and the number of patients tested in transmissions published in the literature from other countries.

transmission of HCV, 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 approximately from 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 the risks for HBV and HIV transmissions. For known infected surgeons, the calculated risks per operation are from one in 1750 to one in 16 000 for HCV, one in 400 for HBV, and one in 40 000 for HIV [26]. A simpler approach to estimating transmission risk was adopted by the UK Department of Health. On the basis that one in 200 surgeons currently practicing in the United Kingdom 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 38.5), 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 10 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. 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 United Kingdom, as discussed in this section). 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).

2. Screen all HCWs who perform EPPs for HCV infection, and exclude all those who are chronically infected. This would result, in the United Kingdom, 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), 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) [23]. Thus, there would be no alternative but to continue screening all HCWs performing EPPs at intervals, most likely on an annual basis. This 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 also 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 [27]. 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" [28], 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 enrolment, but not preventing any HCW found to be HCV infected from performing their duties. A European Consensus Group discussed the issues surrounding both HBV- and HCV-infected HCWs [18]. National guidelines for HCV-infected HCWs were identified for only five (Belgium, Germany, Italy, the United Kingdom, and the United States) 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 recommendation 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

HCWs who know themselves to be infected with HCV are required to inform their patients of this in advance.

In the United Kingdom, the recommendations of the Advisory Group on Hepatitis, subsequently adopted by the Department of Health, represent a traditional compromise [29]. 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 or 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 that 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. In addition, the Department of Health also recognizes the problem of workers from countries with higher prevalence rates of HCV infection than exist in the United Kingdom coming to work in the National Health Service (NHS). There is therefore a requirement for any HCWs who will perform EPPs as part of their job and are "new" to the NHS (this includes students from within the United Kingdom as well as incomers from abroad) to be tested for evidence of HCV infection [30]. Those found to be positive will not be allowed to take up their post. Implicit in these policies is the realization that a small number of patients (5–10 per year) will acquire HCV infection from infected HCWs.

Hepatitis A

Hepatitis A virus (HAV) is transmitted by the fecal-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 a potentially increased risk of acquisition of HAV infection are those who come into contact with raw sewage. Studies in Singapore, France, and the United States have reported seroprevalence rates in sewage or wastewater workers more than double those of non-occupationally exposed population groups, and outbreaks of HAV infection have been reported in sewage workers [31].

HCWs are another potential risk group. Nosocomial outbreaks of HAV infection involving HCWs have been reported in neonatal and pediatric intensive care units and other ward settings. However, seroprevalence studies in HCWs have generated conflicting data. Increased rates have been reported in general HCWs, physicians and dentists, dental workers, and nurse aides. However, others have found no increase for physicians or nurses. Staff who work with children (e.g., in daycare centers and kindergartens) may also be at risk.

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. The UK guidelines for HAV vaccination [8] for occupationally at-risk workers recommend vaccination for the following occupational groups:

- laboratory workers who may be exposed to the virus in the course of their work, for example in microbiology laboratories or clinical infectious diseases units;
- staff of some large residential institutions – outbreaks of HAV are well recognized in this setting, for example in institutions for those with learning difficulties or other institutions where standards of personal hygiene among clients or patients may be poor; in these cases, vaccination should be offered to both staff and residents;
- sewage workers – for reasons explained in this section; and
- people who work with primates.

In addition, the guidance suggests consideration of vaccination under certain circumstances for food packagers and handlers (e.g., where a case or outbreaks occurs), and for staff in daycare facilities working with children who are not yet toilet trained, again especially in the context of a community outbreak. The statement on vaccination of healthcare workers reads, "[M]ost healthcare workers are not at increased risk of hepatitis A and routine immunisation is not recommended" [8].

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, and in a serosurvey in Brazil, six 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.

Compensation for occupationally acquired infection

Virus infections may be transmitted in the workplace through precisely the same routes of infection as in the wider community. 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 United Kingdom 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 noncontributory 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 of 1992, states that the secretary of state may prescribe a disease where he or she 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 be prescribed only 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 that 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 HCWs but does require that the infected person demonstrates that his or her work does involve exposure to blood, 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 [32]. Hence, PID8a would be hepatitis A linked to jobs involving exposure to raw sewage, whereas 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 PID to match what is known about occupational transmission.

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Chapter 39
Neonatal and pediatric infection

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Summary

Viral infections in neonates and children range from being an asymptomatic carrier to experiencing end-stage acute or chronic liver failure. Neonates are usually infected *in utero* or perinatally with TORCH (toxoplasmosis, rubella, cytomegalovirus [CMV], and herpes) infections and could develop acute liver failure due to herpes simplex viruses, enteroviruses, or rarely adenoviruses.

Acute hepatitis in older children is due to hepatitis A virus (HAV), hepatitis E (HEV), or Epstein–Barr virus (EBV) in adolescents. Hepatitis B may present with fulminant hepatitis in neonates of hepatitis B e antigen (HBeAg)-negative mothers as well as in older children. Acute hepatitis from any source may progress to fulminant hepatitis requiring liver transplantation.

Chronic hepatitis in childhood is due to either HBV or HCV. The main route of infection is perinatal, and children have a low natural seroconversion rate and a lifetime risk of chronic liver disease and liver cancer.

Treatment for HBV is unsatisfactory, with only a 30% success rate with either interferon or nucleoside analogs, while combination therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV) clears between 70% and 90% of HCV infections dependent on the genotype.

As mentioned, viral infections in neonates have a wide clinical spectrum ranging from asymptomatic-carrier status to end-stage acute or chronic liver failure. This chapter will highlight the clinical and therapeutic differences in children as compared to adults. Table 39.1 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 and acute liver failure.

Intrauterine infection

The commonest intrauterine infections are the TORCH infections (Table 39.2). Less common infections include human herpes virus 6, varicella zoster virus, enteroviruses (include Coxsackie A and B viruses, echoviruses, and enteroviruses), human parechoviruses, and adenoviruses [1].

Clinical presentation and diagnosis

The clinical presentation is similar for all forms of congenital infection that affect the liver. Most infants are small for gestational dates or are born prematurely. Jaundice and hepatosplenomegaly are constant features. Conjugated hyperbilirubinemia occurs within 24 hours of life. Serum aminotransferases are frequently elevated

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Table 39.1 Infections associated with hepatitis in childhood.

Etiology	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; household contact (e.g., father or siblings HBsAg-positive); blood or blood products	HBsAg	Rash or 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 viremia (>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 or CMV DNA detection (via PCR) in urine 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 or detection of rubella RNA (via RT-PCR) from pharyngeal secretions, urine, or cerebrospinal fluid (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 or detection of HSV DNA (via PCR) from vesicular fluid, CSF, or liver	Skin vesicles; keratoconjunctivitis; encephalitis	
Varicella zoster virus (VZV)	Maternal varicella from less than 5 days before delivery to 7 days after	VZV-specific IgM; viral isolation or detection of VZV DNA (via PCR) 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 or detection of adenovirus DNA (via PCR) in the liver	Thrombocytopenia; coagulopathy; pneumonitis	
Enterovirus (EV)	Epidemics of enteroviral disease in the community	Viral isolation or detection of EV RNA (via RT-PCR) in feces, blood, or CSF	Myocarditis; meningoencephalitis; pneumonitis; generalized myositis; thrombocytopenia	
Human parechovirus (HPeV)	Outbreaks often occur in a two-yearly cycle	Detection of HPeV RNA (via RT-PCR) in feces, blood, or CSF	Neonatal sepsis syndrome; abdominal distension; necrotizing enterocolitis; meningoencephalitis	
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	

HBsAg: hepatitis B surface antigen; PCR: polymerase chain reaction; and RT: reverse transcriptase.

Table 39.2 Intrauterine infection: diagnosis and treatment.

Disease	Diagnostic test	Treatment
Rubella	IgM-specific antibodies	None
Cytomegalovirus	Urine for viral culture or PCR; IgM antibodies	Ganciclovir
Herpes simplex	Viral culture or PCR	Aciclovir
Human herpes virus 6	Serology or PCR	Aciclovir
Herpes zoster	Serology or PCR	Aciclovir
Human immunodeficiency virus (HIV)	Anti-HIV, PCR (EDTA blood), or CD4 count	Retroviral therapy
Parvovirus B19	IgM antibodies or PCR	
Syncytial giant-cell hepatitis	Giant-cell hepatitis on liver biopsy	Ribavarin
Toxoplasmosis	IgM-specific antibodies	Spiramycin

CD: cluster designation; and EDTA: ethylenediaminetetraacetic acid.

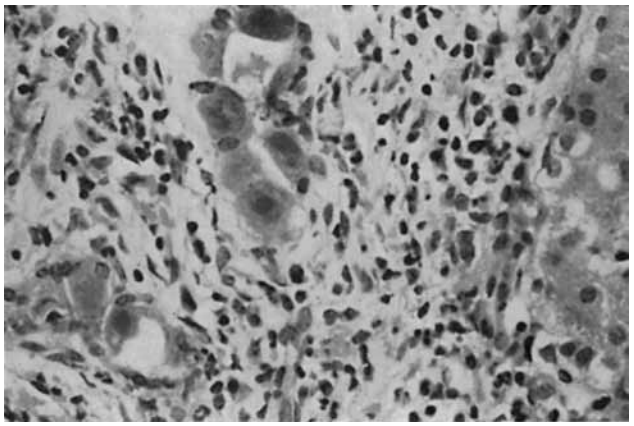

Figure 39.1 Intranuclear inclusion bodies in CMV infection.

(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. Liver histology demonstrates a neonatal hepatitis with multinucleated giant-cell transformation and rosette formation. There may be extramedullary hematopoiesis and cholestasis.

Toxoplasmosis

Toxoplasmosis is not a viral infection but is an important differential diagnosis. Infection occurs in the third trimester. Neonatal hepatitis is associated with chorioretinitis, hydrocephaly, or microcephaly. Intracranial lesions lead to convulsions, nystagmus, and raised intracranial pressure. Spiramycin therapy may prevent maternal transmission of toxoplasma to the fetus and may reduce the severity of central nervous system (CNS) and liver disease in established congenital toxoplasmosis [2].

Rubella virus

Rubella is a toga virus that causes a benign infection in older children. Infants of mothers infected in the first trimester of pregnancy either are stillborn or have congenital malformations. Although congenital infection with rubella virus is rare because of widespread immunization in developed countries, it is a significant problem in the developing world. The clinical features include congenital cataracts; hepatitis; hepatosplenomegaly; 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 IgM antibodies to rubella. Liver disease is self-limited, but occasionally patients have persistent cholestasis and progress to cirrhosis.

Cytomegalovirus

CMV, a DNA virus of the herpes family, 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. Seropositivity for CMV increases with age and is higher among urban children, in those who were breastfed, and among daycare center attendees.

Transmission

In maternal primary CMV infection, the fetal transmission rate during the first and second trimesters is between 30% and 40%, but reaches 70% during the third trimester. However, the risk of symptomatic neonatal CMV disease is lower for third-trimester infection [3]. Perinatal infection may occur through infected maternal genital secretions, urine, saliva, or breast milk [4], and may cause sepsis in premature babies. Infected infants excrete CMV for many years in their urine and saliva, and are a risk to other children and to pregnant women. (Figure 39.1.)

Clinical features

Most infected infants have intrauterine growth retardation. Clinical features include fetal ascites, jaundice, hepatosplenomegaly, and a petechial rash in 60–80%. CMV infection may affect the CNS, with microcephaly, intracranial calcification, and chorioretinitis. Progressive sensorineural deafness, developmental delay, or cerebral palsy may develop later. 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. Although usually self-limiting, CMV infection may progress to hepatic fibrosis or cirrhosis.

Diagnosis

Diagnosis is made by isolation or molecular detection (of CMV DNA) of the virus from any site or by the presence of a specific immunoglobulin M (IgM) antibody to CMV in the neonate. Samples must be collected within 3 weeks of birth to eliminate the possibility of postnatally acquired infection. Disease progression and treatment response are monitored by measuring CMV DNA in blood.

Liver biopsy demonstrates giant-cell hepatitis with multinucleated giant-cell transformation. Hepatocyte necrosis, cholestasis, extramedullary hematopoiesis, and fatty infiltration with minimal inflammatory cells are common.

Vaccination is not available, and thus perinatal transmission cannot be prevented. Treatment is not usually necessary, but severe cases may respond to ganciclovir. CMV persists in a latent form that can be reactivated later.

HIV infection

Infants with congenital HIV infection may present with hepatosplenomegaly, but jaundice is rare. Neonatal hepatitis is more likely with co-infection with CMV.

Acute liver failure in the neonate

Acute liver failure in neonates is rare but can be potentially fatal without liver transplantation. The etiology 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, hypoglycemia, jaundice, coagulopathy, and encephalopathy within hours or weeks of birth. It is important to exclude other causes of liver failure and to treat with the appropriate therapy (Table 39.3).

Table 39.3 Causes of fulminant viral hepatitis in children.

Etiology	Disease	Incidence
Neonates		
	Herpes virus 6	Rare
	Enteroviruses	Rare
	Adenovirus	Rare
	HBV	Occasional
	Parvovirus	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 D virus; and NA–G: hepatitis 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 often presents with a severe multisystem disorder with encephalitis, severe hepatitis, or acute liver failure [5] due to either type 1 or type 2 virus. Infants have progressive jaundice, hepatosplenomegaly, raised hepatic transaminase levels, and coagulopathy during the second week of life.

Diagnosis

Diagnosis is made by the isolation of virus or detection of HSV DNA from vesicle swabs or other tissue or aspirates. Intranuclear inclusion bodies and multinucleate giant cells in scrapings or biopsy tissue are diagnostic, as is a specific IgM response. Maternal first-episode (primary) genital HSV infection within 6 weeks of delivery is a risk, although infection can occur in the absence of visible maternal lesions.

Treatment

Aciclovir (neonatal dose is 20 mg/kg 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 without multi-organ failure [6].

Enteroviruses

The enteroviruses (EV) are a rare cause of severe hepatitis or acute liver failure. Presentation is in less than 5 weeks, with lethargy, jaundice, high levels of hepatic aminotransferases, and severe coagulopathy. Meningitis may be present. Echovirus serotypes 3, 6, 7, 9, 11, 14, 19, and 21 have been reported in severe infections with hepatitis [7], although serotype 11 is the most virulent. Coxsackie viruses cause a self-limiting gastrointestinal disease, but in neonates, Coxsackie A and B may cause fulminant hepatitis, myocarditis, or heart failure. There is no specific treatment, but liver transplantation is effective for acute liver failure.

Human parechoviruses

Human parechoviruses (HPeV) types 1 and 2 were previously classified as echoviruses 22 and 23. They cannot be detected by molecular tests for EV and require specific HPeV reverse-transcriptase polymerase chain reaction (RT-PCR) for diagnosis. Fourteen different types of HPeV have been described, of which HPeV types 1 and 3 are the most common. HPeV1 infection occurs in older children and with mild gastrointestinal and respiratory symptoms, whereas HPeV3 causes severe sepsis in newborns and young infants with hepatitis and meningoencephalitis. Like EV, there is no specific treatment other than supportive management [8].

Adenovirus

Adenoviruses are respiratory-tract pathogens that also cause acute gastroenteritis, hemorrhagic cystitis, meningoencephalitis, hepatitis, and myocarditis in newborn and immunosuppressed patients [9].

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%. There is widespread necrosis of the liver with Feulgen-positive intranuclear targetlike inclusions in the hepatocytes. 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. In neonates, it presents with severe hepatitis, and may progress to chronic cholestasis and decompensated cirrhosis over 6–12 months. Some infants may have chronic hepatitis with autoimmune hemolytic anemia. 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 [10] on liver histology and electron microscopy. Treatment with RBV may be effective, but 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 socioeconomic status. The mean age of infection increases with improvement in sanitation. In the United Kingdom, an oral fluid-based survey in 2001–2002 found only 4% of children aged 1–4 had serological evidence of past HAV infection, increasing to 26% in those aged 25–44 [11].

Transmission of the disease is by fecal contamination of drinking water or food and by person-to-person contact. Outbreaks of hepatitis A have been described in daycare centers and institutions for children with learning disabilities.

Clinical features

Most clinical cases are asymptomatic. In an epidemic in the United Kingdom, only one of 43 children aged <5 years became icteric compared with one of five between eight and 10 years of age [11].

After a prodromal period of 4–10 days, clinical features include jaundice (99%), dark urine (85%), anorexia (83%), lethargy (81%), vomiting (72%), abdominal pain (64%), fever (57%), diarrhea (18%), pale feces (17%), rash (7%), and arthritis (5%). Most children recover spontaneously.

Diagnosis

Diagnosis is by detection of hepatitis A–specific IgM class antibody in serum. Virus particles can be demonstrated by immune electron microscopy in feces in the incubation period and the early acute phase of infection. Virus RNA can be amplified by genome amplification techniques from feces and acute-phase sera for epidemiological studies.

Complications

Fulminant hepatic failure is the most severe complication and may require liver transplantation. 3–20% of

patients with icteric hepatitis may relapse, which is associated with HAV in the stools. Patients usually recover completely [12].

Prevention

Active immunization is now effective with the use of formalin-inactivated hepatitis A vaccine derived from the HM175 or CR 325F′ virus strains [13].

HAV vaccine is also effective in the control of HAV outbreaks. A randomized controlled trial (RCT) conducted in Kazakhstan confirmed non-inferiority of HAV vaccine versus immunoglobulin in preventing laboratory-confirmed symptomatic hepatitis A infection when given within 14 days of exposure [14].

Immunity lasts for at least 10 years, so there is no need for booster doses in healthy patients.

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. Infection in the perinatal period or early infancy occurred in 40% of infants in Taiwan, but in only 1–5% in Sub-Saharan Africa, which is explained by the higher prevalence of HBeAg in Chinese (40%) than African (15%) mothers. In Africa, the majority of infections are acquired between 6 months and 6 years of age from horizontal transmission.

In areas of intermediate endemicity (Italy, Japan, Spain, Greece, and 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%. In areas of low endemicity (Northwest Europe, North America, and Australia), infection in infancy and childhood is uncommon, and <1% of the population has chronic HBV infection [15].

Transmission

Infection in children is predominantly through maternal-infant transmission. HBsAg can be detected in 5% of cord blood samples taken at delivery from HBsAg-positive mothers, which is an indication of infection *in utero*. The risk of infection is 15–40% if the mother is HBsAg-positive, but increases to 70–90% if the mother is HBeAg-positive. In the presence of maternal anti-HBe, the risk of viral transmission is only 10–20%. However, if these children are infected, they may develop fulminant liver failure due to a mutant virus. Seventy percent of mothers with acute hepatitis B may transmit the virus during the third trimester of pregnancy, and 90% if it occurs within 8 days of delivery [16].

Clinical features and prognosis

More than 80% of infants born to HBeAg- and HBsAg-positive mothers become chronic carriers [15].

The clinical features are similar to those of other forms of hepatitis, as described in this chapter, but the clinical course is more insidious than hepatitis A infection. Extrahepatic manifestations include arthritis (14%) and skin eruptions (10%).

The majority (>90%) of children with acute hepatitis B infection recover completely within 6–12 months. Fulminant hepatitis may occur in <1% or in neonates born to mothers who are anti-HBe-positive. Mutations in the pre-core region of the HBV DNA have been implicated [17].

Diagnosis

Acute hepatitis B is diagnosed serologically by detecting HBsAg and IgM antibody to HBcAg in serum. Most acute HBV will resolve with clearance of HBsAg and development of anti-HBs and natural immunity.

Hepatitis E virus (HEV)

Prevalence

The disease occurs as a waterborne epidemic in the Indian subcontinent, Southeast and Central Asia, West Africa, and Mexico. There are four genotypes: genotypes 1 and 2 are responsible for outbreaks in developing countries affecting adults and children, whereas genotypes 3 and 4 are zoonotic. Two patterns of infection occur in developed countries: returning travelers (genotype 1 or 2) or autochthonous infection (genotype 3). In the latter case, the virus is similar to that harbored in pigs [18] and tends to affect older adult males rather than children. The incubation period is 6 weeks (range: 2–9 weeks) after primary exposure. It has a low secondary attack rate among exposed household members.

HEV infection is infrequent among children <10 years of age. In India, only five of 103 (5%) children were anti-HEV IgG-positive, and the sporadic genotype 3 HEV infection is not reported [19].

Clinical features

The clinical features of the disease are similar to those of acute hepatitis A or B with complete recovery. Vertical transmission has been described with significant perinatal morbidity and mortality.

Diagnosis

Acute HEV is diagnosed by detecting HEV RNA and IgM and IgG antibodies to recombinant HEV antigen. 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 are being evaluated in adults but not in children [20].

Non-A–E hepatitis

Acute or fulminant hepatitis may be caused by other, unidentified viruses, labeled non-A–E hepatitis. Non-A–E hepatitis is the commonest viral cause of fulminant hepatic failure requiring liver transplantation in children in the United Kingdom and United States. It may be complicated by transient bone marrow suppression or aplastic anemia in up to 28% of 32 children [21].

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. Spontaneous resolution is usual [22].

Diagnosis

Measles virus can be identified in throat swabs and saliva samples by virus isolation or detection of RNA using RT-PCR.

Parvovirus B19

Human parvovirus (HPV) B19 infection in childhood presents with erythema infectiosum and may lead to an aplastic crisis in patients with an underlying hemoglobinopathy or hemolytic anemia. Infection during pregnancy may lead to fetal ascites in the infant. HPV B19 infection is also associated with hepatic dysfunction with raised hepatic aminotransferase enzymes. Fulminant liver failure (with and without aplastic anemia) in association with HPV B19 is described.

Herpes viruses

The herpes viruses are a family of icosahedral double-stranded DNA viruses that 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

CMV usually occurs in neonates (as discussed in this chapter) 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 that 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. Transmission is by oropharyngeal secretions with an incubation period of 30–40 days [23].

Clinical features

In childhood, EBV infection is asymptomatic or associated with mild nonspecific 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% of cases, with hepatomegaly in 20% and jaundice in 5%. Although elevated aminotransferase enzymes (up to five times the normal level) are detected in up to 80% of cases, hepatitis is usually mild with a complete recovery.

Diagnosis

The diagnosis is made by finding atypical lymphocytes in the peripheral blood, or by serological assays. Regarding an antibody to viral capsid antigen (VCA), IgM is positive in early acute infection, whereas 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.

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, permit specific detection of EBV in infected tissues. In transplant recipients, PCR detection and quantitation of EBV DNA in blood monitor 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.

Treatment

Treatment is supportive in most cases, but the combination of intravenous aciclovir and prednisolone may improve pharyngeal symptoms and fever in 12 of 15 (90%) patients with fulminant mononucleosis.

EBV-related lymphoproliferative disease post transplantation is a serious disease that is treated by decreasing immunosuppression, intravenous rituximab, surgical removal of localized disease, autologous T cell therapy, or chemotherapy in selected cases. (Figure 39.2.)

Human herpes virus 6

Children are infected with human herpes virus 6 (HHV6) in the first year of life 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 includes hepatitis and fulminant hepatic failure. Diagnosis is based on the specific HHV6 IgM antibody.

About 1% of the population have chromosomally integrated HHV6 genome, which is vertically transmitted in the germ line; this means that they have high levels of HHV6 viral load but do not develop an illness [24].

Antiviral therapy is usually not required, except in immunosuppressed patients. Aciclovir, ganciclovir, cidofovir, and famciclovir are all effective, although none have any effect on the plasma DNA level of chromosomally integrated HHV6.

Varicella zoster virus

Varicella zoster virus occasionally affects the liver. The infection is spread by direct contact. The incubation period is 10–21 days. In older children, liver disease is unusual except in immunosuppressed children with HIV infection or posttransplant recipients who require intravenous aciclovir and famciclovir.

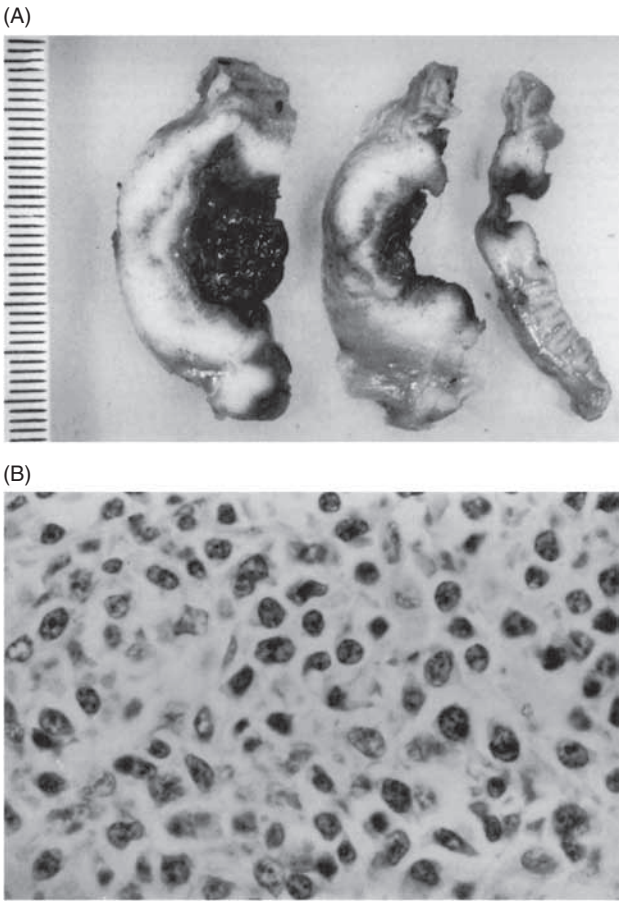

Figure 39.2 EBV-induced lymphoproliferative disease of the gut in a child after liver transplantation. (A) Macroscopic and (B) microscopic appearance.

Prevention

Varicella zoster immune globulin (250 mg for infants aged 0–5 years) is recommended for newborns whose mother had varicella zoster infection 7 days before or 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 hematological 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 or

inducer (CD4) lymphocytes and binding to the CD4 receptor. Transmission may occur by the transplacental or perinatal route, through breast feeding, or by exposure to infected blood or blood products.

The initial manifestation may be cholestatic hepatitis with liver failure, or it may be mild hepatitis. Prognosis is worse if there is associated liver disease.

Histological findings include giant-cell transformation (23%), CMV inclusion (17%), Kaposi's sarcoma (13%), lymphoplasmacytic infiltrate (7%), and granulomatous hepatitis secondary to fungal infection (3%). Nonspecific liver abnormalities include steatosis (47%), portal inflammation (24%), and periportal necrosis (10%). Treatment with retroviral therapy has transformed the outcome for these children [25]. Co-infection with other hepatitis agents is uncommon except in Africa and is treated with combination therapy [26].

Flavivirus

Dengue hemorrhagic fever

Dengue hemorrhagic fever (dengue shock syndrome) is a mosquito-borne infection (*Aedes aegypti or Aedes albopictus*) common in the Western Pacific and Southeast 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 titers to dengue antigen.

Hepatitis, with hepatomegaly and transaminase elevation, is present in 90% of patients as part of multiorgan involvement. Aspartate aminotransferase (AST) levels increase on the third day of illness, reach a peak level on days 7–8, and normalize 3 weeks after the onset of illness. Pathological lesions in the liver include fatty change, focal midzonal necrosis, Kupffer cell hyperplasia, and nonnucleate cells with vacuolated acidophilic cytoplasm, resembling Councilman bodies in the sinusoids. The dengue viral antigen may be demonstrated in the Kupffer cells. Fulminant hepatitis is rare but fatal [27].

Survival is directly related to correction of fluid and electrolyte loss. Eradication of the mosquito vector is essential for prevention.

Yellow fever virus

Yellow fever virus is a member of the flaviviruses (group B arbovirus); it is a spherical 35–45 nm particle containing a single-stranded RNA genome. 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 viremia for 5–10 days. Diagnosis is made by viral isolation or by RT-PCR of viral RNA in the acute-phase blood samples.

Clinical features

The disease may be subclinical or severe with jaundice, renal and cardiovascular symptoms, and bleeding diathesis 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 midzonal coagulative necrosis with eosinophilic degeneration of hepatocytes (Councilman bodies), microvesicular steatosis, and minimal inflammation. For patients who survive, hepatocellular regeneration is rapid and postnecrotic 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. Travelers to endemic areas should be vaccinated at least 10 days before arrival.

Viral hemorrhagic fevers

This includes yellow fever, lassa fever, Crimean-Congo hemorrhagic fever, and the Marburg and ebola fever viruses. Each of these is endemic in specific geographical areas such as Sub-Saharan Africa, South America, and Asia. They are characterized by massive hepatocellular necrosis, disseminated intravascular coagulation (DIC), and very high mortality.

Arenavirus (lassa fever)

The lassa fever virus is a single-stranded RNA virus that infects the African house rat, *Mastomys natalensis*. Humans become infected by consumption of food or water contaminated with infected rat urine, or by close contact with infected secretions and excretions. Diagnosis is confirmed by viral isolation from the throat, urine, or blood or by detection of specific lassa antibodies in the second week of illness by immunofluorescence or by rising antibody titers.

Lassa fever infection during pregnancy may cause an 87% fetal and neonatal loss. The risk of maternal death is higher if the infection occurs in the third trimester of pregnancy.

Clinical features

The clinical features vary from an asymptomatic infection to a fatal disease. Children present with a rising temperature, headache, conjunctivitis, pharyngitis,

abdominal pain, and diarrhea. Patients either recover spontaneously or die from liver failure. Ribavirin may be effective.

Acute liver failure (ALF)

Viral hepatitis is responsible for most cases of fulminant hepatic failure in children of all ages [21]. In a recent study from the US 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.

Hepatitis A

Acute HAV infection was a common cause of fulminant hepatic failure, varying from 1.5% to 31% in both developing and developed countries. Since improved environmental conditions, it is less common [21].

Hepatitis B

Fulminant hepatic failure in acute HBV infection is about 1% in Western Europe and the United States. It is very rare in infants [21], although it has been reported in babies born to HBsAg-positive, HBeAg-negative mothers. Mortality without transplantation is about 20%.

Hepatitis C, D, E, and non-A–E

HCV, HDV, and HEV are unusual causes of fulminant hepatic failure in children. The main cause (discussed in this chapter) is non-A–E hepatitis. The prognosis is poor, with spontaneous recovery in only 5% to 30%, indicating the need for liver transplantation.

Other viruses

HSV, VZV, and EBV may cause fulminant hepatic failure in immunocompromised hosts, with EBV most frequently implicated. Paramyxovirus, parvovirus B19, and toga virus have been identified in some cases.

Chronic hepatitis

Hepatitis B

Chronic hepatitis B is defined as failure to clear HBsAg after 6 months; it is likely if infection occurs early in life, if it is asymptomatic, or if there is a general defect in the immune system. 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 [28].

In perinatally infected children, HBeAg clearance was 30% by the age of 10 years, but only 4% had HBsAg clearance. Liver histology showed that 60% had mild hepatitis and fibrosis, while 18% had moderate to severe fibrosis despite almost normal transaminases. Boys have a higher risk of developing cirrhosis and HBV-related hepatocellular carcinoma (HCC) [28].

Perinatal transmission

The risk of perinatal transmission of hepatitis B is directly associated with the infectivity of the mother (discussed in this chapter). Breastfeeding does not affect the outcome in babies of HBV carrier mothers, especially in the postvaccination era [15].

Prevention of perinatal infection

Prevention of perinatal transmission of hepatitis B depends on antenatal screening for hepatitis B and selective vaccination using recombinant hepatitis B vaccine at birth (at 0, 1, and 6 months; or 0, 1, 2, and 6 or 12 months) with hepatitis B immunoglobulin (HBIG) in babies of HBeAg-positive mothers only. This is effective in up to 95% of infants of HBsAg carrier mothers if properly administered [29].

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 program, and the numbers of children with HCC has also fallen [30].

Universal vaccination 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 high-risk behavior. Many countries have included HBV vaccine in their routine childhood immunization program under the World Health Organization's Expanded Programme on Immunization (EPI).

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 with an additional booster [31].

For pregnant women with very high hepatitis B viral load (>10^6 IU/ml), the combined active and passive immunization of newborns may not be sufficient to prevent transmission as the fetus could be infected *in utero*. Other suggested preventative approaches include the use of specific immunoglobulin or antiviral treatment such as lamivudine during late pregnancy. This has prompted studies to investigate [32]. A systemic review of the use of lamivudine during late pregnancy showed a 13–23.7% lower incidence of intrauterine HBV infection in newborns and a 1.4–2% lower rate of infection at 9–12 months compared to standard management of vaccine and immunoglobulin to the baby at birth,

with or without immunoglobulin therapy to the mother. Due to the low potency of lamivudine and its susceptibility to development of resistance, the use of newer generation antiviral agents such as tenofovir is now being considered.

Management of chronic HBV infection

Chronic HBV carriers should be cared for at a specialist center in order to identify natural seroconversion and the progress of liver disease, consider antiviral therapy, and detect 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 [33].

Treatment

Success rates for viral clearance of hepatitis B are approximately 30% in children. The reasons for this include the immune tolerant state in which there is insufficient innate or adaptive immune response to clear the virus and the development of viral resistance to therapy. Chronic hepatitis B in children is associated with HBV-specific T cell hyporesponsiveness and an inadequate CD8 response. A partial immune response leads to hepatocyte injury and progression of liver disease, detectable by elevated hepatic transaminases and fibrosis on liver biopsy.

Viral resistance arises because the HBV virus replicates very rapidly and the HBV reverse transcriptase does not have the function to repair incorrectly incorporated nucleotides, leading to the development of viral mutations. The rate of development of mutations is related to how effectively the drug reduces HBV DNA. Thus, monotherapy with lamivudine, which only partially reduces HBV DNA, is more likely to produce mutations than other drugs such as entecavir.

Aim of treatment

Treatment aims consist of short-term aims that include the eradication of replicative infection by clearance of HBeAg, reduction in HBV DNA levels, and normalization of hepatic transaminases; and long-term aims include preventing progress of liver disease, reducing the risk of morbidity and mortality from cirrhosis and HCC, and reducing the pool of carriers. In children and young people, it is important to take into consideration the effect of HBV on their career and marriage prospects and the potential psychosocial stigma.

Indications for treatment

Consensus guidelines for the treatment of chronic hepatitis B in children have not been established, although the European Medicines Agency is currently developing them. However, lack of effective therapy means that treatment is limited and should be confined to randomized clinical trials [33].

The indications for treatment are persistent infection, being HBeAg-positive >6 months, and evidence of hepatic inflammation either by elevated aminotransferase enzymes or by liver biopsy. Treatment options include interferon, lamivudine, and adefovir dipivoxil. Factors that are associated with a positive response to interferon or nucleoside analogs include high pretreatment levels of aminotransferase (>2× ULN), low pretreatment HBV DNA levels (<10^5 copies/ml or 20 000 IU/ml), late acquisition of HBV infection, and higher hepatocellular inflammation.

Antiviral therapy

Interferon

In children, the response rates to interferon alpha (IFNα) therapy are 30–50% depending on route of infection, ethnic origin, disease activity, and treatment regime. Predictors of IFN responsiveness include active hepatitis on histology, low HBV DNA levels (<1000 pg/ml), high serum aminotransferase enzymes, short duration of disease, Mediterranean origin, and horizontal transmission [33].

Interferon has many unpleasant side effects, although children tolerate treatment better than adults. Fever and "flulike" symptoms are common when treatment is started, as is bone marrow suppression. Autoimmune thyroid disease, alopecia, and mental disturbance including severe depression may also occur. The major side effect is the effect on growth, as a number of studies have demonstrated that both height and weight are affected during treatment with interferon or PEG-IFN. Although catch-up of growth is rapid once treatment is discontinued, some children do not regain their pretreatment height percentile.

Interferon is not recommended in children with decompensated liver disease, bone marrow depression, or severe renal, cardiac, or autoimmune disease.

PEG-IFN has not yet been approved for HBV treatment in children, but it is more effective than interferon in adults. In view of the convenience of administration and the similar side effect profile, future studies of treatment are likely to be based on PEG-IFN.

Lamivudine

Lamivudine is a pyrimidine nucleoside analog that prevents replication of HBV in infected hepatocytes. It is incorporated into viral DNA, leading to chain termination, and competitively inhibits viral reverse

transcriptase. It leads to a rapid reduction in plasma HBV DNA in most patients within 2 weeks of commencing treatment. Response rates are related to low HBV DNA levels pre-treatment and evidence of hepatic inflammation (raised ALT and AST). A double-blind placebo-controlled trial of 286 children showed a complete response in 23% of children compared to 13% in the placebo group. Treatment for up to 3 years increased the seroconversion rate to 62% in children who did not develop viral mutations, but the high rate of development of tyrosine–methionine–aspartate–aspartate (YMDD) mutant variants of HBV in the majority precludes long-term treatment with lamivudine [34].

Adefovir dipivoxil

Adefovir dipivoxil is a purine analog that inhibits viral replication by binding to DNA polymerase. A recent randomized controlled trial in 173 children has demonstrated that adefovir, in doses up to 10 mg, reduced HBV DNA most effectively in children over 12 years of age (23%) compared to younger children, which is a similar outcome to that for adults. Seroconversion rates were 20% in children aged <12 years, but there was no significant difference compared to placebo. Viral resistance did not occur in the study but is reported in 1% of adults at one year, rising to 29% at 5 years. Viral resistance is treated by combination therapy with lamivudine, but there is increased resistance to adefovir in patients who were previously resistant to lamivudine. Adefovir is not first-line therapy in young children [35].

Combination therapy

In adults, the combination of PEG-IFN (180 mcg weekly) and lamivudine (100 mg daily) for 48 weeks produced greater viral suppression but no real difference in seroconversion rates. There are few studies to date of PEG-IFN in children with HBV with or without lamivudine, and the results are inconclusive so far.

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 or tenofovir either as pre-emptive treatment or in the treatment of acute or fulminant hepatitis.

Future therapy for hepatitis B in children

Telbivudine is an L-nucleoside analog that is more effective than lamivudine (in adults, 26% compared to 23%) but has a high rate of viral resistance compared to adefovir and is not recommended as monotherapy. A pharmacokinetic study in children aged 2–18 years is in progress.

Tenofovir disoproxil fumarate is a nucleotide analog similar to adefovir that was originally licensed for treatment of HIV. *In vitro* studies demonstrated activity against HBV, and clinical studies suggest increased potency compared to adefovir. A randomized, placebo-controlled multicenter study has just begun recruitment.

Entecavir, a carbocyclic analog, inhibits HBV replication at three different steps: the priming of HBV DNA polymerase, reverse transcription, and synthesis of HBV DNA. It is more potent than lamivudine in suppressing wild-type HBV, but less effective in adults with lamivudine resistance. Viral resistance is rare. An open-label multicenter trial is underway in children with promising results.

Choice of therapy for hepatitis B

The management of chronic hepatitis B in asymptomatic children remains a challenge. It is essential that children with HBV be referred to specialized centers to benefit from counseling, from information, and for inclusion in multicenter trials of antiviral therapy in order that natural history and outcome of treatment be appropriately monitored. At present, there is no completely effective therapy, so all children should be treated within the context of a clinical trial. In children who require compassionate therapy, treatment with lamivudine, which is licensed for children, with or without PEG-IFN is a possibility. If viral mutations develop, then adefovir could be added.

Liver transplantation

Liver transplantation is effective treatment for children with acute or chronic liver failure. Recurrence is unusual following transplantation for fulminant hepatitis but is usual following transplantation for chronic hepatitis B unless prevented with a combination of oral lamivudine and HBIG [34].

Hepatitis C virus (HCV)

Prevalence

HCV is endemic in most parts of the world, with an estimated 130–170 million people infected worldwide. In the United States, the seroprevalence is 0.2% for those <12 years and 0.4% for those 12–19 years [36]. The prevalence in children in the United Kingdom is not known; however, the prevalence of HCV in pregnant women is estimated to be 0.16%.

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 viremia. It may also be undetectable in patients with HCV infection who are immunocompromised [36].

Mode of transmission

The main mode of transmission in children has changed over time. Prior to 1990, when HCV had not been identified, transfusion of blood and blood products and organ transplantation were the usual modes of transmission. Currently, perinatal transmission accounts for the majority of cases [37].

Mother-to-baby transmission of HCV occurs in <10% of pregnancies. The seroprevalence of HCV infection in pregnant women in the United States is estimated to be 0.2%, with an estimated 1150 pregnancies occurring each year in women infected with HCV and 70 infants acquiring infection. In a study of infants born in the United States and Ireland, the vertical transmission rate was 6.7% overall, and 3.8 times higher in women co-infected with HIV than in HIV-negative women. Hepatitis C antibody is passively transmitted, and infants will have HCV antibody for up to 13–18 months. Measurement of HCV RNA, which is unreliable before 3 months of age, is necessary to detect active infection. Breastfeeding is safe in mothers with low titers of HCV RNA [37].

Prevention of hepatitis C

There is no vaccine to prevent or treat HCV infection; currently, prevention is achieved through screening of blood, organ, and tissue donors; high-risk behavior modifications; and blood and body fluid precautions.

Natural history of HCV

Acute hepatitis C is uncommon in childhood [36]. Most chronically infected children are asymptomatic with normal growth and development, and they present with mild biochemical evidence of liver injury. The majority will have chronic hepatic inflammation, with a minority progressing to fibrosis or cirrhosis in childhood, particularly those with multiple transfusions, previous chemotherapy, or an underlying disease such as thalassemia.

In a recent study of 60 children with chronic HCV infection, fibrosis was absent or mild in 88%, and none had cirrhosis [38]. Spontaneous resolution of HCV infection in children varies between 20% and 40% with lower rates in perinatally acquired HCV infection [36].

Clinical features

Children with chronic HCV infection are usually asymptomatic. 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. Although (HCV) infection does not impair quality of life in most children, it is associated with higher caregiver stress, particularly in mother-to-child transmission [39].

Management of chronic HCV

As for hepatitis B, children with chronic viral hepatitis C should be referred to specialized centers for information, counseling, and family support. Annual review for children includes HCV serology to detect natural seroconversion, evidence of progressive liver disease, and consideration for antiviral treatment [40].

Antiviral therapy

The goal of the treatment is to prevent chronic liver disease and the development of HCC, and improve quality of life by achieving a sustained viral response (SVR): HCV RNA undetectable for 6 months post treatment [41].

When to treat children

As spontaneous viral clearance may occur within the first 3 years of life, treatment should be delayed until the age of 3 years.

Treatment regimen

IFNα monotherapy has a response rate in children of up to 36 percent of SVR, but combination therapy with RBV (a guanosine nucleoside analog) resulted in a better SVR41 rate in children of up to 50%.

In 2008, the US Food and Drug Administration approved the combination therapy of PEG-IFNα2b and RBV for the treatment of chronic HCV in children ages 3–17 years. There are several studies reporting overall SVR rates of 53–59% in children treated with this therapy, and it is now standard of care. The response rate depends on viral genotypes, with a higher response among children with HCV genotypes 2 and 3 (treated for 6 months) compared to genotype 1 (treated for 12 months). A reduced response is associated with a higher mean Body Mass Index (BMI) and liver steatosis. Careful medical and psychological monitoring by the provider and a supportive and motivated family contribute to the success of treatment in children [41].

The better response rate in children might be related to lower viral loads, less inflammation and fibrosis, shorter duration of disease, and absence of drug or

alcohol consumption. PEG-IFN and RBV were generally well tolerated in younger children, but dose reductions and discontinuation of treatment occurred because of neutropenia or psychiatric side effects. Detectable antithyroid antibodies were common, but clinical thyroid disease only rarely became permanent. The main concern is the effect on growth as weight loss and reduction in linear growth velocity are common. The effect on weight is reversible, but children on 12 months of PEG-IFN do not regain their pre-treatment height centile. Relapse is uncommon [41].

Future therapies for hepatitis C

Direct-acting agents (telaprevir and boceprevir) are currently being evaluated in children infected with HCV genotype 1, both untreated and nonresponders.

Liver transplantation

Liver transplantation for hepatitis C is children is rarely required, but it is a common indication in adults. Reinfection of the graft is almost 100%, despite prophylactic measures such as treatment with antiviral agents and modification of immunosuppression.

Hepatitis D virus (HDV)

Prevalence

HDV is unusual in children except those from Eastern Europe or South America [42].

Clinical features

Although most children remain asymptomatic, there is a higher incidence of chronic hepatitis (71%) and cirrhosis (12%) compared to HBV mono-infected children. Fulminant hepatitis also occurs.

Prevention and treatment

HDV–HBV co-infection can be prevented by HBV vaccination, though this is ineffective in preventing HDV superinfection of HBV carriers. Treatment with interferon may be successful, but oral antivirals are not effective.

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Chapter 40
Management of hepatocellular carcinoma

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Summary

The management of hepatocellular carcinoma (HCC) has greatly improved following the standardization of therapeutic algorithms and the establishment of evidence-based criteria for prognostication (Barcelona Clinic Liver Cancer [BCLC] staging). Patients with an early detected cancer (<5 cm in size) in a well-compensated liver (BCLC-A) represent the best prognostic subgroup, with an expected 5-year survival of up to 75% following radical therapies, including transplantation, resection, and percutaneous ablation. The best prognostic groups for liver transplantation are patients within the radiological Milan criteria, whereas for patients undergoing limited hepatic resection, prognosis depends on the degree of portal hypertension and biochemical derangement of the liver. Patients with biochemically compensated cirrhosis, low portal hypertension, and a single <2 cm HCC nodule may have comparably high survival rates by local tumor ablation with alcohol or radiofrequency and with hepatic resection. The latter, however, is more effective than ablation in patients with HCC nodules between 2 and 5 cm in size, whereas radiofrequency ablation is superior to alcohol injection in 3–4 cm tumors due to better control of local recurrence. Transhepatic arterial chemoembolization is the standard of care for patients with an intermediate tumor (BCLC-B): it prolongs patient survival from 16 to 20 months on average. Survival of patients with advanced HCC (BCLC-C) is prolonged by treatment with the multikinase inhibitor sorafenib: this drug has therefore become the standard of care for patients with an advanced HCC and stable liver function. Multimodality treatments are an option for any treatable stage of HCC: however, these are of unproven value. The fourth stage (BCLC-D) includes patients with an average survival of 6 months: these patients should be given supportive care only. In the near future, prognostication based upon genetic fingerprints of the tumor may change the therapeutic scenario, thereby fulfilling an important unmet therapeutic need.

Background

HCC is the sixth most common cancer and the dominant cause of death in patients with compensated cirrhosis [1]. While the incidence of HCC is growing in many areas of the world as a consequence of an increased number of individuals with chronic viral hepatitis and alcoholic or metabolic liver disease, other factors like dietary exposure to aflatoxin B1 and tobacco smoking may have synergistic carcinogenic effects [2]. Better knowledge of the etiology and natural history of HCC has allowed improved strategies not only for prevention but also for early diagnosis of HCC aimed to improve treatment outcome. Owing to the frequent association between HCC and cirrhosis, the management of most patients with HCC is challenging because portal hypertension increases the risk of mortality and morbidity and limits the application of curative treatments.

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Currently, a majority of HCC patients are being treated with a multidisciplinary approach, whereby protocols for diagnosis and therapy are jointly determined by surgeons undertaking liver resection and transplantation, oncologists, and interventional radiologists in conjunction with hepatologists.

The diagnostic algorithm

The majority of patients with HCC are identified with advanced disease that almost invariably prevents the application of curative treatments. Conversely, early diagnosis through surveillance improves both the applicability and outcome of HCC therapy. Carriers of hepatitis B and patients with chronic liver disease or cirrhosis are the target populations for twice-yearly surveillance with abdominal ultrasound (US), a strategy that reflects the average tumor volume-doubling time [3, 4] (Table 40.1). Owing to its unacceptably low (60%) diagnostic accuracy for HCC and the lack of a standardized recall policy, alpha-fetoprotein (AFP) is no longer a standard-of-care assay for screening. Surveillance involves more than simply a screening test. It is a test framed in a program where testing frequency, recall policies, and quality control procedures are standardized, with significant economic consequences [5]. The recall policy varies according to the size of a liver nodule (Figure 40.1). A less than 10 mm HCC nodule is difficult to diagnose by contrast CT scan, MRI, or echo-guided liver biopsy. However, an enhanced follow-up with US every

Table 40.1 Recommendations for hepatocellular carcinoma surveillance: categories of adult patients in whom surveillance is recommended (Source: EASL-EORTC Clinical Practice Guidelines. J Hepatol 2012; 56:908–943 [3]).

1. Cirrhotic patients, Child–Pugh stage A and B*
2. Cirrhotic patients, Child–Pugh stage C awaiting liver transplantation**
3. Noncirrhotic HBV carriers with active hepatitis or family history of HCC***
4. Noncirrhotic patients with chronic hepatitis C and advanced liver fibrosis F3****

* Evidence 3A; strength B1;
** Evidence 3D; strength B1;
*** Evidence 1B; strength A1 for Asian patients; and Evidence 3D; strength C1 for Western patients; and
**** Evidence 3D; strength B1 for Asian patients; and Evidence 3D; strength B2 for Western patients.

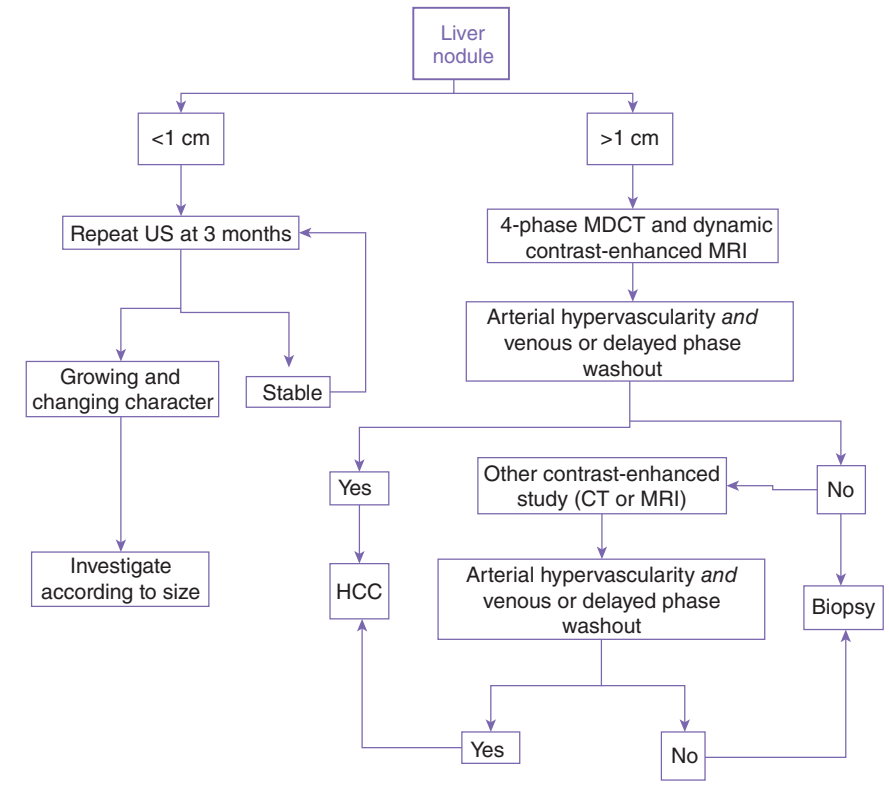

Figure 40.1 Algorithm for investigation of small nodules found on surveillance in patients at risk for hepatocellular carcinoma (Source: Bruix J, Sherman M. Hepatology 2011;53:1020–1022 [4]).

3 months to detect any increase in size or pattern may guide further investigations with radiology or liver biopsy [3, 4]. Nodules greater than 10 mm in diameter, which represent 80% of tumors detected during surveillance, can be diagnosed by CT or MRI imaging whenever the specific pattern of an intense contrast uptake during the arterial phase (wash-in) is followed by contrast wash-out during the venous or delayed phase [3, 4]. In both Europe and the United States, contrast-enhanced US is not recommended as the sole diagnostic imaging technique, because it does not distinguish intrahepatic cholangiocarcinoma from HCC [6]. While a typical "wash-in + wash-out" pattern suffices to diagnose an HCC >10 mm using a single imaging technique in a sequential study, a liver biopsy is required to confirm the diagnosis for the nodules that do not display these characteristic features at radiology [3, 4]. The noninvasive diagnostic criteria are valid only for investigation of screen-detected lesions in the liver with cirrhosis or with long-lasting chronic hepatitis B that may not have yet fully developed cirrhosis [3, 4, 9].

Tumor staging

Tumor staging is of strategic importance for the allocation of patients to the best treatment option available. This is generally achieved by evaluating the volume and number of tumors and their relationship to portal vein branches, in combination with the degree of liver impairment and presence of cancer-related symptoms. Since the prognosis of HCC largely depends on whether the patient will tolerate curative therapies such as liver resection or is suitable for tumor ablation, a valid staging system should enable healthcare providers not only to accurately inform patients and relatives about long-term life expectancy but also to predict treatment outcome. The BCLC staging system serves the purpose and has been externally validated (Table 40.2 and Figure 40.2) [7–9]. Owing to the fact that it includes clinical variables related to tumor stage like the tumor-node metastases (TNM) staging system, liver function in terms of Child–Pugh score, physical status (performance status [PS]), and cancer-related symptoms, the

Table 40.2 Barcelona Clinic Liver Cancer (BCLC) staging classification for hepatocellular carcinoma (Source: Modified from Llovet JM, Bru C, Bruix J. Semin Liver Dis 1999;19:329–338 [7]).

BCLC stage	Performance status	Tumor volume, number, and invasiveness	Child–Pugh score
0, very early	0	Single <2 cm	A
A, early	0	Single or 3 nodules <3 cm each	A or B
B, intermediate	0	Large, multinodular	A or B
C, advanced	1–2	Vascular invasion and/or extrahepatic spread	A or B
D, end-stage	3–4	Any of the above	C

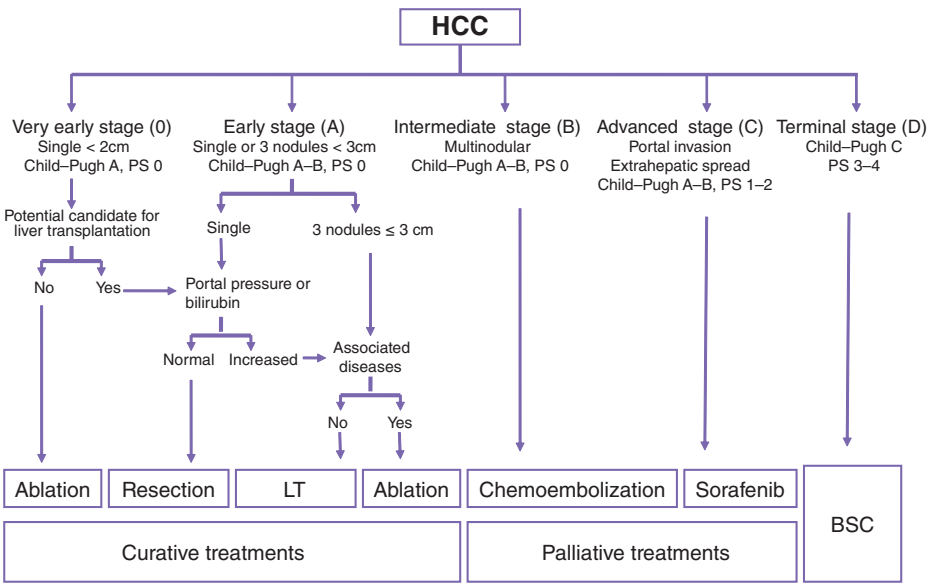

Figure 40.2 Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy (Source: Forner A, Llovet JM, Bruix J. Lancet 2012;379:1245–1255 [9]).

BCLC system accurately separates patients with an early cancer who may benefit from radical therapies from those who may receive palliative treatments only, and those with a poor outlook served best by supportive care. Circumstantial evidence, however, indicates that a single staging system is not deemed necessary for all patients with HCC, due to substantial geographical differences in the epidemiological and clinical presentation of the tumor and resource availability. The paradigm-shifting SHARP trial [10], which established HCC as a treatable target for molecular therapy, also boosted the search for a molecular classification of HCC. Gene signatures from the tumor and adjacent tissue may predict outcome in HCC, thereby providing the basis for depicting genomic predictors at each stage of HCC and their specific influence in determining patient prognosis [10, 11].

The treatment algorithm

Potentially curative interventions include hepatic resection, orthotropic liver transplantation (LT), and percutaneous tumor ablation in carefully selected patients. Palliative treatments are transhepatic arterial chemoembolization (TACE) and systemic therapy with molecularly targeted drugs [3, 4]. Figure 40.2 summarizes the criteria for selection of the patients [9]. This requires diagnosis of the accompanying liver disease and its severity, too, since patients with normal liver or noncirrhotic chronic hepatitis are ideal candidates for liver resection. However, owing to the frequent association of HCC with cirrhosis, hepatic resection is applicable in only a minority of patients with a small tumor and well-compensated liver function (less than 15% in Europe). Patients with poorly compensated cirrhosis are definitively contraindicated for hepatic resection, because the operation may precipitate liver failure. LT is the first-line option for decompensated cirrhotic patients who fit the general criteria of operability in terms of age, comorbidity, tumor burden, and extrahepatic disease. Pretreatment patient assessment is best served by computed tomography (CT) scan or magnetic resonance imaging (MRI_, a chest CT scan, and a bone scan. The presence of portal invasion by the tumor or extrahepatic localization of the tumor indicates a bleak prognosis and precludes curative interventions. The general conditions of the patient as reflected by the PS score are also essential parts of pretreatment patient evaluation [9, 12].

Patients with a noncirrhotic liver

Resection is the treatment of choice for noncirrhotic patients with an HCC who, however, account for less than 10% of all HCC patients in the West compared to 40% of patients in Asia, where the leading risk factor, hepatitis B, may cause HCC during the precirrhotic phase of the infection [13]. Survival largely depends on tumor volume at diagnosis. The worst prognosis group is patients with a tumor developing in a normal liver who are identified outside screening programs, and who often present with a large, symptomatic HCC.

Patients with cirrhosis

Early and very early HCC

Very early HCC (BCLC stage 0) is a single <20 mm tumor without vascular invasion or satellites developing in patients with a good health status (PS-0) and well-preserved liver function (Child–Pugh A). Approximately 10% of patients in the West and up to 30% in Japan are currently diagnosed in this stage as a consequence of the widespread implementation of surveillance programs. Pathologically, these tumors appear as vaguely nodular, without local invasiveness by tumor cells (carcinoma *in situ*). The 5-year survival rates of these patients are greater than 70% following resection or percutaneous local ablation.

Early HCC (BCLC stage A) is a single tumor of 20–50 mm or three nodules each <30 mm of diameter, PS-0, and Child-Pugh A/B. The 5-year survival in accurately selected patients is between 50% and 70% following hepatic resection, liver transplantation, or percutaneous local ablation. Resection is the best option for patients with a single tumor of any size, though the risk of vascular invasion and dissemination by tumor cells, causing postoperative recurrence of the tumor, increases with tumor size. In the few patients, however, in whom HCC may slowly grow to reach a >50 mm size without causing liver invasion or satellite nodules, an effective and safe hepatic resection is feasible with a moderate risk of tumor recurrence as in patients with smaller tumors. Following hepatic resection of a ≤50 mm HCC in a compensated patient, the 5-year survival rates approach 50%, with, however, a 70% risk of tumor recurrence. While in more than one-half of the patients undergoing radical resection, tumor will recur during the first 18 months as a consequence of pre- and postoperative tumor cell dissemination [14], in 30% of resected patients, tumor will recur between 2 and 5 years after the operation as a consequence of *de novo* primary tumors developing in the cirrhotic liver. The most powerful predictors of early postoperative recurrence are microvascular invasion by the tumor, tumor cell grading, and satellite nodules [15], but currently there are no effective neoadjuvant or adjuvant treatments to reduce the risk of recurrence. Hepatic resection is also associated with an increased risk of postoperative (3 months) fatal liver failure caused by reduced functional reserve of the remnant liver. The risk is greater for

Table 40.3 Treatment of early stage HCC: liver transplantation in cirrhotic patients selected by the Milan criteria.

Center	HCC	Cases	5-year survival	Recurrence	Reference
Milan	Single ≤5 cm, ≤3 nodes ≤3 cm	48	75%*	8%	[17]
Barcelona	Single ≤5 cm	79	75%	4%	[7]
Paris	≤3 nodes ≤3 cm	45	74%	11%	[31]
Berlin	Single ≤5 cm, ≤3 nodes ≤3 cm	120	71%	16%	[32]

* 4-year survival.

patients with a >10 mmHg porto-caval gradient, like those with esophageal varices detected by endoscopy, and patients with a serum level of bilirubin >1 mg/dL. In patients with both adverse predictors of survival, hepatic resection may result in a 5-year rate of survival of only 25%. Conversely, the 5-year survival rate of patients with neither adverse predictor may be as high as 75% [16].

HCC is the sole solid cancer for which organ transplantation is an established treatment modality. Theoretically, LT has the advantage of simultaneously leading to eradication of both tumor and underlying cirrhosis, whereas the efficacy of the treatment is only marginally influenced by the degree of liver impairment. The Milan criteria of patient selection based on a single HCC ≤5 cm or up to three nodules, each ≤3 cm in size, without vascular invasion or extrahepatic spread, resulted in a fair balance between a 4-year survival rate of 75% and a limited risk (8%) of tumor recurrence [17]. Following external validation in several liver centers worldwide, the Milan criteria are now the benchmark for patient selection in both the United States and Europe (Table 40.3). Since the success of LT in HCC patients is challenged by a shortage of donors that results in increased waiting times and risk of tumor progression, the effectiveness of LT needs to be evaluated in terms of intention-to-treat analysis [18]. Whenever the waiting time exceeds 6 months, bridge treatments with tumor ablation and TACE aimed to delay disease progression are currently offered, whereas hepatic resection is the first choice for patients with favorable predictors of survival, leaving LT to patients with adverse predictors of response to resection. While access to LT has been successfully improved by strategies based on split-liver and living related liver donors, the strategy of expanding listing criteria beyond Milan is not endorsed by all centers, being highly debated in terms of cost-effectiveness, particularly in regions with a limited donor supply. Using the "up-to-seven" criteria, which allows for a total tumor diameter of 12 cm compared to a maximum of 9 cm for Milan and 8 cm for the UCSF criteria, a continuous range of survival probabilities is provided rather than a dichotomous "in or out" basis for patient selection [19, 20]. However, it should be noted that the 5-year survival of the patients who were transplanted within the up-to-seven criteria but outside the Milan criteria was only 54%.

Percutaneous tumor ablation is a consolidated treatment option for patients with an early HCC who do not fit criteria for surgery [21]; radiofrequency ablation (RFA) and ethanol injection (PEI) are the most widely adopted therapeutic approaches in nonresectable small tumors. Both percutaneous techniques achieve a complete tumor necrosis in almost 100% of patients with ≤20 mm nodules, whereas RFA is more effective than PEI in 30 mm tumors due to a better control of the periphery of the tumor nodule. Both techniques, however, lose efficacy in tumors larger than 30 mm due to the presence of satellites to the point that tumor ablation with RFA or PEI is no longer recommended for nodules greater than 30–40 mm [22] (Figure 40.3). In properly selected patients with compensated cirrhosis, percutaneous tumor ablation may confer 5–year survival rates of 50% to 75%, thus mimicking in these patients the satisfactory rates of efficacy of hepatic resection as a first-line therapeutic option. Despite several RCTs comparing the outcome of percutaneous ablation and resection in early HCC, there is no definite demonstration of the clinical superiority of one procedure over the other. Main reasons for discrepancies between studies were the inadequate sample size, treatment allocation, and trial conduction. Novel techniques such as microwave or electroporation are under evaluation to treat early HCC. Adjuvant approaches with interferon and cytotoxic anticancer agents failed to demonstrate activity against HCC. A RCT (STORM) is in progress to assess the efficacy of adjuvant therapy with sorafenib to improve disease-free survival following hepatic resection or percutaneous ablation of tumors at risk of recurrence.

Intermediate HCC (BCLC stage B)

The BCLC-B stage involves patients exceeding Milan criteria, but in the absence of portal vein invasion or

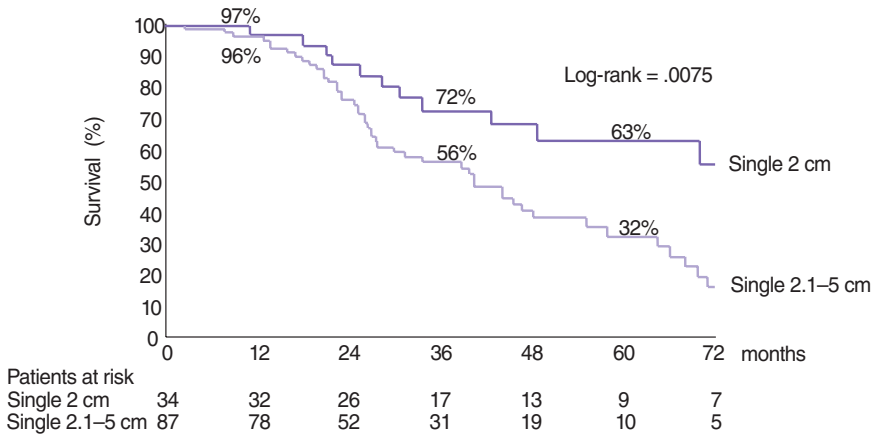

Figure 40.3 Treatment of early HCC: the initial tumor volume predicts survival after percutaneous ablation (Source: Sala M, Llovet JM, Vilana R *et al.* Barcelona Clinic Liver Cancer Group. Hepatology. 2004 Dec;40(6):1352–60 [30]).

extrahepatic spread of the tumor. In these patients, TACE, combining the injection of cytotoxic drugs like oxalyplatinum and doxorubicin with embolic obstruction of the arterial blood supply of the tumor, causes more than 50% of the patients to have an objective response that translated into an improved average survival from 16 to 22 months [23]. The injection of microspheres (DEB TACE) to increase the local concentration of the drug allows standardization of the procedure of TACE while reducing drug-related adverse events caused by the absorption of cytotoxic drugs [24]. The ideal candidates to TACE are compensated patients with an asymptomatic, large, and multifocal HCC restricted to the liver that is not amenable to curative treatments. TACE should be delivered in more than one course either as protocol treatment or *on demand*, whenever after each successful procedure the tumor nodules become revascularized. To delay tumor revascularization, an antiangiogenic agent like sorafenib is being assessed in combination with DEB TACE. Radioembolization with Yttrium-90 (Y90)-labeled microspheres is an attractive option for patients with intermediate or advanced HCC, including patients with portal invasion by the tumor who are contraindicated to DEB TACE [25]. However, no controlled data on survival of patients treated with Yttrium-90 are available.

Advanced HCC (BCLC C)

Patients with symptomatic tumors (PS 1–2), macrovascular invasion, or tumor spread in lymph nodes, lung, and skeleton have an expected median survival of 6 months (25% at 1 year). While interferon, hormones, and cytotoxic anticancer drugs failed to demonstrate any activity against HCC in controlled trials [3, 4, 9], targeted therapy with sorafenib is now the standard of care for BCLC-C patients with clinically compensated cirrhosis, since it can attenuate tumor progression and invasiveness. Sorafenib, in fact, through its activity against RAF signaling as well as VEGF, PDGF, and c-kit, is the sole agent proven to improve survival in patients with advanced HCC. In the registration SHARP study carried out in patients with hepatitis C or alcohol-related HCC, sorafenib prolonged patients' survival from 7.9 months to 10.7 months, causing a 31% decrease in the relative risk of death [10]. Survival benefits have also been obtained in hepatitis B–related tumors [26] and were accompanied by a limited number of adverse events that were usually manageable. Sorafenib is the standard of care not only for compensated patients with advanced HCC (BCLC-C) but also for compensated patients with a tumor not responding to locoregional therapies. No second-line drugs for nonresponders to sorafenib are available as yet (see www.clinicaltrial.gov).

End-stage HCC (BCLC D)

Patients with end-stage disease who have a very poor performance status (PS 3–4), resulting in a median survival of 3–4 months (11% at 1 year) only, will receive best supportive care.

Assessment of tumor response

Though patient survival is the endpoint in cancer therapy, objective tumor responses are necessary surrogates both to predict treatment efficacy in registration trials and to monitor treatment effectiveness in practice. The Response Evaluation Criteria in Solid Tumors (RECIST) criteria, based on morphometric changes of the tumor volume [27], do not accurately evaluate HCC response to therapy as they do not sense arterial

vascularity of the tumor nodule. Paradoxically, nodules treated with invasive procedures may be enlarged at radiology as a consequence of edema accompanying tumor tissue necrosis. A modified RECIST evaluates a response to both invasive and drug-related therapy of HCC, based on the viable tumor cells identified by contrast imaging [28]. By modified RECIST, the rates of objective responses in patients with percutaneous ablation or TACE were as high as 80% compared to 15% by RECIST [29].

Conclusions and perspectives

HCC is a common malignancy with a particularly grim prognosis, since the majority of patients are recognized at an intermediate or advanced stage that precludes delivery of curative treatments like hepatic resection, LT, and percutaneous local ablation. The only hope for a cure rests on early diagnosis through surveillance with abdominal US of patients at risk, an endpoint that is achieved in a minority of patients, most of them clustering in the developed world. A majority of patients therefore are receiving palliative treatments with repeat TACE courses, multimodality interventions, or antiangiogenic drugs. In perspective, neoadjuvant and adjuvant treatments with molecularly targeted drugs might improve the therapeutic scenario of HCC by enhancing the efficacy of curative and palliative antitumor treatments. Molecular cell biology investigations to unravel the pathogenic mechanisms of HCC might help develop targeted therapeutic strategies for this tumor.

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Chapter 41
Application of molecular biology to the diagnosis of viral hepatitis

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Summary

Molecular biology techniques are used routinely to diagnose and monitor treatment of chronic viral infections such as hepatitis B and hepatitis C, and now hepatitis D and E. These tools serve mainly to detect and quantify viral genomes, and also to analyze their sequence, in order to determine their genotype or subtype and to identify clinically relevant nucleotide or amino acid substitutions such as those associated with resistance to antiviral drugs. New methods are now available for detecting and quantifying viral genomes and for analyzing their nucleotide and amino acid sequences. The available molecular biology methods and their current clinical applications to hepatitis B, C, D, and E virus infections are reviewed.

Introduction

Virologic techniques are now used routinely to diagnose and monitor chronic viral infections such as those by hepatitis B virus (HBV) and hepatitis C virus (HCV). Molecular biology tools serve mainly to detect and quantify viral genomes, and also to sequence them, in order to ascribe them to a phylogenic clade or subclade (genotype or subtype) and to identify clinically relevant nucleotide or amino acid substitutions such as those associated with resistance to antiviral drugs.

Molecular biology methods

Detection and quantitation of viral genomes

Viral genome detection and quantitation are required for antiviral treatment decision making and monitoring. They can be achieved with two categories of molecular biology–based techniques: signal amplification methods such as hybrid capture and the branched DNA (bDNA) assay, and target amplification methods such as polymerase chain reaction (PCR) and transcription-mediated amplification (TMA). Recent real-time target amplification methods offer vastly improved viral genome detection and quantitation for both clinical and research purposes.

Signal amplification methods

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, 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 using multiple antibodies

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(resulting in signal amplification) conjugated to a revelation system based on chemiluminescence. Luminescence is proportional to the initial amount of the template, and quantitation 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, viral genomes are captured specifically 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. Like for hybrid capture, luminescence is proportional to the initial amount of the template nucleic acid, and quantitation is based on a standard curve generated simultaneously with known standards.

Classical target amplification methods

Classical target amplification techniques have been used widely for the diagnosis of hepatitis virus infections and to monitor antiviral drug responses. In these assays, PCR or TMA amplicons are detected at the end of the amplification reaction by means of specific hybridization to immobilized oligonucleotide probes and detection of amplicon-probe hybrids in an enzymatic reaction. 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, and the results are interpreted with a standard curve established in parallel.

Polymerase chain reaction

The PCR method 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. In contrast, a reverse transcription step is required for RNA viruses (such as HCV, hepatitis D virus [HDV], or hepatitis E virus [HEV]) in order to synthesize a complementary double-stranded DNA (cDNA) for use as a template in the PCR. 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 synthesized theoretically. The reaction is saturable and reaches a plateau, generally after 35 to 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 colored or luminescent signal. Quantitation 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.

Transcription-mediated amplification

In TMA, 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, 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 reenters 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 colored or luminescent signal. Quantitation can be performed by competitive amplification of the viral template with a known amount of synthetic standard added to each reaction tube.

Real-time target amplification methods

Because amplification reactions reach a plateau, these methods suffer from a narrow dynamic range of quantitation. As a result, high viral levels are not always accurately quantified and necessitate retesting after dilution, while low viral levels during antiviral therapy are not even detectable. A solution to this problem was found with the development of real-time target amplification techniques, in which quantitation takes place during the exponential phase of the amplification reaction. In addition, the reaction is run in a closed system, thus preventing carryover contamination and improving specificity. Real-time target amplification techniques are recognized as the methods of choice for viral hepatitis diagnosis and monitoring in recent international liver society guidelines [1–6].

Real-time PCR

The principle of real-time PCR is to detect amplicon synthesis during the PCR reaction and hence to deduce the starting amount of viral genome in the clinical sample. A fluorescent probe linked to a "quencher" is used to anneal to the target sequence between the sense and antisense PCR primers. During each PCR reaction, as the DNA polymerase extends the primer, its intrinsic nuclease activity degrades the probe, releasing the reporter fluorochrome. Thus, the amount of fluorescence released during the amplification cycle and detected by the system is proportional to the amount of amplicons generated in each PCR cycle. Software is used to calculate the "threshold cycle" (Ct) in each reaction, which bears a linear relationship with the initial amount of nucleic acid. In each run, parallel processing of a panel of quantified standards is used to establish a standard curve for quantification. Real-time PCR assays avoid false-positive results due to carryover contamination. They are fully or partly automated (in the latter case, the extract has to be transferred to automated PCR amplification and quantification after automated extraction).

Real-time TMA

The amplicons created during amplification are detected in real time by using probes that contain both a reporter dye (fluorophore) and a quencher. In the absence of amplicons, these probes are present in the "closed" configuration, in which the fluorescence output of the fluorophore is quenched because it is in close proximity to the quencher. During amplification, when these probes bind to amplicons, the fluorophore and quencher are separated spatially, allowing the generation of a fluorescence signal. Software is used to calculate the amount of viral genome in the initial sample by comparison with a panel of quantified 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 and subtypes. It is also possible to identify amino acid substitutions known to be associated with viral resistance to antiviral drugs or with particular forms of liver disease (e.g., precore and core promoter mutations in HBV). The entire genome of hepatitis viruses cannot be analyzed routinely, and available techniques therefore study particular genomic regions. Genome sequence analysis is based on sequencing techniques that provide the full sequence of the analyzed fragment, or on alternative techniques that identify specific sequences at given positions.

Direct sequencing or "population" sequencing

Direct sequencing, also called "population" sequencing of PCR amplicons, is performed after the sequencing reaction in an automated DNA sequencer. The Sanger method, first described in 1977 [7], was improved by the use of fluorescence and capillary electrophoresis [8, 9]. This approach involves three steps: denaturation, annealing, and elongation. During synthesis, the polymerase randomly incorporates deoxynucleotides (dNTPs) or dideoxynucleotides (ddNTPs) labeled with different fluorochromes, resulting in chain elongation or termination, respectively. Chains of different lengths are therefore generated, each ending with a labeled nucleotide. The amplicons are then migrated in a capillary system that separates them according to their respective lengths. Bioinformatic software measures the fluorescence peak at each position of the analyzed sequence and identifies the corresponding ddNTP in order to determine a consensus sequence. The presence of multiple peaks at the same position indicates sequence mixtures.

Population sequencing 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 coexistence of variant viral populations in the same blood sample (quasi-species distribution of viral genomes) may lead to sequence ambiguities. 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 or population sequencing is the gold standard for genomic sequence analysis.

"Next-generation" sequencing

Viruses that establish chronic infections, such as HBV and HCV, behave as quasi-species (i.e., complex mixtures of genetically related but distinct viral populations in equilibrium in a given replicative environment). This quasi-species distribution of viral genomes implies that, at any given time point, each patient harbors a very large number of different genome sequences. "Population" sequencing techniques are able to identify only the consensus sequence of viral quasi-species at a given time point, while most intermediate and minor viral populations go undetected. In addition, this approach is

unable to establish linkages between substitutions present in the same viral variant or to distinguish substitutions present in different variants. Cloning of PCR products followed by sequencing of multiple clones has been used as an alternative method for identifying intermediate viral populations and for determining linkages between substitutions. However, this approach is cumbersome and time-consuming, and has thus been restricted to the research setting.

Next-generation sequencing methods have recently been developed to increase sequencing capacity while generating clonal sequences. Current methods use a three-step sequencing workflow after PCR amplification of the viral sequence [10]:

1. Library preparation, which consists of preparing DNAs of appropriate lengths and labeling them with primers required for subsequent isolation and sequencing;

2. DNA capture and clonal DNA enrichment; and

3. A sequencing reaction, which can be based on two different approaches: polymerization combined with detection based on pyrophosphate (pyrosequencing), H+ ions (pH variation), or fluorescence; or ligation combined with fluorescence detection.

The detected signals are transformed into readable sequences by integrated browser and specific software, thus yielding biologically relevant information.

The main advantage of next-generation sequencing techniques for studying chronic viral infections is their improved "depth" relative to the Sanger method, as this ensures that minor viral populations with possible clinical significance are sequenced. However, the clinical significance of very small viral populations remains unclear, and it is still difficult to distinguish between true rare substitutions and errors generated by the sequencing reaction; specificity thus needs to be improved. In addition, there is a need for bioinformatic tools to analyze the generated sequences in the context of viral infections. Therefore, next-generation sequencing methods should be used as a research tool until standardized commercial assays become available.

Reverse hybridization

Alternatives to sequencing 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. 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. It can be used for HCV and HBV genotyping, for the identification of amino acid substitutions associated with resistance to antiviral drugs, and to identify HBV variant sequences, such as in the core or HBs antigen region.

Point-of-care tests and alternatives to laboratory tests that require whole-blood samples

Point-of-care tests are used directly at the site of patient care, outside of the biology laboratory. They include assays for nucleic acid detection and quantitation. Point-of-care tests can be particularly useful for large-scale screening and for improving access to care in regions without molecular biology laboratories.

Alternatives to laboratory tests that require whole-blood samples, obtained by venipuncture, are being developed. They can use blood collected from a finger stick into a capillary tube. Blood can also be collected on filter paper, known as the dried blood spot (DBS) method, which makes it possible to store desiccated samples for transport as nonhazardous material via regular mail or courier services. DBS collection and storage are easy, and although standard procedures must be carefully followed, little training is required for preparation. HBV DNA and HCV RNA can be detected and quantified from DBS by classical or real-time PCR methods, although at a cost of reduced analytical sensitivity compared to the whole-blood-based methods described in this chapter. The reduction in sensitivity results from small input volumes, less efficient nucleic acid extraction, and nucleic acid degradation in extreme storage conditions. There is a high level of concordance in HBV genotype analysis of DBS and serum samples, and also in detection of precore mutants and drug-resistant variants [11]. Concordant results were also obtained from HCV quantification and genotype analyses of serum and DBS [12]. In theory, the analytical sensitivity of viral genome detection from DBS could reach levels similar to those obtained from serum and plasma samples.

Clinical use of virologic methods in hepatitis B

Available molecular biology assays for HBV

HBV DNA detection and quantitation

Table 41.1 shows the performance of current and upcoming commercial real-time PCR and TMA assays. These assays have been shown to accurately quantify HBV

Table 41.1 Commercially available real-time target amplification assays for HBV DNA detection and quantification. IU/mL: international units per milliliter.

Assay	Manufacturer	Method	Automated extraction device	Amplification device	Volume required (μL)	Lower limit of detection (IU/mL)	Dynamic range of quantification (UI/mL)
COBAS® TaqMan® HBV Test, v2.0	Roche Molecular Systems, Pleasanton, CA	Real-time PCR	Manual with High Pure System Viral Nucleic Acid Kit	COBAS® TaqMan	650 μL	6–10 IU/mL	29 (1.5 Log_{10}) – 1×10^8 (8.0 Log_{10}) IU/mL
COBAS® Ampliprep-COBAS® TaqMan® (CAP/CTM) HBV Test, v2.0	Roche Molecular Systems, Pleasanton, CA	Real-time PCR	COBAS® AmplipPrep®	COBAS® TaqMan	650 μL	20 IU/mL	20 (1.3 Log_{10}) – 1.7×10^8 (8.2 Log_{10}) IU/mL
RealTi*me*™ HBV	Abbott Molecular, Des Plaines, IL	Real-time PCR	$m2000_{SP}$	$m2000_{RT}$	200 or 500 μL	10 IU/mL (for 500μL) 15 IU/mL (for 200 μL)	10 (1.0 Log_{10}) – 1.0×10^9 (9.0 Log_{10}) IU/mL
Artus HBV QS-RGQ Assay*	Qiagen, Hilden, Germany	Real-time PCR	QIAsymphony RGQ	Rotor-Gene Q	1000 μL	10.2 IU/mL	31.6 (1.5 Log_{10}) – 2×10^7 (7.3 Log_{10}) IU/mL
APTIMA® HBV Quantitative Assay	Hologic Gen-Probe, San Diego, CA	Real-time TMA	Panther®		Available in 2013		

* Available only for ethylenediaminetetraacetic acid (EDTA)-plasma specimens.

DNA in clinical practice [13, 14]. The World Health Organization has established an international standard for universal standardization of HBV DNA quantitation units, and an HBV DNA international unit (IU) has been defined. This IU must be preferred to any other quantitative unit and is now implemented in all commercial HBV DNA quantitative assays. This has proven to be particularly useful for establishing clinically relevant thresholds and universal recommendations for clinical decisions based on HBV DNA level.

All of the HBV DNA assays have been shown to be specific and accurate within their respective dynamic ranges of quantification, regardless of the HBV genotype. The dynamic range of quantification can be extended by diluting and retesting high viral level samples, frequently encountered in HBV-infected patients. It is generally recommended not to take into account HBV DNA level variations of less than ±0.2 Log_{10}, whereas variations of more than 0.2 Log_{10} can reliably be considered to reflect significant changes. However, in the case of HBV infection where the HBV DNA level may spontaneously fluctuate, it is recommended that baseline viral levels 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 basal core promoter mutations, and identification of amino acid substitutions associated with resistance to nucleos(t)ide analogs. All of them can be achieved by means of "in-house" "population" sequencing or commercial assays based on "population" sequencing or reverse hybridization (Table 41.2).

With "population" sequencing, genotype determination is based on sequence comparison with banks of reference sequences, eventually through phylogenetic analysis, which 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. Finally, amino acid substitutions known to confer HBV resistance to nucleos(t)ide analogs are identified by analyzing the sequence of the HBV reverse transcriptase at known amino acid positions.

Line probe assays based on reverse hybridization are available to determine the HBV genotype, identify precore and basal core promoter mutations, and identify mutations of the HBV polymerase known to confer resistance to lamivudine, telbivudine, adefovir, and entecavir (Table 41.2). Compared to population 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.

Table 41.2 Commercially available assays for HBV genome sequence analysis (precore and basal core promoter mutations, genotype determination, and nucleos(t)ide analog resistance testing).

Assay	Manufacturer	Method	Device and interpretation software	Application	Approval as of March 2012
INNO-LiPA HBV PreCore	Innogenetics, Gent, Belgium	Reverse hybridization (line probe assay)	*Auto*-LiPA48 or AutoBlot 3000H, then LiRAS™ (Line Reader & Analysis Software) for interpretation	Detection of precore and basal core promoter mutations	RUO Not CE-marked
INNO-LiPA HBV Genotyping	Innogenetics, Gent, Belgium	Reverse hybridization (line probe assay)	*Auto*-LiPA48 or AutoBlot 3000H, then LiRAS™ (Line Reader & Analysis Software) for interpretation	Determination of the HBV genotype (A to H)	RUO CE-marked
INNO-LiPA HBV DR v2	Innogenetics, Gent, Belgium	Reverse hybridization (line probe assay)	*Auto*-LiPA48 or AutoBlot 3000H, then LiRAS™ (Line Reader & Analysis Software) for interpretation	Identification of amino acid substitutions associated with resistance to lamivudine, telbuvidine, emtricitabine, adefovir, and entecavir	RUO CE-marked
TRUGENE® HBV Genotyping Assay	Siemens Healthcare Diagnostics, Tarrytown, NY	Direct sequencing (CLIP chemistry)	OpenGene® DNA Sequencing System	Determination of the HBV genotype and identification of amino acid substitutions associated with resistance to nucleos(t)ide analogs	RUO Not CE-marked
Abbott HBV Genotyping Assay	Abbott Diagnostics, Chicago, IL	Direct sequencing	ABI sequencer	Determination of the HBV genotype and identification of amino acid substitutions associated with resistance to nucleos(t)ide analogs	RUO CE-marked

CE-marked: approved in the European Union; RUO: research use only in the United States (i.e., not FDA approved).

Practical use

Clinically useful virologic tests in HBV infection include enzyme immunoassays for HBV antigen and antibody detection, assays to detect and quantify HBV DNA, and methods to identify amino acid substitutions associated with HBV resistance to nucleos(t)ide analogs. The clinical interest of hepatitis B surface antigen (HBsAg) quantitation is debatable. Tools for HBV genotype determination and core and core promoter mutant detection are valuable for research but have no clinical uses.

Diagnosis of persistent HBV infection

Persistent HBV carriage is defined by the persistence of HBsAg in blood for more than 6 months. Chronic hepatitis B is characterized by an HBV DNA level of >2000–20 000 IU/mL (i.e., >3.3–4.3 Log_{10} IU/mL), with persistent or intermittent aminotransferase elevation and relevant signs on liver biopsy [1, 5, 6]. Although this threshold remains controversial, viral loads below 2000 IU/mL are generally considered to indicate inactive carriage.

Prognosis of HBV infection

The HBV DNA level has prognostic value. A chronically high HBV DNA level is associated with a significant risk of progression to cirrhosis and hepatocellular carcinoma [15, 16]. However, no HBV DNA threshold has been established to have a strong predictive value on the occurrence of clinical outcomes at the individual level.

Decision to treat

The decision to treat chronic hepatitis B is based on multiple factors, including the HBV DNA level. The European Association for the Study of the Liver (EASL) Clinical Practice Guidelines state that HBV therapy is indicated for noncirrhotic patients with an HBV DNA level >2000 IU/mL (>3.3 Log_{10} IU/mL), and/or serum alanine aminotransferase (ALT) activity above the upper limit of normal (ULN), and a liver biopsy (or a non-invasive test) showing moderate to severe activity (METAVIR grade ≥A2) and/or fibrosis (METAVIR stage ≥F2) [1]. Patients with compensated cirrhosis and detectable HBV DNA should be considered for treatment, and

patients with decompensated cirrhosis require urgent antiviral treatment, whatever the level of viral replication [1]. The Clinical Practice Guidelines of the American Association for the Study of Liver Diseases (AASLD) and the Asian-Pacific Association for the Study of the Liver (APASL) state that antiviral treatment should be considered for noncirrhotic patients with an HBV DNA titer >20 000 IU/mL (>4.3 Log_{10} IU/mL) if they are HBeAg-positive, or >2000 IU/mL (>3.3 Log_{10} IU/mL) if they are HBeAg-negative, and if they have elevated serum ALT activity (>2×ULN) and/or a liver biopsy showing inflammation and/or fibrosis [5, 6]. HBV resistance testing is not recommended prior to therapy.

In patients with normal ALT activity, serum HBV DNA and ALT levels should be measured at 3- to 6-month intervals. More frequent monitoring should be considered during the first year for hepatitis B e antigen (HBeAg)-negative patients with normal ALT and an HBV DNA level <2000 IU/mL (<3.3 Log_{10} IU/mL) in order to confirm their inactive carrier status.

Choice of therapy

Pegylated interferon alpha (PEG-IFNα) is still considered the first-line treatment option for noncirrhotic HBeAg-positive patients who have elevated ALT levels and a low HBV DNA level (<2×10^6 IU/mL, i.e., <6.3 Log_{10} IU/mL). The indication is more controversial in HBeAg-negative patients with the same characteristics [1]. The HBV genotype is a significant predictor of the response to PEG-IFNα. However, the individual predictive value of the HBV genotype for the outcome of PEG-IFNα therapy is weak and could be biased by the strong relationship between this parameter and ethnicity [17, 18]. Thus, HBV genotype determination is not indicated to guide therapeutic decisions.

For most patients, treatment is based on nucleos(t)ide analogs. The international liver society guidelines recommend the use of either tenofovir or entecavir monotherapy as first-line treatment for chronic hepatitis B [1, 5, 6, 19]. The HBV genotype has no influence on the effectiveness of nucleos(t)ide analogs and should not be determined prior to therapy [18].

Treatment monitoring

The ideal endpoint of therapy is a sustained loss of HBsAg, with or without seroconversion to HBs [1]. However, this is rarely achieved with current drugs. The practical aim is thus to reduce HBV DNA levels as far as possible, ideally below 10–20 IU/mL, the lower limit of detection of real-time PCR assays, in order to achieve biochemical remission, histological improvement, and prevention of HBV-related liver disease [1, 5, 6]. HBV treatment monitoring is therefore based on HBV DNA and ALT assays every 3 to 6 months and HBeAg and anti-HBe antibody assessment every 6 months in HBeAg-positive patients.

In HBeAg-positive patients, treatment can be stopped after a consolidation period of 6 to 12 months following HBe seroconversion [1, 5, 6]. However, durable HBeAg seroconversion associated with low-level HBV replication is rare with nucleos(t)ide analogs. In HBeAg-positive patients who do not develop anti-HBe antibodies, and in HBe-negative patients, nucleos(t)ide analogs must be administered for life, with the aim of achieving profound and durable HBV DNA suppression. If HBV DNA is still detectable (>10–20 IU/mL) after 48 weeks of therapy, current guidelines based on studies with modestly potent nucleos(t)ide analogs with a low barrier to resistance recommend adding a second drug with no cross-resistance to the first in order to prevent the emergence of HBV resistance. However, this is not necessary with entecavir or tenofovir if the HBV DNA level continues to decline after week 48, because it is likely to become undetectable thereafter [20]. Serial measurements of HBsAg levels in treatment may help identify patients who will subsequently clear HBsAg.

In patients who adhere and respond to their treatment, selection of resistant HBV variants should be suspected if virologic breakthrough occurs, defined as a re-increase in the HBV DNA level by 1 Log_{10} or more above the nadir or as HBV DNA that becomes detectable after being undetectable [6, 21]. A "blip," defined as one detectable low-level HBV DNA that was preceded and followed by undetectable HBV DNA, should not be considered a virologic breakthrough. If resistance occurs, the only effective strategy is to add a second drug without cross-resistance [1, 6]. Such drug combinations include tenofovir and a nucleoside analog. Identification of amino acid substitutions associated with resistance has little clinical value, as it rarely influences the choice of therapy; the only exception is the rtA181V/T substitution: such viruses are fully sensitive only to entecavir, which must be combined with tenofovir.

Clinical research

Cohort studies, surveillance studies, and clinical trials of new drugs or treatment strategies all require serial measurements of HBV DNA levels. In the research context, resistance testing should be performed systematically when antiviral therapy fails, in order to characterize the nature and dynamics of selected viral populations and to identify new HBV variants selected by antiviral therapies. HBV genotype determination and the identification of core and core promoter

Table 41.3 Commercially available real-time target amplification assays for HCV RNA detection and quantification.

Assay	Manufacturer	Method	Automated extraction device	Amplification device	Volume required (µL)	Lower limit of detection (IU/mL)	Dynamic range of quantification (UI/mL)
COBAS® TaqMan® HCV Test, v2.0	Roche Molecular Systems, Pleasanton, CA	Real-time PCR	Manual with High Pure System Viral Nucleic Acid Kit	COBAS® TaqMan	650 µL	20 IU/mL	25 (1.4 Log_{10}) – 3.0×10^8 to 3.9×10^8 (8.5–8.6 Log_{10}) IU/mL
COBAS® Ampliprep-COBAS® TaqMan® (CAP/CTM) HCV Test, v2.0	Roche Molecular Systems, Pleasanton, CA	Real-time PCR	COBAS® AmplipPrep	COBAS® TaqMan	650 µL	15 IU/mL	15 (1.2 Log_{10}) – 1.0×10^8 (8.0 Log_{10}) IU/mL
RealTi*me*™ HCV	Abbott Molecular, Des Plaines, IL	Real-time PCR	$m2000_{SP}$	$m2000_{RT}$	200 or 500 µL	12 IU/mL (for 500 µL) 30 IU/mL (for 200 µL)	12 (1.1 Log_{10}) – 1.0×10^8 (8.0 Log_{10}) IU/mL
Artus HCV QS-RGQ Assay*	Qiagen, Hilden, Germany	Real-time PCR	QIAsymphony RGQ	Rotor-Gene Q	1000 µL	36.2 IU/mL	67.6 (1.8 Log_{10}) – 17.7×10^6 (7.2 Log_{10}) IU/mL
VERSANT HCV RNA 1.0 Assay (kPCR)	Siemens Healthcare Diagnostics, Tarrytown, NY	Real-time PCR	Sample Preparation (SP) Module	Amplification and Detection (AD) Module	500 µL	15 IU/mL	15 (1.2 Log_{10}) – 1.0×10^8 (8.0 Log_{10}) IU/mL
APTIMA® HCV Quantitative Assays	Hologic Gen-Probe, San Diego, CA	Real-time TMA	Panther®		Available in 2013		

*Available only for ethylenediaminetetraacetic acid (EDTA)-plasma specimens. IU/mL: international units per milliliter.

mutations may be useful for epidemiologic and pathophysiological studies.

Clinical use of virologic methods in hepatitis C

Available molecular biology assays for HCV

HCV RNA detection and quantitation

Table 41.3 shows the performance of current and future commercial real-time PCR and TMA assays for HCV RNA [22–24]. The first-generation Cobas Ampliprep®/Cobas Taqman® (CAP–CTM) assay was found to substantially underestimate HCV RNA levels in approximately 30% of patients infected with HCV genotype 4, and occasionally failed even to detect this genotype, due to nucleotide polymorphisms at the hybridization site of the PCR primers or TaqMan probe in the 5′ noncoding region of the HCV genome [22, 23, 25, 26]. This issue has been resolved in the second-generation assay, released in 2012 [27].

The World Health Organization has established an international standard for universal standardization of HCV RNA quantitation units, and an HCV RNA IU has been defined. It is used currently in all commercial HCV RNA quantitative assays. All available assays have a lower limit of detection of 10–15 IU/mL and satisfactory specificity. They accurately quantify HCV RNA within their respective dynamic ranges of quantification, with no difference among the various HCV genotypes. It is generally recommended not to take into account HCV RNA level variations of less than ±0.2 Log_{10}, which may be related to the intrinsic variability of the assays; in contrast, variations of more than 0.2 Log_{10} can reliably be considered to reflect significant differences in the HCV RNA level.

HCV genome sequence analysis

HCV genome sequence analysis has two potential applications: HCV genotype determination, and identification of amino acid substitutions associated with resistance to direct-acting antiviral drugs (DAAs). All of them can be achieved by means of "in-house" population sequencing. Genotype determination is based on sequence comparison with banks of reference sequences, eventually through phylogenetic analysis that allows

Table 41.4 Commercially available assays for HCV genotype determination.

Assay	Manufacturer	Method	Device	Application	Approval as of March 2012
VERSANT® HCV Genotype 2.0 Assay	Siemens Healthcare Diagnostics, Tarrytown, NY	Reverse hybridization (line probe assay)	*Auto*-LiPA 48 or AutoBlot 3000H	Determination of the HCV genotype and subtype	RUO CE-marked
TRUGENE® HCV Genotyping Assay	Siemens Healthcare Diagnostics, Tarrytown, NY	Direct sequencing (CLIP chemistry)	OpenGene® DNA Sequencing System	Determination of the HCV genotype and subtype	RUO Not CE-marked
RealTi*m*e HCV Genotype II	Abbott Diagnostics, Chicago, IL	Real-time PCR	$m2000_{SP}$	Determination of the HCV genotype and subtype	RUO CE-marked

CE-marked: approved in the European Union; RUO: research use only in the United States (i.e., not FDA approved in March 2012).

classification of the typed HCV strain based on sequence similarities. Amino acid substitutions known to confer HCV resistance to a given DAA or DAA combination are identified by analyzing the sequence of the HCV protein targeted by the drug(s).

Table 41.4 describes current commercial assays for HCV genotype determination. These assays are based on population sequencing, reverse hybridization, or real-time PCR with primers and probes specific for the different HCV genotypes. No commercial assay is yet available to identify amino acid substitutions associated with HCV resistance to DAAs.

Practical use

Anti-HCV antibody detection, HCV RNA detection and quantitation, and HCV genotype determination are useful for diagnosing and treating HCV infection. HCV core antigen detection and quantification are alternatives to HCV RNA testing, but lack sensitivity for treatment monitoring and response-guided therapy. Resistance testing is not useful for patient management but is widely used in clinical trials of new DAAs and host-targeted agents (HTAs) in cases of treatment failure.

Diagnosis of chronic HCV infection

Diagnosis of chronic HCV infection is based on the detection of anti-HCV antibodies by enzyme immunoassay and of HCV RNA by a sensitive molecular biology–based technique with a detection limit of about 10 to 15 IU/mL.

Decision to treat

All treatment-naïve patients with compensated liver disease and detectable HCV RNA should be considered for therapy [2, 4]. Assessment of liver disease severity is important for decision making [2, 4]. Patients infected with HCV genotype 1 in whom PEG-IFNα and ribavirin combination therapy failed to eradicate the virus should be considered for retreatment, because they can now benefit from the triple combination of PEG-IFNα, ribavirin, and a protease inhibitor.

Selection of optimal therapy

The HCV genotype should be determined before starting treatment. Patients infected with non-1 HCV genotypes should be treated with PEG-IFNα and ribavirin alone. For patients infected with genotype 2 or 3, the dose of ribavirin is 0.8 g/day and treatment duration 24 weeks; however, patients with genotypes 2 and 3 with baseline factors suggesting low responsiveness should receive weight-based ribavirin [2]. In patients infected with genotypes 5 and 6, the dose of ribavirin is based on body weight (1.0–1.4 g/day) and treatment should last up to 72 weeks, depending on the on-treatment virologic response.

Patients infected with HCV genotype 1 who qualify for therapy should receive the triple combination of PEG-IFNα, weight-based ribavirin, and either telaprevir or boceprevir. Accurate identification of HCV subtypes 1a and 1b is crucial for clinical trials of new drugs, in order to interpret efficacy and resistance data. However, subtype determination is not mandatory with the combination of PEG-IFNα and ribavirin, or with triple-drug combinations including a protease inhibitor, as it has no influence on the treatment modalities [28]. Further trials are needed to improve the selection of patients infected with HCV genotype 1 who might not need protease inhibitors, such as young patients with mild fibrosis, a low baseline HCV RNA level, and the CC *IL28B* genotype [29].

Treatment monitoring

The endpoint of HCV therapy is the sustained virologic response (SVR), characterized by undetectable HCV RNA (<10–15 IU/mL) 24 weeks after the end of treat-

ment, which corresponds to viral eradication in more than 99% of cases.

Treatment monitoring with the PEG-IFNα and ribavirin combination

HCV RNA levels should be measured at baseline; at weeks 4, 12, and 24; at the end of treatment; and 24 weeks after treatment withdrawal. A rapid virologic response (RVR) is defined as undetectable HCV RNA at week 4; an early virologic response (EVR) as HCV RNA detectable at week 4 but undetectable at week 12; and a slow or delayed virologic response (DVR) as HCV RNA detectable at week 12 but undetectable at week 24 [2]. Patients who have an RVR probably need no more than 24 weeks of therapy, except for those who are infected by an HCV genotype other than 2 or 3 and who have a high baseline HCV RNA level (>400 000–800 000 IU/mL) [30]. In patients infected with genotype 2 or 3 who have a low baseline HCV RNA level and who experience an RVR, 16 weeks of therapy could be sufficient [31]. Patients who achieve an EVR require 48 weeks of therapy, whereas patients with a DVR appear to benefit from 72 weeks of treatment [32–34]. Patients with less than a 2 Log_{10} decline in HCV RNA at week 12 are unlikely to experience sustained viral eradication and should be taken off therapy [2].

Treatment monitoring with the triple combination of PEG-IFNα, ribavirin, and a protease inhibitor (telaprevir or boceprevir)

Telaprevir is administered for 12 weeks in combination with PEG-IFNα and ribavirin, followed by PEG-IFNα and ribavirin alone for a duration that depends on the virologic response. HCV RNA levels should be determined at baseline; at weeks 4, 12, and 24; at the end of treatment; and 24 weeks after the end of treatment. An extended rapid virologic response (eRVR) is defined as undetectable HCV RNA at weeks 4 and 12. According to the European and American labels, treatment-naïve patients and responder-relapsers must stop therapy at week 24 if they achieve an eRVR; if not, they should continue on PEG-IFNα and ribavirin until week 48. Treatment-naïve patients with cirrhosis who have undetectable HCV RNA at weeks 4 and 12 may benefit from an additional 36 weeks of PEG-IFNα and ribavirin (48 weeks total). Partial responders and null responders to a first course of PEG-IFNα and ribavirin should receive 12 months of the triple combination followed by 36 weeks of PEG-IFNα and ribavirin alone, regardless of their virologic response. In all instances, it is recommended to halt treatment if the HCV RNA level is >1000 IU/mL at week 4 or 12, or if HCV RNA remains detectable at week 24.

The European and American labels indicate that boceprevir must be administered after a 4-week "lead-in" period with PEG-IFNα and ribavirin alone. As with telaprevir, the duration of treatment depends on the virologic response. HCV RNA levels should be determined at baseline; at weeks 8 (i.e., week 4 of boceprevir administration), 12, and 24; at the end of treatment; and 24 weeks after the end of treatment. Noncirrhotic, treatment-naïve patients should receive the triple combination of PEG-IFNα, ribavirin, and boceprevir until week 28 if they have undetectable HCV RNA at week 8, or otherwise until week 36 and continue on PEG-IFNα and ribavirin until week 48. The European label recommends that responder-relapsers without cirrhosis and partial responders receive the triple combination of PEG-IFNα, ribavirin, and boceprevir until week 36 and then continue on PEG-IFNα and ribavirin until week 48, whereas the American label recommends that patients receive the triple combination until week 36 and continue on PEG-IFNα and ribavirin only if HCV RNA is still detectable at week 8. The European and American labels agree that null responders to a first course of PEG-IFNα and ribavirin (whatever their fibrosis score) and cirrhotic patients should receive the triple combination until week 48. Treatment should be stopped if the HCV RNA level is ≥100 IU/mL at week 12 or if HCV RNA is detectable at week 24.

Failure of triple combination therapy to clear HCV is associated with an outgrowth of HCV variant populations that are resistant to the protease inhibitor used. However, HCV resistance testing based on sequence analysis of the protease region has no utility in clinical practice [35]. Indeed, resistant viral variants preexist at baseline in virtually all infected patients, but currently available technologies in clinical virology laboratories are not sensitive enough to detect them [36], meaning that negative results are not interpretable. In addition, the profile of HCV variants at the outset of therapy does not appear to affect the outcome of treatment when the protease inhibitor is combined with PEG-IFNα and ribavirin. At the time of treatment failure (virologic breakthrough or relapse), the resistant population is always enriched, and phase II and III trials suggest that it is dominant in 50% to 70% of patients [37, 38]. Resistance testing is still not indicated in this situation, however, because the results will have no impact on subsequent treatment decisions [35].

Clinical research

In cohort studies, surveillance studies, and clinical trials of new HCV DAAs or HTAs used alone or in combination, serial HCV RNA assays are needed to characterize the virologic response and to detect virologic breakthroughs and relapses. Resistance testing should be

used systematically in cases of treatment failure, in order to characterize the nature and dynamics of the selected viral populations and to identify new amino acid substitutions selected by HCV DAAs. When several antiviral drugs with different target sites and mechanisms of action are combined, all the target sites should be studied. Sensitive sequencing methods such as next-generation sequencing are particularly useful to characterize the dynamics of sensitive and resistant viral populations over time.

Clinical use of virologic methods in other types of hepatitis

Hepatitis D

Available molecular biology assays for HDV

The diagnosis of HDV infection is based on the detection of HD antigen, total anti-HD antibodies, and anti-HD immunoglobulin M (IgM) in the presence of markers of acute or chronic HBV infection. HDV RNA detection and quantification are based on real-time PCR. It is useful to diagnose active infection and monitor the virologic response to antiviral therapy. A limited number of commercial assays are available, and current methods are not standardized. In particular, the genotype influences the performance of HDV RNA detection and quantification, including in commercial assays. HDV genotype determination is not useful in clinical practice.

Practical use

HDV infection should be sought in patients with acute hepatitis B or in chronic HBV carriers, especially in at-risk groups such as patients originating from areas of high endemicity or intravenous drug users. In most cases, the HBV DNA level is low or undetectable, because HDV inhibits HBV replication. The presence of HD antigen is a sign of recent infection, which is also suggested by the presence of anti-HD IgM. Anti-HD IgG is present at the acute stage of infection and persists only if infection becomes chronic. Chronic hepatitis D is characterized by the persistence of HDV RNA 6 months after infection. HDV RNA level monitoring is used to assess the efficacy of antiviral therapy with PEG-IFNα.

Hepatitis E

Available molecular biology assays for HEV

Total anti-HEV antibodies and anti-HEV IgM can be sought by means of enzyme immunoassays. Detection of HEV RNA in stools or blood by classical or real-time PCR is required to establish the diagnosis of active infection. No commercial assay is available thus far. Thus, most available techniques lack standardization.

Practical use

The diagnostic strategy must be tailored to the prevalence and type of HEV infections in the area. In Europe, HEV must be suspected in cases of unexplained acute hepatitis, even in the absence of any travel to an area of high endemicity. HEV infection must be suspected in the presence of anti-HEV antibodies, but should ideally be confirmed by means of HEV RNA detection in stools or blood. Chronic cases are characterized by the presence of HEV RNA in blood.

Conclusion

New methods are now available for detecting and quantifying viral genomes, and for analyzing viral genome nucleotide and amino acid sequences. Real-time target amplification (PCR or TMA) methods are well standardized and are widely used in clinical practice to diagnose and monitor HBV and HCV infection. Various techniques based on population sequencing, reverse hybridization, or real-time PCR can be used to determine the HBV or the HCV genotype, identify HBV precore or basal core promoter mutations, and characterize HBV or HCV resistance to antiviral drugs. Next-generation sequencing techniques are restricted to research laboratories, because they generate vast quantities of data; however, software capable of providing clinicians with clinically meaningful information will become available in the near future. Overall, 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, organ, tissue, and cell donations; diagnose active infection; help to establish a prognosis; guide and tailor treatment decisions; and assess the virological response to therapy.

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Chapter 42
Disinfection and sterilization

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Summary

Effective methods for disinfection and sterilization of pathogenic microbes are essential in healthcare settings, including the laboratory and environmental surfaces. The available methods, as well as methods for decontamination, are reviewed with particular emphasis on the hepatitis viruses. These processes are defined, and a number of physical and chemical methods are described for inactivating the hepatitis viruses. While the resistance of each of the individual species of the hepatitis viruses to heat and chemical reagents varies, none exceeds the level of resistance of bacterial spores or *M. tuberculosis*, so conventional procedures can be employed, including application to medical devices.

Introduction

The purpose of this chapter is to discuss disinfection and sterilization strategies used in healthcare and laboratory settings to prevent the transmission of infectious agents from contaminated medical devices and environmental surfaces to patients and healthcare workers. Although these strategies are those used generally in healthcare facilities to accomplish disinfection and sterilization for all groups of pathogenic microorganisms, 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 healthcare 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 or 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, antisepsis, or 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) the materials compatibility of the device with the

Unfortunately Dr Favero has been unable to update his chapter for this new edition, so this is reproduced from the third edition of *Viral Hepatitis*.

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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, healthcare worker, and environment. As will be pointed out in this chapter, 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 a 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. hemodialysis machines, endoscopes, and high-speed dental hand-pieces) will be regulated by the FDA. The EPA requires manufacturers of chemical germicides formulated as sanitizers, disinfectants, hospital disinfectants, or sterilants or 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 manufacturers submit a premarket 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, which are used to inhibit or kill microorganisms 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, healthcare 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 healthcare 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 forever. 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; and
3. the *application* of sterilization.

Any item, device, or solution is considered to be sterile when it is completely free of all living microorganisms. 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 microorganisms, 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 microorganism surviving on an item subjected to the sterilization procedures is less than one in 1 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 labeling of a sterile device are listed in Table 42.1.

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 healthcare facilities have operational protocols that are validated by the manufacturer to accomplish sterilization, and all the variables that control for the inactivation of microorganisms 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 (discussed in [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 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 cleaned and then disinfected with an intermediate-level disinfectant, sanitized with a low-level disinfectant, or simply cleaned with soap and water. These instruments and other medical surfaces are termed (with respect to their need to be sterile at time of use) "critical," "semicritical," or "noncritical," respectively. Selection of the appropriate disinfecting procedure in the last category (noncritical) will include consideration of the nature of the surface, as well as the type and degree of contamination, as shown in Table 42.2.

In the context of these categorizations, Spaulding [1] also classified chemical germicides by activity level. The activity levels are listed in Table 42.3 and are as follows.

1. *High-level disinfection.* This is a procedure that kills all viruses, fungi, and vegetative microorganisms 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 sterilants and disinfectants or simply high-level disinfectants.

2. *Intermediate-level disinfection.* This is a procedure that kills vegetative microorganisms, including comparatively resistant vegetative microbial forms such as *Mycobacterium tuberculosis*. All fungi and most viruses are also killed. These chemical germicides often correspond 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.

Table 42.1 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
Poststerilization testing of devices for function

Table 42.2 Relationship of germicide type, type of device or surface, and process.

Type of germicide	Type of device or surface	Process
Sterilant and disinfectant	Critical (heat-sensitive rigid endoscopes)	Sterilization (sporicidal chemical and prolonged contact time)
	Semicritical (medical instruments, re-usable heat-sensitive anesthesia circuits, endotracheal tubes, and laryngoscope)	High-level disinfection (sporicidal, chemical, and short contact time)
Hospital disinfectant (with label claim for tuberculocidal activity)	Noncritical (medical equipment, and blood-contaminated control knobs of medical equipment)	Intermediate-level disinfection
Hospital disinfectant or sanitizer	Noncritical (environmental surfaces: exteriors of machines, floors, walls, and other housekeeping surfaces)	Low-level disinfection or soap-and-water washing

Table 42.3 Levels of disinfectant action according to type of microorganism.

	Bacteria			Virus		
Level of action	Spores	*Mycobacterium* spp.*	Vegetative cells	Fungi**	Nonlipid, small	Lipid, medium-sized
High	+***	+	+	+	+	+
Intermediate	–†	+	+	+	±††	+
Low	–	–	+	±	±	+

+: A killing effect can be expected; –: little or no killing effect.
Adapted from [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 versus 5–45 minutes for high-level disinfection).
†Some intermediate-level disinfectants (e.g., hypochlorites) may show some sporicidal activity, while others (e.g., alcohols and phenolics) have none.
††Some intermediate-level disinfectants (e.g. certain phenolics and isopropyl alcohol) may have limited virucidal activity, even though they readily inactivate *Mycobacterium* spp.

Comparatively resistant vegetative forms such as *M. tuberculosis* are *not* killed. These chemical germicides are often ones that are approved in the United States by the Environmental Protection Agency (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 42.4 [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 microorganism 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 an one in 1 million probability of a surviving microorganism if the procedure 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 healthcare 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, device, or 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 microorganisms. Quite often, the distinction

Table 42.4 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‡	0.5–3.0%	Intermediate
Quaternary ammonium compounds‡‡	0.1–2.0%	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 harbor organic material. Although alcohols (e.g., isopropanol and 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 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 vapor containment (e.g., disinfection of certain hemodialysis equipment).

†† Only those iodophors designed as hard surface disinfectants should be used, and manufacturer's instructions regarding proper use and 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 nonlipid viruses.

††† See this chapter's text.

‡ Check the product label claims for demonstrated activity against *Mycobacterium* spp. (tuberculocidal activity) as well as a spectrum of lipid and nonlipid viruses.

‡‡ Quaternary ammonium compounds are not tuberculocidal and may not have significant effects against a variety of nonlipid viruses. This class of germicide is used primarily for routine housekeeping throughout healthcare facilities.

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 as either 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

Microorganisms 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 microorganisms approach the broad resistance of these organisms to heat, chemicals, or radiation. A number of factors, some of which are associated with microorganisms 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 microorganism

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 microorganisms

All other factors being equal, the greater the number of microorganisms 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 microorganisms

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 nontuberculous 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 microorganisms. 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. The resistance of certain nonlipid viruses is similar to that of mycobacteria [8].

Amount of organic soil present on the item to be disinfected or sterilized

Blood, feces, 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 discussed in this chapter, care should be taken to examine closely and follow the 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 42.5 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 healthcare 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 patients (sometimes as high as 10^8–10^9/mL) and its 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, and 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

Table 42.5 Effects of chemical agents and heat on HAV viability.

Inoculum and exposure conditions	Agent (exposure time)	Result (log reduction)
Tissue culture–derived HAV with 10% feces 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 feces, 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 [9], Tables 26–13, 26–14, 26–16.

*McCaustland KA, Bond WW, Spelbring JA, unpublished data.

** 104 MID per test; both animals inoculated with treated material were not infected.

***From [10].

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 microorganisms). 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 ppm free chlorine from sodium hypochlorite, an iodophor disinfectant, and isopropyl or ethyl alcohol [11, 19]. Table 42.6 gives a summary of some of the inactivation potentials of various physical agents and germicides in tests using titered 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.

Table 42.6 Complete inactivation of HBV inoculum by chemicals and heat*.

Inoculum	Treatment	Reference
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]
2.0×10^5 CID	5 min, 24 °C: 0.1% glutaraldehyde	[19]
3.3×10^5 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; titered inocula.

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 Pekin duck virus resembles HBV closely in viremia, 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 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 diarrhea 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 quantity of known concentration of OPA solution was added. After an exposure of 5 minutes at 20 °C, the virus was recovered and titrated on monolayers of either duck hepatocytes 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 °C.

Testing against HCV is also difficult because the virus cannot be visualized or effectively grown in tissue culture. HCV replicates 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 in this chapter, 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 those 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 those of *M. tuberculosis* var. *bovis* and nonlipid viruses (e.g., poliovirus), but much less than those 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 healthcare 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 42.4).

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, hemodialysis units, and other spill- or splash-prone healthcare 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 bloodborne 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, with an 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 chem-

ical 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 also 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 as, 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 feces. Furthermore, cleaning personnel must be alerted to the potential hazards associated with blood, serum, and fecal 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 here, 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 42.4).

Conclusions

Strategies for disinfection and sterilization used in hospitals and other healthcare institutions are based on relatively conservative criteria and do not need to be changed because of concerns 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 43
Evolution of hepatitis viruses

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Summary

The evolution of human hepatitis viruses A, B, C, D, and E (HAV, HBV, HCV, HDV, and HEV, respectively) can be studied over very different time scales. Very rapid sequence change occurs in response to a variety of selection pressures driven by the immune escape from B and T cell responses and immunization and, in treated individuals, by antiviral therapy. Each shows more gradual accumulation of largely neutral nucleotide substitutions that, over a much longer term period, lead ultimately to their differentiation into genetically distinct types. Thus, hepatitis A, B, C, and E viruses are currently divided into 6, 8, 7, and 4 genotypes, respectively, with HCV showing the greatest genetic divergence (>30% nucleotide sequence over the length of the genome) and HBV the least (11–15%). Some hepatitis viruses infect nonhuman primates (NHPs) (HBV and HAV) and other mammals (HEV), while HCV infections are restricted to humans. Sequence variability influences their antigenicity, biology, and host interactions with implications for vaccine development and antiviral therapy.

Introduction

This review of the evolution of human hepatitis viruses will contrast information about two time scales: their evolution over short time scales such as weeks, months, or years, as well as over much longer periods from thousands to tens of millions of years. The reason for the wide scope of the chapter is that very different descriptions of the characteristics of virus evolution are obtained by recent studies of short- and long-term evolution, and these have implications for our understanding of the origin and variation of human pathogenic viruses. The viruses that will be discussed within this chapter are HAV, HBV, HCV, and HEV.

The scientific investigation of human hepatitis viruses has a history of not quite 50 years, amounting to perhaps 0.025% of our existence as a species. This means that clinical samples and therefore our perspective on virus evolution are biased toward contemporary samples and sequences. There is no shortage of such sequences. The ease with which virus genomes can be isolated, amplified, and sequenced means that thousands of sequences are now available for HAV and HEV. There are almost 50 000 HBV sequences and more than 120 000 for HCV. The study of variation among these sequences has produced a large literature concerning virus taxonomy, the use of sequence relationships to classify viruses into meaningful categories of genotype (or type or group) and subtype and to explore their evolution and relationship to origins, transmission patterns, and epidemiology. Understanding the extent and processes underlying the genetic variability of hepatitis viruses has considerable scientific, clinical, and practical importance for each that we hope to explain in this chapter.

Genetic variability of hepatitis viruses – classification into genotypes

As a direct result of difficulties in *in vitro* culture that prevent traditional serology-based classifications, variants of each of the hepatitis viruses have been characterized by nucleotide sequence comparisons, although in the case of HBV typing was originally carried out through antigenic reactivity of the surface antigen.

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Currently, each virus is classified into distinct types (genotypes) and subtypes (Figure 43.1), but these designations have different meanings for individual viruses.

Hepatitis A virus

HAV is classified as the sole member of the *Hepatovirus* genus in the virus family *Picornaviridae*. There are seven currently defined genotypes of HAV, I–VII, which differ from each other at >15% of nucleotide positions across the genome [1]. Within these are series of subtypes, such as IA and IB, which show >7.5% divergence from each other. HAV genotypes I–III are widely distributed in human populations worldwide, while genotypes IV–VII have been recovered sporadically from NHPs (cynomolgus macaques and an African green monkey). For all HAV variants, most sequence variability occurs at silent sites (that do not alter the encoded amino acid) and as a result are antigenically similar (or monotypic).

Hepatitis B virus

HBV is the type member of the virus family *Hepadnaviridae*, a group that contains structurally similar although highly genetically divergent viruses infecting humans and NHPs, rodents, and a series of avian species. Variants of HBV infecting humans can be classified into a total of approximately 7 or 8 genotypes [2]. The genotypes, designated A to E and G, differ from each other by around 9% in nucleotide sequence (Figure 43.1); genotype F and the related genotype H, however, are outliers and more divergent in sequence (approximately 13%). Certain genotypes show specific geographical distributions; B and C in East and Southeast Asia, E in West and Sub-Saharan Africa, A predominantly in Central Africa, and genotypes H and F largely confined to aboriginal Indian populations in Central and South America. 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 and Southeast Asia [B, C], and South America [F]). A distinct set of HBV genotypes is known to infect a range of nonhuman ape species (see "Origins of hepatitis viruses," this chapter).

Hepatitis C virus

HCV is a member of the *Hepacivirus* genus in the family *Flaviviridae*. HCV shows quite extensive genetic diversity with seven major genetic groups (Figure 43.1). As an average over the complete genome, these differ in sequence at 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 nonstructural protein genes such as *NS5* are more conserved. Each of the seven major genetic groups of HCV contains a series of more closely related subtypes, that typically differ from each other by 20% in nucleotide sequences compared with >30% between genotypes (Figure 43.1) [3]. 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 infecting drug users (IDUs) over the past 30–70 years (Chapter 17) 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 25).

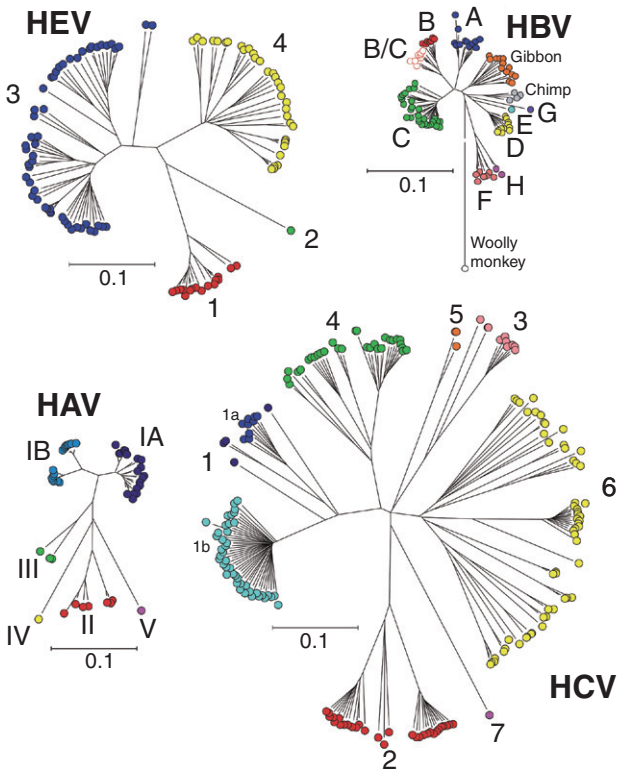

Figure 43.1 Phylogenetic trees of complete genome sequences of human hepatitis viruses drawn to the same scale to highlight their differing degree of naturally occurring sequence variability. Their current classification into genotypes is indicated by labels. Trees were constructed by neighbor joining of Jukes–Cantor corrected distances using the program MEGA5 [82]. (Color plate 43.1)

Hepatitis E virus

HEV is the only member of the recently designated family *Hepeviridae*. Four genotypes of hepatitis E virus differing from each other by a maximum of 26% have been described to date in a range of human populations

worldwide [4]. Despite this genetic diversity, variants are antigenically monotypic. Additional variants of HEV, not currently known to infect humans, have been described in rabbits and wild boar, but these are no more divergent from human isolates than the human genotypes are from each other [5]. In addition, HEV-related viruses have been detected in rats and chickens, but these are substantially more divergent (45% and 50%, respectively).

Virus evolution and selection pressures

Evolution during chronic infection

Although the diversification of hepatitis viruses into genotypes is likely to have occurred over long periods of time, sequence changes have also been observed in each virus over short time intervals. In the case of HCV and HBV, this includes a number of adaptive changes in response to immune selection and drug treatment.

Several studies have described the occurrence of measurable sequence drift of HCV over periods as short as 5–10 years, 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 1.44×10^{-3} nucleotide changes per site per year over the whole genome, or 4.1 and 7.1×10^{-4} changes per site per year in the *NS5* and *E1* regions [6, 7]. 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.

Of more interest are those substitutions that alter the sequence of virus proteins such as those occurring in the "hypervariable" regions (HVRs) of *E2* and *NS5A*. As discussed below, 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. Supporting this hypothesis is the observation that HCV sequence change was slower in individuals with defects in T or B cell immunity [8], interpreted as indicating reduced immune selection on cytotoxic T lymphocyte (CTL) or B cell epitopes. Indeed, recovery from HCV infection is associated with strong and sustained CTL responses around the time of primary infection [9, 10], a time when there is evidence for specific changes in CTL epitopes. Accelerated sequence change in the HCV coding sequence was observed in those who became chronic carriers [11, 12].

[For HBV, the situation is much less certain. The rate of fixation of sequence changes of HBV has been calculated by sequence comparison of HBV variants recovered from sequential samples from chronically infected individuals [13]. The average rate was 2.1×10^{-5} substitutions per site per year for those who were HBV e antigen (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 10). 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 driven by immune pressure.

Comparable is the observation that treatment of immunosuppressed transplant recipients with anti-HBs antibodies frequently generates HBV mutants with mutations in the immunodominant "a" determinant that escape from antibody binding [14, 15]. Less certain are the selection pressures that drive sequence change in the precore region and inhibit the expression of HBeAg [16]. 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 messenger RNA. The regularity with which precore mutants emerge during the course of chronic infection, particularly in HBV genotypes B, 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. 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 HBeAg, likely originating through some kind of immunological selection.

Evolution induced by drug treatment

Of particular clinical interest are the characteristics of virus evolution during drug treatment. The hope of such studies is that the identification of resistance mutations or of associations with virus diversity might allow the prediction of drug responses or the improvement of therapies.

For hepatitis C virus, most attention has been focused on the *NS5A* gene following an initial report in 1996 by Enomoto and coworkers that mutations in a defined region of this gene were associated with the response of patients infected with genotype 1b to interferon treatment. Further studies have generally confirmed this finding and extended it to patients infected with

genotype 2 virus, and to additional regions of the *NS5A* gene for non-Japanese cohorts (reviewed by [17, 18]).

Several recent studies have examined the accumulation of virus mutations in the small number of cases where an initial response to treatment is followed by recovery of HCV levels during treatment, so-called breakthrough cases [19, 20]. An increased number of mutations were observed in the breakthrough virus compared to virus from nonresponders. However, some of these sequence changes may be the result of putting the virus through a bottleneck rather than selection for substitutions *per se*.

More recent studies have been complicated by the use of combination therapies using pegylated interferon, ribavirin, amantadine, and NS3/4A serine protease inhibitors. For example, a Japanese study of individuals infected with type 1 virus showed a correlation between a mutation in the core gene and resistance to treatment with pegylated interferon, ribavirin, and telaprevir, a NS3/4 protease [21] Several studies have also examined the effect of preexisting variability in regions such as the HVR of the E2 envelope glycoprotein on treatment response.

A very different pattern of variation is observed in HBV-infected individuals treated with lamivudine and other antiviral agents targeting the HBV reverse transcriptase (RT). Drug resistance is observed in a high proportion of individuals, for example up to 80% of individuals after 5 years of treatment with lamivudine, and the resistant virus usually has a mutation within the YMDD RT domain, along with an upstream compensatory mutation (reviewed in [22]). Resistance is slower to develop with other drugs such as entecavir and tenofovir, but again a limited range of RT mutations are associated with this resistance.

Hypervariable regions

The genomes of both HCV and HEV contain regions that are hypervariable (i.e., HVRs) in comparison to other regions of the genome. For neither virus is there a satisfactory explanation of why this should be the case.

For HCV, most attention has focused on a potential role of the HVR in immune escape during chronic infection. Strong evidence for this comes from a study in which the neutralization ability of patient sera was tested *in vitro* against contemporaneous and historical viruses expressed as retroviral pseudoparticles [23]. The timing and specificity of neutralizing responses against the HVR were consistent with their being responsible for the emergence of HVR mutants. The presence of neutralizing antibodies also correlated with virus clearance. These conclusions are supported by experimental infection of chimpanzees, although with the usual caveats about experimental versus natural infection. In contrast, very clear information about HCV evolution has been obtained from a study of a cohort of women infected from a common source of anti-D immunoglobulin in 1977–1978 [24]. Analysis of partial virus genomes (core to NS3) present in the infectious source and in samples collected from chronically infected recipients 18–22 years after infection revealed an excess of nonsynonymous changes in the HVR region and, to a lesser extent, in the *NS2* gene [43]. Of particular note, where the HVR sequence of the inoculum differed from the consensus for subtype 1b, the recipients were more likely to revert to the consensus at that position. In addition, when the recipient HLA type was taken into account, several amino acid sequence changes outside the envelope genes were strongly correlated with HLA type. In both of these cases, there is evidence for immune-directed sequence change in the HCV genome, but it is interesting to note that these changes are not progressive; many of the amino acid substitutions involve switching between one or more alternative residues at a particular site. This constraint on virus evolution is also evident from the strong bias toward synonymous substitutions observed for the HCV genome, as well as for many other viruses.

A very different situation is observed for HEV that also contains a HVR but is present within the ORF1 polyprotein rather than the capsid protein. Variation of this region therefore seems unlikely to be driven by immune selection. In addition, HEV is an acute infection except in immunocompromised individuals, and infection can be prevented by vaccination with the capsid protein, encoded by ORF2. Dissection of the HVR *in vitro* suggests that the HVR can be partially deleted without impairing virus replication [25, 26]. This is consistent with the length variation of the HVR observed between and within virus genotypes. Extreme examples of this length variation have recently been described in virus from individuals chronically infected with HEV in which the HVR contains an in-frame insertion of human ribosome protein S17 or S19 [27, 28]. However, the absence of similar HVR insertions among virus sequences derived from acutely infected individuals suggests that different evolutionary processes may occur during chronic infection. In addition, the amino acid composition of the HEV HVR in virus from acutely infected individuals is strongly biased toward proline and serine, biases that cannot be explained merely by a local bias toward cytosine [29]. These biases are not observed for the human sequence insertions, again suggesting that viruses circulating during chronic HEV infection are atypical. The consensus at present is that the HVR acts as a linker or spacer between flanking regions of the ORF1 polyprotein. Whether this limited structural requirement is sufficient to explain its extreme plasticity between isolates remains to be proven.

Virus recombination

Recombination between different genotypes of HBV frequently leads to the appearance of viable hybrid viruses with highly variable and frequently complex combinations of genetic elements derived from the parental viruses [30]. Although many recombinant viruses have been observed only sporadically, others may be widely distributed, such as a C/D recombinant virus in Tibet [31], A/C in Vietnam [32], and B/C recombinant frequently found in East Asia [33]. Infection with the latter is more frequently associated with HBeAg expression than the nonrecombinant form of genotype B, and it may therefore differ in long-term pathogenicity, transmissibility, and treatment response from nonrecombinant forms [34].

Recombination has also been described for HCV, with intergenotypic, intragenotypic, and intrasubtype recombinants described (reviewed in [35]). However, the frequency of recombinants appears to be low (1%) [36], possibly because the frequency of mixed infection is also low (1%), even among high-risk groups such as intravenous drug users [37]. Only a highly restricted set of recombinants may be biologically viable, a suggestion supported by the observation that almost all intergenotype chimeras described to date have a core–E1–E2 region derived from genotype 2, a breakpoint between *p7* and *NS3* and nonstructural gene blocks derived from other genotypes (reviewed in [38]). Indeed, why genotype 2 is specifically favored to provide structural genes in such recombinants remains entirely unclear at this stage.

There are several reports of recombinant HEV genomes, the most striking of which represents an intertypic recombinant [39]. However, three of the recombinant viruses identified in the first two studies were not confirmed in the most recent and most comprehensive study. We also note that the proposed recombination events all relate to the interpolation of short genome segments (~500 nt), and [40] point out that in two instances, the interpolated segment was very similar to a virus previously sequenced in the same laboratory. A suspicion must remain that some or all HEV recombinants represent artifacts arising from laboratory contamination of polymerase chain reaction (PCR) products.

No examples of intertype recombination in HAV have been described, although recombination within HAV type I has been documented in the capsid region [41] and more extensively within types I and III [42].

Virus evolution – fast and slow

The cumulative effect of some of the evolutionary processes described in this chapter may be to limit virus sequence evolution to a small number of sites. Although substitutions might be observed over short periods of time, these would rapidly become saturated over longer periods of divergence. An illustration of this comes from the sequence analysis of HCV in a common-source infected cohort, in which immune selection could be shown to concentrate nonsynonymous substitutions to particular regions or residues. In the case of the HVR, the selection was normative, with residues differing from the genotype consensus in the infectious source reverting back to the consensus in virus from many of the recipients [43]. Much of the phylogenetic signal between virus isolates derives from substitutions at synonymous sites, and once these are saturated, long-term rates of evolution would become much lower, requiring the coincidence of multiple substitutions to produce a viable virus. These considerations make it very difficult to estimate how long HCV has been present in human populations.

For example, attempts have been made to reconstruct the time and place for the origin of HCV from the current geographical distribution of its genotypes. In parts of Africa and Southeast Asia, there are close associations between HCV genotypes and specific geographical regions (Figure 43.2). For example, HCV infections in Western Africa are almost invariably by genotype 2,

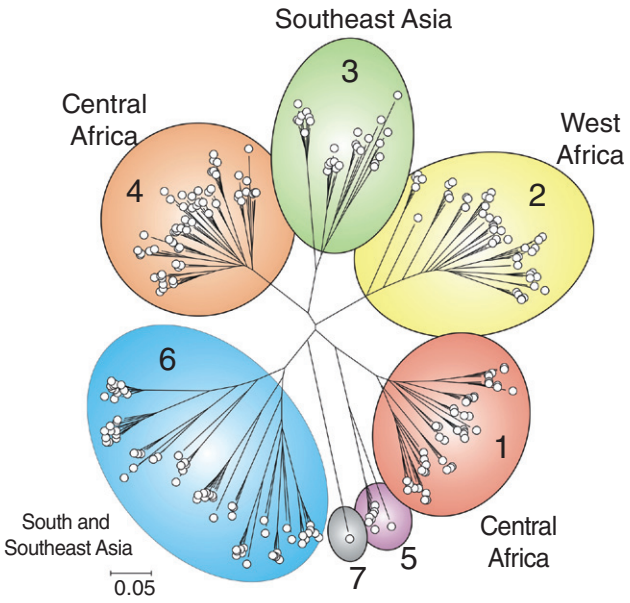

Figure 43.2 Phylogenetic tree of *NS5B* sequences of HCV (positions 8276 to 8615 as numbered as in the H77 reference sequence). High-diversity areas in Sub-Saharan Africa and Southeast Asia contain a large number of variants additional to subtypes such as 1a, 1b, and 3a (found in Western countries), displaying an endemic pattern of diversity. The tree was constructed as described in Figure 43.1. (Color plate 43.2)

while those in Central Africa, such as the Democratic Republic of Congo (DRC) and Gabon, are by genotypes 1 and 4. 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 different and previously undescribed subtypes [44]. 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 [45]. These field observations reflect both the huge genetic diversity of HCV genotypes 1, 2, and 4, and also their 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 East Asia.

Since 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 the use of published rates of HCV sequence change over time [6, 7]. However, extrapolation of contemporary rates of evolution to 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 [7]. This seems recent for such a widely distributed virus infecting often relatively isolated human populations in equatorial Africa and Southeast Asia.

We have argued that hidden constraints of HCV sequence change, such as the extensive RNA secondary structure in coding regions of the genome [46], 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 [46, 47]. 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 time scale 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.

For HBV and HEV, an additional constraint is imposed by the existence of overlapping reading frames in one or more genes. Given that the remaining single-coding and noncoding regions of the genome are packed with RNA structural elements and transcriptional and translational regulatory sequences, it is doubtful whether there are any sites where sequence changes are selectively neutral. Combined with the evidence for intense immune system–mediated selection for sequence change, a large part of the sequence diversification over time observed in individuals chronically infected with HBV or following transmission events must represent virus immune evasion. If such sequence changes incur fitness costs, they would be expected to revert on transmission of infection to individuals with different HLA types. Thus, the apparently relatively rapid sequence change observed in chronic infection, rather than being accumulative, may simply involve flip-flopping certain key amino acid residues that are most immunogenic. The consequence of this is that viruses might be capable of rapid change during the course of infection or following transmission between individuals, yet remain phylogenetically close over long periods of time. There may therefore be no net sequence evolution over longer periods, each genotype gravitating toward a particular phenotypically optimized master sequence constrained in a relatively rugged fitness landscape. Such convergent changes may conceal the evolutionary history and divergence time of primate HBV isolates from nucleotide sequence comparisons alone.

Origins of hepatitis viruses

As alluded to previously, nucleotide sequences of hepatitis viruses are necessarily biased toward recent isolates. Nevertheless, it is possible to infer some aspects of virus evolution from current patterns of virus diversity and distribution around the world and, for some hepatitis viruses, their relationships with homologous viruses infecting primates and other mammals. A significant recent advance has been the discovery of virus "fossils" within vertebrate genomes, a finding that has greatly expanded our perspective on the time scale of virus evolution.

Global genotype epidemiology

A great deal of effort has been expended over the last few decades in documenting the extent of virus variation and the distribution of these variants around the

world. Although primarily driven by the necessity to ensure that screening methodologies apply to the full range of virus variants, these studies have also provided detailed information about their recent epidemiology.

For example, the global distribution of HCV variants described in preceding sections is informative about recent epidemiological events, suggesting a model in which HCV has been endemic in Sub-Saharan Africa and Southeast Asia for a considerable time, with the occurrence of HCV infection with genotypes 1a, 1b, and 3a in Western and other nontropical countries representing a relatively recent emergence of infection in new risk groups for infection. In the twentieth century, parenteral exposure to bloodborne 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.

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 Southeast Asia where the greatest genetic diversity is observed. Transmission either by sexual contact or from mother to child is uncommon (see Chapter 15), and there is little evidence historically for the type of widespread parenteral exposure that fueled the HCV epidemic in Western countries. However, recent field investigations in southern Burkina Faso (Central or Western Africa), where HCV genotypes 1 and 2 are prevalent and highly diverse in sequence, have shown associations between HCV infection and previous sexually transmitted diseases (STDs), circumcision, and scarification practices [48].

For HBV, geographical regions where infections are prevalent and endemic have characteristic genotypes: genotypes B and C predominate in East and Southeast Asia, genotype E in Western and Sub-Saharan Africa, genotype A in central Africa, and genotypes H and F in aboriginal Indian populations in Central and South America (reviewed in [49]). In contrast, genotypes A and D are globally distributed, while there is still little information about genotype G. Similar geographical correlations are seen for variants within some HBV genotypes. For example, while both genotype C and D variants are widely distributed in Southeast 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 [50, 51]. Variants of genotype A (but not E) are similarly segregated between Central and West Africa, while Central American variants designated as genotype H [52] could perhaps be more consistently classified as geographical variants of genotype F found in South America.

The previous evolutionary history of HBV in humans therefore remains difficult to reconstruct because we lack clear information of the long-term rate of sequence change of HBV over which genotypes or subtypes have diverged. Extrapolating the short-term rates of sequence change to longer periods leads to relatively recent estimates for the time of divergence of human HBV genotypes. For example, the estimate of about 8×10^{-4} substitutions per site per year [13], measured in HBeAg carriers who are most likely transmitters of infection, predicts a most recent common ancestor of human HBV genotypes A–E and G of around 200–400 years ago [53]. This does not fit in easily with their geographical distributions and potential routes of HBV transmission between continents in previous centuries. The existence of more divergent F and H genotypes in South America and species-associated genotypes infecting wild populations of apes in Sub-Saharan Africa and Southeast Asia that are interspersed within the human HBV tree is similarly difficult to reconcile with a model of such recent global spread. This issue is discussed further below in relation to zoonotic origins (see the "Zoonoses" section).

The current distribution of HEV is also rather perplexing. Genotype 3 viruses are globally distributed, with human and porcine isolates intermingling on phylogenetic trees. Type 4 viruses show the same species distribution, but are confined to Asia. Type 1 viruses are the cause of HEV epidemics in the Indian subcontinent and have only been isolated from humans there, or in other parts of Asia or Africa. Apart from type 2, for which only a few isolates have been described (from Mexico and Nigeria), HEV types appear to have a primarily Asian origin, with type 3 viruses perhaps having spread globally through export of infected pigs or pork.

Zoonoses of human hepatitis viruses

While there is a natural tendency to view human virus infections as privileged, the reality is that they represent a sample from a much larger catalog of mammalian viruses. Understanding the relationship between human and animal isolates can reveal the evolutionary history of the virus and also the epidemiological context, for example whether human infections can be acquired directly from animal sources or whether these represent distinct populations.

HBV infection is highly prevalent in chimpanzees and gorillas in Sub-Saharan Africa, and in gibbons and orangutans in Southeast Asia, with each species showing frequencies of HBsAg carriage ranging from 10% to 30% [54, 55]. 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 orangutan-specific genotypes. This may be an oversimplification, as further analysis of HBV variants in Southeast Asian primates showed that phylogenetic grouping correlated better with geographical range than host species [55, 56]. Variants from orangutans more closely resembled HBV variants from gibbon species at the southern end of their geographical range, thus overlapping their natural habitat rather than forming a separate species-specific phylogenetic group. Similarly, HBV variants infecting gorillas appear most similar to HBV variants recovered from Central African chimpanzees [56], rather than forming a separate species-associated lineage. A more divergent variant has been detected in the woolly monkey, a species confined to South America. This distribution is partly consistent with a long period of co-evolution between virus and hosts and a consequent extremely slow rate of long-term sequence drift, but the interspersed nature of human and other ape-associated genotypes necessitates several cross-species transmission events in multiple geographical regions. Nevertheless, greater diversity is observed among human HBV genotypes than within or between isolates from other primates, consistent with the alternative possibility that HBV was transmitted to NHPs from humans. However, despite large surveys, there is no evidence for cross-infection of human and primate HBV variants in Central or West Africa or with gibbon and orangutan variants in Asia.

In contrast to HBV, there is currently no evidence that HCV or HCV-like variants infect Old World ape or monkey species [54]. Strangely, those areas where HCV is endemic and highly diverse are precisely those where HBV is endemic, and also represent regions where human and ape population ranges overlap. Analysis of the diversity of subtype 6 in East Asia has led to the suggestion that the virus may have been present there for more than 1000 years [57]. There are tempting analogies between the introduction and spread of HIV-1 and -2 infections in human through cross-species transmission of simian viruses from chimpanzees and mangabeys [58, 59] and a comparable primate origin of HCV in humans. This hypothesis is attractive because it fits directly with the existence of much more diverse and older populations of HCV in regions such as Sub-Saharan Africa and Southeast Asia where human and NHP habitats overlap. It also fits with the existence of a virus distantly related to HCV, termed the GB virus B, in a laboratory-housed tamarin, a New World primate [60, 61] that perhaps represents, in evolutionarily terms, the NHP homolog of HCV. However, both published [54, 62] and unpublished surveys for infections with HCV or HCV-like viruses in chimpanzees, other apes, and a range of Old World monkey species [63] have failed to find evidence for infection with either HCV or more distant related homologs of HCV in any primate species, findings that contrast markedly with the high rates of infection of simian immunodeficiency viruses (SIVs) in most Old World monkey species and in African apes.

Despite the ongoing focus on primates for viruses related to HCV origins, it has recently been discovered that a virus much more closely related to HCV than GBV-B infects domestic dogs [64] and, more commonly, horses where current and past infection frequencies approach 30% of domestic horse populations [65]). The host range and possible existence of further nonprimate hepaciviruses are currently active areas of research.

The situation is more straightforward for HAV where human infections comprise viruses belonging to genotype I and, in rarer cases, genotypes II and III. Genotype III virus has also been isolated from New World monkeys, while three additional genotypes have been identified in Old World monkeys. This distribution is consistent with HAV having arisen in primates, with only a subset of strains being capable of infecting humans.

HEV presents further complexities with four distinct genotypes infecting humans. Two of these genotypes (3 and 4) have also been detected in pigs, wild boar, and deer, and the sequences of these viruses are interspersed with those of human viruses on phylogenetic trees. A detailed survey of human and animal isolates from China over the last 25 years reveals a complex situation with geographical and temporal variation in the distribution of virus types and subtypes [66]. Some virus subtypes were shared between human and porcine isolates. This suggests that there is some exchange of virus between humans and pigs. A more divergent genotype 3 virus has been detected in farmed rabbits in China, these being distinct from isolates from humans or pigs, while an additional genotype has been described in wild boar [67]. An even more divergent HEV has recently been described in rats [68], but there are no reports of this variant being found in humans or pigs. Instances of HEV detection in shellfish presumably reflect environmental contamination rather than infection. These zoonotic relationships are consistent with human HEV variants being derived from wild mammals, still to be identified in the case of genotypes 1 and 2.

Ancient viruses

An unexpected consequence of the ease with which a eukaryotic genome can now be sequenced is the finding of virus "fossils," sequences related to those of modern viruses that have become integrated within their host genome. Such viral fossils often contain multiple mutations that would be expected either to impair or to prevent expression of a functional virus protein, and the presumption is that these sequences have been

evolving within their hosts in the absence of selection. The date of such integration events can be inferred from the phylogenetic relationship between the virus fossil sequences and those of their hosts. A striking recent finding is that sequences related to hepatitis B virus can be detected in the genome of the zebra finch [69]. Multiple copies of these sequences are found at different chromosomal locations in the genome, and some of these are also found in homologous chromosomal positions in other finch species. This implies that the integration of these virus sequences occurred before radiation of this group, estimated at more than 20 million years ago. A distinct group of hepadnaviruses are still present in many avian species, although there is much less diversity among isolates from different species than is observed among the multiple versions represented among the virus fossils.

Unfortunately, no such "fossils" have been described for the other hepatitis viruses, but this is not simply because the HBV genome passes through a DNA stage; "fossils" of both positive- and negative-strand RNA viruses have also been described [70]. The full paleontological history of viruses may be revealed only when a wider range of eukaryote genomes have been sequenced.

The existence of virus fossils raises the question of how nucleotide sequences can be conserved among actively replicating and evolving viruses over such long periods of time. Viruses are usually considered to have high error rates and short replication periods, a combination that accounts for their rapid evolution and extensive strain variation. How can such characteristics be combined with the retention of basic features over geological time?

One interpretation is that the process of virus evolution over short time scales (weeks, months, and years) cannot be simply extrapolated to longer periods of time. Viruses are obviously constrained in their evolution by the need to produce functional structural and nonstructural proteins, and also to conserve RNA sequences involved in the initiation or control of transcription and genome packaging into virions. There is also evidence that the genome structure of RNA viruses is constrained throughout their length [47]. These constraints appear to be particularly strong for the genomes of HCV and nonprimate hepaciviruses.

Practical implications of virus variability

Virus diagnosis

An important consequence of virus sequence variation is that immunological and nucleic acid amplification assays need to be optimized to ensure that all virus strains of hepatitis viruses are detected with equal efficiency. That this may not be the case for HEV was recently revealed in a survey of in-house PCR assays for HEV in 20 laboratories around the world. A 100–1000-fold difference in sensitivity between different assays, probably reflecting the different extraction and amplification methods and the different primers used, was observed [71]. The same variation in sensitivity was also observed within a laboratory when detecting viruses of different genotypes (type 3 vs. type 4 in the cited study) or even between subtypes of the same genotype (subtype 3a vs. subtypes 3b or 3f).

The use of an insufficiently generalized assay might lead to the conclusion that, for example, all HEV infections in a particular region were of type 3, whereas it might be that only type 3 viruses could be detected by the assay. In the case of HEV, most cases in the developed world are type 3, except for Northeast Asia where type 4 is also present. Type 1 is largely confined to the Indian subcontinent and North Africa, while type 2 has been isolated on only a few occasions in Mexico and West Africa [4]. However, as the frequency and range of travel increase, geographical restrictions in virus diversity can be expected to break down, and so diagnostic tests must be designed so that they can detect the full range of known virus variants. For example, both genotype 1 and genotype 3 HEV isolates have been described in the United Kingdom.

Even with such a well-characterized virus as HCV and the effort that has been put into characterizing virus variation and designing general assays, similar problems persist. Although the seven major genetic groups differ in sequence by 30–35% of nucleotide sites over the genome, the much greater sequence conservation in the 5′ untranslated regions (UTRs) has been exploited in the development of PCR-based screening assays. Assay development continues with attempts to improve detection reliability by targeting the conserved 3′ untranslated region [72], and by developing alternatives to 5′ *NCR*–core typing assays that are based on the analysis of variation in the *NS5B* region [73].

Such issues have been less problematic for HBV, although some HBsAg immunoassays may fail to detect certain HBsAg variants [74]. Such variants are relatively uncommon in the developed world, but can comprise more than one-quarter of variants in chronically infected individuals and regions where HBV is endemic.

Vaccine design

Vaccines currently exist for HAV and HBV, and both are considered to be effective against the range of subtypes currently known to infect humans (see Chapters 4 and 6). There is less experience with vaccination against HEV; two vaccines, both containing recombinant

proteins derived from type 1 virus, have been tested in regions where type 1 [75] and type 4 [76] viruses predominate. Vaccine efficacy was 95% or greater in the heterologous challenge, confirming previous suggestions that human HEV genotypes comprise a single serotype. Although attempts continue to produce a vaccine against HCV infection, this may prove to be a greater challenge than it has been for the other hepatitis viruses. Factors contributing to this are greater divergence between different HCV genotypes, the persistence of virus infection in the presence of humoral and cellular immune responses, and the presence of HVRs in immunogenic regions of the virus envelope proteins that may help the virus evade immune attack.

Investigation of virus transmission

While virus variation creates problems for diagnostic assays and broadly effective vaccines, that same variation is fundamental in the investigation of virus transmission. Even within a virus subtype, there is often sufficient divergence between co-circulating variants to allow robust identification of specific strains and their routes of transmission.

This approach has proven very informative for HCV in tracking infections following iatrogenic exposure. Some instances of nosocomial transmission have been identified retrospectively because of an unexpected frequency of a particular subtype, for example of subtypes 1a and 3a among Rh-negative women in Ireland and of type 2a among Israeli surgical patients. Subsequent phylogenetic analysis of virus from these cohorts has shown a close relationship between virus sequences, consistent with their having been infected from a common source [24, 43, 77]. A similar approach has been used to reach the conclusion that patient–patient transmission of HEV occurred in a hematology ward [78], to identify uncooked meat as a source of infection [79], and to characterize the relationship between viruses in a localized outbreak [80].

However, such studies are less informative for HBV since the extent of within-subtype variation is more restricted. For example, a recent study of HBV in men having sex with men in Amsterdam identified the predominant strain as genotype A, and 15 out of 40 viruses had identical HBV-S sequences [81]. However, due to the limited variability within this gene, the epidemiological relationship of this group to other isolates from this and other high-risk groups was unclear. Nevertheless, phylogenetic analysis has been used as forensic evidence in criminal cases involving HBV.

Summary

The evolution of hepatitis viruses comprises highly dynamic processes, occurring through multiple processes of adaptive selection that drive sequence change (such as those resulting from the host immune response and from antiviral treatment), sequence drift, and their longer term differentiation into genetically distinct types. However, despite their potential to evolve rapidly, the longer term evolution of hepatitis 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 any of the hepatitis viruses, despite the likely extremely long periods over which this viral diversity has developed. Hepatitis viruses thus appear to have filled very specific, different ecological niches in human populations successfully. 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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Page numbers in *italic* refer to figures.
Page numbers in **bold** refer to tables.
Sorting is in letter-by-letter order so that, for example,
"B cells" comes before "BI201335".
Terms starting with numerals are sorted according to pronunciation of initial numeral so that "5BSL3.2" comes after "fitness cost".

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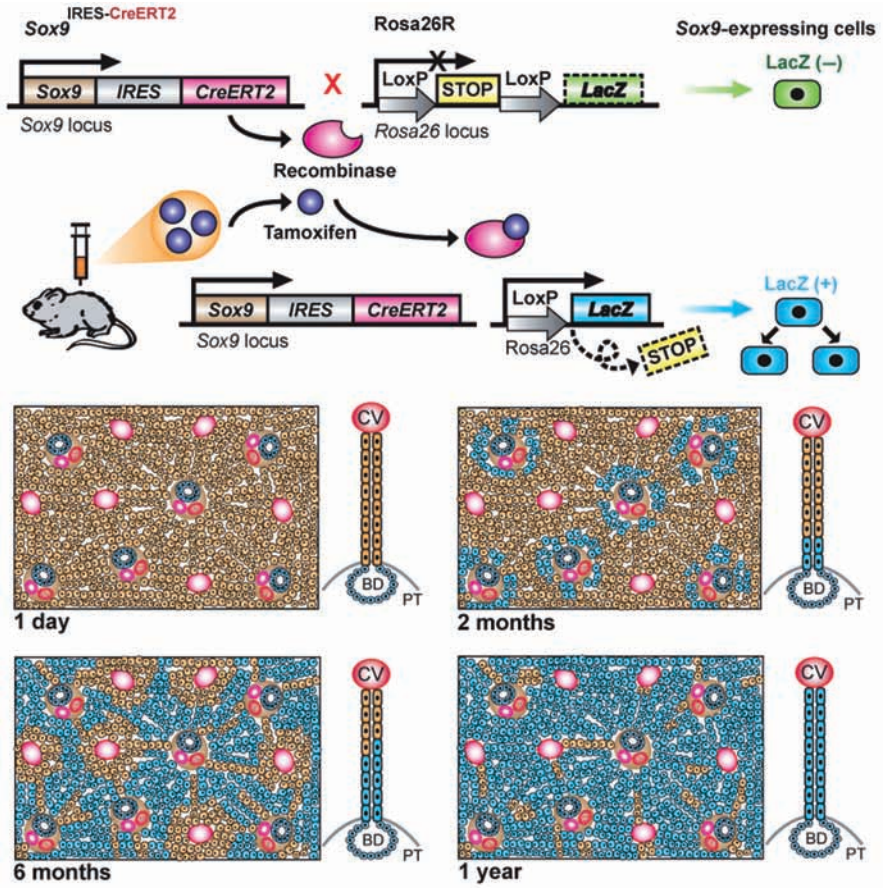
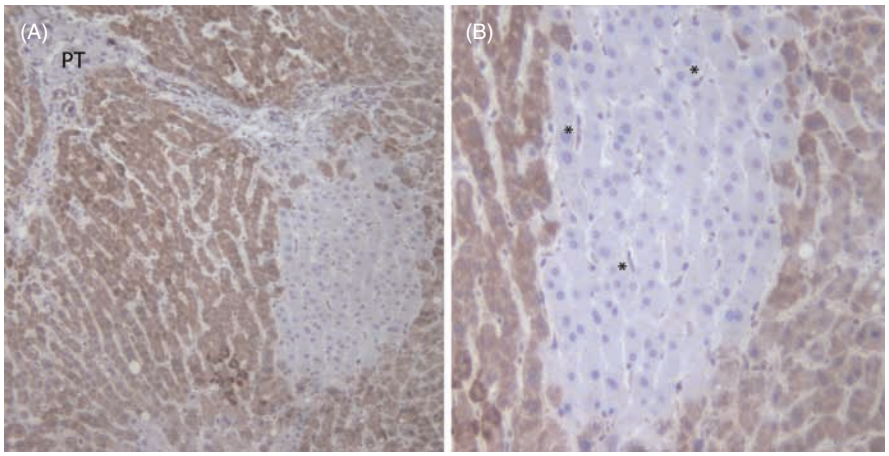


Color plate 1.1 Top: Strategy of the genetic lineage-tracing study employed by Furuyama *et al.* [3] using tamoxifen-induced Cre-mediated cell tracking using Sox9IRES-CreERT2; Rosa26R mice. Bottom: Schematic illustrating the spread of X-gal staining after 8-week-old mice were injected with tamoxifen. After one day, only intrahepatic bile duct cells are labeled, but later X-gal-positive hepatocytes gradually spread from the portal tracts to the central veins, thus supporting the streaming liver hypothesis. See Alison and Lin [4] for further details. (Source: Alison and lin. Hepatology 2011, 53:1393–1396 [4]).



Color plate 1.2 (A) A single cytochrome c oxidase (CCO)–deficient patch, appearing to emanate from the portal tract. (B) High-power magnification illustrates that within the patch there are CCO-positive sinusoid-lining cells (asterisks) indicative of different cells of origin from hepatocytes. See Fellous *et al.* [7] for further details. (Source: Fellous TG *et al.* Hepatology 2009, 49:1655–1663 [7]).

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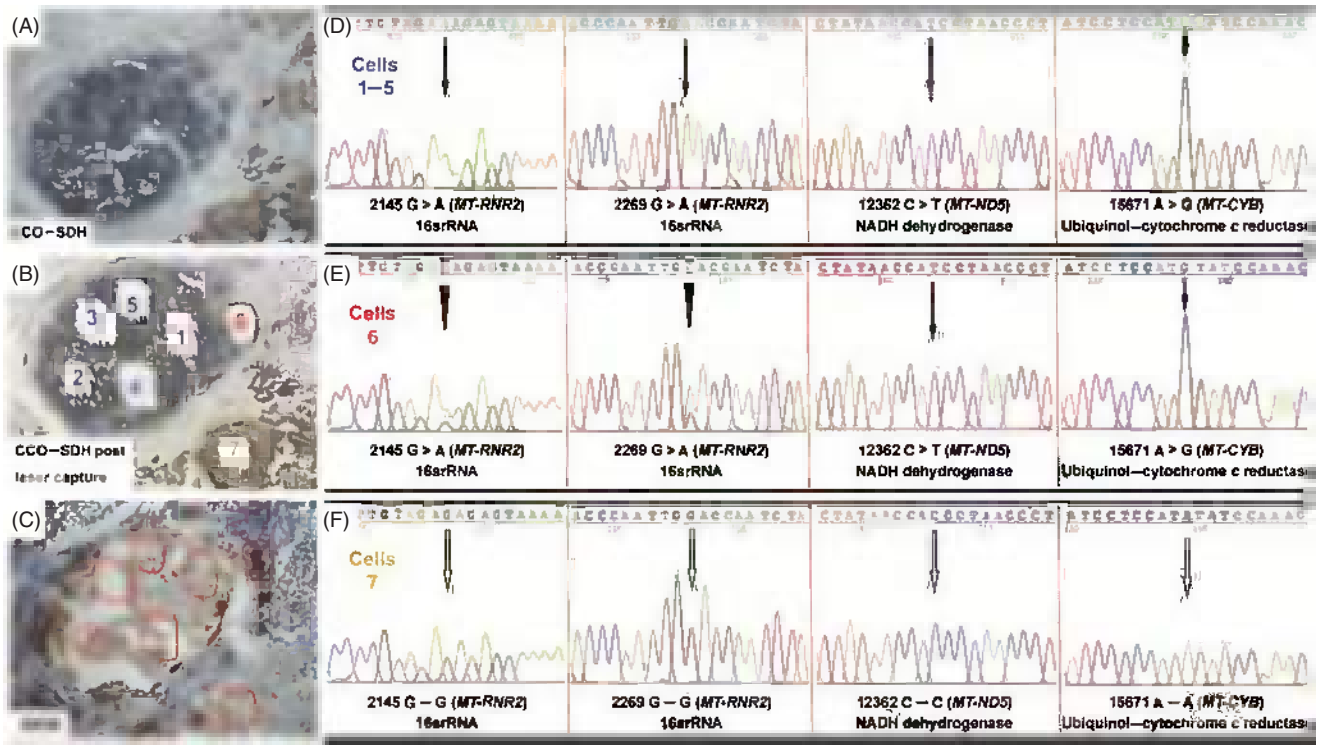
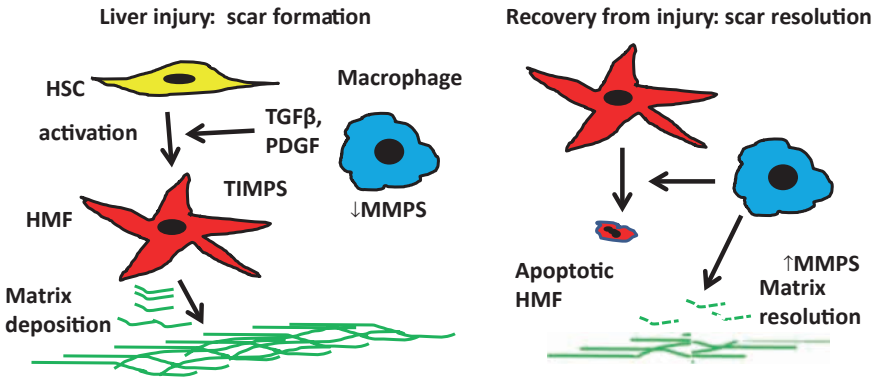
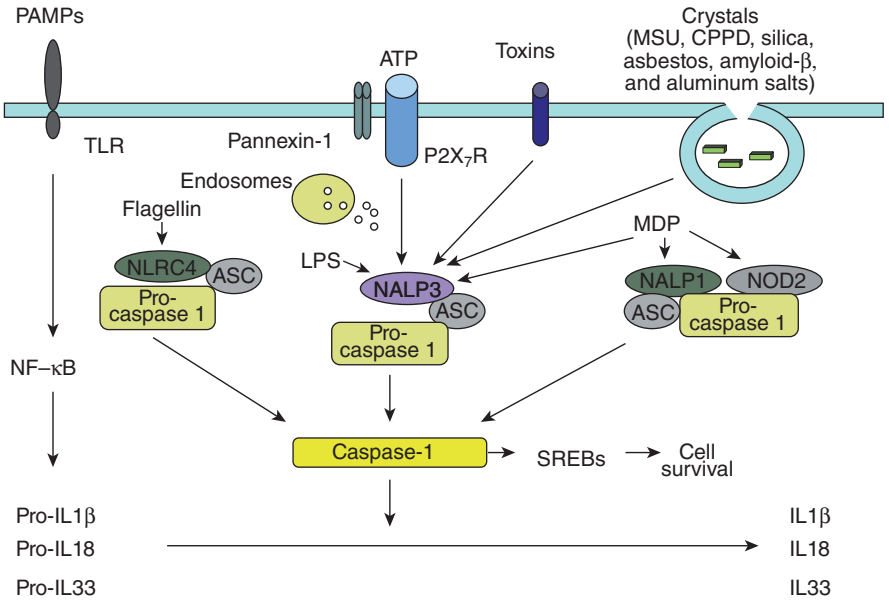


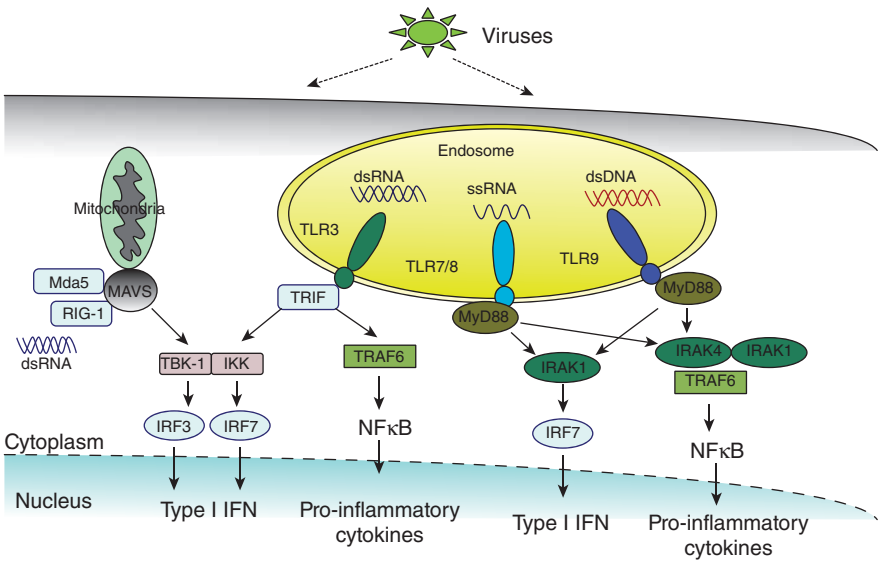
Color plate 1.3 Mitochondrial DNA genotyping indicates that regenerative nodules can be derived from CK19-positive HPCs. (A) An entirely CCO-deficient nodule (stained blue for succinate dehydrogenase activity). (B) Five groups of cells (1–5) from the same CCO-deficient nodule; cells (6) from the adjacent CCO-deficient ductular reaction, confirmed by CK19 IHC on the next serial section (C, brown staining), and cells (7) from the CCO-positive nodule were laser capture-microdissected, and the entire mitochondrial genome was sequenced. (D) Cell areas 1–5 all contained four different transition mutations: 2145G>A, 2269G>A, 12362C>T, and 15671A>G (black arrows). (E) Cell area 6 from the abutting CCO-deficient ductular reaction had exactly the same mutations. Heteroplasmy was detected at locations 2145 and 2269 (arrowheads), while the mutations at locations 12362 and 15671 were homoplasmic (black arrows). (F) Cell area 7 from the CCO-positive nodule had no mutation (white arrows). See Lin *et al.* [52] for further details. (Source: Lin WR *et al.* Hepatology 2010, 51:1017–1026 [52]).



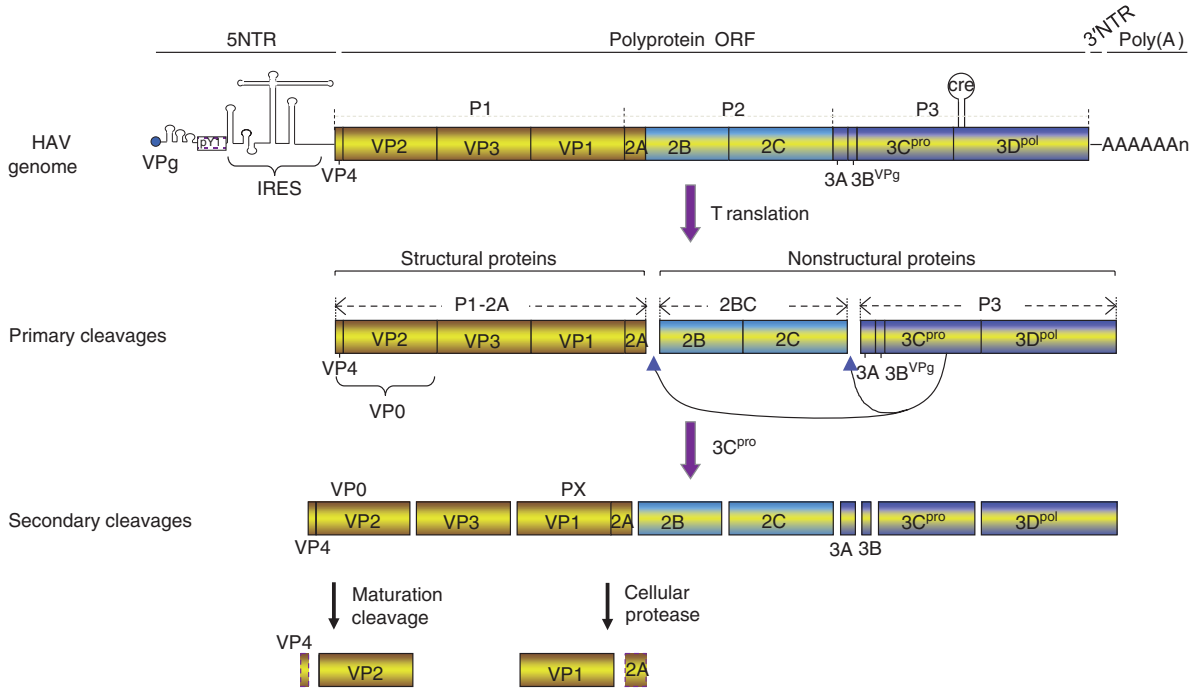
Color plate 1.4 During liver injury the hepatic macrophages help, along with other factors, to stimulate quiescent fat-storing hepatic stellate cells (HSCs) into activated hepatic myofibroblasts (HMFs). The activated HMFs deposit collagen scar and secrete tissue inhibitors of metalloproteinases (TIMPS), which inhibit the degraders of scar matrix, the matrix metalloproteinases (MMPs). If the cause of liver injury is removed (e.g., viral eradication), then a recovery phase commences and, to a variable degree, the quantity of liver fibrosis lessens. In this phase, HMFs reduce their TIMP secretion, and the hepatic macrophages secrete MMPs that aid the degradation of scar and promote the apoptosis of HMFs.



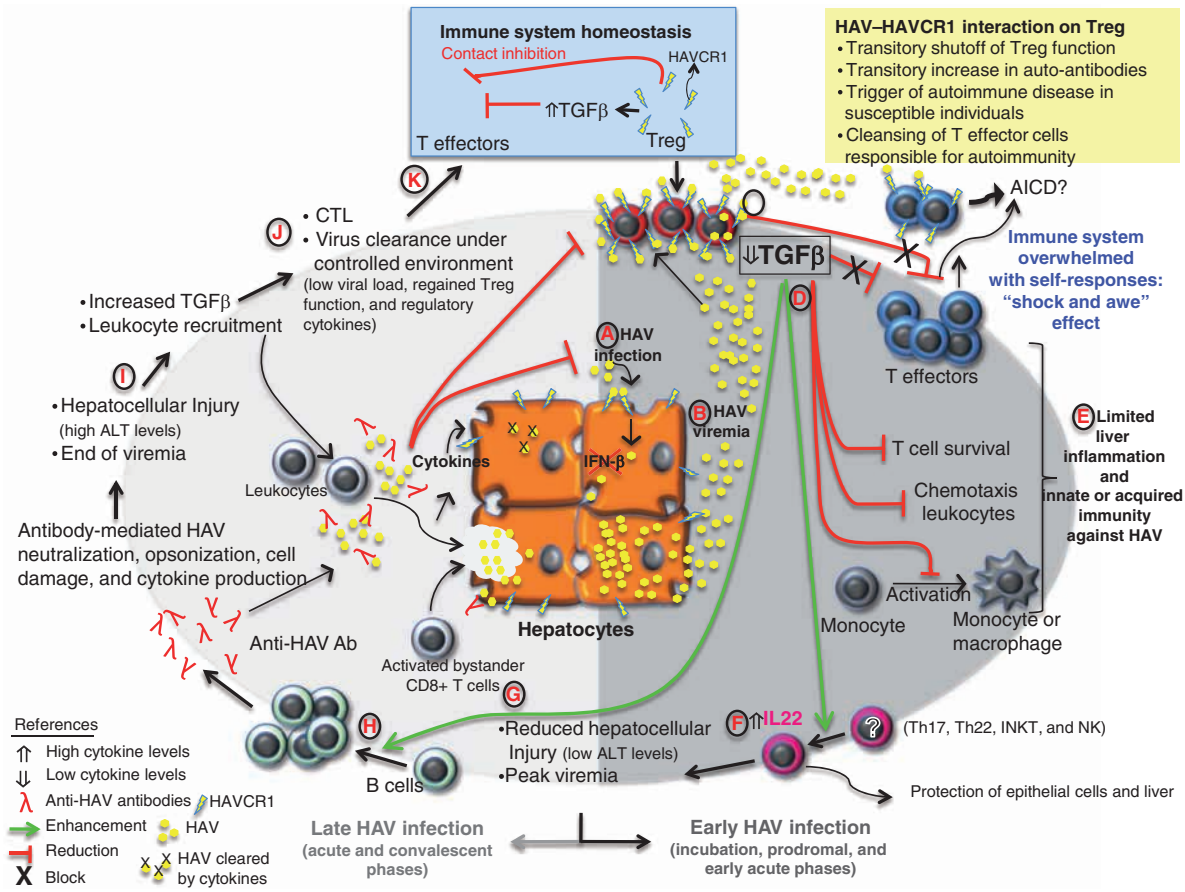
Color plate 2.1 Activation of the inflammasome by PAMPs and DAMPs. PAMPs: pathogen-associated molecular patterns; ATP: adenosine triphosphate; MSU: monosodium urate; CPPD: calcium pyrophosphate dihydrate; TLR: Toll-like receptor; LPS: lipopolysaccharide; MDP: muramyl dipeptide; ASC: apoptosis-associated speck-like CARD domain-containing protein; NALP: NACHT-LRR-PYD-containing protein; NOD: nucleotide-binding oligomerization domain-containing protein; SREB: sterol regulatory element-binding protein.



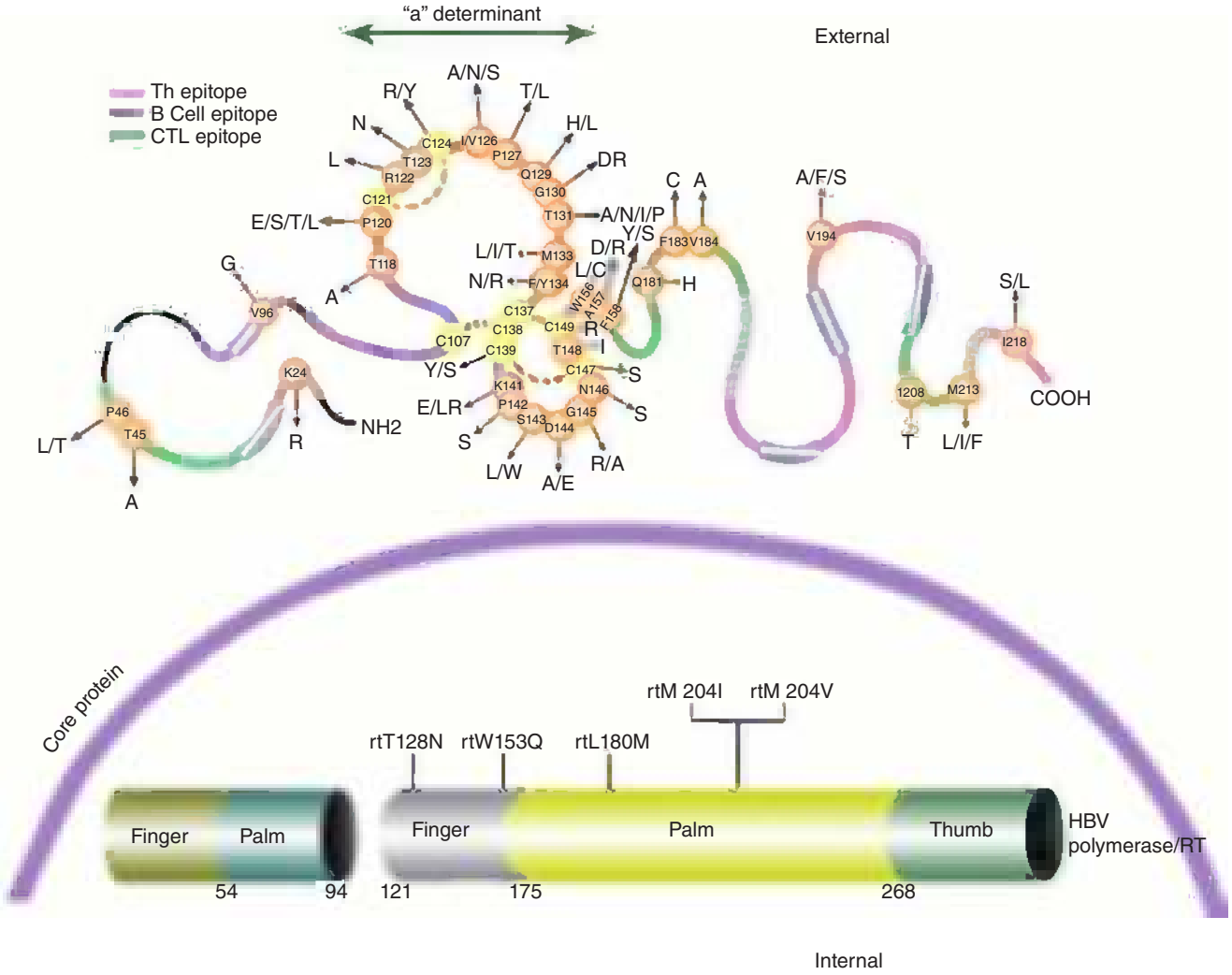
Color plate 2.2 Intracellular sensors of viral infection. dsRNA: double-stranded RNA: ssRNA: single-stranded RNA; dsDNA: double-stranded DNA; MAVS: mitochondrial antiviral-signaling protein; TRIF: TIR domain-containing adapter-inducing interferon-β; MDA5: melanoma differentiation-associated protein 5; IRAK: interleukin-1 receptor-associated kinase; TRAF: TNF receptor-associated factor; IRF: interferon regulatory transcription factor; TBK: TANK binding kinase; IKK: IkappaB kinase.



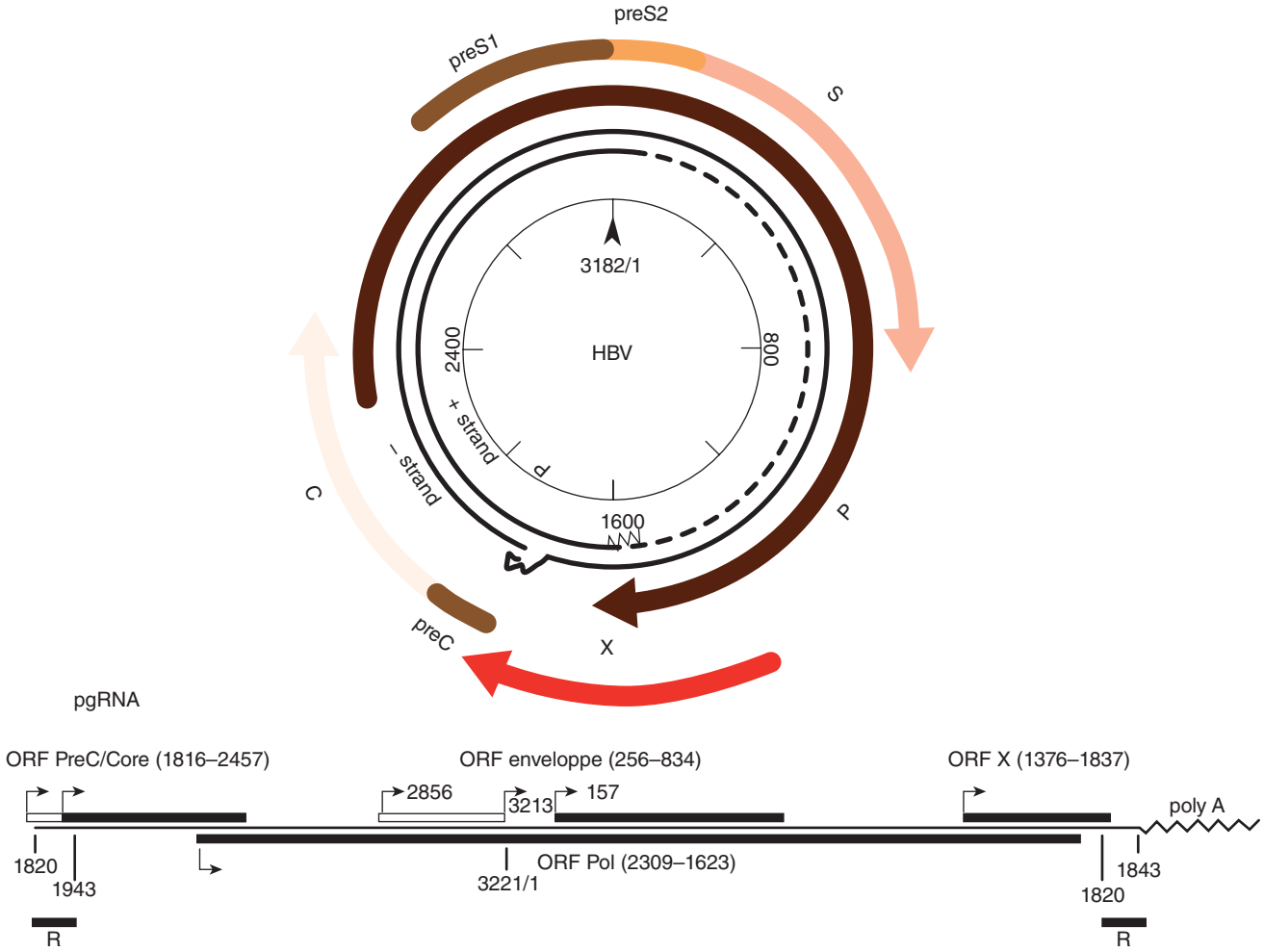
Color plate 3.1 Genomic organization and cleavage of the HAV polyprotein. The HAV genome contains a 5′ nontranslated region (NTR) of approximately 750 nucleotides (nts) containing a polypyrimidine tract (pY1) of approximately 40 nts that separates the internal ribosomal entry site (IRES) from the rest of the 5′ NTR. A small viral VPg protein ($3B^{VPg}$) is covalently linked to the 5′ end. The HAV genome contains a long open reading frame (ORF) that codes for the HAV polyprotein of approximately 230 kDa. Within the $3D^{pol}$ gene, there is a *cis*-acting replication element (*cre*). A short 3′ NTR is followed by a poly(A) tract. The HAV genome has three regions: P1 encodes the capsid proteins, and P2 and P3 encode the functional proteins. The viral $3C^{pro}$ protease processes all cleavages, except for the cleavage of PX into VP1 and 2A and the cleavage of VP0 into VP2 and VP4.



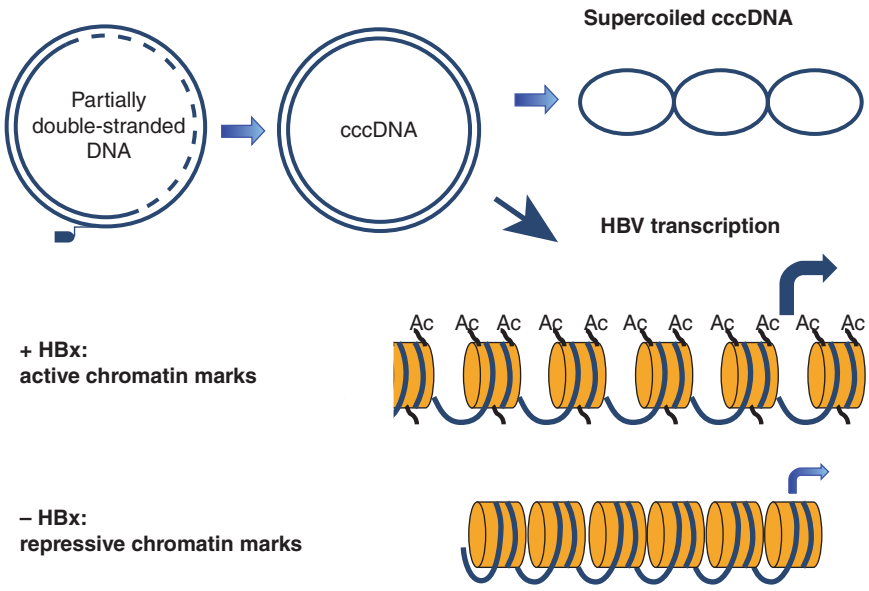
Color plate 3.2 Model of the pathogenic process of HAV. Schematic representation of the cycle of immune events leading to a typical course of HAV infection characterized by an immunopathogenic process with limited inflammation that culminates in complete clearance of the virus. This cycle is described in detail in this chapter, guided by the A–K circled reference points.



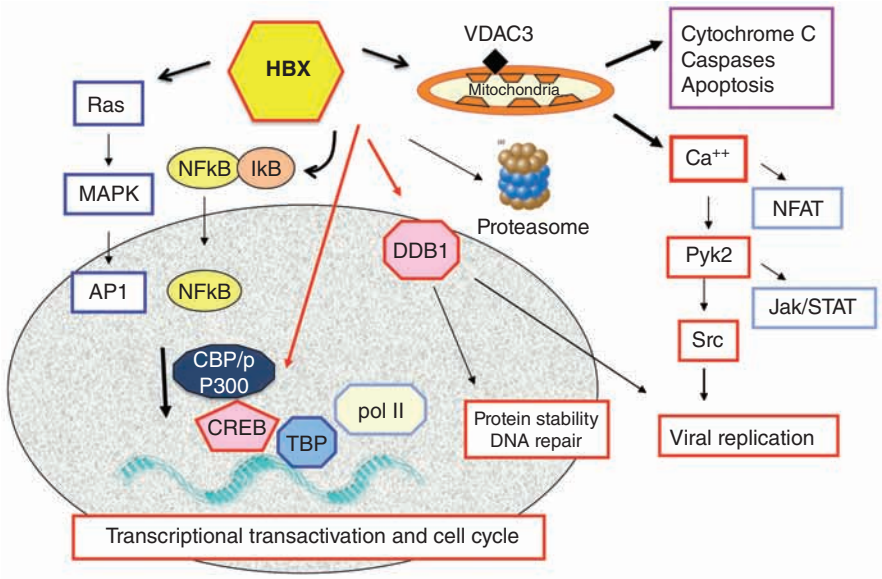
Color plate 8.1 Schematic of the HBsAg immune epitopes and MHR (including *a* determinant). Epitope numbering based on the proposed antigenic epitopes dedicated from Table 8.5. Cysteine residues are shown as yellow circles, and disulfide bridges are indicated as dashed lines. The changes selected by antibodies (natural, used in assays, and vaccine induced) that occur within, upstream, and downstream of the *a* determinant are indicated. The alpha helices are shown as cylindrical structures.



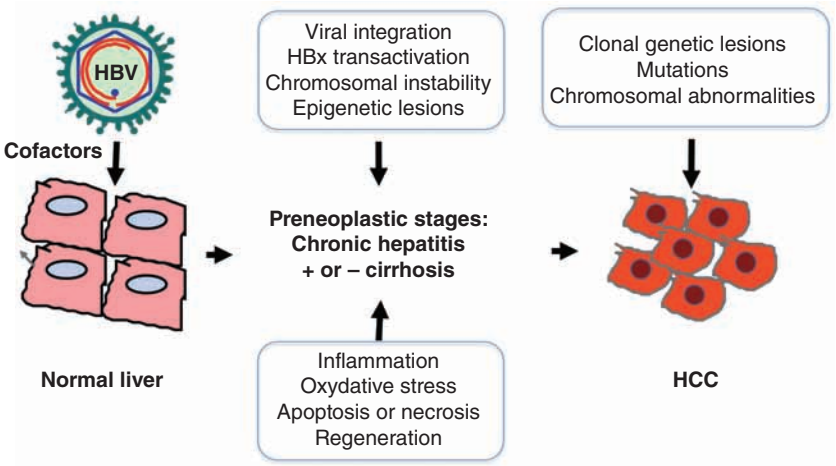
Color plate 12.1 Genetic organization of HBV. The viral genome is made of two linear DNA strands (thick black lines) maintained in circular configuration by their cohesive ends. The minus strand is around 3.182 kb in length, while the plus strand has variable length with a fixed 5′ end position and variable 3′ end (dashed line). The protein bound to the 5′ end of the minus strand corresponds to the N-terminus of the HBV polymerase, also called primase. The broad arrows surrounding the viral genome represent four ORFs that encode seven different proteins. The lower panel depicts the organization of the pregenomic RNA (pgRNA). Nucleotide positions are given for HBV subtype *ayw* (Source: Gailibert F, Mandart E, Fitoussi F, *et al.*, Nature 1979;281:646–650 [15]).



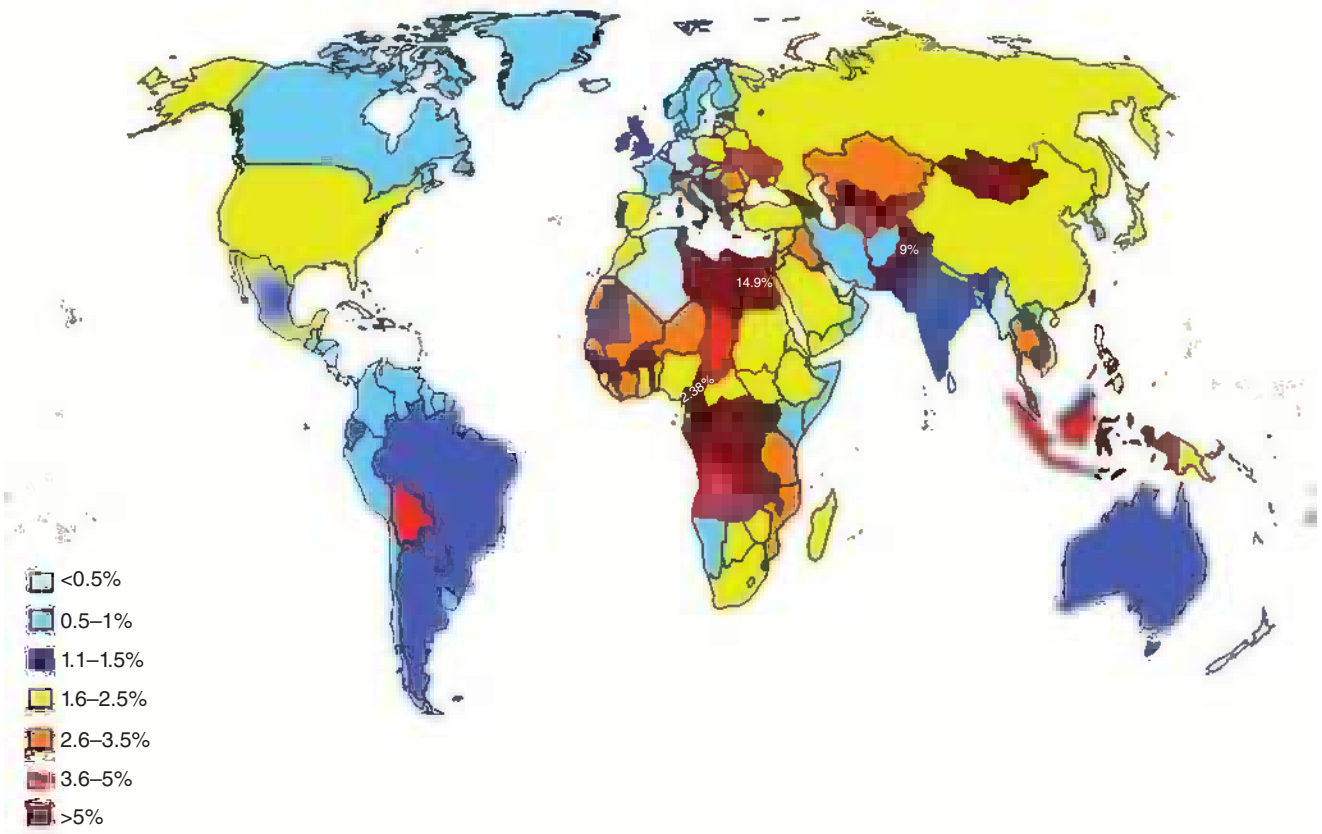
Color plate 12.2 Epigenetic regulation of HBV transcription. After viral entry, the HBV genome is delivered into the nucleus. It is then converted into covalently closed circular DNA (cccDNA) by the cellular machinery. The cccDNA is organized into a mini-chromosome with bound histone and nonhistone proteins including HBx and the core protein. By recruiting different chromatin-modifying factors such as CBP/p300, HBx plays a pivotal role in the modulation of epigenetic marks such as histone tail acetylation and methylation that result in active HBV transcription.



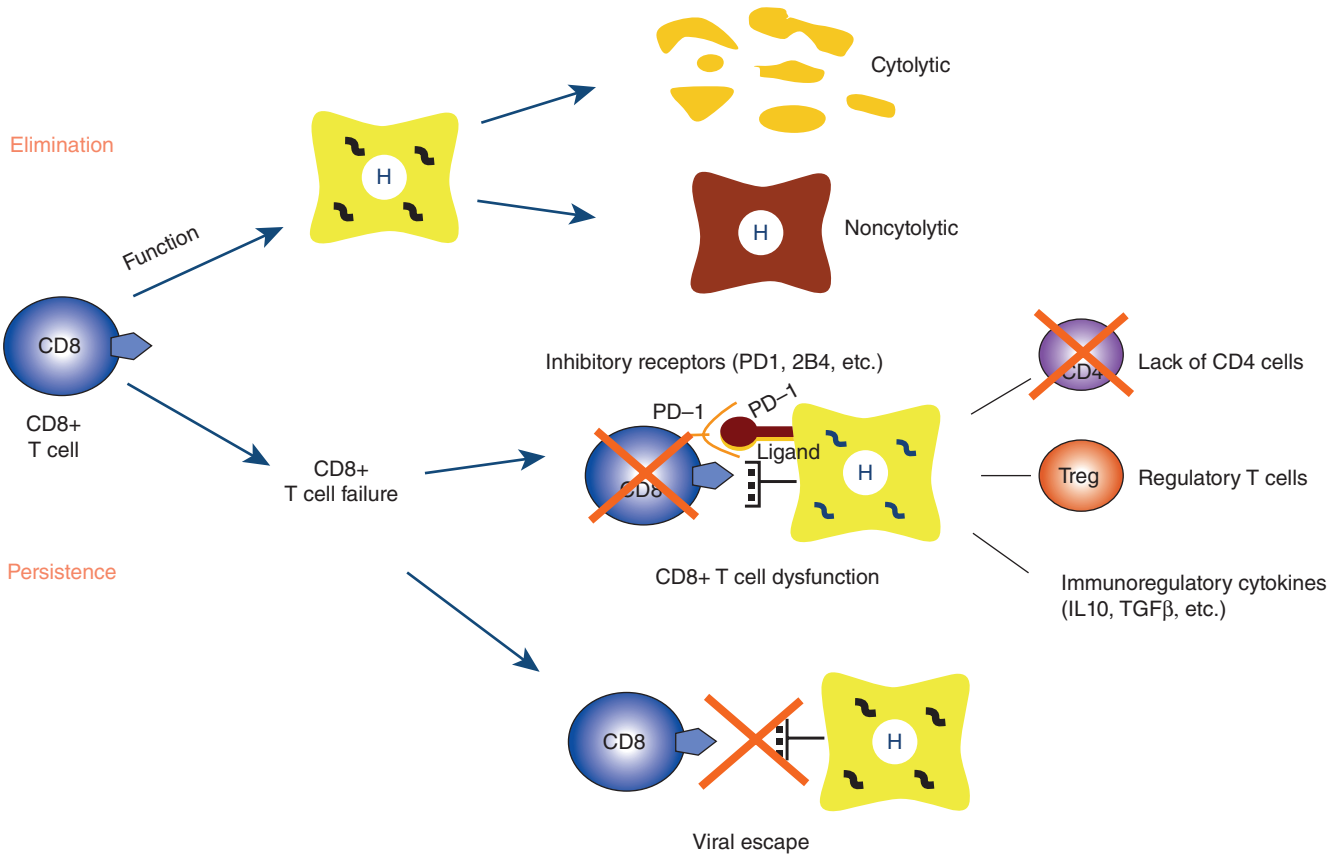
Color plate 12.3 Multiple interactions of HBx with cellular partners from cytoplasmic and nuclear locations. Interactions of HBx with a large array of cellular partners may be relevant for HBx functions in cellular transformation, including stimulation of virus replication, cell cycle deregulation, activation of signaling pathways, induction or repression of apoptosis, and interference with DNA repair. Most of these functions are achieved through the transcriptional transactivation activity of HBx mediated by its interactions with cellular partners in the nucleus (transcription factors, co-activators, and/or basal machinery) or in the cytoplasm through the stimulation of the Src, Ras, MAPK, NFKB, and NFAT signaling pathways. Importantly, the binding of HBx to DDB1 is crucial for viral replication.



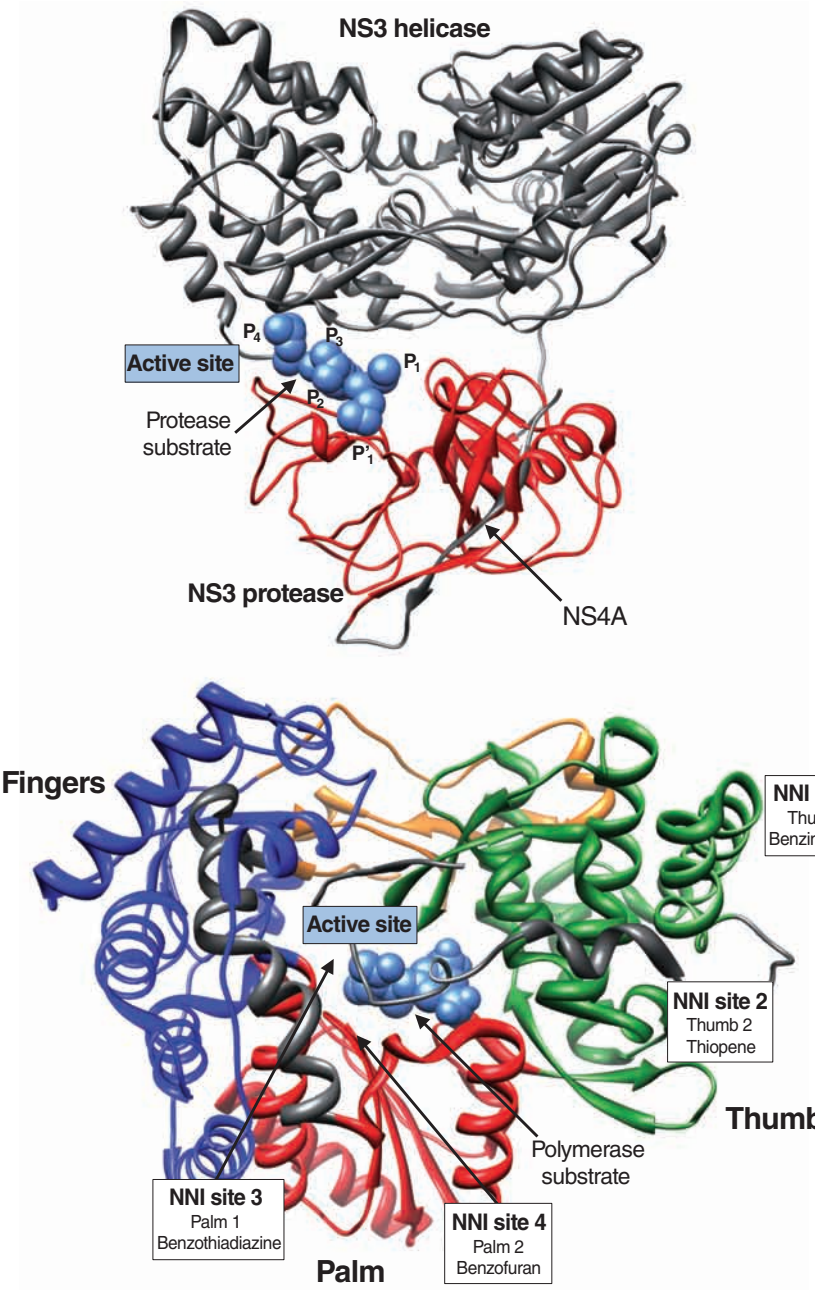
Color plate 12.4 Multistep process of HBV-induced liver tumorigenesis. Chronic HBV infection, alone or in combination with cofactors such as aflatoxin B1 exposure or alcohol consumption, triggers a cascade of events ultimately leading to transformation. At pre-neoplastic stages, the immune response induces a necroinflammatory disease that creates a suitable environment for the occurrence of genetic and epigenetic lesions. Integration of HBV DNA sequences into the host genome as well as long-term production of the viral HBx and surface proteins may contribute to the tumoral process via multiple mechanisms.



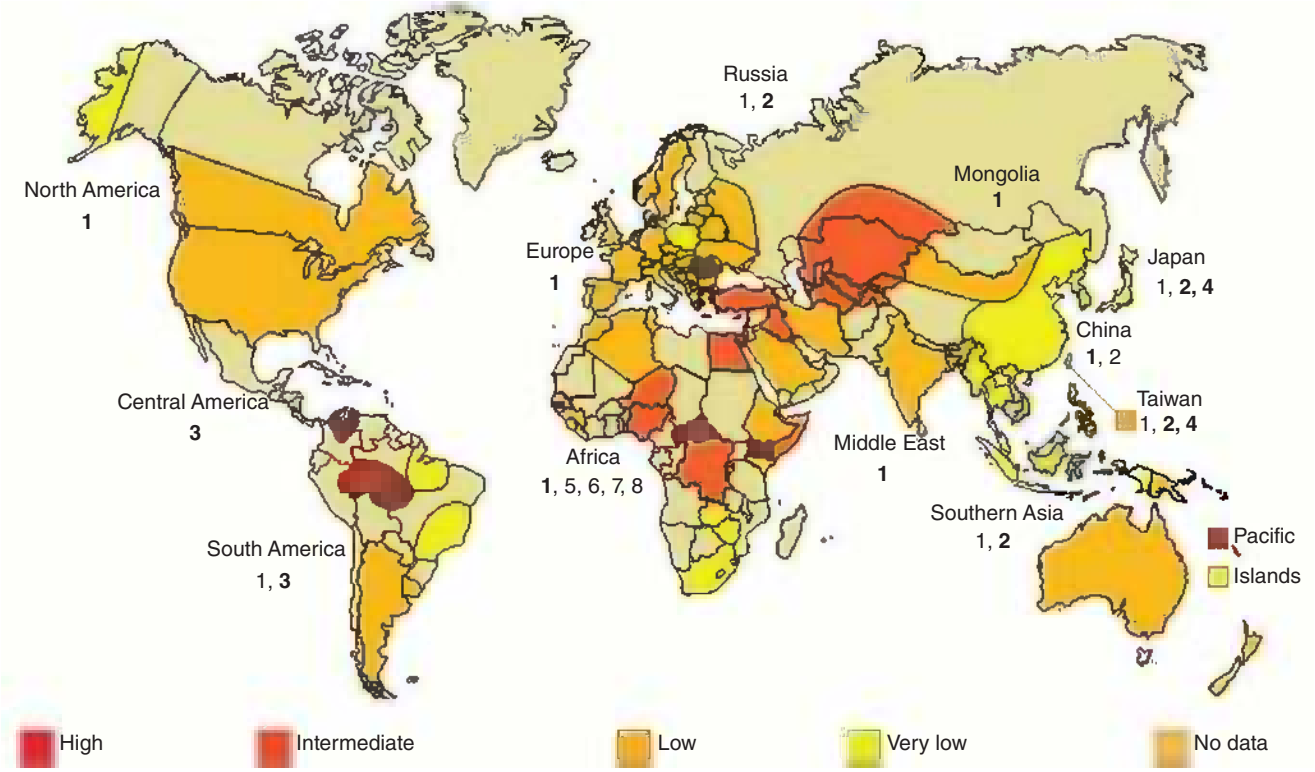
Color plate 17.1 Hepatitis C virus (HCV) prevalence (%) in the general population.



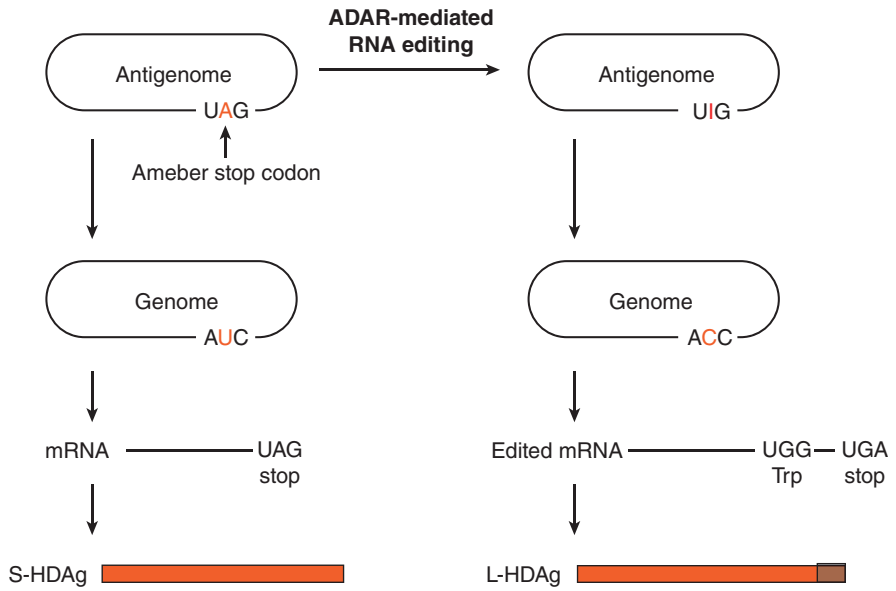
Color plate 18.1 Elimination and escape from the CD8+ T cell response. Virus-specific CD8+ T cells can inhibit HCV replication by cytolytic (killing of infected hepatocytes) and noncytolytic (IFNγ-mediated) effector functions. Virus-specific CD8+ T cell failure is mediated by two major mechanisms: CD8+ T cell dysfunction and the emergence of viral escape mutations. Several pathways contribute to CD8+ T cell dysfunction, such as inhibitory receptors, lack of CD4+ T cell help, the action of regulatory T cells, and immunoregulatory cytokines.



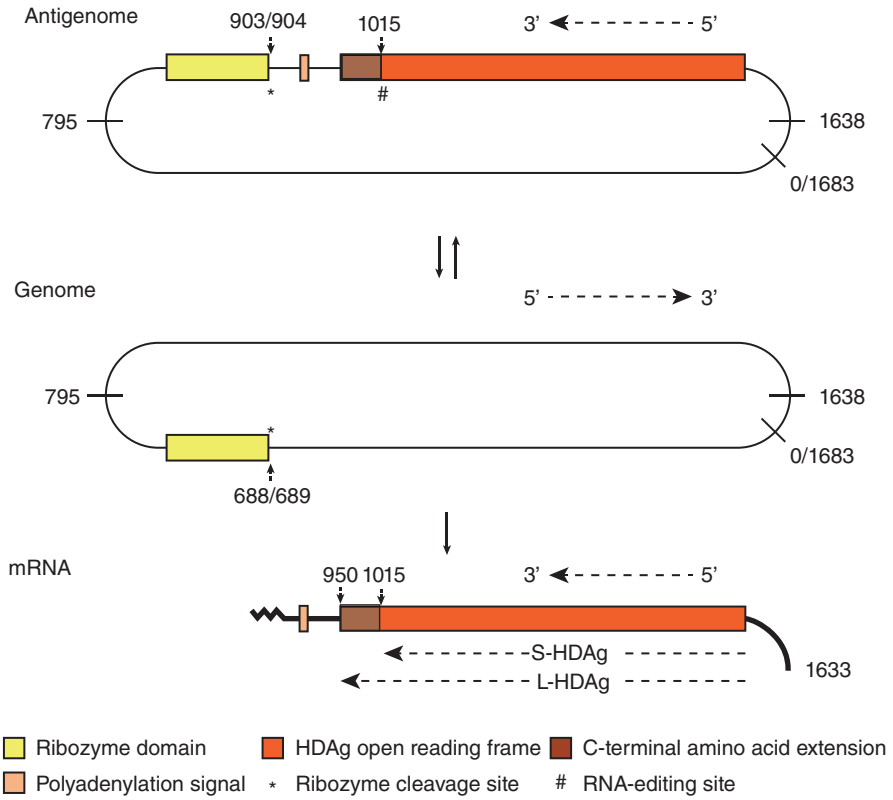
Color plate 25.1 NS3 protease-helicase and NS5B polymerase structure and molecular target sites. Top: Protease active site with natural substrate of proteolysis. Bottom: Polymerase palm, thumb, and fingers in different gray with active site bound inhibitor and NNI sites 1 to 4 (Source: Welsch C, Jesudian AB, Zeuzem S, *et al.*, Gut 2012;61(Suppl 1):136–146 [8]).



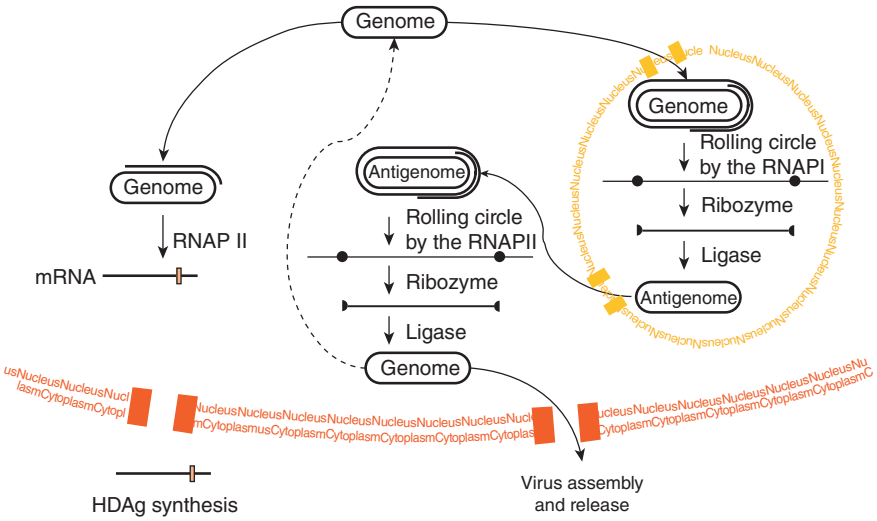
Color plate 27.1 Schematic representation of the main areas of HDV distribution in the world. In bold, the predominant HDV genotypes for each geographic area (Source: Pascarella S, Negro F. Liver Intl 2011;31:7–21 [2]).



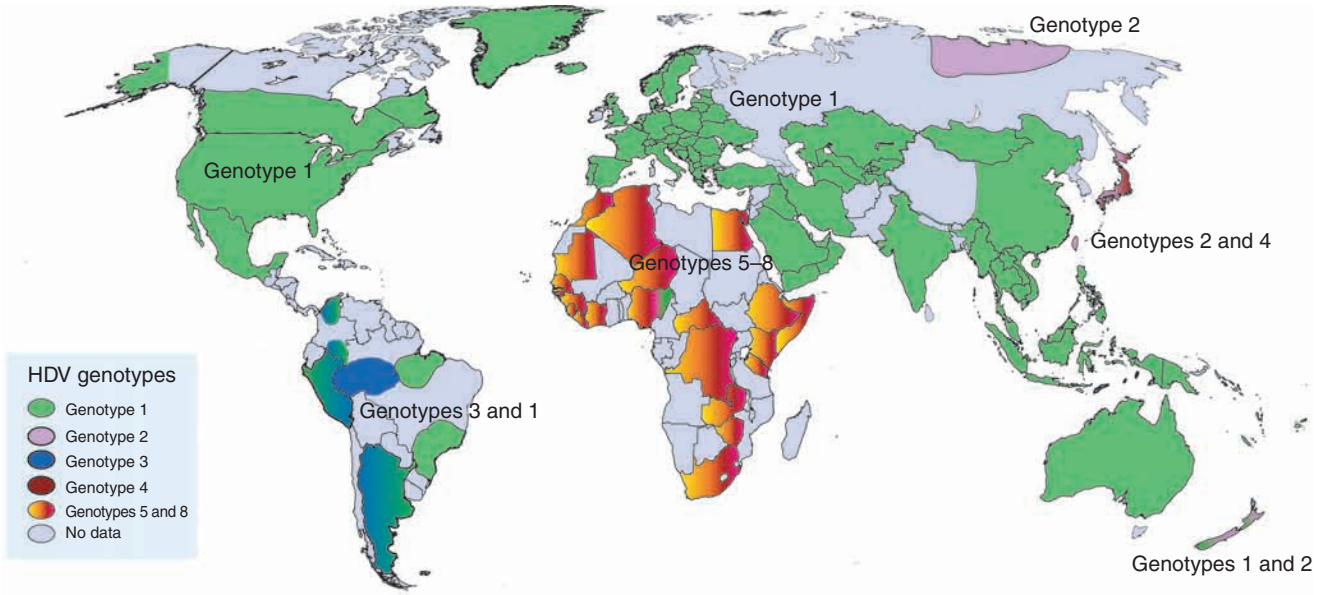
Color plate 27.2 Editing of the HDV antigenomic RNA (Source: Pascarella S, Negro F. Liver Intl 2011;31:7–21 [2]).



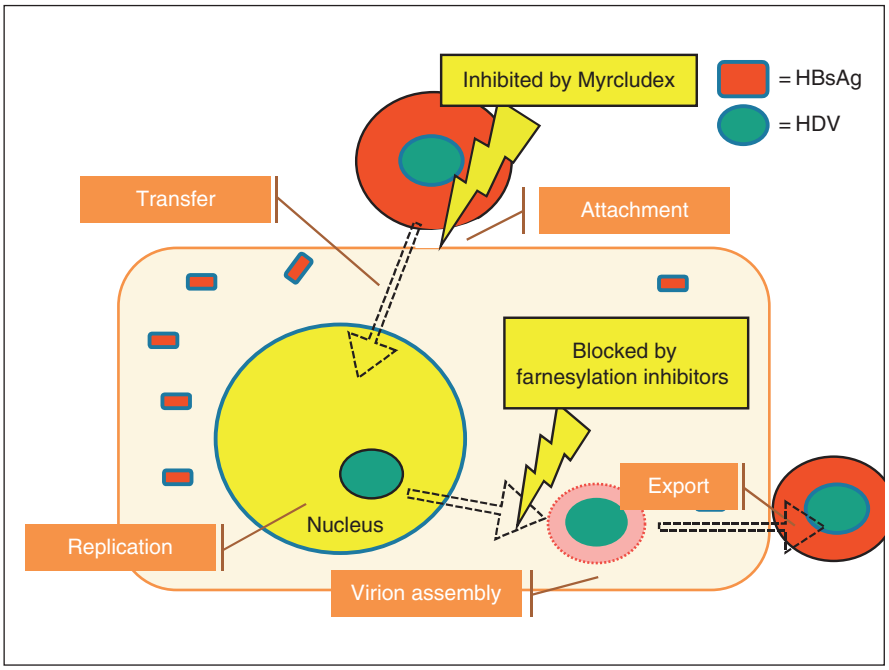
Color plate 27.3 Schematic representation of the HDV antigenomic and genomic RNAs, as well as of the HDAg mRNA (Source: Pascarella S, Negro F. Liver Intl 2011;31:7–21 [2]).



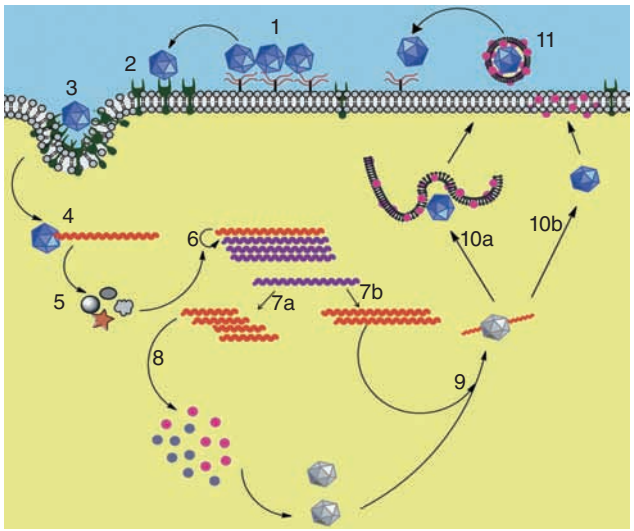
Color plate 27.4 Double-rolling circle replication of HDV and localization of the different replication events (Source: Pascarella S, Negro F. Liver Intl 2011;31:7–21 [2]).



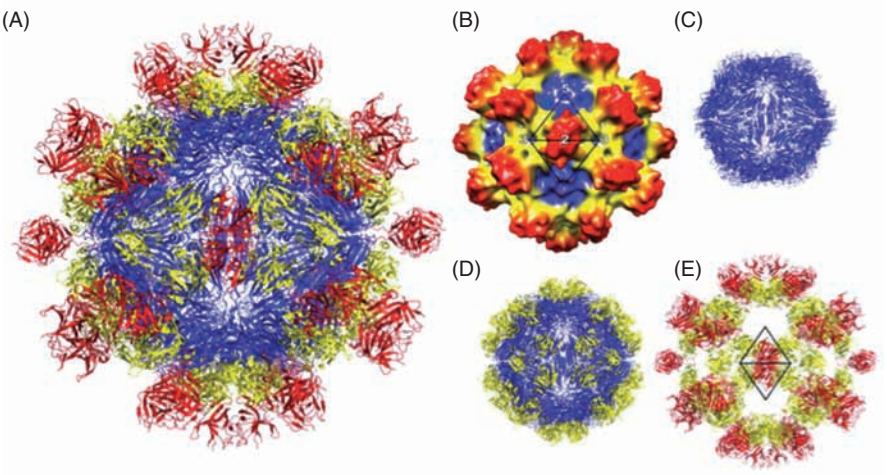
Color plate 28.1 Geographic distribution of hepatitis delta genotypes. (Source: Adapted from Hughes SA, Wedemeyer H, Harrison PM. Lancet 2011;378:73–85 [1]).



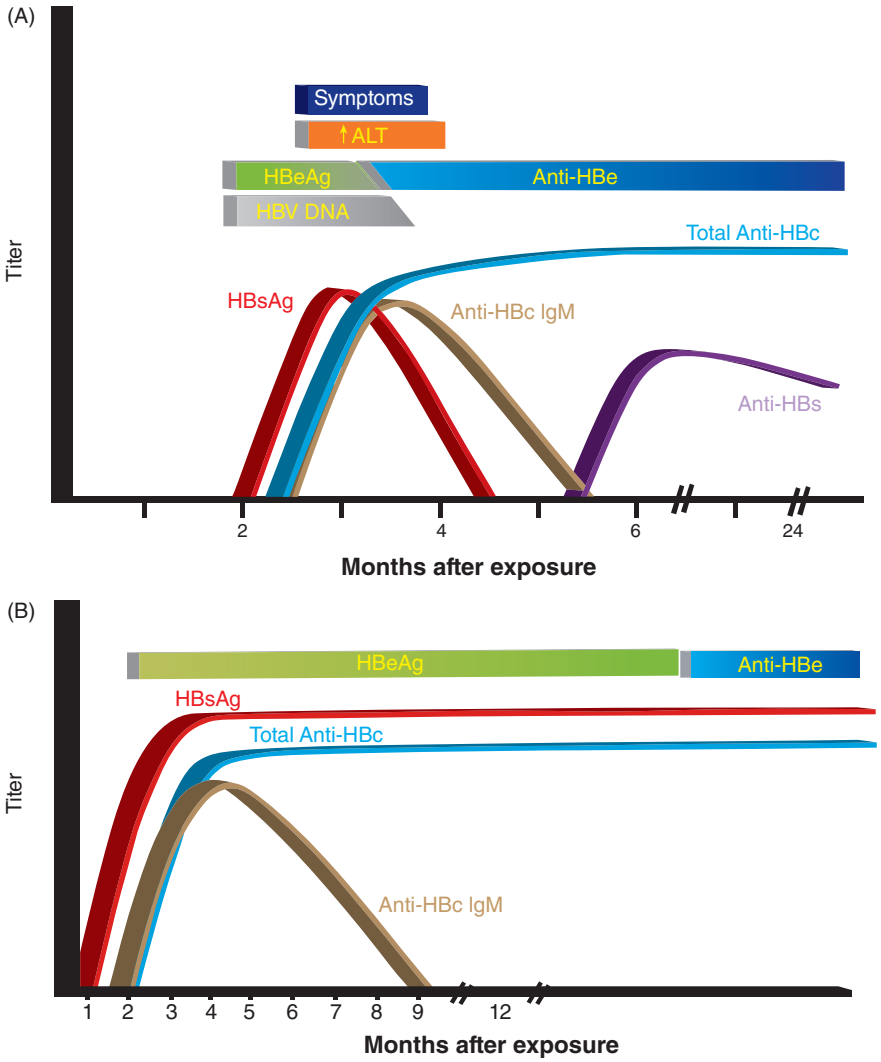
Color plate 29.1 Targets for HDV therapy. Steps in the life cycle of the HDV are the attachment of the virion to the liver cell through an HBsAg receptor, transfer to nucleus, replication, virion assembly, and export. Potential therapeutic targets are the prevention of attachment of HDV to the liver cell membrane through modifications or disruptions of the HBsAg envelope and receptor, and of the process of HD virion assembly, which requires farnesylation (prenylation) of the large HD-Ag and the integrity of critical regions of the HBsAg. Myrcludex B has been proposed for the inhibition of HBV entry into hepatocytes, and prenylation inhibitors for the disruption of HD virion assembly.



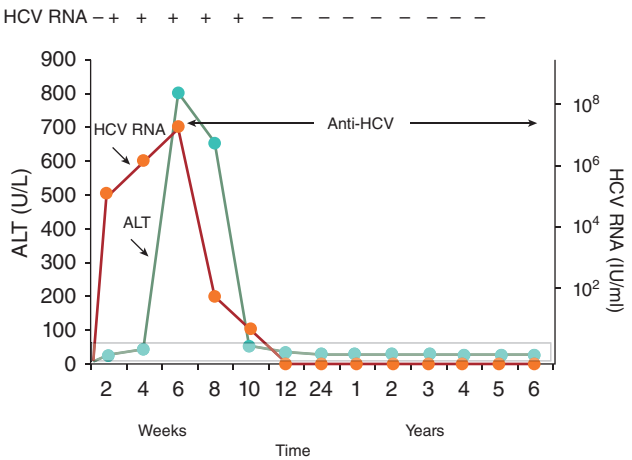
Color plate 30.1 Proposed replication cycle of HEV. (1) HEV particles are concentrated on the surface of target cells through heparin sulfate proteoglycans (HSPGs) acting as attachment factors (red wavy lines). (2) HEV particles bind to an unknown cellular receptor. (3) The HVE particles are internalized by unknown mechanisms. (4) The virus uncoats to release genomic RNA (red noodles). (5) Positive-strand viral genomic RNA is translated in the cytoplasm into nonstructural proteins. (6) The nonstructural proteins, including the viral RNA-dependent RNA polymerase (RdRp), replicate the positive-strand genomic RNA into intermediate negative-sense RNA genomes (purple noodles). (7) The intermediate negative-sense RNA genomes then serve as the templates for the synthesis of 2.2 kb subgenomic RNAs (step 7a) as well as full-length positive-sense genomic RNAs (step 7b). (8) The positive-sense subgenomic viral RNA is translated into ORF2 (blue) and ORF3 (crimson) proteins. (9) The ORF2 capsid protein packages the viral genomic RNA to assemble new progeny virions, while the ORF3 protein may optimize the host cell environment for viral replication. (10) The ORF3 protein is associated with endomembranes (step 10a) or plasma membranes (step 10b) and may aid in viral egress. (11) Mature virions are associated with the ORF3 protein and lipids, which are subsequently removed by a unknown mechanism before the virus begins a new cycle of replication. (Source: Ahmad I, Holla RP, Jameel S. Virus Res 2011;161:47–58 [7] (the high resolution drawing is courtesy of Dr Shahid Jameel, International Centre for Genetic Engineering and Biotechnology, New Dehli, India)).



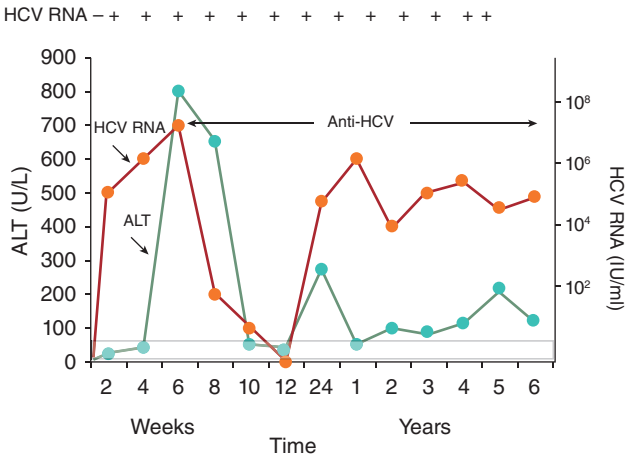
Color plate 30.2 Structure of the hepatitis E virus (HEV)-like particle (viruslike particle [VLP]) ($T=1$). (A) Crystal structure of an HEV VLP. The three domains, S, P1, and P2, are colored blue, yellow, and red, respectively. The VLP is positioned in a standard orientation with the three twofold icosahedral symmetry axes aligned along the vertical, horizontal, and viewing directions, respectively. (B) Cryo-EM reconstruction at 14 Å resolution. The surface is colored by radial depth cue from blue to yellow to red. (C) HEV VLP with only the S domain. (D) VLP with S and P1 domains. (E) HEV VLP with P1 and P2 domains. (Source: Guu TS, Liu Z, Ye Q, *et al.* Proc Natl Acad Sci USA 2009;106:12992–7 [56] with permission from the National Academy of Sciences, USA).



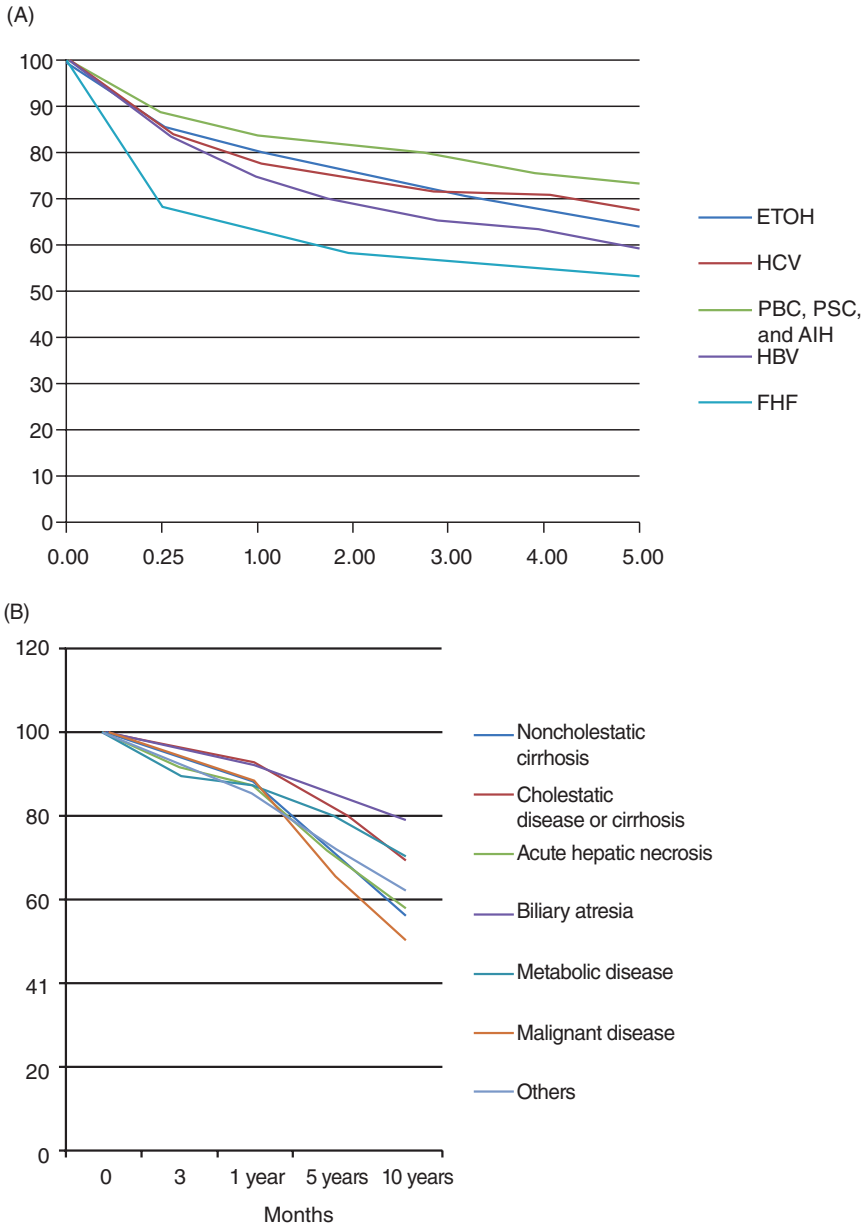
Color plate 33.1 (A) Serological profile of acute hepatitis B infection with recovery. (B) Progression to chronic HBV. (Source: Adapted from Centers for Disease Control and Prevention. MMWR 2008;57(RR08):1–20 [51]).



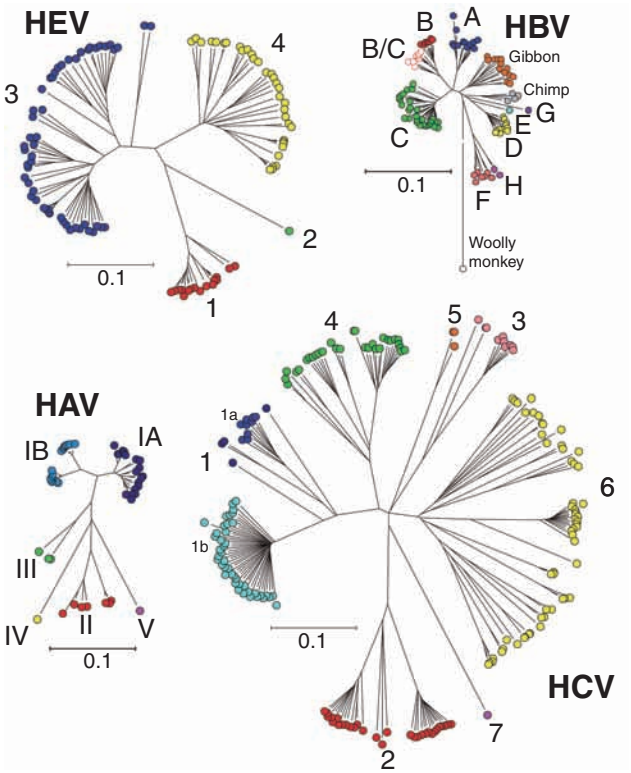
Color plate 33.2 The clinical course of acute HCV. (Source: Hoofnagle JH 2002. Hepatology 36:s21–s29 [52]).



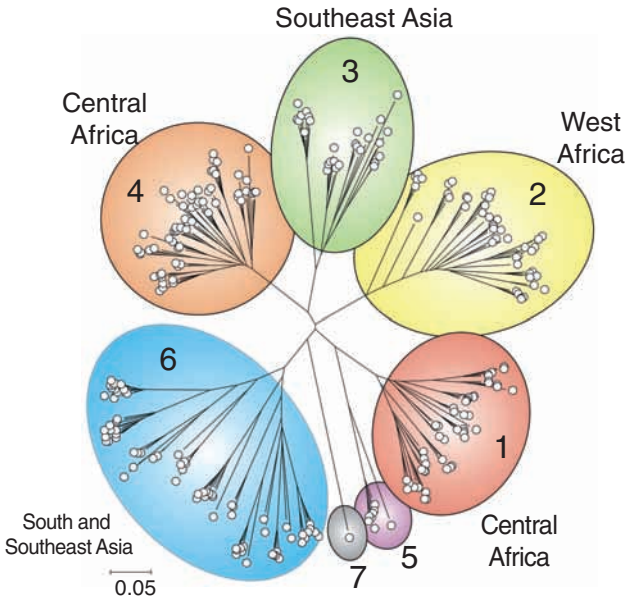
Color plate 33.3 Illustration of the evolution from acute to chronic HCV infection. (Source: Hoofnagle JH 2001. Hepatology 36:s21–s29 [52]).



Color plate 34.1 (A) Five-year survival according to etiology based on Pittsburghs first 4,000 transplants prior to the year [77]. (B) Survival according to etiology based on an 2009 OPTN/SRTR report for patients transplanted in the US [78].



Color plate 43.1 Phylogenetic trees of complete genome sequences of human hepatitis viruses drawn to the same scale to highlight their differing degree of naturally occurring sequence variability. Their current classification into genotypes is indicated by labels. Trees were constructed by neighbor joining of Jukes–Cantor corrected distances using the program MEGA5 [82].



Color plate 43.2 Phylogenetic tree of *NS5B* sequences of HCV (positions 8276 to 8615 as numbered as in the H77 reference sequence). High-diversity areas in Sub-Saharan Africa and Southeast Asia contain a large number of variants additional to subtypes such as 1a, 1b, and 3a (found in Western countries), displaying an endemic pattern of diversity. The tree was constructed as described in Figure 43.1.