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Hepatitis B Virus in Human Diseases

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Hepatitis B Virus in Human Diseases

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Foreword

The publication of "Hepatitis B virus in Human Diseases" coincides with the fiftieth anniversary of the discovery of the virus [1]. This is a good time, therefore, to reflect on where we have come from, and are going.

We now know that Hepatitis B virus (HBV) is a small DNA virus that has chronically infected hundreds of millions of people worldwide and is responsible for nearly a million liver disease-related deaths a year [2, 3]. However, it is worth noting that the discovery of hepatitis B was not an obvious one, but was made in a very exciting time. Although science progressed with great speed in the 1940s, 1950s, and early 1960s, most of this progress was in molecular biology, as compared with medical science. For example, and to put the period in context, DNA was identified as the information molecule in all cells [4] and its structure of DNA was solved by Watson and Crick [5]. The genetic code was translated by Nirenberg and Matthaei [6] and the genetic regulation of protein synthesis was discovered by Jacob and Monod [7]. These fundamental discoveries were made possible by rigorous application of the scientific method. If medical science progress was slower, it may have been because it focused on detailed descriptions of disease rather than applications of the scientific method to discover etiology. Discovery of HBV came after a series of clever insights standing on what could have been taken as unrelated discoveries and observations.

Baruch S Blumberg, who received the 1976 Nobel Prize in Medicine or Physiology for his role in the discovery of hepatitis B and chronic viral diseases, had studied biochemistry with Alexander Ogston at Oxford University in the late 1950s. There he learned that British research used a "scientific" method to investigate medical and biological problems. His work began with the study of protein polymorphisms in peripheral blood. Initially, Blumberg and Allison determined if people who received transfusions made antibodies to antigens on polymorphic blood proteins [8]. Later Blumberg and Alter continued to test this hypothesis using serum from multiply transfused patients, to identify more polymorphic proteins. One such protein, they called "Australia" antigen, named for the location of individual in whose blood it was found [1]. Careful testing of sera from patients with a variety of diseases eventually led to finding the association of Australia antigen with

one type of viral hepatitis, once called "serum hepatitis," and is now called hepatitis B. Surprisingly the Australian antigen was located on the surface of the virus itself and is now called HBsAg. The complicated natural history of hepatitis B disease in people made the discovery of its etiology all the more remarkable. Blumberg viewed it as a vindication of both non-goal-oriented research and the application of the scientific method to human disease.

Realization that Australia Antigen, originally recognized only by precipitin lines in Ouchterlony gel plates [1], was associated with hepatitis [9] and the hepatitis B virus envelope protein [10, 11] led to development of an assay to screen donor bloods and to invention of an effective vaccine [12]. The screening assay rapidly led to clearance of the blood supply of virally contaminated blood. Harvey Alter led the call to test all blood to be used for transfusions for the presence of "Australia" antigen based on these early observations and thus affected an important translation of basic scientific findings into an important clinical application in an unprecedentedly brief time.

The first HBV vaccine was produced from viral antigen derived from chemically inactivated virus, isolated from the blood of infected carriers [12], and approved for use by the US FDA in 1981. This was replaced in the 1990s with vaccines made from HBV recombinant envelope protein, isolated from yeast or mammalian cell culture (CHO cells), thus avoiding the concerns surrounding use of human HBV carrier blood [13]. These vaccines have been effective in interrupting perinatal transmission (which is a form of "post-exposure" protection), as well as other "horizontal" transmissions of the virus [14]. The effectiveness of these vaccines, all of which are formulations of purified HBsAg proteins (rather than live, replicating virus), is, and in itself, as surprising as it is important and instructive. Indeed, the discovery of HBV and development and use of an effective vaccine is one of the great accomplishments in medical and public health of the last century.

Realization that HBV is associated with hepatocellular carcinoma (HCC) must also be considered a great scientific accomplishment [15–17]. The demonstration that vaccination against HBV resulted in reducing the incidence of HCC both showed the public health benefit of vaccination [18] and providing final, definitive evidence of a cause and effect relationship between the virus and the cancer.

However, how HBV causes HCC remains elusive. The mechanism of oncogenesis almost certainly involves the necro-inflammatory pathogenesis associated with most chronic hepatitis B, but there is no specific viral oncogene, and replication of the virus in hepatocytes does not usually kill the infected cell [14]. Indeed, how the virus replicates has also generated some surprises. For example, the discovery that, although HBV is a DNA virus, it replicates through an RNA intermediate, and uses a virus-specified reverse transcriptase, is one of the major non-obvious findings in virology of the last part of the twentieth century [19]. It is also the molecular basis for the action for the small molecule direct acting hepatitis B antivirals [20] that are changing the natural history of the disease.

Parenthetically, one of the more dramatic demonstrations of how intervention with only polymerase inhibitors (in this case, lamivudine) can affect the natural history of chronic hepatitis was reported by this book's co-editor, Dr. Liaw [21].

The discovery of hepatitis D, a "viroid" that requires hepatitis B co-infection for it to complete its replication cycle, and exacerbates chronic hepatitis B, must also be considered to be another enormously significant medical and scientific finding that is a part of the hepatitis B story [22]. Hepatitis D continues to be a major, although often overlooked health threat.

Hepatitis B remains a vital topic for study: with somewhere between 250 and 350 million people chronically infected with the virus, and as many as 25 % may die from liver disease (liver cirrhosis or hepatocellular carcinoma), without beneficial intervention [3].

Thus, chronic hepatitis B (but not hepatitis D) is now treatable. The polymerase inhibitors, and interferons, are enabling achievement of viral suppression and improvement of liver function. Indeed, discussion about these advances is found in this book. However, a medical cure for hepatitis B is not yet available, and continued research is still needed to achieve a cure of the infection and prevent HCC, its most deadly outcome. We went 25 years from the discovery of hepatitis C to a definitive cure. The part of the story that tells of a cure for hepatitis B, even after 50 years, still needs to be written.

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Preface

The discovery of Australia antigen 50 years ago, with subsequent link to hepatitis B virus (HBV), has opened up a golden era of hepatitis research. Tremendous advances in both basic and clinical aspects of HBV have been achieved in the past five decades. However, HBV remains a major public health problem worldwide and is still the leading cause of hepatocellular carcinoma, one of the most deadly cancers. The field of HBV infections continues to evolve, allowing maximal benefit for patients. This is manifest in higher response rate to antiviral therapy and prevention of liver disease complications in infected patients, and better prophylaxis for non-infected ones. The HBV research field is currently living a new momentum with major discoveries on its life cycle for instance with the discovery of the receptor for virus entry or the identification of key cellular enzymes involved in the formation of viral cccDNA, and research efforts for the identification of novel treatment targets towards a real cure of the infection.

To mark and celebrate the fiftieth anniversary of HBV discovery, world renowned HBV experts have reviewed the development/advancement in their respective fields, as compiled in this book. Thanks to the contribution of these experts, this textbook has provided a comprehensive, state-of-the art review of this field. The different chapters review new data about basic and translational science including the viral life cycle, the immunopathogenesis of virus-induced chronic hepatitis, the mechanism of virus-induced liver cancer, and their potential applications for the clinical management of patients. The book also provides a comprehensive review of the clinical aspects of this chronic viral infection with important chapters on the global epidemiology, the natural history of the disease, and the management of special patient populations. Important chapters on the management of antiviral therapy and the recent international guidelines for the treatment of hepatitis B should help clinicians in their daily decisions when treating patients. Finally, the book reviews the current state of the art regarding immunoprophylaxis to prevent the spread of the virus and its major clinical consequences. The new advances and perspectives in the development of improved antiviral treatments are discussed as they may pave the way towards novel therapeutic concepts which, together with mass vaccination programs, should significantly impact the disease burden hopefully in a near future.

The content of this book may have shed light on and may help in the development of new viewpoints and approaches in hepatitis B research and clinical hepatology. Hopefully this book should serve as a valuable resource for students, clinicians, and researchers with an interest in hepatitis B. In this regard, we would like to express our deep appreciation to the authors of this book. We would also like to acknowledge our collaborators who helped in the reviewing of the chapters:

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Chapter 1
Hepatitis B Virus Virology and Replication

Jianming Hu

The Virus and Classification

Discovered 50 years ago as an antigenic "polymorphism" in an Australian aborigine—the "Australia antigen" [1], the hepatitis B virus (HBV) remains today a major global pathogen that causes acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2]. Owing to its unique replication strategy, as will be detailed below, HBV is classified into its own family, *Hepadnaviridae* [3], along with related animal viruses. The latter include the woodchuck hepatitis virus [4], particularly useful as a model for studying HBV pathogenesis, and the duck hepatitis B virus (DHBV) [5], particularly useful for studies on viral replication. HBV and DHBV represent, respectively, the type member of the two separate genera within the family, the mammalian and avian hepadnaviruses that infect a number of mammalian and avian species. All hepadnaviruses share strict species and tissue tropism—mostly restricted to hepatocytes in their respective hosts, and a unique life cycle—replicating a double-stranded (DS) DNA genome via an RNA intermediate and are thus sometimes called retroid viruses or para-retroviruses [3, 6].

The Virions and Subviral Particles

The complete HBV virion is a sphere with a diameter of ca. 40–45 nm, first visualized using transmission electron microscopy (EM) (the so-called Dane particle) [7] and more recently, and with much greater detail, using cryo-EM [8, 9].

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The virion has an outer envelope, which is studded with viral envelope (surface) proteins and surrounds an icosahedral capsid that, in turn, encloses the DS DNA genome. The complete virion is extremely infectious, with one virion being able to cause productive infection in chimpanzees that, in addition to humans, are susceptible to HBV [10]. On the other hand, a huge excess (100–100,000-fold over the complete virion) of noninfectious viral particles that contain no viral genome are also produced during infection. These so-called subviral particles include the classical spheres and filaments, which are ca. 22 nm in diameter and contain only the outer envelope layer of the virion, and the recently discovered empty virions, which contain both the outer envelope and the inner capsid shell but no viral DNA or RNA [11, 12].

Viral DNA Structure and Genome Organization

All hepadnaviruses share a peculiar virion DNA structure (Fig. 1.1) [13–15]. The DNA is small (3.2 kbp for HBV and 3.0 kbp for DHBV) and is held in a circular configuration via complementarity at the 5′ ends of both DNA strands, the length of complementarity being ca. 200 nucleotides (nt) in HBV and 60 nt in DHBV. Neither of the two strands of this relaxed circular DNA (rcDNA) is covalently closed. Whereas the (−) strand, i.e., the DNA strand complementary to pgRNA, has a short (ca. 9 nt) terminal redundancy (r), the other strand, the (+) strand, is heterogeneous in length with 3′ ends terminating hundreds of nt before completion. The rcDNA is further modified by a covalently linked protein (the terminal protein or TP) [16], later shown to be part of the viral reverse transcriptase (RT) or polymerase (P) protein [17] that is used to prime (−) DNA synthesis, and a capped, 18 nt-long RNA oligomer attached to the 5′ end of (+) DNA resulting from its use as a primer to initiate (+) strand DNA synthesis [14, 15] (see section "Reverse Transcription and NC Maturation" below).

Four distinct classes of viral mRNAs, all 5′ capped and 3′ polyadenylated, are encoded by the viral DNA. The genomic PreC/C mRNA is in fact longer than the DNA template (i.e., overlength), being 3.5 kb in length. The subgenomic PreS1, PreS2/S, and X mRNAs are approximately 2.4 kb, 2.1 kb, and 0.7 kb long, respectively. All viral mRNAs share the same 3′ sequences as represented by the shortest X mRNA, since they all terminate at the single polyadenylation signal. Transcription of these four groups of mRNAs is driven by four different viral promoters, respectively, the core, PreS1, PreS2/S, and X promoters that are further regulated by two viral enhancers, enhancer I upstream and overlapping with the X promoter and enhancer II located upstream of the core promoter (Fig. 1.1). A total of seven viral proteins are produced from these mRNAs using four open reading frames (ORF) (Fig. 1.1). The PreC/C mRNAs encode the viral core or capsid (C) protein and the slightly longer PreC protein using the same ORF, and the P protein using an alternative reading frame. As will be detailed below, the shortest of these genomic RNAs also serves as the template for reverse transcription to reproduce rcDNA

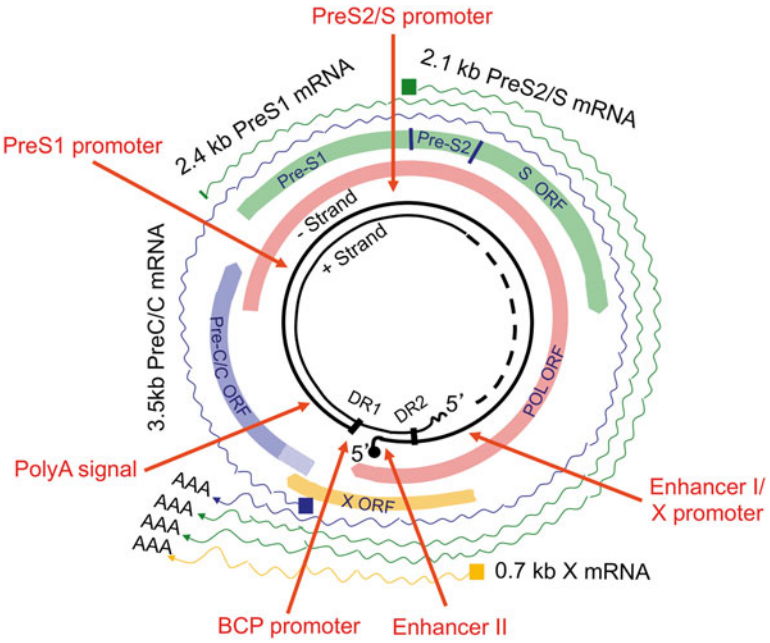

Fig. 1.1 HBV DNA structure and genome organization. The *inner circle* represents the virion rcDNA, and the *dashes* represent the region of the (+) strand DNA that is yet to be synthesized. The 5′ ends of the (−) and (+) strands are indicated. The *small*, *filled circle* represents the P protein covalently attached to the 5′ end of the (−) strand, and the *short wavy line*, the capped RNA oligomer attached to the 5′ end of the (+) strand. The *vertical bars* on rcDNA denote the direct repeats 1 and 2 (DR1 and DR2). The short terminal redundancy (r) on the (−) strand is denoted by the flap attached to the P protein (for clarity, it is not labeled in the figure). The promoter and enhancer positions are indicated. The *middle circle* of *shaded boxes* represent the four open reading frames (ORFs) corresponding to the precore/core, X, polymerase, and surface proteins, with their C-terminal ends denoted by the *arrows*. The *outer circle of wavy lines* represent the viral RNAs. *Filled squares* at one end of the mRNAs denotes heterogeneous 5′ ends (PreC/C, PreS2/S, and X mRNAs), the *thin vertical line* represents the precise 5′ end of the PreS1 mRNA. The *arrows* at the other end of the lines denote the 3′ ends of the mRNAs with polyA tails (AAA). *BCP* basal core promoter, *Pol* polymerase, *PolyA* polyadenylation

during replication and is thus termed pregenomic RNA or pgRNA. The PreS1, PreS2/S, and X mRNAs encode, respectively, the large (L) envelope protein, and the middle (M) and small (S) envelope proteins, and the X protein. All three envelope (or surface) proteins are encoded within a single ORF, which is entirely embedded within the alternative P ORF (Fig. 1.1). The P gene also overlaps with the 3′ end of the C gene and 5′ end of the X gene at its 5′ and 3′ ends respectively. In addition, all transcriptional regulatory elements including the promoters, enhancers, and the polyadenylation signal overlap with the protein-coding sequences. The genomic organization of hepadnaviruses is thus characterized by extreme economy.

Structure and Functions of Viral Proteins

The Envelope Proteins

Of the three HBV envelope proteins, the smallest, S, is 226 residues long and is the most abundant. M contains a N-terminal extension, relative to S, called the PreS2 region, which is 55 residues long (Fig. 1.1). L is the longest and contains yet another N-terminal extension called the PreS1 region, which is 108 (or 119 depending on the strains) residues long. In addition to being major constituents of the virions, the envelope proteins are also secreted in large excess to the blood stream of infected people as spheres and filaments in the absence of capsids or genome, as mentioned above. Indeed, it was the abundance of these particles that allowed the discovery of HBV as the Australian antigen, i.e., hepatitis B surface antigen (HBsAg). The spheres contain mostly S and some M, and the filaments have in addition some L, which is enriched in virion particles [18]. Both L and S are required for virion secretion but M is dispensable [19]. In particular, the PreS1 region in L contains determinants required for both capsid envelopment during virion formation as well as receptor binding during entry (see below) [20]. This dual role of PreS1 is facilitated by its dynamic dual topology in the virions [21, 22]. Immediately following translation in the endoplasmic reticulum (ER) membrane, all PreS1 is located on the cytosolic side allowing it to interact with the capsids to fulfill its role in virion formation; as the virions traffic through the cellular secretory pathway, ca. 50 % of PreS1 is translocated from the interior of the virions to the exterior to allow it to bind the cell surface receptor. How this dramatic gymnastic feat is accomplished remains an enigma.

The C Protein and e Antigen

The C protein is 183 (or 185 depending on the strains) residues long. C can be divided into two structural and functional domains. The N-terminal 140 residues form the assembly domain (NTD) that is sufficient to mediate capsid assembly [23, 24]. The C-terminal domain (CTD) is dispensable for capsid assembly but plays essential roles in packaging of pgRNA into replication-competent nucleocapsids (NCs) and in reverse transcription of pgRNA to rcDNA. The C protein rapidly forms dimers, which are the building blocks for capsid assembly. Two morphological capsid isomers, with either 120 ($T=4$, the major isomer) or 90 dimers ($T=3$), are formed [25, 26]. The functional significance, if any, of this dichotomy is unknown. The arginine-rich CTD is highly basic and has nonspecific nucleic acid binding activity [27]. It also harbors multiple nuclear localization signals (NLSs) [28–30] that may be important for delivery of NCs to the nucleus (see section “Intracellular Trafficking and Uncoating” below).

Moreover, CTD is heavily phosphorylated when expressed in mammalian cells, with three major sites of phosphorylation all displaying the Ser-Pro motifs [28, 31] plus three to four additional minor sites of phosphorylation [32]. As will be described below, CTD phosphorylation plays critical roles for C functions in viral replication. As HBV does not encode any viral kinase, it has to usurp host protein kinases for C phosphorylation. A number of cellular kinases, including protein kinase C (PKC) [33], cyclin-dependent protein kinase 2 (CDK2) [34], serine-arginine protein kinase (SRPK) [35] have been reported to phosphorylate C or specifically its CTD. Among these, CDK2 has been shown to associate with and phosphorylate the CTD, in particular, its Ser-Pro sites (consistent with the known substrate specificity of CDK2 as a well-known proline-directed kinase), and is incorporated into the capsids (see below) [34, 36]. As will be detailed below, CTD phosphorylation is highly dynamic and a dramatic dephosphorylation of CTD is shown to accompany viral DNA synthesis in the DHBV NCs. The cellular phosphatase(s) responsible for C dephosphorylation remains to be identified.

The precore (PreC) protein is translated from its own mRNA (PreC mRNA), which differs from the C mRNA (pgRNA) by a 5′ extension some 30 nt long. The sequence of PreC is thus essentially the same as C, except for an additional 29 amino acids at its N-terminus [37, 38]. However, these two proteins are functionally very different; unlike C, PreC is entirely dispensable for viral replication and mutants unable to express this protein are frequently selected late during persistent infection [38]. The first 19 residues of PreC comprise a secretion signal that induces its translocation into the lumen of the ER, where the signal sequence is cleaved off by a host cell signal peptidase. The remainder of PreC undergoes further proteolytic processing (e.g., by furin) in the host cell secretory pathway to remove the highly basic CTD in C, resulting the secretion of a heterogeneous population of soluble, dimeric proteins [39, 40], defined serologically as the hepatitis B e antigen (HBeAg) (Fig. 1.2, 9c) [41]. While dispensable for viral replication, PreC/HBeAg appears to play an important role in vivo for establishing persistent infection by regulating host immune response against the related and highly immunogenic C protein [42]. Also, serum HBeAg has proven to be a useful marker to monitor viral replication as its presence tends to correlate with high levels of viral replication and its loss usually signifies a decrease in viral replication [38].

The Reverse Transcriptase

The HBV RT or P protein is a multifunctional protein that plays a central role in viral replication. P is 832 or (or 845 depending on the strains) residues long and can be divided into four separate domains, from the N-terminus: TP, the spacer, the RT domain, and the RNase H domain [43–47]. TP harbors the invariant Tyr residue essential for priming reverse transcription [48–50] (see section “Reverse

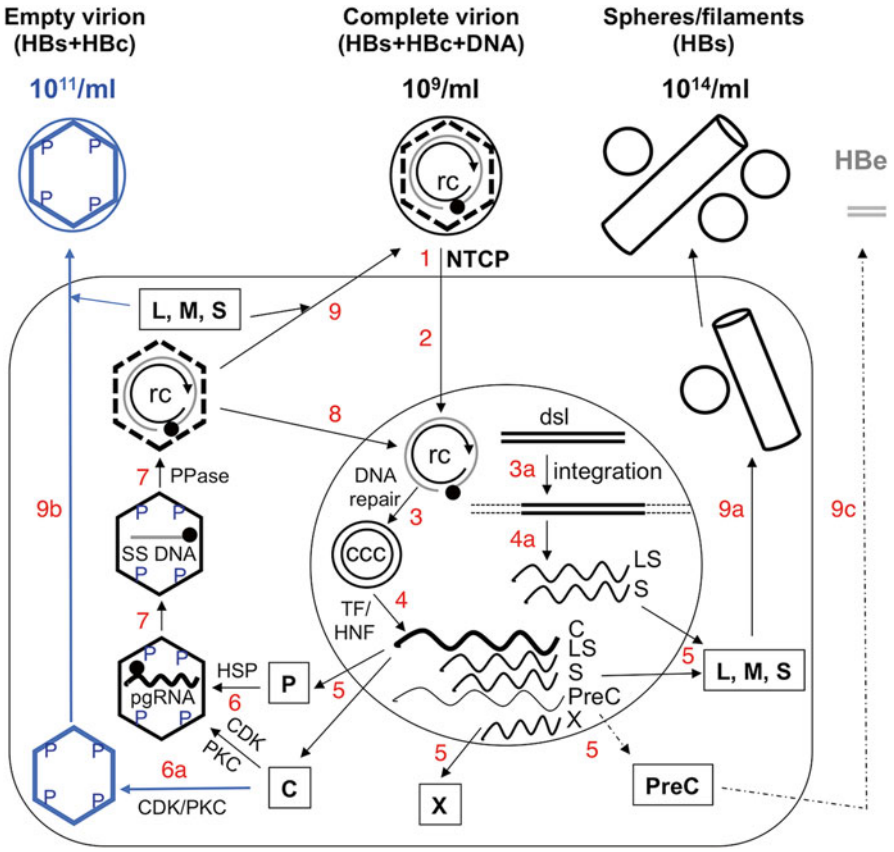

Fig. 1.2 HBV life cycle. The replication cycle of HBV is depicted schematically. (*1*) Virus binding and entry into the host cell (*large rectangle*). (*2*) Intracellular trafficking and delivery of rcDNA to the nucleus (*large circle*). (*3*) Repair of rcDNA to form cccDNA, or integration of dslDNA into host DNA (*3a*). (*4* and *4a*) Transcription to synthesize viral RNAs. (*5*) Translation to synthesize viral proteins. (*6*) Assembly of the pgRNA-containing NC, or alternatively, empty capsids (*6a*). (*7*) Reverse transcription to make the (−) strand DNA and then rcDNA. (*8*) Nuclear recycling of progeny rcDNA. (*9*) Envelopment of the rcDNA-containing NC and secretion of complete virions, or alternatively, secretion of empty virions (*9b*) or HBsAg spheres and filaments (*9a*). Processing of the PreC protein and secretion of HBeAg are depicted in (*9c*). The different viral particles outside the cell are depicted schematically with their approximate titers indicated: the complete or empty virions as *large circles* (outer envelope) with an *inner diamond shell* (capsid), with or without rcDNA inside the capsid respectively; HBsAg spheres and filaments as *small circles* and *cylinder*. Intracellular capsids are depicted as *diamonds*, with either SS [(−) strand] DNA (*straight line*), viral pgRNA (*wavy line*), or empty, and the letters "P" denoting phosphorylated residues on the immature NCs (containing SS DNA or pgRNA) or empty capsid. The *dashed lines* of the *diamond* in the rcDNA-containing mature NCs signify the destabilization of the mature NC, which is also dephosphorylated. The soluble, dimeric HBeAg is depicted as *grey double bars*. The *dashed line* and *arrow* denote the fact that HBeAg is not always secreted during viral replication. The *wavy lines* denote the viral RNAs: C, mRNA for the C and P protein (and pgRNA); S and LS, mRNAs for the S/M and L envelope proteins, respectively; PreC, mRNA for the PreC protein and following

Transcription and NC Maturation"), and together with the RT domain, are required for specific binding of pgRNA for its encapsidation into NCs. pgRNA packaging requires also the RNase H domain but none of the known enzymatic activities of P [51, 52]. The spacer region is the least conserved of the four domains and is dispensable for all known functions of P. However, its coding sequences have to be retained to encode the PreS1 region in the overlapping S ORF, which is essential for the virus as discussed above. The RT domain harbors the polymerase active site essential for DNA polymerization [44, 45], particularly the Tyr-Met-Asp-Asp motif conserved across all RT proteins including those in retroviruses and retrotransposons. The RNase H domain is responsible for degrading the pgRNA template during (−) strand DNA synthesis [44, 45, 53].

The HBV DNA polymerase activity was discovered early on, before it was realized that HBV replicates via reverse transcription, via the so-called endogenous polymerase assay [54] whereby a DNA polymerase activity in the virions was shown to carry out DNA synthesis using the endogenous virion DNA as a template. It was only a decade later that Summers and Mason made the landmark discovery that a DNA virus (i.e., DHBV) replicates through reverse transcription of an RNA intermediate [6]. However, biochemical studies on this important enzyme have proven difficult to date, and no high-resolution structures of P are yet available. As will be detailed below (section "NC Assembly"), the discovery that P requires specific host factors for its folding and functions provides at least a partial explanation to this difficulty.

The X Protein

The 154 residue-long hepatitis B X protein (HBx) is the smallest HBV protein but is arguably the least understood. There is general agreement that X is required for viral replication in vivo [55] and perhaps contributes to viral pathogenesis (for a recent review, see ref. [56]). Numerous reports have suggested a large number of functions for X in the regulation of viral and host gene expression [57], DNA damage repair [58], Ca^{2+} signaling [59], cell cycle [60], apoptosis [61], and autophagy [62, 63]. As there is no evidence that X has DNA binding activity, it is thought to affect gene expression through host protein interactions, which are probably also

Fig 1.2 (continued) processing, HBeAg. *Boxed letters* denotes the viral proteins translated from the RNAs. The *filled circle* on rcDNA denotes the P protein attached to the 5′ end of the (−) strand (*outer circle*) of rcDNA and the *arrow* denotes the 3′ end of the (+) strand (*inner circle*) of rcDNA. *ccc* cccDNA, *dsl* double stranded linear DNA, *HNF* hepatocyte nuclear factor, *HSP* heat shock protein, *PPase* phosphatase, *rc* rcDNA, *TF* transcription factor. For simplicity, the synthesis of dslDNA (the minor genomic DNA form) in the mature NC, its secretion in virions, and infection of dslDNA-containing virions are not depicted here, as are the functions of X. See text for details

important for the various other viral or cellular effects attributed to X. It remains to be clarified how the various activities attributed to X are related to each other, and how they in turn relate to viral replication and/or pathogenesis (esp., hepatocarcinogenesis). As these activities are uncovered usually using different systems and assays, which are often less than physiologically optimal due to experimental limitations, and the role of X in viral replication or pathogenesis is likely regulatory and indirect, the interpretation of the results obtained, which can be in apparent conflicts, is by no means straight-forward. Recent attempts at standardization of experimental systems and assays and the development of more physiologically relevant systems will hopefully help clarify the functions of HBx in viral replication and pathogenesis [64].

Viral Life Cycle

As a para-retrovirus, the HBV life cycle (Fig. 1.2) shares a number of similarities to conventional retroviruses, including, of course, the central role of reverse transcription. However, HBV and hepadnaviruses in general have indeed a rather unique replication strategy, which is different from the conventional retroviruses in a number of important aspects including the initiation of reverse transcription and NC assembly, genome maintenance, and virion morphogenesis.

Entry

The strong species and tissue tropism of hepadnaviruses are in part underpinned by viral entry. Until recently, the only cells in culture that are reported to support HBV infection reproducibly are primary hepatocytes from humans [65] and the small primate tupaia [66], and one human hepatoma cell line HepaRG, which requires differentiation in vitro for even the rather low infection efficiency achieved [67]. Very recently, hepatocyte-like cells differentiated from induced human pluripotent stem cells [68], and a newly established human hepatoma cell line HLCZ01 [69], are reported to support limited HBV infection.

After many false starts, the primary entry receptor for HBV was finally identified in 2012 as a hepatic bile acid transporter, sodium taurocholate cotransporting polypeptide (NTCP) (Fig. 1.2, step 1) [70]. This breakthrough allows the establishment of convenient hepatoma cell lines such as HepG2 and Huh7, which have been the mainstay for studying other aspects of HBV replication and can now support infectious entry via NTCP reconstitution. On the other hand, NTCP is insufficient to render mouse hepatocytes susceptible to HBV infection [71, 72]. This result, though disappointing, is not unexpected given previous observations that another essential, intracellular, stage in the viral life cycle, the formation of the covalently closed circular DNA (cccDNA), is also defective in mouse hepatocytes (see section "Nuclear Recycling of rcDNA and Amplification of cccDNA"). It is clear that

additional host factors are required for the early stages of the viral life cycle beyond cell attachment and entry.

The viral requirements for entry are much better defined. Specifically, the N-terminal 48 residues of L as well as its N-terminal myristylation are both required for NTCP binding and infection [20, 70, 73, 74]. In addition, a region in S within the conserved "a" determinant (the major antigenic loop) is also required for infection via binding to the cell surface heparin sulfate proteoglycans and mediating the initial (nonspecific) viral attachment to the cells [75, 76]. This observation helps explain the high conservation of this antigenic determinant among HBV genotypes/strains. A role for glycosylation of the envelope proteins has also been reported recently [77]. Intriguingly, HBV binding to NTCP, in addition to mediating viral infection, may also inhibit the transport function of NTCP and alter cellular gene expression [78], raising the possibility that this initial virus-host interplay may contribute to viral pathogenesis.

Intracellular Trafficking and Uncoating

The next essential step in the HBV life cycle after entry into susceptible host cells is to deliver the virion rcDNA into the nucleus (Fig. 1.2, step 2). Relatively little is understood here due to the lack of convenient and efficient infection systems until very recently. It is thought once the viral envelope and cellular membrane fuse to release the internal NC, the latter traffics towards the nuclear membrane, through interactions with cellular importins mediated by NLSs located on the C CTD [36, 79, 80]. As HBV can efficiently infect nondividing hepatocytes in the liver and NC is too large to pass through the nuclear pore complex (NPC), it has been proposed that NC interacts with components of the NPC leading to the arrest of NC and release of its rcDNA content into the nucleus [81] for cccDNA formation (see next).

cccDNA Formation

cccDNA is the first new viral DNA species detected upon infection [82] and is essential to initiate and sustain viral replication, as it is the only viral transcriptional template that can direct the expression of all viral RNAs and proteins. As with NC trafficking/uncoating, little is currently understood about this critical stage of HBV infection due to the lack of convenient experimental systems, until recently, that can support efficient HBV infection (for a recent review, see ref. [83]). It is clear, however, that the conversion of rcDNA to cccDNA in the nucleus (Fig. 1.2, step 3), as well as the preceding stages of entry and uncoating, must be highly efficient during natural infections since one DNA-containing virion particle is able to establish a productive infection in susceptible hosts [10, 84]. As will be detailed below (section "Nuclear Recycling of rcDNA and Amplification of cccDNA"), cccDNA can also be formed from progeny rcDNA synthesized de novo, via an intracellular amplification

pathway. This process, which bypasses the entry process, has been used, with limited success so far, to study cccDNA formation.

Viral RNA Synthesis

Once formed, the nuclear episomal cccDNA functions as the equivalent of a provirus in retroviruses and is used as the template to transcribe, by the host RNA Pol II, all the viral RNAs (Figs. 1.1 and 1.2). Viral transcription is dependent on ubiquitously expressed as well as liver-enriched transcriptional factors (hepatocyte nuclear factors or HNFs) (Fig. 1.2, step 4), which contributes to the liver (hepatocyte) specificity of viral replication [85–94] (for a recent review, see ref. [95]). cccDNA is organized into mini-chromosomes with host cell histones and potentially other host and viral proteins [96, 97]. Transcription from the cccDNA mini-chromosomes, like that from host chromosomes, is subject to epigenetic regulation, which may be further modulated by the viral regulatory protein, X [98–100]. X has been reported to be critical for transcription from cccDNA during infection [57], and apparently functions only on episomes (like cccDNA) but not integrated DNA, in a DNA sequence-independent manner [101]. This sequence independence is consistent with the lack of DNA binding activity of X but if and how X specifically affects viral transcription remains an enigma. It has been suggested that HBx is recruited onto the cccDNA mini-chromosomes [98] but how this is accomplished in a DNA sequence specific fashion also remains unclear.

An interesting feature of HBV transcription is its dimorphic response to sex hormones, being stimulated by androgen [102, 103] and suppressed by estrogen [104]. Why HBV has evolved such a sexual dimorphism is an interesting unresolved question but this phenomenon likely contributes to the well-known male predominance of HBV replication and carcinogenesis.

All HBV mRNAs described above are unspliced, which have to be exported from the nucleus to the cytoplasm in order to be translated or packaged into NCs in the case of pgRNA. As eukaryotic mRNA export is usually coupled to splicing, HBV has evolved a mechanism of exporting its mRNAs out of the nucleus in a splicing-independent manner, which relies instead on a *cis*-acting RNA sequence called the post-transcriptional regulatory element (PRE) [105] encoded by viral DNA sequences overlapping enhancer I (Fig. 1.1).

Viral Protein Synthesis

Another interesting feature of HBV gene expression is that all viral promoters, except PreS1, lack an canonical TATA box and as a result, all viral RNAs, except the PreS1 mRNA, have heterogeneous 5′ ends, which is used to translate distinct proteins from closely related mRNA species. The heterogeneous 5′ ends of the

over-length genomic RNAs, PreC/C mRNA, bracket the translation initiation codon of the PreC protein. The longer PreC mRNAs containing the PreC initiation codon are translated to produce the PreC protein and ultimately the secreted HBeAg (Fig. 1.2, step 5), as described above. The shortest, C mRNA, missing the PreC initiation codon, is translated to produce both the core and RT proteins, the latter from an internal AUG in a different reading frame from C (Fig. 1.1). As mentioned above, the C mRNA is also pgRNA, serving as the template for reverse transcription to produce progeny viral DNA (section "Reverse Transcription and NC Maturation"). The M and S envelope proteins are similarly translated from the PreS2/S mRNAs, which have heterogeneous 5′ ends bracketing the PreS2 initiation codon. Thus, the longer RNAs containing this initiation codon are translated to produce M and the shorter ones lacking it are translated to produce S. This gene expression strategy effectively increases further the coding capacity of the highly compact HBV genome.

NC Assembly

The next stage in the viral life cycle ensues once C and P are translated from their shared mRNA, which doubles further as the template for reverse transcription (pgRNA) as alluded to above. These three components, the C and P protein and their shared mRNA (pgRNA) are all the viral factors needed for intracellular HBV replication (i.e., in the absence of virus secretion or infection). Assembly of the replication-competent NC requires the incorporation, into the same capsid, of both pgRNA—the template for reverse transcription, and P—the catalyst for DNA synthesis, by assembling C protein dimers. HBV has evolved to satisfy this dual (the P protein and pgRNA) packaging requirement by initiating NC assembly via the formation of a specific pgRNA-P ribonucleoprotein (RNP) complex, which then serves to trigger NC assembly (Fig. 1.2, step 6). A short structured RNA signal, called ε, located at the 5′ end of pgRNA (Fig. 1.3, step 1) was identified as the RNA packaging signal that mediates the packaging of pgRNA into NCs [106, 107]. ε was later found to be recognized specifically by the P protein (Fig. 1.3), not C, and P and pgRNA packaging are mutually dependent [51, 108, 109].

ε forms a conserved stem-loop structure with an apical loop and two short stems separated by an internal bulge (Fig. 1.3). To form the RNP complex, the internal bulge but not the apical loop is required [50, 110, 111]. However, for ε to serve its RNA packaging function, both the internal bulge and apical loop are required [112–114]. Furthermore, a closely spaced 5′ cap next to ε is also critical for pgRNA packaging in HBV [115] but dispensable for RT-ε interaction [50, 110, 111]. This requirement for a closely spaced 5′ cap in pgRNA packaging provides a satisfying explanation for the failure of the other copy of ε, which is located at the 3′ end of all viral RNAs (Figs. 1.1 and 1.3), to serve as a functional RNA packaging signal. Similarly, the requirements from P for pgRNA packaging go beyond those required for ε binding. Whereas only parts of the TP and RT domains are required for ε

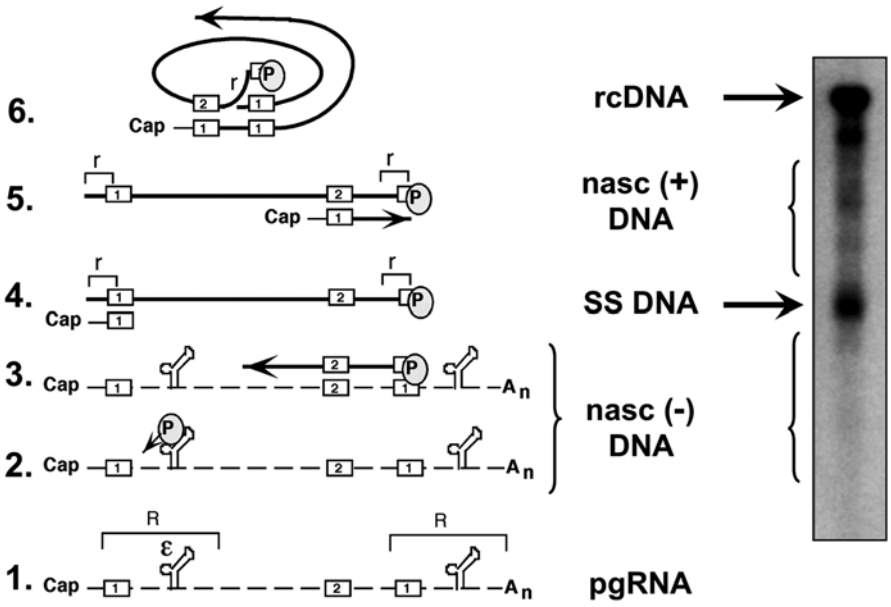

Fig. 1.3 HBV reverse transcription pathway. The pathway is depicted schematically from *bottom* to *top* on the *left* so as to match the Southern blot image of replicative viral DNAs extracted from intracellular NCs shown on the *right*. (*1*) The pgRNA (*dashed line*) harbors a large terminal repeat (R) that bears the RNA packaging signal, ε, and DR1. (*2*) P protein binding to ε triggers protein-primed initiation of (−) strand DNA synthesis at ε and packaging of pgRNA into NCs (not depicted). (*3*) (−) strand template switch to the 3′ DR1 and continuation of (−) strand DNA synthesis. (*4*) Degradation of pgRNA as (−) strand DNA synthesis proceeds, leaving a capped RNA oligomer containing DR1. (*5*) Translocation of the RNA oligomer to DR2 to prime (+) strand DNA synthesis. (*6*) (+) strand template switch from the 5′ to the 3′ end of the (−) strand DNA facilitated, in part, by the short terminal repeat (r) on the (−) strand DNA, circularizing the DNA, and continuation of (+) strand DNA synthesis for a variable length generating rcDNA. The *boxed numerals 1* and *2* denote DR1 and DR2. A_n polyA tail, *nasc* nascent

binding [110, 111, 116–118], pgRNA packaging additionally requires P sequences extending into most of the RNase H domain [51, 52, 116, 119]. Interestingly, four conserved Cys, three within the C-terminal portion of the initially defined spacer region and one in the RT domain, were found to be required for both ε binding and pgRNA packaging [116, 120].

In addition to the viral P protein and ε RNA, host cell factors play an important role in RNP formation, and thus in pgRNA packaging (and protein-primed initiation of viral reverse transcription or protein priming, see section “Reverse Transcription and NC Maturation” below). In particular, the chaperone proteins heat shock protein 90 (Hsp90) and Hsp70, and other co-chaperones, are required to establish and maintain a P conformation active in ε binding (Fig. 1.2, step 6) [121–125]. One structural effect on P elicited by chaperone action is the exposure of a site in TP that may directly bind ε RNA [126]. Deletion of the RNase H domain and the C-terminal

portion of the RT domain in the DHBV P protein leads to a "mini" P that is active in ε binding (and protein priming) independent of the cellular chaperones. This suggests a model for conformational activation of P whereby the chaperones act, during P-ε interaction, to relieve the auto-inhibitory effects on ε binding exerted by these C-terminal sequences of P [127], which are nevertheless essential for the later stages of viral replication (see section "Reverse Transcription and NC Maturation").

Another essential viral protein for NC assembly, of course, is the C protein itself, which forms the capsid shell enclosing the P-pgRNA complex. As discussed above, the NTD of C alone is able to form empty capsids and when the CTD is present, those capsids can incorporate nonspecific RNAs when assembled in bacteria or in vitro [23, 24, 26, 128]. On the other hand, for the capsids to package the P-pgRNA RNP complex, the CTD is required and furthermore, has to be phosphorylated in HBV (Fig. 1.2) [129, 130]. Precisely how the assembling C dimers recognize the P-pgRNA complex remains unknown. A recent cryo-EM study revealed that P is located at a unique position inside NCs [131], consistent with the suggestion that the initial site of interactions between the P-pgRNA complex and first C dimers may mediate the nucleation of NC assembly. The kinetics of capsid assembly also influences P-pgRNA packaging as either C mutants or small molecules binding to C that perturb assembly kinetics inhibit P-pgRNA packaging [132–134].

As in bacteria, HBV capsids also assemble in authentic human host cells without incorporating the RT-pgRNA complex. These empty capsids are produced in large excess relative to the replication competent NCs both in cell cultures and in the liver (Fig. 1.2, step 6a) [11, 135] and are indeed secreted as empty virions upon envelopment (i.e., enveloped capsids without DNA or RNA) (section "NC Envelopment and Virion Secretion"). In contrast to assembly in vitro or in bacteria, the nonproductive assembly in mammalian cells leads to the formation of truly empty capsids, packaging little to no RNA. One reason to account for this difference may be the fact that CTD is heavily phosphorylated in mammalian cells (Fig. 1.2, steps 6 and 6a) (but not in bacteria), which leads to the inhibition of its nonspecific RNA binding activity as described above.

It has long been known that HBV capsids package a cellular protein kinase(s), the so-called endogenous kinase [136]. Kinase packaging into capsids is evidently independent of either the P protein or pgRNA [34]. While early work suggested that the endogenous kinase was possibly PKC [33], recent work indicates that the cellular CDK2 may be the major endogenous kinase and it can phosphorylate specifically the CTD in capsids (Fig. 1.2, steps 6 and 6a) [34].

Reverse Transcription and NC Maturation

Once both the P protein and pgRNA are packaged into NCs, viral DNA synthesis occurs converting the RNA pregenome to the characteristic rcDNA genome (Fig. 1.2, step 7). This process of reverse transcription in hepadnaviruses is also defined as NC maturation. NCs, as initially assembled and containing pgRNA, as well as those

containing the SS DNA intermediates are considered to be immature as they are not secreted in virions, in contrast to those containing the rcDNA that are secreted in virions and are therefore considered mature.

Protein-Primed Initiation of Reverse Transcription

Initiation of viral reverse transcription is triggered by the same P-pgRNA interaction described above that triggers NC assembly. Indeed, initiation of viral DNA synthesis could occur before, during, or after NC assembly, since C is dispensable for initiation of DNA synthesis [48–50, 137]. In an unusual reaction, very different from conventional retroviruses and so far, unique to hepadnaviruses, initiation of (−) strand DNA synthesis occurs via protein priming and the catalyst of DNA synthesis, i.e., the P protein itself, is also used as a specific protein primer. In addition to being essential for pgRNA packaging, the ε RNA element, specifically its internal bulge, also serves as the template for protein-primed DNA synthesis [50, 138–142]. The result of protein priming is a short (3–4 nt long) DNA oligomer covalently attached to the P protein (Fig. 1.3, step 2), specifically, a Tyr residue within its TP domain.

Protein priming is a highly complex reaction that requires multiple determinants of both the ε RNA and P protein and additionally, host factors. The ε RNA, in addition to serving as the template for DNA synthesis, also functions as an allosteric activator of the P enzymatic activity during protein priming [143, 144]. Similarly, upon RNP formation, the ε RNA also undergoes significant structural rearrangement thought to be critical for protein priming [145, 146]. As discussed above, HBV protein priming, like pgRNA packaging, also requires a 5′ cap near the ε RNA signal and thus only the 5′, but not the 3′ copy of ε on pgRNA can support protein priming (Fig. 1.3, step 2) [50, 147]. Therefore, host factors binding the cap structure are potentially needed for protein priming, in addition to the host chaperones discussed above that play a critical role in facilitating RNP formation. The apical loop of ε, like the cap structure, is dispensable for P binding (above) and is not part of the template sequence, but yet is essential for protein priming [50]. While the exact role of these RNA elements in protein priming (or pgRNA packaging, see above) remains to be defined, the similar requirements for these two related reactions, both being critically dependent on P-pgRNA interaction, may suggest these two processes are mechanistically linked to ensure that viral DNA synthesis will only occur using an RNA template that can also be packaged into NCs.

The Tyr residue used to prime (−) strand DNA synthesis resides in the N-terminal TP domain of P (Y63 in HBV and Y96 in DHBV) [50, 148–150]. The structural basis for selecting the particular Tyr residue as the primer remains ill-understood. “Cryptic” sites, i.e., other Tyr (or even Ser/Thr residues) in both the TP and RT domains, can also serve to initiate protein-primed synthesis of short DNA strands, albeit inefficiently [151, 152]. However, they cannot support viral replication. In addition to the priming Tyr residue, other sequences in TP are also critical for protein priming by participating in ε RNA binding and possibly helping to present the primer to the RT active site [50, 116, 126, 153].

Protein priming in HBV also requires the RT domain and most of the RNase domain. The RT domain bears the Tyr-Met-Asp-Asp polymerase catalytic center, which is required to form the initial phosphotyrosyl linkage between the 5′ dGMP residue and the priming Tyr residue in TP during priming as well as for all subsequent DNA polymerization [48, 50, 150]. Additional RT domain sequences, beyond the polymerase active site, are required for ε binding and DNA synthesis during protein priming [116, 153]. Also, although both protein priming and pgRNA packaging (see section "NC Assembly") require additional sequences and structures from the ε RNA and P protein beyond RNP formation, distinct requirements also exist for these highly related reactions [116]. In particular, the very C-terminal portion of the RNase H domain is required for pgRNA packaging but not for protein priming, suggesting that these RNase H sequences may interact with the viral C protein, which is similarly required for NC assembly but not protein priming. In contrast, other sequences in the TP and RT domains are required for protein priming but not pgRNA packaging, suggesting that these may play a role in positioning Y63 in the RT active site to allow priming or in some aspect of catalysis per se.

DNA Synthesis Following Protein Priming

Following protein priming, which occurs at the 5′ end of pgRNA, the short (−) strand DNA oligomer attached to P is translocated to a site (acceptor) overlapping the short (ca. 12 nt) sequence motif DR1 (Fig. 1.1) near the 3′ end of pgRNA before DNA synthesis continues (Fig. 1.3, step 3) [14, 138, 147, 154]. In addition to the short (3–4 nt) sequence complementarity between the nascent (−) strand DNA and the pgRNA sequence at the acceptor site, this (−) strand template switch reaction is facilitated by other *cis*-acting elements on pgRNA, which help bring together spatially the 5′ donor site at ε and the 3′ DR1 acceptor site on pgRNA via base-pairing [155, 156]. As (−) strand DNA synthesis continues, the RNase H activity of P degrades pgRNA except its extreme 5′ end (Fig. 1.3, step 4) [157]. The preserved 18-nt long capped RNA oligomer is subsequently used as a primer to initiate (+) strand DNA synthesis, but in most cases, only after the RNA primer is first translocated from the 3′ end to near the 5′ end of the (−) strand DNA (Fig. 1.3, step 5) [14, 15, 158]. This (+) strand primer translocation is facilitated by sequence complementarity between the other DR1 motif at the 5′ end of the terminally redundant pgRNA (part of the RNA primer) (Fig. 1.3, step 1) and an identical sequence motif called DR2 near the 5′ end of the (−) strand DNA. (+) strand DNA synthesis soon reaches the 5′ end of the template (−) strand DNA, when another template switch occurs resulting in the translocation of the elongating 3′ end of the (+) strand DNA from the 5′ to the 3′ end of the (−) strand DNA and the circularization of the DNA product (Fig. 1.3, step 6). This (+) strand template switch is facilitated by the short (ca. 9 nt) terminal repeat (r) at both ends of the (−) strand DNA, as well as multiple other *cis-acting* sequences on the template (−) strand DNA, which function, as in (−) strand template switch, to bring the donor and acceptor sites together spatially [159–162]. (+) strand DNA synthesis then continues to at least half completion

before the maturing NC is enveloped and secreted or recycles its rcDNA content to the nucleus for amplifying the cccDNA pool (see below).

Failure to translocate the RNA primer during (+) strand DNA synthesis leads to the production of the double stranded linear DNA (dslDNA) via an alternative, minor pathway of DNA synthesis when the primer is elongated in situ [163]. dslDNA is the predominant viral DNA substrate for integration into host chromosomes via nonhomologous recombination (Fig. 1.2, step 3a), which occurs early during acute infection and accumulates over time during the chronic phase of infection [164–166]. Integrated dslDNA is unable to support viral replication as it cannot direct the expression of the genomic RNA species. However, it can drive the expression of the viral envelope proteins (Fig. 1.2, step 4a) and has diagnostic implications (see section "NC Envelopment and Virion Secretion" for more detail).

Whereas C appears to be dispensable for the protein priming stage of viral reverse transcription, it clearly functions as a critical *trans*-acting factor, in addition to the enzyme P itself, in all subsequent stages of viral DNA synthesis. In particular, the phosphorylation state of its CTD plays an integral role in facilitating reverse transcription (Fig. 1.2, steps 6 and 7), possibly via regulating the charge state of the maturing NC or the CTD function as a nucleic acid chaperone [167–173]. Recent structural studies suggest indeed that the phosphorylation state of CTD can influence pgRNA organization in the NCs [174]. In DHBV, CTD is heavily phosphorylated in immature NCs, which is required to facilitate (−) strand DNA synthesis, but it is completely dephosphorylated in mature NCs, which is required to facilitate (+) strand DNA synthesis and to stabilize mature NCs once rcDNA is synthesized (Fig. 1.2, step 7) [36, 169, 175, 176]. Also, the NTD, in addition to forming the capsid shell, may play an active role in facilitating reverse transcription [133, 134, 177].

It is likely that additional host factors regulate viral reverse transcription, other than those required for protein priming and the host kinase(s) (e.g., CDK2) and phosphatase(s) that regulate the state of CTD phosphorylation, as described above. For example, induction of the early stage of autophagy was reported to facilitate HBV DNA replication [62]. On the other hand, the antiviral deaminase proteins, the Apobec3 proteins, can be incorporated into NCs and block the early stage of (−) strand DNA synthesis when overexpressed ectopically [178–180], although whether levels of Apobec3 proteins under physiological conditions in vivo ever reach those needed for viral inhibition remains uncertain.

Like the RT enzymes encoded by retroviruses (e.g., the human immunodeficiency virus or HIV) and RNA-dependent RNA polymerases of RNA viruses (e.g., the hepatitis C virus or HCV), the HBV P protein lacks proofreading activity. As a result, HBV DNA replication is associated with a much higher (by ca. 10^4-fold) error rate as compared to host cell DNA replication, resulting in viral genetic variations. However, its compact genetic organization means that HBV variations that are viable (thus observable) are not nearly as great as HIV or HCV, since many of the variations will be lethal due to their detrimental effects on multiple overlapping coding sequences and/or *cis*-acting sequences important for viral gene expression or replication (Fig. 1.1). Still, HBV can be classified worldwide into eight to ten genotypes, with inter-genotype differences being 8 % or higher [181]. Furthermore, the

sequence variations provide opportunities for selecting drug resistant or vaccine escape mutants under drug treatment or immune pressure (see section "NC Envelopment and Virion Secretion" also).

Nuclear Recycling of rcDNA and Amplification of cccDNA

As alluded to above, cccDNA can be derived from de novo synthesized rcDNA in intracellular mature NCs, in addition to rcDNA in the incoming virion. In this process, mature NCs deliver (recycle) their rcDNA content into the nucleus (instead of secretion extracellularly as virions, see section "NC Envelopment and Virion Secretion") to make more cccDNA (Fig. 1.2, step 8), which amplifies the cccDNA reservoir for production of more pgRNA and other viral RNAs. Discovered initially in DHBV infected primary duck hepatocytes [182, 183], this process also occurs in hepatoma cells replicating DHBV and HBV [184–188]. Through this intracellular amplification pathway (and possibly super-infection as well), the steady state level of cccDNA is maintained at ca. 1–17 copies per cell as shown using DHBV-infected duck livers [189].

The viral envelope protein, L, directly regulates cccDNA amplification through an apparent negative feedback mechanism. Thus, when cccDNA levels are low, e.g., during the early stage of infection, L protein levels are low and rcDNA in the mature NCs is recycled to the nucleus to form more cccDNA. Later during infection, when the cccDNA levels are raised, more L proteins are produced that block this recycling pathway and instead direct the mature NCs for envelopment and secretion extracellularly [184, 185, 190]. The C protein is also involved in this recycling process, and the recently revealed destabilization of mature NCs, relative to immature ones [191], likely facilitates the uncoating of mature NCs and release of rcDNA for cccDNA formation. The much lower efficiency of cccDNA amplification by HBV compared to DHBV in the same cells [187, 192] also indicates that viral specific factors can affect the efficiency of cccDNA formation. The differential binding of host factors by CTD in a phosphorylation state-dependent manner [193] may play a role in the nuclear recycling of mature NCs or virion formation (see section "NC Envelopment and Virion Secretion"), as mature NCs, as opposed to immature ones, are dephosphorylated.

The involvement of host factors in cccDNA formation is also suggested by the age-related difference in DHBV cccDNA formation kinetics (being more rapid in young ducklings) [194]. Moreover, as mentioned above, normal mouse hepatocytes fail to accumulate cccDNA [195] and the elimination of the liver specific transcription factor, HNF1α, increases (albeit only weakly) levels of cccDNA in these cells [196], again indicating that host cell factors, related to the physiological or differentiation state, can influence cccDNA levels. In particular, host DNA damage repair factors probably play a direct role in the conversion of rcDNA to cccDNA [187, 197], which involves a number of distinct biochemical reactions. These include the completion of (+) strand DNA synthesis, removal of the capped RNA primer from the 5′ end of the (+) strand DNA, removal of the covalently attached P protein from

the 5′ end of the (−) strand DNA, removal of precisely one copy of the terminal repeat (r) from the (−) strand DNA, and the ligation of both DNA strands. To date, a role for the P protein, the only viral protein with any enzymatic activities, in cccDNA formation has not been conclusively demonstrated although it could play a role conceivably in completing the (+) strand DNA synthesis. This and all the other reactions required for cccDNA formation are instead likely carried out by host DNA repair enzymes.

HBV cccDNA levels can reach five to ten copies per cell in hepatoma cell cultures, exclusively via intracellular amplification as those cells are not susceptible to HBV infection; however, over-amplification in the absence of the viral envelope proteins is limited, reaching only a few fold higher than the wild type (WT) virus [187, 188, 192, 198]. This is in contrast to DHBV, which can reach hundreds of copies of cccDNA per cell in either avian or human cells [36, 185]. It remains unclear if HBV cccDNA can be amplified to the same extent as DHBV in the liver or culture systems that would more closely mimic human hepatocytes in vivo. Even for DHBV, cccDNA amplification can be saturated, suggesting the need for rate-limiting host factors [36]. Whatever these host factors may be, they are unlikely to be strictly species- or hepatocyte-specific. DHBV forms cccDNA efficiently in duck, chicken and human cells [187, 192] and the human embryonic kidney cell line HEK293 supports cccDNA formation by both HBV and DHBV [36, 187].

To date, no clear intermediate in the conversion of rcDNA to cccDNA has been identified conclusively, which would facilitate studies on the mechanism of cccDNA formation. However, a rcDNA species, called protein-free (PF) or deproteinated (dp) rcDNA, accumulates in established cell lines that support HBV replication [187, 192, 198] but not in normal human hepatocyte in vivo [199] or in primary culture [65], nor in the chimpanzee liver [200]. PF-rcDNA resembles grossly the rcDNA in mature NCs except that the P protein is removed [187, 198]. However, the precise structure of PF-rcDNA, particularly, the structure of the 5′ end of the (−) strand DNA, from which the P protein (or at least the bulk of it) has been removed, remains to be more clearly defined. PF-rcDNA also accumulates in mouse hepatocytes when HNF1α is eliminated [196], suggesting that the accumulation of PF-rcDNA, like cccDNA, is subject to regulation by the host cell physiology. Although the PF rcDNA has been suggested to be a precursor to cccDNA (and thus a true intermediate during rcDNA to cccDNA conversion) [79, 187, 198], the possibility exists that it could instead represent a dead-end processing product from rcDNA and cannot be converted further to cccDNA. How the P protein is removed during the formation of the PF-rcDNA (or cccDNA) remains unknown. However, the host DNA repair factor, tyrosyl-5′ DNA phosphodiesterase 2 (Tdp2), has been shown to cleave precisely at the P protein-(−) strand DNA junction [50, 201, 202], which is not entirely surprising given that an important cellular function of Tdp2 is to remove covalently trapped topoisomerase II (Topo II) from Topo II-DNA adducts [203] with exactly the same phosphotyrosyl-DNA bond at their protein–DNA junction as that found at the 5′ end of the viral (−) strand DNA. Whether Tdp2 plays a role in HBV cccDNA formation remains to be clarified although a recent report suggests that Tdp2 may play a modest role in facilitating DHBV cccDNA formation in human hepatoma cells [202].

NC Envelopment and Virion Secretion

To complete the viral life cycle, the mature NCs containing rcDNA acquire the host-derived lipid bilayer studded with the viral envelope proteins via budding into the lumen of an intracellular vesicle thought to represent the late endosome or multivesicular body (MVB) [204, 205] and secreted outside of the cells via the cellular secretory pathway. As with many other enveloped viruses, the cellular ESCRT proteins critical for host vesicular trafficking may play a role in HBV virion secretion although pleiotropic effects of these factors on NC maturation makes the interpretation for a specific role of ESCRT proteins in virion formation difficult [204–206].

A particularly interesting aspect of HBV virion morphogenesis is the selection of the "correct" NCs, i.e., only the mature ones containing rcDNA (or dslDNA) but not the immature ones containing SS DNA or pgRNA, for envelopment and virion formation (Fig. 1.2, step 9) [6, 11, 207–210]. A putative maturation signal, which emerges on the mature NCs following rcDNA synthesis, was hypothesized long ago to direct the viral envelope proteins in the selection of the mature NCs for envelopment [6]. The nature of this signal, or the exact timing of its emergence during NC maturation, remains poorly understood. As mentioned above, the (+) strands of HBV rcDNA found in virions in the blood of infected patients are heterogeneous; they can be up to half incomplete but mostly are several hundred nt from completion [11, 13]. Elongation of the (+) strands may stop when the nucleotides that are trapped in the enveloped virion particles are exhausted and no additional nucleotides can get into the virions. Indeed, dissolution of the virion membrane and provision of nucleotides allow further (+) strand DNA elongation of the virion rcDNA during the endogenous polymerase reaction [54], and blocking envelopment can increase the length of the (+) strands in rcDNA [188].

The C protein likely plays an integral role in the selection of mature NCs for virion formation as it forms the NC shell and is thus situated appropriately to transmit the nature of the nucleic acid inside NC (rcDNA vs. SS DNA or pgRNA) to its exterior so as to allow the viral envelope proteins to differentiate NCs with different maturity. In other words, the envelope proteins have to sense, indirectly, the interior content of the maturing NC through maturation-associated structural changes on the capsid surface, which could constitute the elusive maturation signal. Indeed, NTD mutants have been identified that remain competent for rcDNA synthesis but are defective in virion formation [211–213]. Furthermore, other NTD mutations lead to the secretion of SS DNA in virions ("immature secretion") [214, 215]. Interestingly, the snow goose hepatitis B virus (SGHBV) [216] naturally secrets SS DNA-containing virions, in contrast to all other hepadnaviruses identified to date, and two specific residues [74, 107] in the NTD of the SGHBV C protein have been identified as responsible for this immature secretion phenotype [217]. These results thus all suggest that NTD may contain the maturation signal or is at least involved in generating the signal during NC maturation.

On the envelope side, it has been long known that the L and S, but not M, proteins are required for virion formation [19]. More recently, the C-terminal portion of the PreS1 region of L was identified as a "matrix" domain (MD) that is thought to

recognize the mature NCs [218–221]. As discussed above, the N-terminal portion of the PreS1 region also mediates receptor (NTCP) binding during virus entry, with this dual role in NC and receptor recognition being accommodated by a dramatic shift of the PreS1 topology following NC envelopment. The host chaperone, heat shock cognate protein 70, may play an essential role in virion formation by retaining the PreS1 region on the cytosolic side of the ER membrane during L protein synthesis to allow it to serve its MD function [222].

Secretion of HBsAg Spheres and Filaments

As described above, HBV virion secretion is characterized by the release of a large excess of defective subviral particles containing only the envelope proteins (HBsAg particles, including the spheres and filaments) (Fig. 1.2, step 9a). The cellular pathway for secreting these particles appears to be distinct from that used for virion secretion [204], which is also suggested by the fact that the virions and HBsAg particles contain a different complement of viral envelope proteins (see section "The Envelope Proteins" above). The functions of the HBsAg particles remain to be better defined although they probably act as a decoy for the virions to protect the latter from host neutralizing antibodies that target the envelope proteins.

Secretion of Empty Virions

Given the above discussion on selective HBV virion formation, it was indeed surprising to find that HBV also secrets a large excess (typically >100-fold above the DNA-containing or complete virions) of empty virions in vivo and in vitro, which contain the envelope and the capsid but no genome (Fig. 1.2, step 9b) [11, 12]. In sharp contrast to complete virions, the secretion of these empty virions is completely independent of pgRNA packaging or DNA synthesis [11, 223]. In retrospect, these empty virions were probably detected decades ago, even before the discovery of reverse transcription in hepadnaviruses, as "light" Dane particles [224, 225] but received little attention, perhaps deemed to be an artifact of virion isolation. To reconcile the apparent stringency in selecting mature (but not immature) NCs for complete virion formation and the secretion of empty virions containing no genome at all, it was proposed that a SS DNA (or pgRNA)-dependent "blocking signal" is induced in immature NCs that actively prevents their envelopment [11]. The empty capsids, devoid of any nucleic acid, lack such a negative signal and can thus be enveloped and secreted as empty virions. However, the requirements from either the capsid or the envelope for secretion of these empty virions need to be characterized and it remains possible that the secretion of the complete and empty virions may involve distinct signals and pathways. Similarly, the functions of empty virions remain to be determined.

Like HBsAg particles (Australian antigen), which greatly facilitated the discovery of HBV and the development of both the diagnostics for HBV infection and the

first generation HBV vaccine that was derived from these particles in the human serum [1], the empty virions may also prove to be valuable as a diagnostic marker and perhaps, a new vaccine candidate. On the diagnostic side, a recent pilot study found that the ratios of empty to complete virions in the sera of HBV infected patients vary greatly (50–100,000:1) [12]. Among other factors, this ratio may reflect the efficiency of intrahepatic assembly of empty vs. pgRNA-containing capsids, which, together with the efficiency of reverse transcription and virion assembly, ultimately determine the ratio of empty vs. complete virions in the blood (Fig. 1.2). Furthermore, the empty virions, which can be readily monitored as serum hepatitis B core antigen (HBcAg) (due to the large excess of empty virions relative to complete ones, the contribution of the complete virions to serum HBcAg is negligible), may be useful as an easily accessible biomarker to monitor antiviral responses, in particular, the levels and transcriptional activity of cccDNA in the liver during treatment with inhibitors of viral DNA synthesis. Treatment with a nucleoside analog drug that inhibits the DNA polymerase activity of the P protein effectively blocks the secretion of complete virions in virtually all cases, but the secretion of empty virions is not decreased in most cases [12]. This is exactly as predicted given that DNA synthesis is required for secretion of complete virions, but dispensable for empty virions. On the other hand, if the hepatic cccDNA levels (or its transcriptional activity) is decreased or eliminated, the production and secretion of empty virions will be reduced or eliminated as both C and envelope proteins are required for empty virion production [12]. Although serum HBsAg particles have been suggested as a marker for hepatic cccDNA, they can also be produced from integrated viral DNA (Fig. 1.2, step 4a) [226, 227], which accumulates to high levels during chronic infections [166] and is not decreased by viral polymerase inhibitors [166], and therefore, are not reliable for monitoring cccDNA especially during the later stage of chronic infection [228, 229]. Secretion of empty virions, on the other hand, requires also the viral C protein, which is unlikely to be produced from the integrated DNA due to the disruption of the C gene in the dslDNA, the precursor to the integrated HBV DNA (Fig. 1.2, step 3a) [164], and thus should be a more reliable marker for hepatic cccDNA. Under antiviral therapy, significant decrease of serum empty virions (and thus HBcAg), without HBsAg decrease in parallel, could reflect a reduction of intrahepatic cccDNA level (or its transcriptional activity) leading to a decrease in HBcAg (hence empty virion) production but not serum HBsAg, whose expression may be driven exclusively from integrated HBV DNA [12]. Another potential marker for hepatic cccDNA is the secreted HBeAg, which like empty virions probably can only be produced from cccDNA but not integrated viral DNA. However, HBV frequently mutates to reduce or eliminate HBeAg expression under immune pressure (Fig. 1.2, step 9c) [228], rendering HBeAg less useful or no use at all as a biomarker for cccDNA.

The empty virions could also form the basis for a new generation of HBV vaccine. The current recombinant (second generation) HBV vaccine contains the S envelope protein only. Though it is very safe and effective in most cases, it does not induce sufficient response in some vaccinees. Also, as the vaccine elicits predominantly neutralizing antibodies against a single epitope (the “a” determinant as discussed

above) in S, HBV can evolve mutations in this determinant to escape the vaccine-induced antibodies [230, 231]. To potentially exacerbate the vaccine escape problem, inhibitors of the P protein can also select drug-resistant mutants that are, additionally, vaccine escapees. Due to the overlap of the P and S coding sequences (Fig. 1.1), certain drug resistant mutations in the P gene also encode vaccine-escape S proteins in the overlapping S gene [232, 233]. A potential (third generation) HBV vaccine could be based on empty virions and would contain all the viral structural proteins but no genome. Such as vaccine should be as safe as the current vaccine but may help overcome the limitations of the current vaccine by providing additional antigenic determinants for both humoral and cellular immunity, the latter of which targets mostly the internal C protein and may render an empty virion-based vaccine effective for therapeutic as well as prophylactic purposes.

Perspectives

HBV research is experiencing a renaissance in recent years, along with the advent of effective antiviral therapies that can dramatically suppress viral replication and potentially improve the prognosis for hundreds of millions of chronically HBV-infected patients worldwide [234]. With the exception of type I interferon that is thought to derive its efficacy from immune-regulatory functions as well as direct antiviral activities, both of which are complex and still ill-understood, all other currently approved treatments target the HBV P protein, specifically its DNA polymerase activity in the RT domain, and belong to the same structural class—nucleoside analogs. As the viral life cycle becomes understood in greater detail, it is anticipated that more antiviral therapies targeting different stages of the life cycle and different viral proteins will be forthcoming. Along with better strategies to manipulate the host immune response, these antivirals may be able to bring about the complete elimination of the nuclear cccDNA reservoir and thus cure chronic HBV infection. Viral and host targets that can be potentially exploited include entry by using the PreS1 peptide responsible for NTCP binding as well as using small molecule NTCP ligands to disrupt virus-cell binding [235, 236]; NC assembly using small molecules binding to the viral C protein [132, 237, 238]; additional functions of P such as ε binding and RNase H activity and novel ways of inhibiting its polymerase activity [47, 53, 239, 240]; and inhibition of cccDNA formation [36, 241]; and possibly suppression of cccDNA transcriptional activity [99, 100] or even degradation of cccDNA [242, 243]. These and other antiviral strategies as well as immune modulation approaches will be detailed elsewhere in this volume. Similarly, the large amounts of classical HBsAg particles and HBeAg released into the blood stream, though nonessential for viral replication, have been extremely valuable for monitoring viral infection and as the basis for prophylactic vaccines, and the recent discovery of empty virions may yet spur the development of new diagnostics and vaccine candidates.

On the other hand, important gaps in the understanding of the HBV life cycle persist. The viral entry mechanism remains to be better defined even though the

recent identification of NTCP as the HBV receptor should undoubtedly accelerate studies in this area. The intracellular trafficking and uncoating of the NCs to deliver rcDNA to the host cell nucleus for cccDNA formation, and the process of cccDNA formation itself, remain poorly understood. The X function(s) important for viral replication remains to be clarified, as is the molecular basis of virion morphogenesis. A high-resolution structure of the P protein, central to viral DNA replication and the only viral protein with any enzymatic activities, will help greatly understand its multiple functions and biochemical properties, including the structural basis of chaperone activation, and reveal novel ways of blocking P functions. With the recent resurgence of interest in HBV, it is anticipated that these and other issues in HBV virology will be resolved within the next decade, which should prove great news for both students and victims of this most interesting and deadly virus.

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Chapter 2
Experimental Models: Cell Culture and Animal Models

Maura Dandri, Tassilo Volz, and Marc Lütgehetmann

Introduction

The hepatitis B virus (HBV) is a member of the hepadnavirus family, which are small enveloped DNA viruses that can cause acute and chronic hepatitis in their respective hosts. Although none of these hosts belongs to the typical laboratory animals, most of our knowledge in HBV biology has been gained from studies with the two viruses closely related to HBV: the woodchuck hepatitis virus (WHV) and the duck hepatitis virus (DHBV). Studies with DHBV, which infects Peking ducks, have substantially contributed to our understanding of viral replication [1], while experiments performed with WHV, which infects the Eastern American woodchucks, have provided important insight about factors involved in the establishment of virus infection, persistence, and hepatocarcinogenesis [2–5]. Based on the close phylogenetic relationship between primates and tree shrews [6, 7], the Asian tree shrew species *Tupaia belangeri* has been frequently used for in vitro and in vivo

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infection studies both with HBV and with the woolly monkey hepatitis B virus (WMHV) [8].

Regardless of the animal model used, a hallmark of all hepadnaviruses is their unique genomic organization with asymmetric mechanism of replication and their high species and cell-type specificity. Thus, only chimpanzees and, to a certain extent, tupaias are susceptible to infection with the human HBV. The in vitro susceptibility of primary human hepatocytes (PHHs) to HBV is also limited, since these cells loose their differentiation status, and hence their ability to express the cellular receptor mediating viral entry, shortly after plating [9–13]. Apart from the possibility to infect an in vitro differentiated bipotent progenitor cell line (HepaRG cells), only recently the identification of the sodium taurocholate cotransporting polypeptide NTCP as functional cellular receptor mediating HBV entry [9] has opened new opportunities to establish new in vitro infection systems.

Although mice and rats are not permissive for HBV infection, the availability of mouse models of HBV replication has provided the opportunity to assess the efficacy of antiviral agents and to study some aspects of the antiviral adaptive immune responses to HBV. However, the obvious limitations imposed by the use of great apes and the need to work with inbred, well-characterized and small animal systems have limited our understanding of HBV biology and pathogenesis, as well as the development of more efficient antiviral therapies. As a consequence, and despite the existence of a safe HBV vaccine, persistent HBV infection continues to be a major health problem worldwide (WHO report, update 2015) [14].

The therapeutic strategies currently available can efficiently suppress viral replication, but are unable to eradicate the infection. This is due both to the persistence of the HBV genome, the covalently closed circular DNA (cccDNA) present as a stable minichromosome in the nucleus of infected hepatocytes, and to the poor ability of the immune system to counteract chronic HBV infection [15]. To recapitulate the entire HBV life cycle in the natural target of HBV infection, the human hepatocyte, most researchers have focused on the development of more efficient in vitro infection systems and sophisticated small animal models based, for instance, on the use of human chimeric mice.

Cell Culture Models

Commonly used hepatoma cell lines are not susceptible to HBV infection, since they have lost the capability to express the cellular receptor permitting viral entry (Fig. 2.1). To overcome such limitations, the transfection of human hepatoma cell lines with recombinant HBV DNA constructs carrying overlength HBV genomes (1.1–1.3 unit length) has been used for almost three decades to study mechanisms of HBV replication and morphogenesis [16, 17]. These in vitro assays have also served to assess the efficacy of antiviral compounds directed against the HBV polymerase [18] to determine the replication capacity of clinically relevant drug-resistant HBV strains [19–21] and to characterize specific HBV mutations [22]. Moreover,

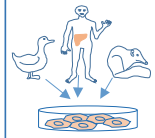
	cell culture			duck	wood-chuck	chimp	mouse		
	 HBV DNA Transfection, HepG2.2.15	 HepaRG, hNTCP-HepG2	 primary hepatocytes				 HBV transgenic	 genome transfer	 human liver chimeric
Virus source									
Applications (studies of mechanisms & drug testing):									
entry	-	++	++	-/+	++	+++	-	-	+++
cccDNA	+	++	++	+++	+++	+++	-	-	+++
HBV replication	+++	+++	+++	+++	+++	+++	++	++	+++
viral clearance	-	+	+	+	+++	+++	-	+	++
Interactions with immune cells	-	-/+	-/+	-/+	++	+++	+	+	-/+
Interactions with hepatocytes	-	-/+	+	-/+	+	+++	-/+	+	+++
Pathogenesis	-	-	-	-	++	++	+	-/+	+
Limitations:									
	Cancer cells: Poorly reflecting hepatocyte function	Cancer cells: High virus titer needed for infection	Rapid loss of cell differentiation Limited availability of human hepatocytes	DHBV shares only 40% homology with HBV	Virus & host Differences Outbred Not easy to breed /housing	Strong ethical restrains high costs No availability for research	Studies with only one virus strain	Transient replication	Complex to generate Human chimerism variability

Fig. 2.1 Comparison of the different in vitro and in vivo systems currently available for HBV research with respect to application and limitation

cell lines harboring stable integration of HBV DNA genomes, like the HepG2.2.15 and HepAd38 hepatoma cells have been shown to be useful for drug screening [23, 24]. A hallmark of HBV infection establishment is the conversion of the incoming HBV DNA genome, the relaxed circular DNA (rcDNA) present in circulating virions, into a covalently closed circular DNA (cccDNA), which serves as template for the transcription of all HBV RNAs. Transfection of plasmid-free monomeric linear full-length HBV genomes in hepatoma cell lines was shown to promote the circularization of linear HBV-DNA genomes, thus permitting the formation of episomal cccDNA molecules in HBV non-permissive cells [25]. Furthermore, by employing recombinant baculovirus- and adenovirus-derived vectors harboring replication-competent HBV constructs, the establishment of a large number of cccDNA molecules could be observed. Using this type of approach, chromatin-immunoprecipitation studies have been performed to demonstrate that these HBV-DNA genomes associate with histone and non-histone proteins to build a minichromosome [26, 27]. Although the episomal HBV DNA molecules generated in these systems are not maintained in rapidly proliferating cell culture systems, these approaches permitted to study virus and host factors that are involved in the epigenetic regulation of the cccDNA [28–30].

In 2002 a bipotent progenitor cell line, the HepaRG cells, that was derived from a liver tumor associated with chronic HCV infection, was found to become permissive for HBV infection after being cultured for some weeks under specific conditions promoting the enhancement of various hepatocyte-specific functions [31]. As observed in PHH cultures, the addition of dimethyl sulfoxide (DMSO), hydrocortisone and polyethylene glycol (PEG) is necessary to increase cell differentiation and hence the efficacy of HBV infection [32, 33]. The use of these cells offered new possibilities to study the infectivity of clinical HBV isolates and variants [34], as well as to explore the capacities of peptides derived from the preS-envelope protein of HBV to block the entry of both hepatitis B and Delta virus in vitro [35]. Moreover, cell entry was shown to occur in a polarized manner in these hepatocyte-like cells [13], whereas additional host factors appeared to mediate virus endocytosis. An in vitro study showed for instance that caveolin-1-mediated endocytosis is required for HBV entry in HepaRG cells [36]. HepaRG cultures were also shown to produce infectious HBV particles for more than 100 days and were successfully used for antiviral studies [37]. Using both transfected hepatoma cell lines and HepaRG cells, non-immunosuppressive cyclophilin inhibitors, such as alisporivir, have recently been shown to affect HBV replication and HBsAg production [38].

Since differentiated HepaRG cells represent a mixture of hepatocyte-like and biliary-like epithelial cells, only a subset of these cells becomes susceptible to HBV infection [13, 39]. It is also worth noting that no intracellular cccDNA amplification or spreading of HBV infection was observed in HepaRG cultures. This may be in part due to the inefficient and very slow conversion of the input relaxed circular DNA into cccDNA determined in these cells [39]. A newly described hepatoma cell line, named HLCZ01, was shown to be permissive for infection with both HBV and HCV clinical isolates [40]. Interestingly, sustained viral replication, for up to 90 days, without evidence of overt HBV and HCV interference could be shown.

A highly variable susceptibility to HBV infection is commonly observed in primary human hepatocyte (PHH) cultures [41, 42]. This may be in part due to divergences in host genetic susceptibility to infection, as well as to the loss of hepatic phenotype shortly after plating [11, 12]. To enhance susceptibility of HBV infection and virion productivity, Shlomai and colleagues showed that micropatterning and coculturing of primary human hepatocytes with stromal cells (MPCCs) maintained prolonged infection that could be further boosted by suppression of the innate immune responses. Moreover, to limit donor variability bias, the authors also obtained HBV infection using induced pluripotent stem cells differentiated into hepatocyte-like cells (iHeps) [43]. Since HBV permissiveness occurred in these cells in a differentiation-dependent manner, this system may be used to study host factors involved in HBV infection establishment and productivity.

Only recently, the discovery of the cellular receptor has opened new possibilities to investigate the initial steps of HBV infection in vitro [9]. In this elegant study, Li and colleagues used a synthetic peptide corresponding to the myristoylated N-terminus of the preS1 protein of the HBV envelope to identify the cellular receptor responsible for viral entry. By using photo-cross-linking and tandem affinity purification procedures, the authors showed that the preS1 peptide specifically interacts with a sodium taurocholate cotransporting polypeptide (NTCP), a transmembrane transporter exclusively localized to the basolateral membrane of high differentiated primary hepatocytes. It is indeed the rapid decrease of NTCP expression observed in cultured PHHs, as well as the lack of this receptor in most of the hepatoma cell lines that account for the rapid loss or lack of HBV susceptibility in these in vitro systems. NTCP mediates the transport of conjugated bile salts and of some drugs from the portal blood into the liver.

Based on the discovery that NTCP is the functional HBV entry receptor, hepatoma cell lines constitutively expressing the human NTCP gene have been created and have demonstrated the successful establishment of HBV infection in a significant proportion of NTCP-transfected hepatoma cells [9, 44, 45].

The availability of in vitro assays permitting investigation of the early steps of infection, as well as rapid screening of new anti-HBV agents, is expected to open new opportunities in HBV research. By using HBV-susceptible cells, it has been observed that DDX3, a member of the DEAD-box RNA helicase family, can affect cccDNA transcription [46]. A sophisticated study involving the use of both human and duck in vitro systems provided evidence that HBV utilizes the cell tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2) to release the terminal protein of the polymerase from the rcDNA to initiate cccDNA biogenesis [47]. NTCP-transduced hepatoma cell lines are also expected to accelerate the acquisition of data revealing the interplay between HBV and host factors, as documented by studies showing that binding of the preS1 domain of the HBV envelope to NTCP inhibits its function [45]. Nevertheless, additional hepatocyte-specific factors appear to be involved in the HBV infection process, since infection rates and virion productivity are generally low in NTCP expressing cell lines. Intriguingly, the establishment of infection with the Hepatitis Delta Virus (HDV) but not productive HBV infection could be demonstrated in murine cells engineered to express the human NTCP [48]. Since

both HBV and HDV use the same envelope proteins for cell entry, further species-specific differences or the lack of essential cellular factors within the murine hepatocytes may be responsible for such discrepancies [44, 49].

Primary hepatocytes isolated from *Tupaia belangeri* represents a good alternative to carry out in vitro infection studies with HBV [8]. It should be noted that the use of in vitro systems based both on primary tupaia hepatocytes and hepatoma cell lines have been fundamental even to identify the cellular receptor and other factors involved in the HBV attachment process [9, 50]. In general, being faster and more convenient than in vivo experiments, cell culture studies are very useful to carry out high throughput antiviral screening approaches and single cell level analyses in well-controlled experimental settings (Fig. 2.1). However, in spite of the existence of highly sophisticated primary hepatocyte-based systems, cultured cells may respond differently to infections and to other stimuli than cells in the intact liver [51]. Having lost the capability to express various hepatocyte-specific genes, discrepancies between data obtained in vitro and in vivo have to be considered. Thus, insights gained using cell-based in vitro systems need to be verified in in vivo systems.

Animal Models

The Duck Model

A major advantage of employing the DHBV model is that primary hepatocytes from ducklings or embryos are easily accessible and domestic Pekin ducks can be used under normal laboratory conditions. Furthermore, ducks show high infectivity rates in vivo [52] and generally reach high levels of DHBV replication and antigen expression. In vitro and in vivo studies with DHBV have contributed substantially to elucidate the replication mechanism adopted by the hepadnaviruses [1, 53–56] and mechanisms involved in the biogenesis of the cccDNA [47]. Furthermore, antiviral studies with polymerase inhibitors indicated that a stronger cccDNA reduction could be achieved in animals displaying higher cell proliferation rates [57], suggesting that hepatocyte turnover may destabilize the cccDNA pool in infected cells. Although various antiviral compounds have been tested in the duck model [58–61], these animals appear to be less sensitive to potential toxic effects than woodchucks [62]. Therefore, the results of antiviral drug screening might be of limited value for human HBV infection due to specific features of DHBV and the corresponding host hepatocytes. For instance, nucleocapsid inhibitors of the HAP family which can potently inhibit HBV replication appeared inactive on DHBV [63, 64]. It should be also kept in mind that the DHBV genome is smaller than HBV and is the most distantly related virus, since it shares little primary nucleotide sequence homology (40 %) with HBV. Moreover, DHBV uses the duck carboxypeptidase D (DCPD) and not like HBV the NTCP as receptor for viral entry and the infection is mostly not associated with liver disease and development of hepatocellular carcinoma (HCC) (Fig. 2.1).

The Woodchuck Model

Because of the higher similarities of WHV to HBV in terms of genomic organization, experimentally induced infection of woodchucks, the American *Marmota monax*, with WHV has been fundamental in the preclinical evaluation of antiviral drugs now in use for treatment of HBV infection [65–71]. Moreover, experimental infection of newborn woodchucks almost invariably leads to chronic infection, whereas animals infected at older ages generally develop acute hepatitis. Since acute and chronic WHV infection in woodchucks show serological profiles similar to those of HBV infection in humans, the woodchuck system has provided important insight about virological factors involved in the establishment of virus infection [2] and persistence [72]. Viral integrations, which frequently lead to proto-oncogene activation of the myc gene [73], are commonly found in woodchucks even after resolution of transient infection with WHV [74], while their frequency increases dramatically in chronically infected animals [5]. Interestingly, viral integrations were used as genetic markers to estimate the fate of infected hepatocytes and the amount of cell turnover occurring in the course of infection [5, 74]. These studies revealed the existence of cccDNA-free hepatocytes containing "traces" of the infection in form of viral integrations, thus indicating that cccDNA loss can occur also without destruction of the infected cells [5]. Since nearly all neonatally infected woodchucks develop hepatocellular carcinoma, this is the most used model of viral-induced HCC and has contributed to the development of new imaging agentsfor enhancement of detection of hepatic neoplasms by ultrasound and magnetic resonance imaging [73].

Numerous nucleoside analogues currently used in the clinic have been first assessed in woodchucks. Lamivudine, for instance, acted as a nontoxic antiviral drug in woodchucks and was shown to operate synergistically with interferon alpha [70, 75]. Using the woodchuck model, an antiviral activity comparable to lamivudine was reported for adefovir [67, 76, 77] and tenofovir [71], while drugs displaying higher antiviral efficacy, like entecavir, induced profound viremia reduction and a stronger delay of viral rebound after drug withdrawal [78]. Moreover, long-term suppression of WHV replication showed to delay the development of HCC [79]. Drug-resistant mutants have been also found after prolonged lamivudine treatment, while supplemental treatment with adefovir could restore viral suppression, thus mimicking clinical observations [73]. Therapeutic strategies involving the inhibition of PD-L1 [80] or gene therapy-based approaches enabling prolonged expression of IL-12 have been employed to study the efficacy of these drugs to break immunological tolerance [81, 82]. The characterization of the transcriptional response of these animals to WHV persistent infection, by performing sequencing of the woodchuck transcriptome and generation of custom woodchuck microarrays, indicated that chronic WHV infection, like CHB, is associated with a limited type I interferon response and induction of markers that, like in CHB infection, are associated with T cell exhaustion [83]. Improved sequencing information is expected to facilitate studies also aiming at developing therapeutic vaccines [84]. Regarding the screening of novel immune modulatory substances, the treatment of woodchucks

with the oral TLR7 agonist GS-9620 has shown to induce sustained antiviral responses and even seroconversion in a substantial proportion of treated animals [85]. Although woodchucks are difficult to breed in captivity and they do not fulfill the requirements for an easy to handle experimental animal, the use of these outbred animals in HBV research plays an important role for the assessment of new immune therapeutic approaches (Fig. 2.1).

The Chimpanzee Model of HBV Infection [63]

The chimpanzee is the only immunocompetent experimental animal model fully susceptible to HBV infection, as demonstrated by the induction of acute infection and hepatitis after injection of serum from human HBV carriers [86]. Even though chimpanzees rarely develop chronic liver disease, they develop cellular immune responses largely resembling those observed in humans acutely infected with HBV [87]. Thus, researchers have relied upon chimpanzees to study the pathogenesis of acute HBV infection [88] and these high primates have played an essential role in the development of a safe vaccine, for the preclinical evaluation of HBV-specific monoclonal antibodies [89] and production of neutralizing HBV-specific antibodies [90–95]. The half-life of circulating HBV virions was also determined first in these animals [96]. Both protective immunity and the efficacy of the hepatitis B vaccine against antiviral drug-resistant HBV mutants have been assessed by rechallenging the chimpanzees either with homologous or heterologous viruses [95]. Because sequential liver biopsies can be obtained throughout the course of infection, chimpanzees represent an extremely valuable infection system for the analysis of intrahepatic virological changes and immune responses (Fig. 2.1). Such studies revealed for instance that non-cytolytic downregulation of HBV replication may play an important role in HBV clearance, because most HBV-DNA could be cleared from the liver and the blood of experimentally infected chimpanzees before T-cell infiltration and liver injury occurred [97]. It was also shown that hepatocellular injury is predominately immune mediated [98] and that a strong and polyclonal CD8 T cell response to HBV proteins characterizes the acute self-limited HBV infection [99]. Moreover, the depletion of CD4 T cells indicated that these immune cells do not directly participate in viral clearance, but rather contribute to the induction and maintenance of B and CD8 T cell responses [100]. Interestingly, low levels of cccDNA were shown to persist in the liver of chimpanzees even after resolution of infection [101], while the presence of HBV DNA integrates could demonstrate the clonal expansion of hepatocytes in livers of chronically infected chimpanzees [102]. Infection kinetic and microarray analyses of serial liver biopsy samples obtained from experimentally infected chimpanzees revealed that HBV does not induce significant changes in the expression of intrahepatic innate response genes and production of type I IFNs in the first weeks following HBV infection and spreading [101]. The relevance of these studies could be confirmed in CHB patients [103]. HBV infection studies in chimpanzees also indicated that the size of the inoculum

affects not only the kinetics of viral spread but also the outcome of infection, since injections with few virions were associated with a strong CD4 T-cell priming delay and development of persistent HBV infection [104]. A recent example of preclinical assessment of antiviral drugs in chronically infected chimpanzees regarded the evaluation of the therapeutic efficacy of TLR7 agonists, where oral administration of this immune modulatory compound reduced viral loads and induced enhancement of antiviral immune responses, such as elevation of interferon stimulated genes (ISGs) in liver and blood [105]. Establishment of HBV infection also appeared to be limited by the antiviral effects and enhancement of type I interferon responses that HCV induced in the liver of chimpanzees previously infected with HCV [106].

Although chimpanzees are a uniquely valuable species for research with human hepatotropic viruses, the strong ethical constraints and high costs encountered by working with primates have increasingly restricted their use (Fig. 2.1). Moreover, the extremely limited number of chimpanzees available for research represents a serious restriction for the evaluation of antiviral drug efficacy, while recent advances in alternate research tools, including cell-based and other animal models, are further reducing the necessity to use chimpanzees as research subjects [107–109].

The Tupaia Model

Inoculation of tree shrews with HBV-positive human serum was shown to result in a transient HBV infection, characterized by low levels of viral replication and production of antibodies to HBsAg and HBeAg [8]. Moreover, HBV virions produced in tupaias were successfully passed through several generations and infection of these animals could be specifically blocked by immunization with hepatitis B vaccine [110]. Infection of adult tupaias causes only a mild, transient infection with low viral titers. Recent data suggest that infection of neonate animals with HBV is inducing chronic infection. Although viremia reached only moderate levels (up to one million HBV DNA copies/ml), immunopathologic changes in the liver of long-term infected animals, including forms of bridging necrosis and fibrosis were observed [111]. It is however worth noting that even if experimental infection of tree shrews causes only a very mild and transient infection in these animals, primary hepatocytes isolated from *T. belangeri* can be efficiently infected in vitro (Fig. 2.1) and hence represent a valuable alternative source of HBV-permissive cells to study the early steps of HBV infection [7, 9]. Cultures of primary tupaia hepatocytes have been successfully used to show that polymerase inhibitors like adefovir and lamivudine can reduce but not prevent the formation of cccDNA upon hepatocyte infection [112]. Moreover, the woolly monkey hepatitis B virus (WMHV) was isolated from an endangered new world primate [113], the woolly monkey *Lagothrix lagotricha*. Phylogenetic analysis of the nucleotide sequences of WMHBV indicated that this virus may represent a progenitor of the human virus [113]. Interestingly, WMHBV is not infectious for chimpanzees [113], but primary tupaia hepatocytes are highly susceptible to infection with this HBV-closely related hepadnavirus [114, 115].

Mouse Models

Mouse Models of HBV Replication

Transgenic Mice

To investigate specific aspects of HBV replication, as well as the role of distinct viral proteins in HBV pathogenesis using convenient inbred animal models, embryo microinjection technologies have enabled the development of mice harboring either single HBV genes or terminally redundant over-length HBV-DNA constructs [116–120]. The first HBV-replicating transgenic mice, which were developed by Chisari and colleagues in 1995 [119], demonstrated the feasibility to produce in murine hepatocytes infectious HBV virions morphologically indistinguishable from human-derived virions [97]. As the immune system of transgenic animals recognizes during embryonic development the virus as "self," these studies provided the first evidence that HBV replication does not induce hepatocellular injury [121]. To show that HBV-related pathogenesis is largely mediated by the host immune responses, induction of acute hepatitis and hepatocellular injury was demonstrated after adoptive transfer of HBV-antigen specific CTLs [122–124]. CTL-mediated release of cytokines also showed to suppress viral replication by non-cytolytic mechanisms [125, 126], while the recruitment of antigen nonspecific inflammatory cells amplified the severity of liver damage initiated by antigen-specific CTLs [127]. CD8+ T cells isolated from mice and engineered to express HBV specific chimeric antigen receptors (S-CARs) were also shown to engraft and expand in immunocompetent HBV-transgenic mice. After adoptive transfer, these cells were shown to control HBV replication, while causing only transient liver damage. Since these effector T cells, can be developed regardless of their HLA type, the adoptive transfer of such genetically modified HBV-specific T cells may represent a promising immunotherapeutic approach deserving further investigations [128, 129].

HBV transgenic mice have been successfully used to evaluate the impact of various polymerase inhibitors, such as lamivudine [130], adefovir dipivoxil and entecavir [131, 132] on HBV replication. Therapeutic approaches involving the use of HBV-specific small interfering RNAs (siRNAs) were also tested in HBV-transgenic mice [133, 134]. To combine gene silencing with the induction of interferon responses, Protzer and colleagues recently employed 5′-triphosphorylated small interfering RNAs targeting highly conserved sequences on HBV RNA transcripts and showed that by triggering RIG-I-mediated innate immune responses these bifunctional antiviral molecules suppressed HBV replication more efficiently than siRNAs lacking a triphosphate group [135].

A major limitation encountered by using HBV transgenic mice is that they do not allow investigation of viral entry and spreading (Fig. 2.1). Moreover, no cccDNA is built in the liver of transgenic mice and the chromosomally integrated HBV genome cannot be eliminated from the host genome. As a consequence, viral clearance and cccDNA eradication cannot be achieved in this model. To break tolerance to HBV antigens and investigate the mechanisms of viral clearance, alternative mouse models

have been developed which rely on transfecting or transducing the viral genome into mouse hepatocytes by different means, such as using recombinant adenoviral vectors, or by hydrodynamic injection of naked DNA in mice.

Vector-Mediated Transfer of HBV Genome

Adenoviral vectors containing a replication-competent HBV genome (Ad-HBV) have been shown to permit efficient transfer of the HBV genome into the liver of immunocompetent mice [136]. After intravenous injection of such adenoviral derived vectors, HBV proteins are produced under the control of endogenous HBV promoters and enhancers and viral replication could be demonstrated for up to 3 months. Viral clearance was accompanied by mild to moderate liver inflammation with elevated serum alanine transaminase activities [137]. After the induction of adaptive immune responses, anti-HBs seroconversion and development of neutralizing antibodies the infection was cleared. Inflammation and liver damage were also shown to be controlled by regulatory T-cells [138]. Because of the acute, self-limiting character of such adenoviral-mediated HBV infection, the system offers good possibilities to investigate the mechanisms of immune-mediated viral clearance also involving intrahepatic expansion of cytotoxic T cells (CTL) and NKT cells [139, 140]. By injecting relative low doses of adenoviral vectors [141] or by injecting the viruses intrahepatically into neonatal mice [142], persistent infection in immunocompetent mice could be established. This type of tolerance resembles immunological features of chronic HBV infection in humans. Moreover, even if mice do not establish cccDNA, using adenoviral vectors the HBV genome is in an extrachromosomal organization and hence its clearance can be achieved. Recombinant adeno-associated viruses (AAV) were also used to transfer replication-competent HBV genomes in a mouse strain carrying human leukocyte antigen A2/DR1 transgenes [143]. In these animals, viremia and antigenemia persisted for at least 1 year. Notably, a higher number of regulatory T-cells and no significant liver inflammation were determined in those livers, while impairment of functional T cell responses indicated the occurrence of tolerance. However, establishment of long-term viral replication with Ad-HBV vectors is limited by the immune responses against these vectors and occurrence of adenovirus-mediated cytotoxic effects may also limit their application. Since a functional cccDNA, associated with histone and non-histone proteins is not built in murine hepatocytes, these models are not suitable for the development of drugs or antiviral strategies targeting the natural template of HBV transcription and replication (Fig. 2.1).

Hydrodynamic Injection of HBV Genome

Hydrodynamic injection techniques, which involve the rapid injection of a large volume (10 % of the animal weight) of a solution containing naked DNA into the tail vein of mice, are quite stressful for the animals but allow crossing species-specific

barriers and permit efficient HBV DNA transfer [144]. Moreover, the rapid injection of liquid induces significant liver damage and ALT elevation shortly after injection. Hydrodynamic injection of replication-competent HBV genomes in mice resulted in viremia titres up to 1×10^7 HBV DNA/ml [144]. Although HBV replication initiated already 1 day post-injection, replication levels decreased after 1 week and HBV was cleared from blood within 2–3 weeks, as soon as specific antiviral antibodies and CD8+ T cells appeared. However, HBV infection persisted for 3 months after hydrodynamic injection of mice lacking adaptive immune cells and natural killer cells, thus demonstrating that the outcome of hydrodynamic transfection of HBV depended on the host immune response [144]. Hydrodynamic injection studies also showed that simultaneous delivery of HBV expressing plasmids and HBV-specific siRNAs prevented HBV replication [133, 134], while by injecting modified HBV DNA plasmids into C57BL/6 mice, a significant immune clearance of HBV could be achieved [145]. A recent report showed that the use of a lentiviral backbone instead of an AAV vector led to increased and prolonged HBV replication (>56 days post injection) [146]. In an attempt to generate cccDNA-like molecules in mice, a monomeric HBV genome precursor plasmid (pr-cccDNA), that can be converted by Cre/loxP-mediated DNA recombination into a 3.3-kb cccDNA, has been recently used [147]. Although such recombinant cccDNA could be detected in the nuclei of murine hepatocytes, the induced immune response rapidly limited viral replication in vivo [147].

To investigate whether cccDNA molecules could be directly targeted for destruction, the CRISPR/Cas9 system has recently been employed both for in vitro [148, 149] and in vivo studies, using the HBV-hydrodynamic-mouse models [150]. In these studies, the levels of HBV-expressing vectors and different markers of viral replication were significantly reduced, and without evidence of toxicity, suggesting that the CRISPR/Cas9 system could be recruited to the HBV-expressing vectors. Although many issues regarding the efficiency and safety of the system remain to be addressed, these findings are the first to demonstrate nuclease-mediated disruption of a HBV expressing vector, as a model of cccDNA, thus opening new possibilities for the development of innovative antiviral strategies aimed at disrupting the cccDNA.

Despite the relatively short span of viral replication available, mice transfected by hydrodynamic injection are suitable not only for short-term antiviral studies but also for testing the consequences of specific mutations within the viral genomes. In comparison to HBV-transgenic mice, hydrodynamic-based procedures permit investigation of immune response emerging during acute infection. Since the viral genome is not integrated into the host genome as a transgene, viral clearance commonly occurs in these systems. A clear advantage of the system is that different HBV genotypes and variants can be injected into mice and analyzed in vivo in relatively short time (Fig. 2.1). Nevertheless, the rapid injection of large volume of fluids causes not only strong discomfort to the animals but also significant damage in the liver, which may alter cell function and signaling analyses. Since reinfection of the mouse hepatocytes is not possible, viral clearance is easier to achieve in these non-transgenic murine models than in humans, and therefore, the efficacy of antiviral treatments should be validated in systems permissive for HBV infection. Interestingly, all results available so far indicate that murine cells engineered to

express the human NTCP do not become susceptible for HBV infection [44]. It appears that species-specific differences or the lack of cellular factors involved in post-entry steps are responsible for these discrepancies.

Mouse Models of HBV Infection

The inability to reproduce the entire infection cycle of HBV in murine hepatocytes has hindered our understanding of the mechanisms by which HBV interacts with hepatocyte-specific functions, as well as to study mechanisms of viral entry, cccDNA formation and spreading in a well-controlled in vivo laboratory system. Because of these restraints, many efforts have concentrated on the development of models based on the use of the natural target of HBV infection: the human hepatocyte. However, the susceptibility of cultured primary human hepatocytes to HBV infection is highly variable and cultured hepatocytes rapidly lose the expression of essential hepatocyte-specific factors, such as the NTCP receptor [9]. The generation of mice carrying human hepatocytes permits to overcome most of these limitations.

The Trimera Mouse

These mice represented the first human–mouse chimeric system that was developed by transplanting human liver fragments under the kidney capsule of normal Balb/c mice. To avoid rejection of the implanted tissue, these mice were preconditioned by total body irradiation and reconstituted with SCID mouse bone marrow cells [151]. After ex vivo infection of the small human liver specimens with HBV, low levels of viremia, that remained detectable for approximately 20 days, could be determined in implanted animals. Interestingly, mice ectopically carrying human liver fragments could also be engrafted with human peripheral blood mononuclear cells (BPMC), so that the effects of polyclonal anti-HBs antibodies against HBV could be assessed [89, 152]. Nevertheless, due to the extra-hepatic location of the implanted tissues, human hepatocytes remained functional only for limited time and in vivo infection with HBV or other human hepatotropic viruses could not be established.

Human Liver Chimeric Mice

To achieve long-term survival of primary human hepatocytes permissive for HBV infection in vivo, isolated cells need to be integrated in the mouse liver. The requirements to achieve this goal are (1) the damage of the endogenous murine hepatocytes to create the space and the regenerative stimulus necessary for transplanted hepatocytes to reconstitute the diseased mouse liver; and (2) the absence of murine adaptive immune cells and NK cells to permit engraftment and survival of transplanted xenogeneic hepatocytes. Different human-liver chimeric mouse models are

currently available. The Alb-urokinase-type plasminogen activator (uPA) transgenic mouse was the first model describing the strong regeneration capacity of healthy transplanted hepatocytes. In this system, over-expression of the hepatotoxic uPA transgene, which is driven by the mouse albumin promoter, induces high levels of uPA in plasma, hypo-fibrinogenemia and subacute liver failure in young mice [153]. To generate mice with human liver chimerism, uPA transgenic mice have been backcrossed with immunodeficient mouse strains, such as the RAG2$^{-/-}$ [154–156], the Severe Combined Immune Deficient (SCID) [157], which lack functional B and T cells or SCID/beige mice, which also lack NK-cell functions (shortly USB mice) [158, 159] and NOD/SCID/gamma(c)(null) (shortly uPA-NOG) [178]. Following intra-splenic injection of one million freshly isolated or cryopreserved-thawed human hepatocytes, the transplanted cells migrate via the splenic and portal veins to the liver, where cells integrate into the liver parenchyma. Engrafted human hepatocytes proliferate to form larger regenerative nodules that eventually merge together to replace the diseased liver parenchyma. Reconstitution of the mouse liver takes around 2 months and the levels of human chimerism can be estimated by determining the concentration of human serum albumin in mouse blood [159, 160]. Within the mouse liver, the transplanted human hepatocytes maintain normal metabolic functions [157, 161]. To delay the production of the toxic transgene, which makes transplantation procedures necessary in the first month of life, alternative mouse models, where the expression of the uPA transgene is inducible [162] or is regulated by the MUP (major urinary protein) promoter, have been also developed [163]. To generate an animal model where hepatocyte failure can be induced at will in adult mice, alternative human-liver chimeric mice, based on the use of fumaryl acetoacetate hydrolase-deficient (FAH) mice, were established [164, 165]. FAH plays a crucial role in the tyrosine breakdown pathway and its deficiency leads to accumulation of toxic tyrosine catabolites and liver failure. However, accumulation of these catabolites can be avoided by administering the drug NTBC (2-(2-nitro-4-trifluoromethylbenzyol)-cyclohexane-1,3-dione), a pathway inhibitor that protects the animals from the occurrence of liver injury until drug administration is withdrawn. More recently, by transplanting higher amounts of human hepatocytes, or by performing repeated hepatocyte transplantation, high rates of human hepatocyte chimerism could be achieved in mice also lacking the Rag2 and the gamma-chain of the receptor for IL-2 genes (shortly FRG mice) [166]. Human hepatocytes were also successfully transplanted in mice expressing the herpes simplex type-1 thymidine kinase (TK) transgene that were backcrossed with NOG (NOD/SCID/gamma(c)(null)) mice [167]. Even in this case, human hepatocyte transplantation can be performed in adult mice, since mouse liver cells expressing the TK-transgene can be selectively destroyed upon administration of ganciclovir [168].

From the first successful transplantation of human hepatocytes into uPA/RAG2 mice and establishment of de novo infection with HBV [156], several groups performed HBV infection studies [160, 169, 170], as well as demonstrated infection with other human hepatitis viruses, like HCV and HDV, in humanized uPA/SCID or uPA/SCID/beige (USB) mice [160, 171–173]. Notably, after intra-peritoneal inoculation of HBV infectious serum or cell culture derived virions, productive HBV

infection, which requires the establishment of a functional cccDNA in hepatocyte nuclei, is first achieved in a minority of human hepatocytes and several weeks are needed to accomplish viral spreading [159]. After that, nearly all human hepatocytes stain HBcAg-positive and viremia reaches a stable plateau which, to a certain extent, correlates with the levels of human chimerism. The use of patient serum samples as virus inoculum allows the functional analysis of distinct HBV genotypes, naturally occurring variants and drug-resistance mutants. Genetically engineered viruses can therefore be used to investigate the role of distinct viral proteins in human infected hepatocytes. Using this type of approach, the expression of the regulatory HBx protein provided *in trans* was shown to be essential for cccDNA-driven HBV replication in infected human hepatocytes [170]. Studies focusing on investigating how HBV may affect cellular pathways [161] and innate immune responses of the human hepatocytes are just starting to emerge and showed that expression of metabolic genes [161] and innate immune response genes in these cells resembles well the expression pattern determined in human livers [158]. To this regard, a recent study indicated that binding of HBV to its cellular receptor alters the hepatocellular uptake of bile salts and the expression profile of genes of the bile acid metabolism [161]. The occurrence of such alterations was also confirmed in patient biopsy samples. Using humanized mice, both HBV and HCV have been recently shown to contribute to the induction and accumulation of aberrant DNA methylation in human hepatocytes through the activation of NK-cell-dependent innate immune responses [174].

Both the antiviral activity of clinically approved polymerase inhibitors [115, 158, 169] and the in vivo efficacy of novel polymerase or capsid inhibitors [175, 176] have been assessed in human liver chimeric mice. The model also served to evaluate the in vivo efficacy of lipopeptides derived from the large envelope protein of HBV to prevent de novo HBV [177] and HDV infection [172], as well as to investigate the ability of the most clinically advanced entry inhibitor, Myrcludex-B, to block HBV spreading post-infection [178]. Moreover, the serial passage of infected hepatocytes isolated from infected mice and transplanted into new recipients has permitted to gain insight about the impact of hepatocyte proliferation on cccDNA stability and activity in vivo [179]. The drastic reduction of intrahepatic cccDNA loads induced by cell division even in the absence of antiviral therapy revealed a weak point in HBV persistence that deserves further investigations. Thus, these systems offer unique opportunities to investigate factors that can affect the stability and/or activity of the cccDNA minichromosome, as well as the direct antiviral effects of cytokines and immune modulatory substances, such as interferons (Fig. 2.1). To this regard, human liver chimeric mice have been used to assess whether HBV can circumvent the induction of antiviral defense mechanisms [158]. Upon administration of regular human IFNα, HBV was shown to hinder the nuclear accumulation of STAT-1, thus providing a potential mechanism for the reduced induction of interferon stimulated genes (ISGs) determined in HBV-infected human hepatocytes [158]. On the other hand, studies in these mice also showed that IFNα can mediate epigenetic repression of the cccDNA minichromosome [29], while the repeated administrations of the longer-active pegylated IFNα was shown to be able

to breach the impairment of HBV-infected hepatocyte responsiveness and induce sustained enhancement of human interferon stimulated genes (ISG) [180]. In this study, the stronger antiviral effects of peg-IFNα exerted on the human hepatocytes were shown to trigger a substantial decline of circulating HBsAg and HBeAg levels in chimeric mice, without claiming the involvement of immune cell responses [180]. Moreover, the comparative analyses of the innate immune responses revealed that type I, II and III IFNs are differently induced in murine and human hepatocytes and that the effects of distinct IFNs may differ between animal species, thus underlying the importance of validating results obtained in murine systems also by employing primary human hepatocytes [181].

Immune Competent Human Liver Chimeric Mice

Human hepatocytes can be very abundant within the chimeric livers but non-parenchymal cells, such as sinusoidal endothelial cells and Kupffer cells, are of murine origin. As a consequence, development of fibrosis and pathophysiologic processes that are commonly associated with chronic viral hepatitis infections but involve a cross talk between the hepatocytes and other liver resident cells are not observed in the above mentioned systems. Moreover, since these chimeric animals are genetically immune deficient they are not suited for vaccine studies and for evaluation of adaptive immune responses. To circumvent these limitations, partially haplotype-identical human peripheral blood mononuclear cells (PBMC) have been recently transferred in uPA/SCID chimeric mice after the establishment of HBV infection [182]. Notably, infiltrating human immune cells caused severe hepatocyte degeneration, while treatment with anti-Fas antibodies or depletion of dendritic cells prevented the death of human hepatocytes.

A different attempt to generate a humanized mouse model harboring both human liver cells and a human immune system was the development of the AFC8 model [183]. These mice were obtained by crossing BALB/c-RAG2$^{-/-}$γc$^{-/-}$ mice, which lack functional B, T and natural Killer cells, with mice carrying a liver-specific suicidal transgene with inducible activity based on the induction of caspase 8 in mouse hepatocytes. These animals were used to transplant simultaneously human fetal hepatocytes and hematopoietic stem cells that were obtained by the digestion of human fetal liver tissues (15–18 weeks of gestation period). Since the fetal liver provides both types of cells, reconstitution of both cell lineages is syngeneic and hepatocyte rejection by the human immune system is not expected. Although HBV infection studies were not performed, after inoculation of hepatitis C virus, low levels of intrahepatic viral replication, as well as T-cell responses and development of fibrosis could be determined. Despite partial success and the ethical restrains encountered by employing human fetal tissues, the generation of chimeric systems equipped with both a human liver and a functional human immune system, with a matched major histocompatibility complex, still remains a major challenge. The latest advances reported by Gutti et al. showed the feasibility to reconstitute an uPA-NOG mouse strain with both adult human hepatocytes and hematolymphoid

cells [184]. In this study, dual reconstitution was achieved by transplanting fetal or even adult hepatocytes and mismatched hematopoietic stem cells (CD34+ HSCs) derived from either a fetal liver or umbilical cord blood. As an alternative and well-tolerated procedure to total body irradiation, mice received 3 days of a treosulfan-based chemotherapeutic conditioning before HSC injection. The presence of CD8+ and CD4+ human cells and of human hepatocyte clusters was observed in the liver of these animals. No cell-mediated rejection but also no evidence of cell interactions were determined in animals reconstituted with mismatched HSCs. Although the applicability of these approaches for the study of HBV and HDV associated immune pathological processes needs further research, these technical advances have paved the way for the development of dually reconstituted humanized systems.

Conclusions

In the recent years fundamental progresses were made concerning the development of both in vitro and in vivo systems that offer new opportunities to researchers to choose between different models on the base of the specific questions addressed. The recent discovery of the long-searched cellular receptor for HBV was a milestone that has permitted the creation of HBV permissive hepatoma cell lines which can recapitulate the entire HBV life cycle. Together with the HBV permissive cell line HepaRG and the new described cell line HLCZ01 these tools shall allow high-throughput screening of large compound collections for innovative substances interfering with the different steps of the life cycle. In the light of the success of direct antiviral substances in HCV field, it can be expected that new direct antiviral substances targeting different steps of the life cycle will arise in the near future. Nevertheless, and in contrast to HCV infection, HBV persistence is guaranteed by the stable episomal cccDNA minichromosome. Thus, the task of curing HBV infection will require development and testing of new substances targeting key steps of HBV life cycle, such as viral entry [177], cccDNA formation [47], stability [185] and activity [29], as well as enhancing immune responses. Since the maintenance of hepatocyte specific functions that are often lost in hepatoma cell lines appears fundamental to identify the factors involved in the infection process, it can be expected that the use of convenient hepatoma cell lines culture systems will keep bearing important limitations. Thus, further development of more sophisticated in vitro HBV infection systems, possibly based on the use of engineered nonhuman hepatocyte-like cells or humanized murine hepatocytes, appears mandatory.

Regarding the in vivo models of HBV infection, the classical reference was the chimpanzee. However, due to stronger ethical restrains this model is practically not available for HBV research and needs to be replaced by different older and newer systems. As a consequence, important research progresses focusing, for instance, on identifying host factors involved in cccDNA formation mechanisms still relay on the use of duck and tupaia hepatocytes, while the woodchuck system, despite its known virological and host-related differences, still offers unique advantages to

accomplish preclinical vaccination studies and testing of immune modulatory substances. Human liver chimeric mice represent already a well establish HBV infection model for preclinical in vivo testing of direct antiviral agents and for studying how human hepatitis viruses interact with their natural target of infection, the human hepatocyte. Moreover, co-transplantation of human immune cells into these mice may further brighten their use by allowing the study of human immune responses to HBV. Ideally, immune competent HBV-permissive murine models may fulfill most of the unmet needs in preclinical research. Nevertheless, species-differences both in term of pharma cokinetics and immune responses may be misguiding and it can be expected that all systems will keep having specific advantages and limitations, depending on the research purpose. Thus, validation of results in multiple systems should always be encouraged.

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Chapter 3
Hepatitis B Virus Genotypes

Hideaki Kato, Masaya Sugiyama, and Masashi Mizokami

Introduction

Hepatitis B virus (HBV) was discovered as the "Australian antigen" by Blumberg et al. in 1965 [1]. This achievement, which contributed to the discovery of and prophylaxis of post-transfusion hepatitis, Blumberg was awarded the Nobel Prize for Physiology and Medicine in 1976. Thereafter, in spite of the development of a vaccine for prophylaxis against HBV infection, more than four billion people have been chronically infected with HBV (HBV carriers). It is estimated that at least 500,000 patients with HBV die annually due to HBV-related diseases. In developing countries, such as Asia and Africa, there are more patients with HBV than with hepatitis C virus infection. Therefore, hepatitis B remains an as-yet unsolved major problem in public health [2].

HBV Genome Mutation Rate in Four Open Reading Frames

The HBV genome harbors four partially overlapping open reading frames (ORFs) encoding the polymerase (P), surface antigen (S), nucleocapsid (C), and X proteins (X) (Fig. 3.1). Gene P accounts for two-thirds of the genome and overlaps all the other genes of the genome (Fig. 3.1). Regulatory sequences are also embedded in these protein-encoding genes. Furthermore, the secondary structure of the HBV RNA might constrain the number and nature of substitutions that can occur in the HBV genome [3–5]. Mizokami et al. [6] and Torres et al. [7] analyzed the influence of these overlapping

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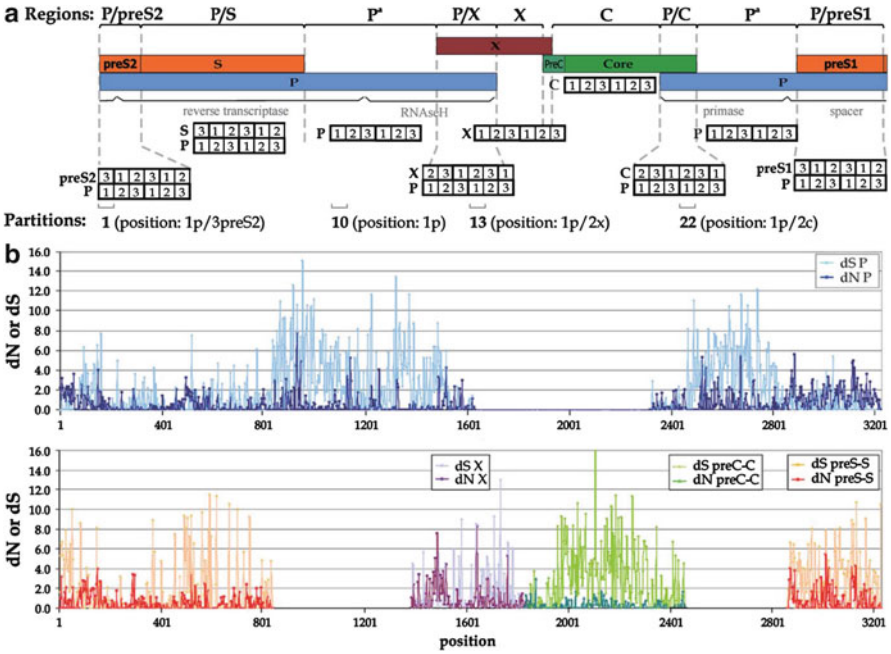

Fig. 3.1 (**a**) Four overlapping open reading frames of hepatitis B virus (HBV) were analyzed. Position: "1p/3preS2" means the nucleotide position is codon 1 in polymerase gene and codon 3 in the preS2 region. (**b**) dN and dS estimates for all HBV genes. Cited from Torres et al. [8] with publishers permission

genes on the evolution of HBV. They evaluated the influence of the overlapping reading frames on the nucleotide variation pattern in the HBV genome and evaluated the relative substitution rates for the first, second, and third codon positions of overlapping and non-overlapping genetic regions (Fig. 3.1a). They found that, in the non-overlapping regions of genes P, C, and X, the respective third positions (3p, 3c, and 3x) were the most variable. The third position of gene P displayed the highest rate of variation. For the P/X overlapping region, position 3p/1x was the most variable. In contrast, in other regions, the third codon positions of genes overlapping with P were the most variable. In the P/preS-S region, the most variable codon position was 1p/3preS-S, whereas in the P/C region, the most variable codon position was 2p/3c. They also studied selective pressure using the Single likelihood ancestor counting (SLAC) method, which is one of the traditional approaches commonly applied to study selective pressure. This method accounts for an independent estimation of dN and dS, which becomes especially important when nonuniform distribution of rates occurs, as in HBV [8].

Classification of HBV Genotypes

In 1988, HBV was classified into four genotypes due to a sequence divergence in the entire genome exceeding 8 %; these genotypes were designated by capital letters A to D (Fig. 3.2) [9]. In 1994, Norder et al. [10] found an additional two HBV

genotypes using the same criteria, and named these E and F. Genotype G was reported in 2000 [11], and genotype H, which is phylogenetically closely related to genotype F, was proposed in 2002 [12]. In 2008, sequence analysis of the complete genome of a single isolate (AB231908) obtained from a Vietnamese male revealed a ninth genotype, I, which was closely related to genotypes A, C, and G [13]. Thereafter, an HBV strain was isolated from a Japanese patient who had resided in Borneo during World War II [14]; phylogenetic analysis of this isolate showed that it was closely related to gibbon HBV, with mean divergences of 10.9 and 10.7 %, and it was provisionally named as genotype J [14].

Subgenotypes of HBV

Each HBV genotype is subdivided into several subgenotypes, based on exceeding 4 % of full genome differences (Fig. 3.3). To date, at least 35 subgenotypes have been reported [15], but no subgenotypes of genotype E, G, H, or J have been reported to date.

Some points should be considered when classifying HBV strains into subgenotypes [16]. (1) Analysis of the full length of the genome, including entire ORFs, at nucleotide level is a prerequisite for determining subgenotypes accurately. (2) Adherence to the ranges of intra-genotypic nucleotide divergence (more than 4.5 % and less than 7.5 %) that define distinct genotypes should be observed. (3) Bootstrap values greater than 75 % are required to support the monophyletic tree for introducing a cluster as an independent subgenotype. (4) Recombinant strains should be excluded from any subgenotyping analysis as far as possible, as these can disrupt the topology of a phylogenetic tree and can falsely increase nucleotide divergence. (5) To introduce novel subgenotypes, strains harboring specific nucleotide and amino acid motifs should be identified. (6) To avoid sampling bias, a minimum of three purported novel strains, together with all available subgenotype strains from the same genotype, should be subjected to evolutionary and phylogenetic analysis. Using random reference sequences, as opposed to selecting some particular reference sequence, is highly recommended for subgenotyping by phylogenetic analysis.

Distribution of HBV Genotypes

HBV genotypes have a distinct geographic distribution (Fig. 3.4). Genotypes A and D are seen frequently in the USA and Europe, while genotypes B and C are the most common in Asia. Genotype E has been reported exclusively from West Africa, and genotype F is reported to cluster in Central America [17].

S Region Mutation and HBV Vaccination

The mutation rate of the HBV genome is 10,000 times faster than that of the human genome and HBV infects humans as quasi-species. The first-generation HBV vaccine that contained polyclonal HBV antibodies could prevent HBV infection;

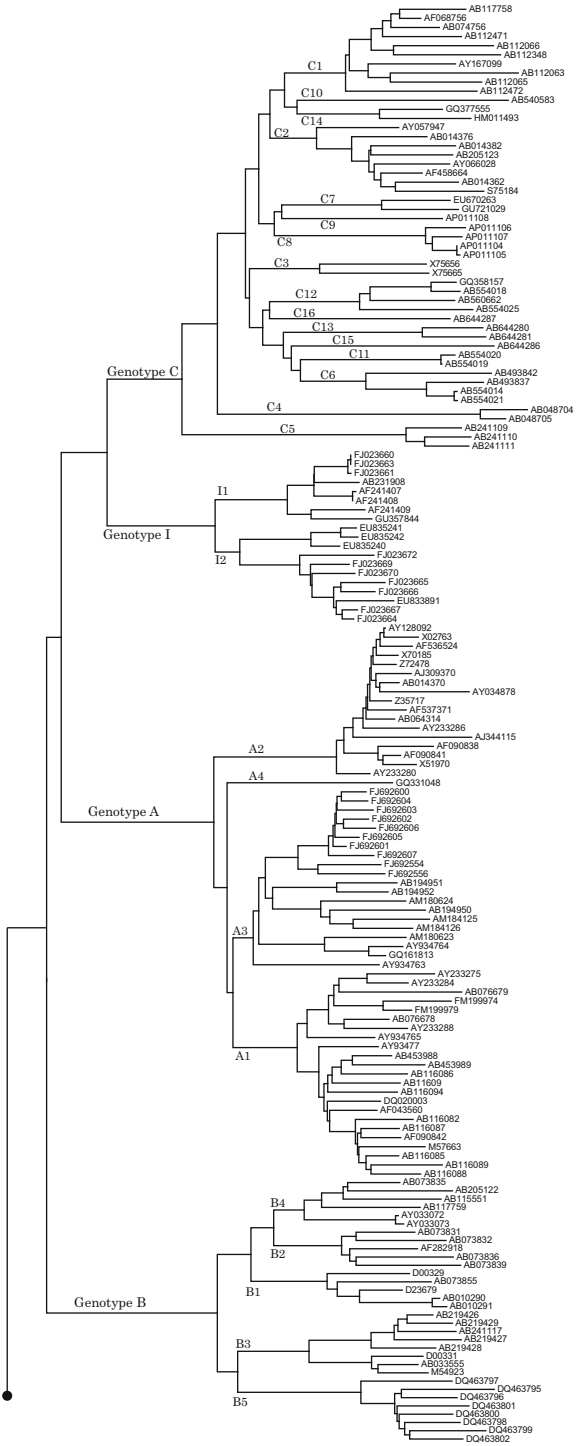

Fig. 3.2 Phylogenetic tree of the full genome of hepatitis B virus (HBV). HBV isolates are represented by GenBank accession number. HBV strains are classified into ten genotypes, designated A to J. Each genotype is further subdivided into 2–16 subgenotypes, except for genotypes E, G, H, and J

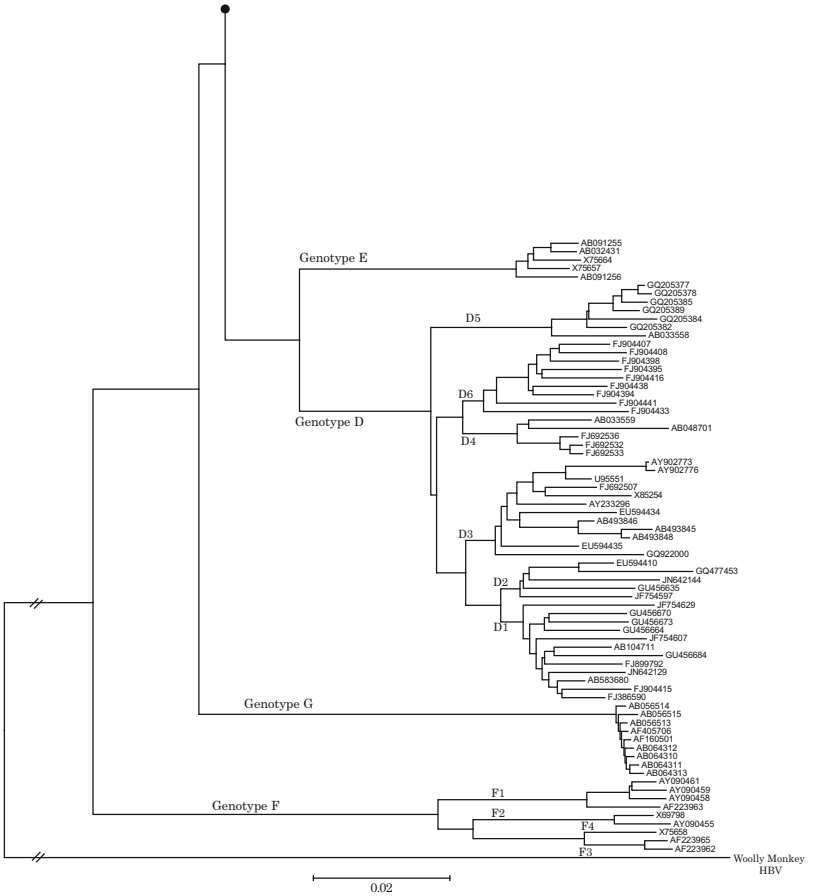

Fig. 3.2 (continued)

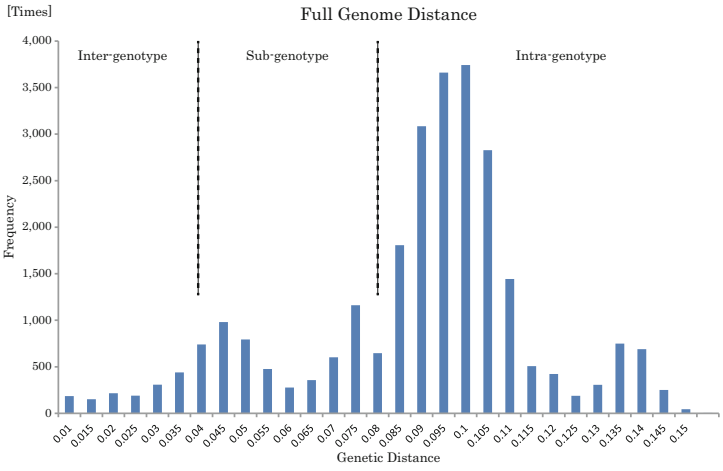

Fig. 3.3 Frequency of genetic distance of the full genome of hepatitis B virus (HBV). In total, 27,261 base pairs of the full genome sequence were analyzed. A genetic distance of <4 was defined as inter-genotype, 4–8 as subgenotype, and >8 as intra-genotype

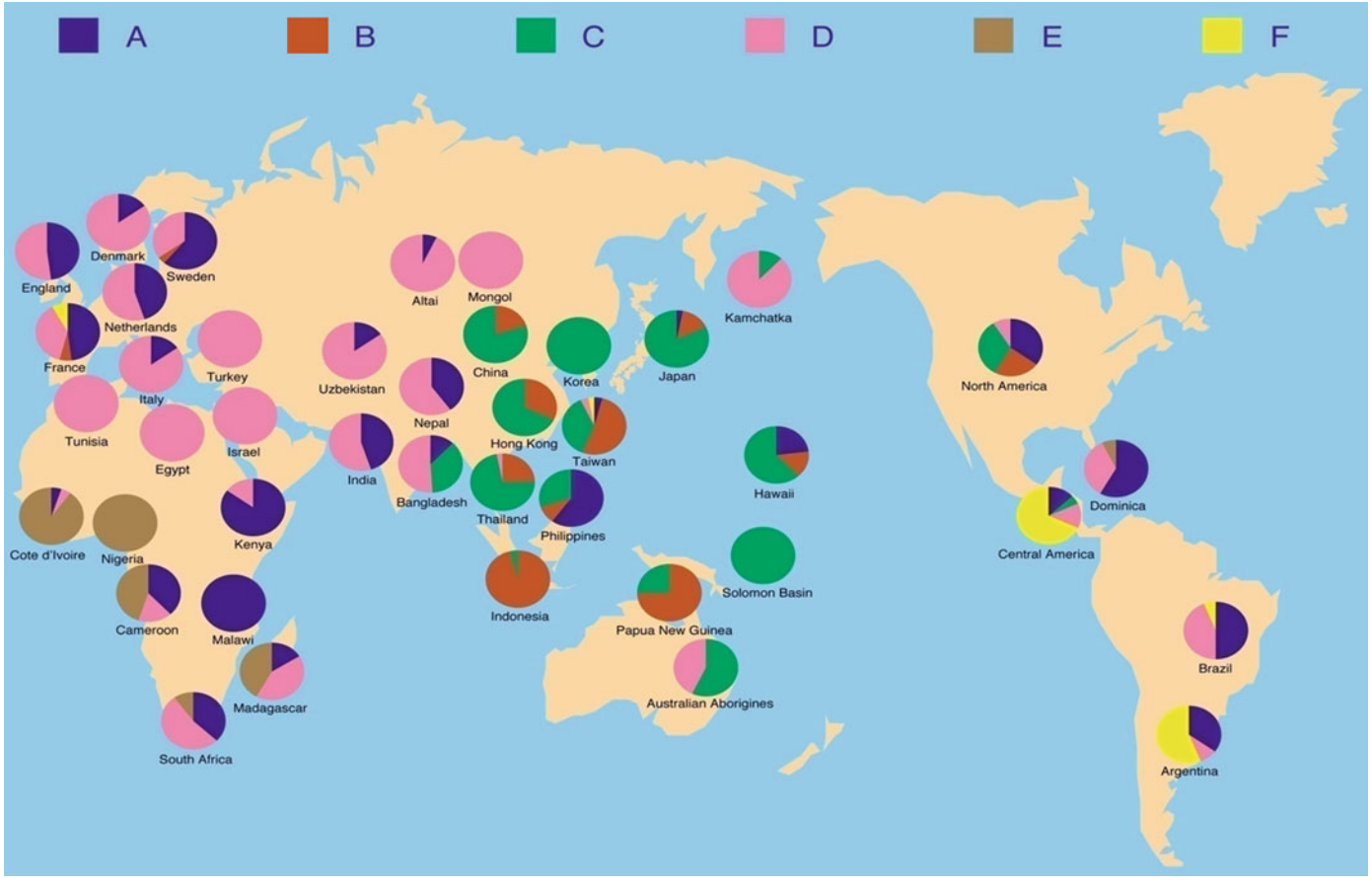

Fig. 3.4 Distribution of hepatitis B virus (HBV) genotypes. HBV genotypes have a distinct geographic distribution. Genotypes A and D are seen frequently in the USA and Europe, while genotypes B and C are the most common in Asia. Genotype E is reported exclusively from West Africa, and genotype F clusters in Central America [17] Cited from Miyakawa and Mizokami [17] with publisher's permission

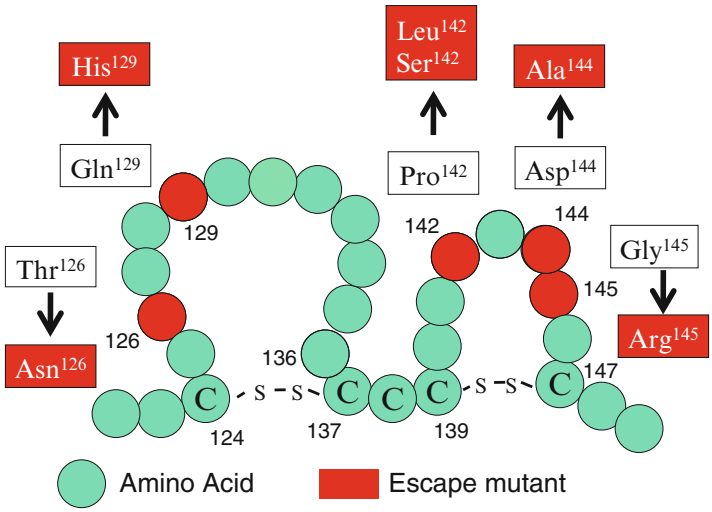

Fig. 3.5 α-Loop structure of the S region of HBV and escape mutations. The Thr[126] to Asn[126], Gln[129] to His[129], Pro[142] to Leu[142] or Ser[142], Asp[144] to Ala[144], and Gly[145] to Arg[145], amino acid mutations are shown above, either solitary or in combination with the induced escape mutant

however, it is possible that the second-generation HBV vaccine, which is made using biogenetic technology and which contains an antigen from a monoclonal sequence, might fail to prevent HBV infection. The existence of a vaccine-induced escape mutant (VEM) was firstly reported by Carman et al. in 1990 [18, 19]. The sequence from amino acid (a.a.) 111 to a.a. 156 of the S region of HBV (the so called α-loop, Fig. 3.5), which is consistently exposed to humoral immunity and cell immunity, was shown to be frequently substituted [20–23]. However, it is controversial whether VEM increases the rate of infection in individuals vaccinated against HBV or whether HBV vaccination could prevent HBV infection even if VEM is detected in the resource. Although the significance of VEM in HBV vaccination has not been fully elucidated, further studies are required for the production of a safe and assured HBV vaccine [24, 25].

Stramer et al. [6] reported six individuals who became infected with HBV despite having received HBV vaccination previously. Moreover, five of the six individuals infected with a non-A2 HBV genotype, although HBV genotype A2 is the primary strain used for producing the HBV vaccine in the USA. Furthermore, HBV was also found in the partners of four of the six individuals. In Taiwan, Lai et al. [26] also reported a follow-up survey of children who had received the HBV vaccine immediately after birth, in which HBsAg, anti-HBc, and HBV DNA were investigated. The data showed that HBsAg, anti-HBc, and HBV DNA were more frequently present in the 18 years and older group than in the younger groups. Thus, the present HBV vaccine cannot completely prevent HBV infection, especially as it is sexually transmitted, and strongly suggests that the HBV genotype should be studied in detail, especially because it differs geographically.

Characteristic Mutations in HBV Genotypes

The HBV genotypes are determined by molecular phylogenetic analysis that takes into account mutations in the entire genome and includes an examination of the various sequences that reveal characteristic mutations in each genotype. In particular, mutations in a sequence known as the core promoter impact the effectiveness of viral replication. In this section, we provide an overview of the mutations observed in this region.

The subgenotype A1 virus has characteristic mutations in the Kozak (1809T/12T) and epsilon sequences (1862T/88A) that immediately precede the open reading frame (ORF) for the HBe antigen [27]. In a basic study, it was found that the presence of the 1809T/12T mutation decreased antigen production and suppressed replication in one case. The presence of the 1862T/88A mutation also reportedly inhibits viral replication and decreases core protein production [28]. These characteristics were especially observed in subgenotype A1.

The subgenotypes B and C have similar characteristics, including a well-known core promoter mutation (1762T/64A) [29]. Not only are mutations in this region known to result in long-term infections in individuals, but also new infections with a virus with such mutations are also reported to carry the risk of fulminant hepatitis [29]. Studies have also shown that such mutations are associated with liver cancer [30]. Basic studies have shown that the 1762T/64A core promoter mutation enhances viral replication.

In the subgenotype D, the mutation pattern around the core promoter varies in different subgenotypes. A different core promoter mutation is observed in HBV/D1 (1764T/66G) than that in the subgenotype B and C [31]. In terms of virological properties, it is thought to bind to different transcription factors apart from those that bind to 1762T/64A. Although viral replication is enhanced, at present there is no evidence of an association with liver cancer, unlike HBV/B and C. In genotypes such as HBV/D2, the key 1762T/64A mutation is observed frequently, similar to HBV/B and C.

Characteristics of HBV Genotypes

The characteristics of the HBV genotypes are given in Table 3.1.

Genotype A

Genotype A is characterized by an insertion of six nucleotides at the carboxyl end of the core gene. Genotype A is dominant in Northwest Europe and North America. Additionally, some strains of genotype A have been found in the Philippines, Hong Kong, and in some parts of Africa and Asia. Subgenotype A2 is dominant in Europe, A1 is prevalent in Asia and most of Africa, A3 is found in the Cameroon and Gambia, A6 (currently named A4) and quasi-subgenotype A3 (which includes the strains previously named A4 from Mali, A5 from Nigeria, and A7 from Cameroon) have been isolated from other regions [32].

Table 3.1 Comparison of virological characteristics of HBV genotypes [14]

Genotype	Length	Differentiating features	Subgenotypes	Serotype
A	3,221	6-nucleotide insert at carboxy end of core region	A1 A2 A3 (A3, A4, A5) A4 (A6)	*adw2/ayw2* *adw2* *ayw1* *ayw1* *adw4*
B	3,215	B1(Bj), the subgenotype without recombination with genotype C in the precore/core region, distributes in Japan	B1 B2 B3 (B3, B5, B7-9), B6 B4 B5 (B6)	*adw2* *adw2* *adw2* *ayw1/adw2* *adw2* *adr*
C	3,215	Presumed to be the oldest HBV genotype [32]	C1 C2 C3 C4 C5 C6-C12 C13-C15 C16	*adr* *adr* *adr* *ayw2/ayw3* *adw2* *adr* *adr* *ayr*
D	3,182	33-nucleotide deletion at the amino terminus of the preS1 region	D1 D2 D3 D4 D5 D6	*ayw2* *ayw3* *ayw2/ayw3* *ayw2* *ayw3/ayw2* *ayw2* *ayw4/adw3*
E	3,212	3-nucleotide deletion at the amino terminus of the preS1 region		*ayw4*
F	3,215	Intra-genotypic diversity is the mostly high	F1 F2 F3 F4	*adw4* *adw4* *adw4* *adw4*
G	3,248	36-nucleotide insert of the core region; 3-nucleotide deletion at the amino terminus of the preS1 region; two stop codons at position 2 and 28 of the precore region		*adw2*
H	3,245	Closely related to genotype F		*adw4*
I	3,215	Genotype A, C, G recombination	I1 I2	*adw2* *ayw2*
J	3,182	33-nucleotide deletion at the amino terminus of the preS1 region		*ayw3*

(This table is cited from Kramvis *et al.*, Intervirology, 2014 [15] partly modified and used with publisher's permission.)

Genotype B

Genotype B is distributed throughout Asia and has been classified into nine subgenotypes to date. B1(Bj), the subgenotype without recombination with genotype C in the precore/core region, is distributed in Japan [33, 34]. Genotype B is mainly prevalent in Southeast Asia, but can also be found in the Pacific islands. Subgenotype

B5, obtained from a Canadian Inuit population [35], represents genotype B without recombination with genotype C in the precore/core region, as opposed to the other subgenotypes of B, which do show this recombination [33]. Subgenotype B1 is the most likely ancestor of B5, which was possibly carried by the indigenous peoples during migration from Siberia and Alaska to North America and Greenland [36, 37].

Genotype C

Genotype C is mainly prevalent in Southeast Asia, but can also be found in the Pacific islands [38]. According to Paraskevis et al. [39], genotype C is the oldest HBV genotype. It has the highest number of subgenotypes, C1–C16 [40, 41], reflecting the long duration of being endemic in humans. A large number of these subgenotypes circulate in Indonesia [40]. Subgenotype C4 is exclusively found in the indigenous people of northern Australia [42], who are descended from a founder group that emigrated from Africa at least 50,000 years ago [43].

Genotype D

Genotype D is the genotype most widely distributed globally. It is found in northeastern Europe, the eastern and central Mediterranean, northern Africa, and the Middle East. Furthermore, it is highly prevalent in the Indian subcontinent and in a group of islands in the Indian Ocean with high endemic levels of HBV (Nicobar and Andaman) [44], and has also been identified in Oceania [43]. Nine HBV/D subgenotypes (D1–D9) have been described to date [45]. D1 is the most prevalent subgenotype in Greece, Turkey, and North Africa [46, 47]; D2 in northeastern Europe (Russia, Belarus, and Estonia) and Albania [48, 49]; and D3 in Italy and Serbia [50, 51]. D4 is the dominant subgenotype in Oceania [40], D5 in primitive tribes living in India, where a number of different D subgenotypes are also found [52], D6 in Papua New Guinea and Indonesia [53], and D7 in Tunisia and Morocco [54, 55]. Finally, the recently described D8 and D9 subgenotypes are found in Nigeria and India.

Genotype E

Genotype E is characterized by a three-nucleotide deletion in the preS1 region. Genotype E is mainly dominant in West Africa [56]. Genotype E is rarely found outside of Africa, except in individuals of African descent. Although it is found over a large geographical area, it is interesting to note that it has a very low degree of genetic diversity: the isolates studied by means of phylogenetic analysis do not segregate into distinct subgenotypes, but are included in a single monophyletic group [57]. This observation suggests that it has a relatively recent evolutionary history among humans and, despite the forced immigration of West African slaves [57], the absence of any significant spread among Afro-Americans indicates that it was probably rare in West Africa at the time of the slave trade and before the

nineteenth century. The only documented finding of its presence in America is a report by Alvarado et al., who identified nine HBV-infected individuals carrying genotype E in 2010 in the relatively isolated Afro-American community of Quibdò, Colombia [58]. All of these strains were identified by means of their two-nucleotide synapomorphy in the S region, thus forming a highly significant monophyletic group.

Genotype F

Genotype F is indigenous to America, and is the most prevalent HBV genotype in Central and South America, and among the Amerindians of the Amazon basin [59, 60]. Genotype F is classified into four subgenotypes (F1–F4), which are further subdivided into different clades [43]. F1 is highly prevalent in Central America, Alaska, and southeast America [61, 62]; F2 is highly prevalent in Venezuela, and is also present in Brazil [61]; F3 is present in central (Panama) and northern Latin America (Colombia and Venezuela); and F4 is present in Bolivia and Argentina [63]. The presence of HBV-F among the Amerindian population suggests the long evolution of this strain. In the study by Alvarado et al. on the molecular epidemiology and evolutionary dynamics of HBV/F in Colombia [64], it was found that HBV/F3 was the most prevalent subgenotype in Colombia, and its origin was suggested to be in Venezuela. This is probably the oldest F subgenotype, as it is closely related to genotype H [61, 64].

Genotype G

In 2000, genotype G was defined as the seventh HBV genotype from a strain isolated from a French patient [11]. Genotype G harbors a 36-nucleotide insertion in the core region and a genome length of 3248 base pairs. The HBe antigen was detected in the sera of individuals infected with genotype G, despite the presence of two stop codons in the precore region, which should not allow production of HBe antigen [65]. Stuyver et al. proposed that genotype G might have a unique mechanism that allowed production of the HBe antigen. However, we revealed that the sera of all the individuals infected with genotype G showed coinfection with genotype A [66]. Thus, the HBe antigen in the sera of individuals infected with genotype G was produced due to the coinfection with genotype A HBV, which does not have a stop codon in its precore region. Much evidence has accumulated showing that genotype G was not exceptionally associated with co-infection with HBV of other genotypes [67, 68]. Genotype G was identified frequently in homosexual men, and demonstrated very low genome diversity.

Genotype H

Phylogenetic analysis has demonstrated that genotype H is closely related to genotype F. Genotype H is prevalent in Mexico in both the indigenous populations and the mestizos (individuals of mixed descent), suggesting that this genotype has a long history among the descendants of the Aztecs, preceding the arrival of Europeans [12, 69].

Considering that genotype F demonstrates a wide range of diversity, it has been proposed that genotype H should be classified as a subgenotype of genotype F [70].

Genotype I

In 2008, sequence analysis of the complete genome of a single isolate from a Vietnamese male showed that it was closely related to three previously described "aberrant" Vietnamese strains [15, 71] and a ninth genotype, I, was proposed [13]. This proposal was not accepted, because the mean genetic divergence of these four strains from genotype C was 7 % and the recombination analysis was not robust [72]. Subsequently, sequences derived from isolates obtained from Laos [73], the Idu Mishmi tribe in northeast India [74], a Canadian of Vietnamese descent [75], and China [76] have expanded the number of these sequences. The nucleotide divergence of most of these sequences relative to genotype C was at least 7.5 %, with good bootstrap support for the group, thus meeting the criteria for genotype assignment [77]. Two subgenotypes, I1 and I2, with serological subtypes adw 2 and ayw 2, respectively, were described [73]. Genotype I is a recombinant of genotypes A/C/G and an indeterminate genotype [73–76], which clusters close to genotype C when the complete genome is analyzed, and with genotype A in the polymerase gene region [76]. The genotype A and C regions are closely related to subgenotypes A3 and C3, respectively [73–76].

Genotype J

Recently, we found a new strain, named HBV genotype J, from a patient with hepatocellular carcinoma [14]. The characteristics of this genotype are that (1) phylogenetically, the pre-S and S region of genotype J are very close to those of viruses infecting non-human primates, especially the orangutan; (2) the core region of genotype J was close to that of human HBV; (3) and a low possibility of recombination in the pre S, S, and core region. The distribution of genotype J has not yet been fully elucidated. However, these lines of evidence strongly suggest that (1) genotype J has infected humans from ancient times and (2) this type of strain infects both humans and non-human primates. There is a marked possibility that the current HBV vaccine could not protect against this type of HBV infection, even if the third-generation vaccine, based on human HBV sequences, is used. It is also likely that this type of HBV would prevail once the use of the typical HBV vaccine is widely dispersed, as our data have indicated that trans-species infection occurs among humans, orangutans, and gibbons.

Conclusion

Genotypes/subgenotypes of HBV are important in both the clinical manifestation of infection and the response to antiviral therapy. Tracking HBV genotypes/subgenotypes and the genetic variability of HBV can facilitate epidemiological studies, tracing human migrations, predicting the risk of development of severe liver disease,

and the response to antiviral therapy. Knowledge of HBV genotype/subgenotype is therefore important and requires further study.

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Chapter 4
Hepatitis B Virus Immunopathogenesis

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Introduction

Hepatitis B virus (HBV) is the prototype member of the Hepadnaviridae family and it is a small, enveloped DNA virus whose host range is restricted to man and chimpanzees and whose tropism is restricted to the parenchymal cell of the liver, i.e., the hepatocyte [1]. Over the last 30 years, fundamental principles of HBV replication and gene expression have been uncovered, infectious viral genomes have been cloned and sequenced, and all of the viral gene products have been basically characterized [1]. Essential aspects of HBV pathogenesis have been also elucidated during this time, namely that HBV replicates noncytopathically in the hepatocyte and that most of the clinical syndromes associated with this infection reflect the immune response [1]. The innate immune response appears not to contribute significantly to the pathogenesis of liver disease or viral clearance, while the adaptive immune response, especially the virus-specific effector CD8+ T cell response, contributes to both [1–3]. Although effector CD8+ T cells are central to HBV pathogenesis, several other liver resident (including Kupffer cells and stellate cells) and nonresident

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(including platelets and polymorphonuclear or mononuclear antigen-nonspecific inflammatory cells) cells play distinctive roles in it, indicating that the host response to this infection is a highly complex but coordinated process [1, 4].

Despite this large body of information, further improvement in our understanding of the molecular and cellular mechanisms that are ultimately responsible for viral clearance and liver disease is required; however, if we are to develop better treatments for chronic HBV infection and its complications. Indeed, our limited capacity to specifically treat liver fibrosis/cirrhosis and HCC makes extremely important to eliminate their most important trigger, i.e., the chronic liver injury associated with persistent HBV infection.

Termination of chronic HBV infection by available antiviral therapies has been associated with significantly reduced occurrence of liver fibrosis/cirrhosis and HCC development [5]. Unfortunately, a large fraction of chronically infected patients do not respond to these therapies with permanent elimination of HBV; while in the case of first-generation antivirals this was primarily due to dose-limiting side effects and, especially, the emergence of drug-resistant mutants [6], in the case of last-generation antivirals (which are safer and seldom confer resistance) this mostly depends on the impossibility to sustain the cost of treatments that, albeit efficacious at inhibiting HBV replication, cannot be discontinued [7]. Since HBV can be naturally (and permanently) controlled by the immune system, there is a general consensus in the scientific community that new immune therapeutic strategies should be explored for the treatment of chronic HBV infection. These strategies comprise the use of therapeutic T cell vaccines and the infusion of virus-specific effector CD8+ T cells previously expanded ex vivo. Undoubtedly, the implementation of these strategies will greatly benefit from a clearer understanding of the mechanisms by which T cells exert their effector functions in vivo. That our current knowledge on HBV pathogenesis needs to be improved is further suggested by the few applications of therapeutic vaccines attempted thus far [8]. These applications—based on either prophylactic surface antigens or T cell vaccines containing different viral peptides/polypeptides—showed some evidence of T cell response restoration in the blood of chronically infected patients, but it remains poorly understood why such responses failed to induce sustained virological or clinical benefits.

HBV Infection and the Innate Immune Response

The recent identification of sodium taurocholate cotransporting polypeptide (NTCP) as relevant entry receptor [9] has started to shed some light onto the mechanisms whereby HBV gains access to hepatocytes. As discussed at length in other chapters of this book, following viral entry, HBV nucleocapsids are released into the cytoplasm and transported to the nucleus, whereupon the relaxed circular viral DNA genome is repaired by cellular enzymes into an episomal "minichromosome" termed covalently closed circular (ccc) DNA [1, 10–12]. The cccDNA molecule

represents the viral transcriptional template and encodes four capped and polyadenylated RNAs producing structural and nonstructural viral proteins [1, 10–12]. The largest HBV transcript is a 3.5 kb greater-than-genome length RNA, which is translated to produce the viral core and polymerase proteins and also serves as a pregenomic RNA [1, 10–12]. Once encapsidated in the cytoplasm, the pregenomic RNA is reverse transcribed to produce a single-strand DNA copy that serves as the template for second-strand DNA synthesis, producing a circular double-stranded DNA genome [1, 10–12]. Viral capsids containing double-stranded DNA traffic either back to the nucleus to amplify the viral cccDNA genome or to the endoplasmic reticulum where they engage the viral envelope proteins, bud into the lumen, and exit the cell as virions that can infect other cells [1, 10–12].

Of note, the double-stranded HBV DNA genome is completely sequestered within cytoplasmic capsid particles [1, 10–12]. This feature renders the virus potentially invisible to the innate sensing machinery of the host and, in fact, HBV appears not to induce early innate defense mechanisms. In many other viral infections these mechanisms include the induction of apoptosis by the virus [13], the production of antiviral cytokines such as IFNαβ by the infected cells [14], and the triggering of effector functions (e.g. the destruction of infected cells and/or the production of antiviral cytokines such as IFN-γ by NK or NKT cells [15]. Most of what is known about innate defense mechanisms during the early phase of HBV infection has been indirectly inferred from the longitudinal analysis of liver biopsies in experimentally infected chimpanzees. Thanks to these studies, it is apparent that HBV replicates and spreads throughout the liver noncytopathically, and that early innate defense mechanisms significantly contribute neither to clear the infection nor to promote liver injury. Indeed, during the initial spread of HBV infection in the chimp liver (i.e., before T cells enter the organ) there is little or no evidence of hepatocyte damage [16]. This notion goes along with the observations that high hepatic levels of HBV replication in patients and transgenic mice are not associated with overt pathological consequences (including the induction of hepatocellular apoptosis) when cellular immune responses are pharmacologically suppressed [1, 10, 17] or deficient [18]. Global gene expression profiling performed on chimp liver RNA samples at multiple time points after infection further indicates that HBV acts like a stealth virus, remaining largely undetected by infected hepatocytes (which do not show signs of IFNαβ production) or NK and NKT cells (which do not show signs of IFN-γ production) until the onset of the adaptive immune response several weeks after exposure [19]. Whether the lack of relevant innate immune cell activation also involves active suppression of NK or NKT cells (as suggested by the temporal association between the inhibition of NK activity ex vivo and the levels of IL-10 observed in the blood of patients undergoing longitudinal studies [20]) remains to be fully determined. Like in the case of NK cells, little is known about the role of NKT cells during the early phase of HBV infection. Studies in human and mouse hepatocytes showed that hepatocellular HBV gene expression has the potential to induce the production of lysophospholipids capable of activating NKT cells [21], and studies in transgenic mice showed that activated NKT cells have the potential

to perform antiviral activities (mostly dependent on the capacity of these cells to produce IFN-γ [21, 22]. These latter results suggest that NKT activation, although not strongly apparent during the initial phase of infection, may be explored therapeutically. The same is true for the activation of NK cells, which are highly abundant in the human liver (representing 30–40 % of the total intrahepatic lymphocytes [23]) and also capable of producing high levels of IFN-γ [22].

Priming and Arrival of the Adaptive Immune Response

Whatever the role of innate defense mechanisms, the initial evidences of viral clearance and liver pathology associated with HBV infection in chimpanzees occur concomitantly with the arrival of effector CD8+ T cells into the liver [16]. In keeping with this, both liver pathology and viral clearance do not occur as a consequence of CD8+ T cell depletion in these animals [24].

For viruses like HBV that seem not to infect professional antigen presenting cells (APC), tissue-derived dendritic cells capable of processing antigen are likely to migrate to regional lymph nodes [25–27]. Within lymph nodes, dendritic cells present antigens to naïve CD4+ and CD8+ T cells, which in turn become activated and differentiate into populations of effector cells. Although very little is known as per when and where T cell priming occurs during HBV infection, it is widely accepted that T cell priming occurring within the liver would likely induce T cell inactivation, tolerance or apoptosis (thus predisposing the host to viral persistence). At any rate, functional effector T cells that have expanded in secondary lymphoid organs need to eventually migrate to infection sites in order to perform antiviral and pathogenic activities. In most circumstances this is made possible because activation-dependent signals program T cells to express a range of homing molecules that are required to enter specific nonlymphoid tissues [28–30]. For instance, effector T cells migrating to the skin, mucosal tissues or the central nervous system express distinct arrays of selectins, integrins, or chemokine receptors [28–30]. While the nature of these and other tissue-specific T cell homing signals has been elucidated in the last few years [30], the in vivo requirements regulating T cell trafficking into the HBV-replicating liver have been addressed just very recently.

Few observations related to leukocytes other than T cells suggest that the liver may be an exception to the classic multi-step leukocyte migration paradigm involving rolling, adhesion and extravasation in and from post-capillary venules [28, 31]. For example, neutrophil or monocyte adhesion is not restricted to the endothelium of post-capillary venules, but it also occurs in sinusoids [31]. Further, neutrophil or monocyte adhesion to liver sinusoidal endothelial cells (LSEC) often occurs independently of any notable rolling [32]. Notably, LSEC lack both tight junctions between cells as well as a basal membrane [33]. This is in sharp contrast to most vascular beds in other tissues and organs, where a continuous endothelial cell layer and a basement membrane physically separate parenchymal cells from circulating leukocytes [33].

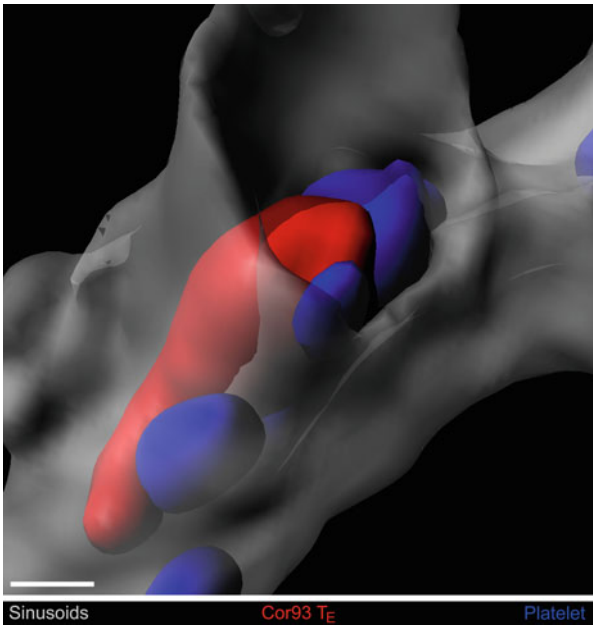

Fig. 4.1 Hepatic effector CD8+ T cell accumulation requires platelets that have adhered to liver sinusoids. Confocal micrographs of the liver of a HBV replication-competent transgenic mouse that was injected 2 h earlier with effector CD8+ T cells specific for HBV core antigen (Cor93 CD8 T_E, *red*). Platelets are shown in *blue* and sinusoids in *gray*. To allow visualization of intravascular event and to enhance image clarity, the transparency of the sinusoidal rendering was set to 45 %. Scale bar represents 2 μm

Recent data indicate that selectins, β2- and α4-integrins, platelet endothelial cell adhesion molecule (PECAM)-1, vascular adhesion protein (VAP)-1, and Gαi-coupled chemokine receptors (all previously thought to be variably relevant for leukocyte trafficking in other organs) are not required for the hepatic homing of effector CD8+ T cells in mouse models of HBV immunopathogenesis [34]. Studies in similar mouse models have instead demonstrated that the intrahepatic recruitment of virus-specific effector CD8+ T cells critically depends on platelets [34–36]. Indeed, the first of those reports showed that platelet depletion is associated with a significant reduction in the intrahepatic accumulation of effector CD8+ T cells and a proportional reduction in liver disease [35]. Both phenotypes are restored upon reconstitution with normal platelets, but not upon reconstitution with platelets that are treated in advance with inhibitors of platelet activation [35]. In vitro findings also indicate that, under the low shear flow conditions likely occurring in the venous circulation of the liver, effector CD8+ T cells tightly interact with platelets [35]. The concept of physical platelet-T cell interaction (Fig. 4.1) leading to hepatic accumulation of the latter cells has been more recently corroborated by in vivo experiments utilizing intravital microscopy; there, it has been shown that the initial sinusoidal arrest of circulating effector CD8+ T cells depends on their capacity to dock onto platelets that have previously adhered to sinusoidal hyaluronan via CD44 [34].

Antiviral Functions of the Adaptive Immune Response

Thanks to additional intravital microscopy studies it was also recently shown that after the initial platelet-dependent arrest, effector CD8+ T cells actively crawl along liver sinusoids (at an average speed of about 10 μm/min) and extend cellular protrusions through sinusoidal endothelial fenestrate to probe underlying hepatocytes for the presence of antigen [34]. Unexpectedly, hepatocellular recognition of HBV antigens leading to cytokine production and hepatocyte killing occurs in a diapedesis-independent manner (Figs. 4.2 and 4.3), i.e., when effector CD8+ T cells are still intravascular and before they extravasate into the parenchyma [34]. Notably, CD8+ T cell antigen recognition and effector functions are inhibited by sinusoidal defenestration and capillarization—two pathological conditions that typify liver fibrosis (see below)—suggesting that the process of liver fibrosis might reduce CD8+ T cell immune surveillance towards infected or transformed hepatocytes [34]. Altogether, the abovementioned studies highlight the notion that rather peculiar mechanisms regulate the ways by which HBV-specific CD8+ T cells recognize hepatocellular antigens and perform effector functions aimed at viral clearance.

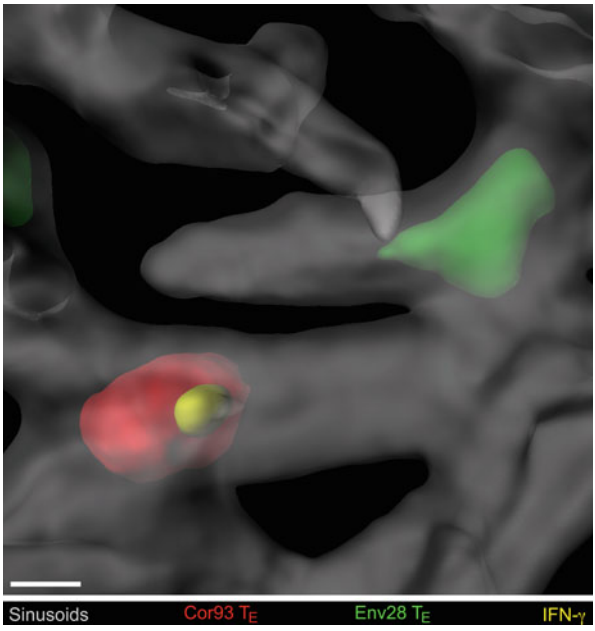

Fig. 4.2 Effector CD8+ T cells recognize hepatocellular antigens and produce antiviral cytokines in a diapedesis-independent manner. Confocal micrograph showing an intravascular HBV-specific effector CD8+ T cell (Cor93 CD8 T_E, *red*) that produces IFN-γ (*yellow*) upon recognition of hepatocellular antigen within the liver of HBV replication-competent transgenic mice. Note that a nearby intravascular MHC-mismatched HBV-specific effector CD8+ T cells (Env28 CD8 T_E, *green*) does not produce IFN-γ. To allow visualization of intravascular events and to enhance image clarity, the transparency of the sinusoidal rendering (*grey*) was set to 70 % and that of the T cell to 60 %. Scale bar represents 4 μm

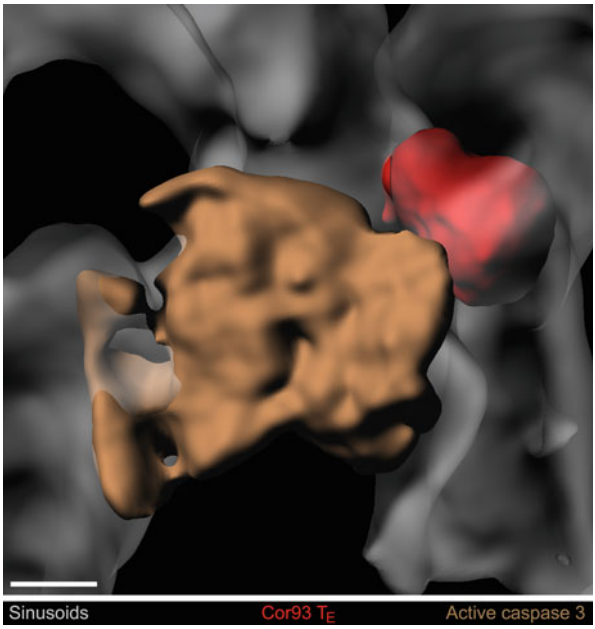

Fig. 4.3 Effector CD8+ T cells kill HBV-expressing hepatocytes in a diapedesis-independent manner. Confocal micrograph showing an intravascular effector CD8+ T cell specific for HBV core antigen (Cor93 CD8 T_E, *red*) juxtaposed to an apoptotic HBV-expressing hepatocyte (*brown*). To allow visualization of intravascular events and to enhance image clarity, the transparency of the sinusoidal rendering (*grey*) was set to 50 %. Scale bar represents 4 μm

There is little doubt that target cell killing by effector CD8+ T cells represents a highly relevant means by which effector CD8+ T cells contribute to HBV clearance. Target cell killing, however, is an intrinsically inefficient process, requiring physical contact between the infected hepatocyte and the T cell. As such, it may not be possible for the effector CD8+ T cells to reach and kill all infected hepatocytes, particularly if one considers that (a) all of the hepatocytes (~10^{11} cells) are routinely infected during HBV infection in chimpanzees and (b) relatively few virus-specific effector CD8+ T cells circulate in the bloodstream of these animals [24]. Thus, viral clearance may require more efficient T cell functions than killing. Important insights into such functions have spawned from studies in HBV replication-competent transgenic mice. There, it was demonstrated that rapid inhibition of HBV replication by effector CD8+ T cells is mostly mediated by noncytolytic mechanisms involving the local production of IFN-γ by these cells [37]. Indeed, IFN-γ, largely via its ability to induce nitric oxide in the liver [38], was shown to prevent the hepatocellular assembly of replication-competent HBV RNA-containing capsids in a proteasome- and kinase-dependent manner [39, 40]. During this process, the levels of viral nucleocapsids in the cytoplasm of hepatocytes rapidly decline, and viral RNAs are destabilized in the hepatocellular nucleus by an SSB/La-dependent mechanism [41–43].

Antibody blocking and knockout experiments in the HBV transgenic mouse model further demonstrated that the cytolytic and antiviral functions of effector CD8+ T cells are completely independent of each other [37]. Thus, effector CD8+ T cells have the potential to inhibit viral gene expression and replication noncytopathically. Similar antiviral activities were recently shown to extend, via a gradient of IFN-γ, more than 80 μm beyond the site of antigen presentation, promoting pathogen clearance in the absence of immunological synapse formation [44].

Of note, additional work in the HBV transgenic mouse model also indicates that, upon entry into the liver, effector CD8+ T cells rapidly lose the capacity of secreting IFN-γ (i.e., the IFN-γ-producing phenotype is maintained only for the few days during which HBV antigens are cleared from the liver), and this is followed by the intrahepatic expansion of IFN-γ-non-producing virus-specific effector CD8+ T cells with unaltered cytotoxic capabilities [45]. These results suggest that sustained antigen stimulation, as occurs during chronic infection, may create an environment in which antiviral (i.e., production of IFN-γ) but not pathogenic (i.e., killing of hepatocytes) functions of intrahepatic virus-specific effector CD8+ T cells are relatively impaired (see below).

Pathogenic Functions of the Adaptive Immune Response During Acute Hepatitis

Even when the adaptive immune response effectively clears a virus, immune-mediated mechanisms can cause significant injury to host tissues. Besides, viruses like HBV can persist in the presence of an active adaptive immune response, predisposing the host to chronic tissue damage (see below). Thus, the balance between the protective and the harmful effects of immunity in some cases clearly shifts to immunity being the primary cause of tissue pathology. In these cases, virus-induced tissue damage is referred to as immunopathology.

Virus-specific T cells can promote immunopathology by directly killing infected cells, by releasing cytokines or other soluble mediators with intrinsic cytotoxic properties and, also, by recruiting antigen-nonspecific inflammatory cells that have the potential to amplify tissue damage. Studies in HBV-infected patients have indeed shown that hepatic infiltrates contain a large antigen-nonspecific component, whose extent correlates with the degree of liver damage [46]. This observation goes along with data obtained in mouse models of HBV immunopathogenesis, where it was shown that the initial apoptotic process triggered by passively transferred virus-specific effector CD8+ T cells involves only a relatively small number of hepatocytes. Using these same models, it was also shown that Kupffer cells (the resident macrophages of the liver) rapidly remove apoptotic hepatocytes in a manner largely dependent on scavenger receptors [47]. As time progresses, though, apoptotic hepatocytes not readily removed by Kupffer cells become secondarily necrotic and release damage-associated molecular pattern molecules (DAMPs) such as the high-mobility group box 1 (HMGB1) protein [48]. HMGB1 is an

abundant nuclear protein acting as an architectural chromatin-binding factor that can be passively released by necrotic, but not apoptotic, cells [49]. Once discharged by necrotic hepatocytes, HMGB1 chemo-attracts mainly polymorphonuclear cells (e.g., neutrophils), the first antigen-nonspecific inflammatory cells arriving at the site of disease [48]. Neutrophil activation leading to production of matrix metalloproteinases (MMPs) rapidly degrades matrix components (e.g., collagen, laminin, fibronectin, and proteoglycans) that are deposited de novo by stellate cells, myofibroblasts and fibroblasts during the process of liver repair [50]. In turn, these matrix-degrading events favor the intrahepatic arrival of numerous antigen-nonspecific mononuclear cells (e.g., antigen-nonspecific CD8+ and CD4 T cells, B cells, monocytes), which respond to their own chemoattractants (mostly chemokines such as CXCL9 and CXCL10 produced locally by parenchymal and non-parenchymal liver cells [51]) and exacerbate disease severity [50]. The pathogenic mechanisms whereby antigen-nonspecific mononuclear cells thus recruited induce organ damage are not well understood and may involve the local production of pro-inflammatory and cytotoxic mediators (including TNF-α, perforin, hydrogen peroxide, superoxide anion, and nitric oxide) by these cells. Moreover, antigen-nonspecific mononuclear cells (in particular NK cells, NKT cells, and T-helper cells) and platelets express Fas-L, a glycoprotein that triggers hepatocellular apoptosis by ligating Fas on the hepatocyte membrane [1].

Observations in acutely infected chimpanzees depleted of CD4+ T cells at the peak of acute HBV infection indicate that the liver disease in this animal is comparable to that detected in immunologically unmanipulated controls [24]. Thus, CD4+ T helper cells may contribute to HBV pathogenesis mainly by facilitating the induction and maintenance of virus-specific effector CD8+ T cells, as has been suggested for other viruses such HCV [52]. In keeping with this, relatively vigorous HBV-specific T helper responses are always associated with quantitatively and qualitatively significant effector CD8+ T cell responses in humans and chimpanzees that resolve HBV infection [1].

Viral and Host Factors Contributing to Viral Persistence

Based on the studies abovementioned, it is apparent that the adaptive immune response to HBV, particularly the CD8+ T cell response, plays key roles in viral clearance and liver disease. Thus, it is reasonable to assume that HBV persistence demands that such response must be either not induced or deficient, or if present it must be overwhelmed, counteracted or evaded.

Notably, both viral and host factors can be involved in the establishment of chronicity. Among the former, it has been suggested that circulating hepatitis B e-antigen (HBeAg) functions as a tolerogenic protein that induces anergy of HBcAg/HBeAg cross-reactive T cells [53, 54]. The capacity of circulating HBeAg to functionally suppress HBcAg/HBeAg-specific T cell responses may explain clinical observations whereby HBeAg-negative variants are frequently cleared following neonatal

exposure and they are usually associated with more severe courses of liver disease in adults [55]. Through its capacity to function as high dose tolerogen, circulating hepatitis B surface antigen (HBsAg) is also considered a viral factor that retains immune suppressive potential [55]. Indeed, the extremely high-serum HBsAg titers observed in certain chronically infected patients are often associated with absence of peripheral HBsAg-specific T cell responses [55]. Mutational inactivation of HBV-derived B cell or T cell epitopes is also thought to facilitate viral persistence [55], albeit this process is likely to play a more prominent role during infection with viruses (such as HCV) that intrinsically possess a much higher mutation rate. Nonetheless, mutations involving epitope residues that anergize or antagonize recognition by the T cell receptor have been reported to arise during HBV infections evolving towards a chronic phase [56].

Among the latter, the notion that immune tolerance is likely responsible for viral persistence in most neonatal HBV infections coupled with the fact that a vigorous, multispecific, and polyclonal cellular immune response is associated with viral clearance in immunocompetent adults strongly suggest that host factors significantly determine infection outcome [1]. Why T cell responses are quantitatively weak and qualitatively inadequate to terminate infection in some adult onset infections remains to be fully determined. An increasing body of studies in HBV infected patients or surrogate animal models suggests that several nonexclusive mechanisms favor viral persistence; they include the inhibition of functional T cell priming as a results of antigen presentation by the hepatocyte [57] or the induction of anergy and exhaustion of initially vigorous T cell responses as a result of (a) antigen overload and excessive T cell stimulation [1], (b) action of regulatory T cells [1], and (c) activation of negative regulatory pathways in T cells (such as those promoted by programmed cell death protein 1 [PD1], cytotoxic T-lymphocyte antigen-4 [CTLA-4], or T-cell immunoglobulin and mucin 3 (Tim-3) [1, 55, 58, 59]. Additional factors contributing to suppression of pre-existing T-cell responses during chronic HBV infection may relate to the relative intrahepatic abundance of selected cytokines (e.g., IL-10 or TGF-β) or enzymes (e.g., arginase) possessing immunosuppressive potential [60–62]. All together, these results indicate that both primary and secondary immunological unresponsiveness to HBV presumably occurs, and this likely contributes to the establishment of persistent infection.

Pathogenic Functions of the Adaptive Immune Response During Chronic Hepatitis

As mentioned above, HBV has the capacity to persist in face of an active, albeit functionally inefficient, adaptive immune response. Indeed, chronic HBV infection could be characterized by a dysfunctional virus-specific CD8+ T cell response that fails to eliminate HBV from the liver but maintains continuous cycles of low-level hepatocellular injury, promoting the development of liver fibrosis/cirrhosis and, ultimately, HCC.

Of note, liver fibrosis and cirrhosis are pathological conditions characterized by an imbalance between fibrogenesis and fibrolysis, resulting in the excessive intrahepatic deposition by stellate cells, myofibroblasts, and fibroblasts of extracellular matrix (ECM) that is qualitatively different in its composition and organization from that of normal liver repair [63]. As a result of this process, a dense, reticulated ECM is initially deposited around the portal areas of the liver and, as a function of time, the fibrosis progressively expands into the lobules with the formation of septa that can eventually connect portal and central veins [63]. Liver cirrhosis represents the final stage of fibrosis in which fibrous septa surround nodules of regenerating hepatocytes, causing profound architectural distortion of the liver, functional insufficiency and diversion of venous blood containing intestinal toxins into the systemic circulation [63]. As mentioned above, liver fibrosis and cirrhosis are also associated with a reduction in number and size of sinusoidal fenestrae (a process often described as "defenestration" of the hepatic sinusoids) and the formation of a basal membrane separating hepatocytes from sinusoidal blood (a process often described as "capillarization" of the hepatic sinusoids) [64]. These events alter the normal exchange of soluble factors between blood and hepatocytes [64] and worsen HBV morbidity (possibly, as stated earlier, because of reduced immune surveillance). The severity and duration of chronic liver disease positively influence liver fibrosis/cirrhosis, and the same is true for HCC where almost all cases take place after many years (usually several decades) of a chronic hepatitis characterized by a sustained liver disease with associated hepatocellular regeneration (i.e., cellular DNA synthesis) and inflammation (i.e., the production of mutagens) [1]. Chronic liver cell injury, therefore, also appears to be a premalignant state promoting cellular processes, like enhanced cellular DNA synthesis and production of inflammatory mutagens, which are oncogenic. Persistence of these events for a sufficiently long period of time results in the random/multiple genetic and chromosomal alterations that contribute to HCC development [1]. Consistent with this, it has been shown in mouse models of immune-mediated chronic HBV infection that the maintenance of low-level liver cell destruction caused by a dysfunctional and detrimental virus-specific CD8+ T cell response is sufficient to cause the development of liver fibrosis/cirrhosis and HCC, and this occurs in the absence of cofactors (e.g., viral integration, HBV X gene expression, or genotoxic agents) that have been proposed to contribute to the development of hepatocellular carcinoma in humans [65, 66].

The notion that a virus-specific CD8+ T cell response, although inefficient and essentially harmful, remains detectable in the liver of patients chronically infected with HBV can be exploited therapeutically. One reasonable approach is to restore the functionality of such response to the levels that are observed in patients undergoing self-limited acute infection. There, a number of different hurdles must be overcome and, in particular, the severe exhaustion that typifies T cells chronically exposed to large amounts of antigens (with the hope that these cells do not carry dysfunctional signatures that are permanent) [67]. Another approach, conceptually different, is to further reduce the capacity of T cells to induce chronic liver damage with the idea that—in so doing—the onset of liver fibrosis/cirrhosis might be prevented or delayed. In keeping with this and building on the observation that platelets are instrumental to

intrahepatic effector CD8+ T cell homing, a recent mouse study demonstrated that clinically achievable doses of the antiplatelet drugs aspirin and clopidogrel, when administered continuously after the onset of liver disease, can prevent the development of advanced fibrosis and HCC, greatly improving overall survival [66]. These outcomes were preceded by and associated with reduced hepatic accumulation of pathogenic virus-specific effector CD8+ T cells and pathogenic virus-nonspecific inflammatory cells, and reduced hepatocellular injury and hepatocellular proliferation [66]. Irrespective of antiplatelet treatment, intrahepatic virus-specific effector CD8+ T cells analyzed at multiple times during chronic liver injury were found to express virtually no IFN-γ [66]; this is consistent with the abovementioned observation that HBV-specific effector CD8+ T cells rapidly abandon the ability to produce this antiviral cytokine after intrahepatic antigen recognition in mice [45] and that IFN-γ-nonproducing HBV-specific CD8+ T cells are commonly present in the liver of chronically infected patients [68]. Altogether, the abovementioned results indicate that the antiplatelet drugs aspirin and clopidogrel effectively prevent or delay the onset of severe liver fibrosis HCC and improve survival, supporting the concept that platelets promote CD8+ T cell-induced liver immunopathology. The results also reinforce the notion that a detrimental CD8+ T cell response is both necessary and sufficient to induce the complications of chronic viral hepatitis and they suggest that future drugs targeting platelet function or other functions linked to disease severity may be a therapeutic option in patients with chronic HBV infection.

Conclusive Remarks

Our comprehension of the immunopathogenesis of HBV infection has significantly advanced over the last 30 years. Regardless of this, however, we are still far away from the clinical application of immune therapeutic approaches capable of terminating chronic HBV infection. As largely discussed in other chapters of this book, last generation antivirals have proven potent efficacy in the absence of significant side effects. Unfortunately, the relative incapacity of these molecules to completely eliminate the virus renders the likelihood of viral rebounds following treatment withdrawal quite high. Future work intended to expand our current knowledge of the complex host-virus relations that determine the immunopathogenesis of HBV infection may guide us to the design of new strategies that, alone or in combination with existing or forthcoming antivirals, will direct the immune system to terminate chronic HBV infection and/or its life-threatening complications.

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Chapter 5
Pathobiology of Hepatitis B Virus-Induced Carcinogenesis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent solid tumors worldwide, with more than 250,000 new HCC cases annually and an estimated 500,000–600,000 deaths/year [1, 2], and because of its very poor prognosis is the second cause of cancer death worldwide [3].

HCC development is driven by multiple viruses (HBV, HCV) and chronic metabolic alterations that lead to chronic inflammation, DNA damage, and epigenetic and genetic changes that affect both "common" and "etiology specific" oncogenic pathways. The clinical and molecular heterogeneity of HCC translates into "molecular signatures" that identify discrete molecular subgroups of HCC and stratify patients according to prognosis.

Numerous signaling modules are deregulated in HCC, including growth factor signaling (e.g., IGF, EGF, PDGF, FGF, HGF), cell differentiation (WNT, Hedgehog, Notch), and angiogenesis (VEGF). Intracellular mediators such as RAS and AKT/mTOR also play a role in HCC development and progression. The use of novel molecular technologies such as next-generation sequencing (NGS) has enabled the identification of pathways previously underexplored in the HCC field, such as chromatin remodeling and autophagy.

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Different molecular mechanisms are involved in aberrant pathway activation, including point mutations, chromosomal aberrations and epigenetically driven downregulation. Importantly, whereas mutations and chromosomal aberrations have been predominantly found in tumor tissues, with the notable exception of the recently reported TERT promoter mutations, deregulation of signaling pathways and epigenetic changes are also detected early in the natural history of HCC development, at the stage of cirrhosis or dysplastic nodules.

Chronic inflammation, double-strand breaks (DSBs) accumulation, epigenetic modifications, chromosomal instability, and early neo-angiogenesis are the major driving forces in hepatocytes transformation, HCC development and progression. All "etiologic" factors (i.e., chronic HBV and HCV infections, chronic metabolic alterations) seem to act through overlapping and non-overlapping mechanisms that finally converge on these pathways.

Recent views on the molecular pathogenesis and classification of HCCs and their impact on the design of new therapeutic approaches can be found in Refs. 4–10. In this chapter, we focus on the molecular characterization of HBV-related HCCs and the role of HBV genetic variability, HBV integration into the host genome and wild-type and mutated/truncated viral proteins to HBV carcinogenesis.

Epidemiology and Risk Factors

Chronic hepatitis B infection remains a major public health problem worldwide despite the availability of the HBV vaccine since the early 1990s and the decreased incidence of HBV new infections in most countries [2]. Over 400 million people chronically infected with HBV are at high risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) [11], making HBV the most common carcinogen after tobacco. Recent estimates attribute to HBV over 50 % of HCC cases worldwide [1, 2]. Because of geographic variations in the incidence of hepatitis B, the fraction of HCC attributable to HBV varies significantly, representing less than 20 % of all cases of HCC in the USA and up to 65 % in China and Far East; Europe is divided into a low-risk (18 %) area (west and north Europe) and a high-risk (51 %) area (east and south Europe) [3]. The role of HBV in HCC may be greater than that depicted by sero-epidemiologic studies, as suggested by the increased risk of developing HCC in patients with occult HBV infection [defined as persistence of free and/or integrated forms of HBV-DNA in the liver in the absence of the viral marker HBsAg in the serum [12]] and after hepatitis B surface antigen (HBsAg) clearance [13–17].

The lifetime risk of developing HCC is 10- to 25-fold greater for chronic HBV carriers, as compared with non-infected populations [18]. Important epidemiologic features of HBV-related HCC include younger age at presentation compared with HCC cases related to alcohol, non-alcoholic steato-hepatitis, and HCV and the absence of cirrhosis in one-third of patients with HCC [11, 18].

Several virus-related, host-related, dietary, and lifestyle factors are associated with an increased risk of HCC in patients who are chronically infected by

HBV. Increasing age, reflecting longer exposure to HBV, and male gender have long been known to enhance the risk for HCC [18]. More recently, evidence has emerged that gender disparity in HCC risk may also reflect protection against this tumor by estrogen via complex networks involving hepatocyte nuclear factor-4a [19] and IL6 signaling [20]. Hepatitis severity and coinfection with hepatitis D virus and hepatitis C virus (HCV), or human immunodeficiency virus (HIV) have been also found to augment the risk of HCC in chronic HBV infection. Alcohol consumption, a well established independent risk factor for HCC, also plays a synergistic role with a more than twofold increase of the carcinogenic risk of HBV [21]. Tobacco smoking is also associated with an increased risk of HCC in patients with HBV-related cirrhosis, with evidence of a quantitative relationship between smoking and cancer risk. HCC frequency is particularly high in Asia and Africa due to the high frequency of viral hepatitis infections and to Aflatoxin B1 (AFB1) exposure [1, 22]. Other known etiological factors of HCC development, including hemochromatosis, steatosis, nonalcoholic fatty liver diseases, and diabetes, often act as co-factors of overt and occult HBV infection for HCC development [19, 21–23].

HBV Viral heterogeneity and HCC

As discussed in the previous chapters, the HBV genome is, in plasma circulating infectious HBV particles, a circularized linear partially double-stranded DNA of about 3200 nucleotides [24]. Once entered the cell, HBV DNA is converted into a covalently closed circular DNA (HBV cccDNA) that accumulates in the nucleus of infected cells as a stable episome and is organized into nucleosomal structures [25–27]. HBV cccDNA is responsible for persistent HBV infection of hepatocytes and is the template for the transcription of all viral mRNAs, including the pregenomic HBV RNA (pgRNA), the obligatory replicative intermediate, which is reverse transcribed by the viral polymerase to produce the first HBV DNA strand and sustain the viral replication in cytoplasmic core particles [28]. Chromatin-modifying enzymes, cellular transcription factors, and the viral proteins HBx and HBc are recruited on the cccDNA mini-chromosome to regulate its transcription and, ultimately, viral replication [25–27, 29–31]. The integration of viral DNA into the host genome, that occurs randomly in regenerating infected hepatocytes [24], contributes to viral pathogenesis both by cis-acting mechanisms and by the continuous expression of *trans*-acting wild type and truncated HBx or truncated preS/S polypeptides bearing enhanced transforming properties [32]. The goal of therapy in chronic hepatitis B (CHB) is the persistent suppression of HBV replication [33]. Due to the lack of direct effect on the cccDNA in the nucleus, a sustained suppression of HBV replication by NUCs does not lead to cccDNA elimination (eradication) [26]. Long-term inhibition of HBV replication by NUCs has resulted into a reduction of the risk of HCC in non-cirrhotic patients by preventing progression to cirrhosis whereas in cirrhotic patients the reduced rates of anticipated liver mortality due to clinical decompensation, has often translated into increased rates

of HCC-related mortality or, at best, a marginal effect on HCC development in long-term follow-ups [33, 34].

The risk of developing HCC also correlates with HBV genotype, HBV genomic mutations, and HBV replication [14, 15]. At least eight different HBV genotypes have been identified (A-H) where the nucleotide sequence varies by at least 8 %. HBV genotype C has been associated with a higher risk of HCC development [35]. However, the findings are not univocal and no firm conclusion can be drawn on whether HBV genotypes harbor different oncogenic potential. HBV replication drives both disease severity and progression and the persistence of high-serum HBV-DNA levels correlate in the clinical setting with liver damage accumulation, evolution to cirrhosis, and HCC development [14].

Variability of HBV genome is basically attributed to lack of proofreading by the HBV polymerase and the high copy number of the virus that lead to the selection of HBV quasi-species containing several mutations within their viral genome. Some of these mutations may provide a replicative advantage to the virus while others are detrimental. The accumulation of mutations reflects the duration of active HBV infection, the strength of the immune response and the selection pressure exerted by exogenous factors such as antiviral therapies and vaccinations [36]. HBV mutations are not distributed randomly over the entire genome but cluster in particular regions such as the basal core promoter (BCP)/preCore region and the preS/S region [37].

The double-mutation T1762/A1764 in the basal core promoter is significantly associated (OR: 6.72) with the development of HCC in both genotypes B and C [38] and can be detected in plasma up to 8 years before HCC diagnosis suggesting a possible use of this mutation as a strong predictive biomarker, at least in some geographical areas [38].

Several HBV variants with point mutations, deletions, or insertions in the preS1 and preS2 sequences are often found in patients with long-lasting chronic hepatitis B (CHB) [37] and variants defective for the M envelope protein are the most frequently selected [39–44]. A role of HBV preS mutants in the pathogenesis of HCC is supported by a large number of experimental and clinical evidence [45–50]. A meta-analysis of 43 studies and ~11,500 HBV-infected patients has shown that infection with preS mutants is associated with a 3.77-fold increased risk of HCC [51] and the predictive value of preS deletion mutants has been recently confirmed in a prospective study [45]. The HBV variants commonly associated with HCC include (a) mutations of the preS2 start codon and/or deletions in the 5′-terminal half of the preS2 region and (b) deletions in the 30-terminal half of the preS1 region [52, 53]. These preS deletion deletions correspond to viral regions containing B or T cells epitopes and are thought to represent HBV immune escape variants [54]. Both preS1 and preS2 mutations may lead to unbalanced production of the different envelope proteins and the accumulation of mutated L protein in the ER of hepatocytes, resulting in the activation of the ER stress signaling pathway [48, 55–61]. ER stress can generate reactive oxygen species and cause oxidative DNA damage, genomic instability, and ultimately favor HCC development [48, 62–66].

HBV mutants with premature stop codon at position 172 or 182 of the S gene have been frequently found in patients with cirrhosis and HCC [67–70] and

nucleos(t)ide analogues (NUCs) can select mutants in the B, C, and D domain of the reverse transcriptase/DNA polymerase (Pol) associated with pharmacological resistance [71]. Since the HBV surface gene overlaps completely, on a different open reading frame (ORF), with the Pol gene, some changes in Pol ORF selected by NUCs can affect the overlapping surface gene. The rtA181T/ sW172* mutation, selected by lamivudine or adefovir, results in the generation of a stop codon in the S ORF and the production of a truncated S protein with a dominant negative secretion defect that accumulates within the hepatocyte leading to ER stress and activation of oncogenic cellular pathways to be cited [71]. Importantly, the emergence of the rtA181T/sW172* mutant associated with an increased risk of developing HCC in patients [72].

Genetic Alterations in HBV-Related HCC

Extensive evidence indicates that HCC is an extremely heterogeneous tumor at the genetic and molecular and genetic level with a complex mutational landscape and multiple transcription and signaling pathways involved [73–75].

Genetic host factors are thought to play an important role in the development of HCC during HBV infection and several studies of family clusters, mostly performed in the Far East, have identified single-nucleotide polymorphisms (SNPs) associated with an increased HCC risk as compared to the control populations [76]. Risk-associated SNPs in chromosome 8p12 have been associated with DLC1 locus (Deleted in Liver Cancer 1) deletion or chromosomal loss in patients with HBV-related HCC [77]. Additional SNPs associated with HCC development in patients with chronic hepatitis B were identified in the CTL-4 (cytotoxic T-lymphocyte antigen 4) gene [78] and the KIF1B locus in chromosome 1p36.22 [79] but, overall, their predictive power seems to be low and need to be validated in larger cohorts of multiple ethnicity.

HBV-related tumors generally harbor a higher rate of chromosomal abnormalities than tumors linked to other risk factors [80], likely due to the ability of HBV to generate genomic instability through both viral DNA integration and the activity of the X protein (see below). HBV-related HCCs are characterized by higher frequencies of TP53 mutations at, as well as outside, the aflatoxin B1-related codon 249 hotspot mutation [74], and AXIN1 [80], whereas activating β-catenin mutations are more frequent in non HBV-related HCCs [80]. IRF2 inactivation, that leads to impaired TP53 function, was found exclusively in HBV-related tumors [81]. A recent whole-exome sequencing analysis of 243 HCCs [82] identified, by integrating mutations, focal amplifications and homozygous deletions, 161 putative driver genes associated with 11 pathways altered in >5 % of the tumors [TERT promoter mutations activating telomerase expression (60 %); WNT/β-catenin (54 %); phosphoinositide 3-kinase (PI3K)-AKT-mTOR (51 %); TP53/cell cycle (49 %); mitogen-activated protein kinase (MAPK) (43 %); hepatic differentiation (34 %); epigenetic regulation (32 %); chromatin remodeling (28 %); oxidative stress (12 %);

interleukin (IL)-6/JAK-STAT (9 %); transforming growth factor (TGF)-β (5 %)]. New genes found to be recurrently mutated in HCC included β-catenin inhibitors (ZNRF3, USP34 and MACF1), hepatocyte-secreted proteins (APOB and FGA), and the TGF-β receptor ACVR2A. TERT promoter mutation were usually an early event, whereas FGF3, FGF4, FGF19, or CCND1 amplification and TP53 and CDKN2A alterations appeared at more advanced stages in aggressive tumors. HCV infection, metabolic syndrome, and hemochromatosis did not show significant associations. Alcohol-related HCCs were significantly enriched in CTNNB1, TERT, CDKN2A, SMARCA2 and HGF alterations. IL6ST mutations were found in HCCs with no known etiology. HBV-related HCCs were more frequently mutated in TP53 [82].

Genome-wide transcriptomic analysis of well-annotated HCCs identifies subgroups of HCC associated with specific clinical and genetic characteristics [73, 83]. In the study from Boyault et al. [83] the G1–G2 subgroups demonstrated overexpression of fetal stage-associated genes and were controlled by parental imprinting; G3 tumors were characterised by TP53 mutations and demonstrated adverse clinical outcome; G4 was a heterogeneous subgroup of tumours; G5–G6 subgroups were strongly related to β-catenin mutations, leading to Wnt pathway activation. G1 and G2 tumors were both related to HBV infection and displayed frequent activation of the PI3K/AKT pathway, but differed for the overexpression of genes expressed in fetal liver and controlled by parental imprinting (G1) and the frequent mutation of the PIK3CA and TP53 genes (G2) [83]. In a more extended study focused on the molecular characterization of HBV-related HCCs Amaddeo and colleagues [84] confirmed that the TP53 gene was the most frequently mutated gene in HBV-related HCC (41 % vs 16 %, in non-HBV tumors) and that inactivation of the IRF2 tumor-suppressor gene, that controls p53 protein activation, was exclusively identified in HBV-HCC (7 %) but also showed that HBV-related HCCs display a wide genomic diversity and were distributed in all G1–G6 transcriptomic subgroups. In particular G2 and G3 profiles were enriched and genes associated with progenitor features (EpCAM, AFP, KRT19, and CCNB1) were significantly overexpressed in HBV-related HCCs compared to HCCs related to other etiologies [84]. G4–G6 profiles characterized a small subset of HBV-related HCCs in older patients with other cofactors such as HCV, alcohol consumption, or NASH.

microRNAs [small noncoding single-stranded RNAs that regulate gene expression, primarily at the posttranscriptional level] are increasingly recognized as key players in the regulation of liver functions and in hepato-carcinogenesis [85]. Using global miRNA profiling of HCC cell lines or liver tissue, the expression of several miRNAs has been found to be either upregulated (miR-18, miR-21, miR-221, miR-222, miR-224, miR- 373, and miR-301), or downregulated (miR- 122, miR-223, miR-125, miR-130a, miR-150, miR-199, miR-200, and let-7 family members) in HCC [85]. Differences between HCV- and HBV-related HCC associated miRNAs are emerging. miR143, miR34, and miR-19 are upregulated in HBV-related HCC and promote the more aggressive biological phenotype of HBV-related HCCs [86, 87]. Downregulation of Let 7a by HBx is associated with upregulation of STAT3-induced cell proliferation [88]. HBx suppression of miR-152 leads to upregulation

of DNMT1, which methylates the promoters of many tumor suppressors [89]. Finally, miR26 expression is low in HBV-related HCCs and lower in man than in women and associate with a poor survival and lower response to adjuvant therapy with interferon-α [90].

Epigenetic Mechanisms in HBV-Related HCC

The principal mechanisms involved in chromatin remodeling and the epigenetic control of gene expression are DNA methylation, enzymatic covalent *histone modifications*(e.g., acetylation, methylation, and phosphorylation) and nucleosomal re-structuring by ATP-dependent*chromatin re-modeling complexes*.

Global hypo-methylation of DNA with selective hyper-methylation of tumor-suppressor genes promoters containing CpG islands, have been shown to modify gene expression patterns in the liver before HCC appearance. A number of tumor-suppressor genes, including RASSF1A, p16/INK4A, APC, E-cadherin, SOCS-1, IGF-binding protein 3 (IGFBP3), and glutathione *S*-transferase P1 (GSTP1), have been shown to be silenced by DNA methylation in a large proportion of liver tumors, and this process often starts at pre-neoplastic (cirrhotic) stages [91]. A higher rate of promoter methylation for specific genes such as pl6INK4A, E-cadherin, ASPP1, and ASPP1 has been observed in HBV-related tumors compared to non-viral tumors [91, 92]. Using genome-wide methylation profiling, Villaneueva and collegues [93] have identified and validated a 36-probes methylation signature able to accurately predict survival in HCC patients. This signature correlated with known predictors of poor outcome and identified patients with the mRNA signatures of proliferation and progenitor cell features. The study confirmed a high prevalence of genes known to be deregulated by aberrant methylation in HCC (e.g., RSSFA1, APC, NEFH, IGF2, RAFF5, NKX6.2, SFRP5) and other solid tumors (e.g., NOTCH3 in acute leukemias; NSD1 in glioblastoma; ZIC1 in colorectal cancer) and described new potential candidate epidrivers in HCCs (e.g., SEPT9, a tumor suppressor described in colon and ovarian cancer; ephrin-B2 ligand EFNB2, reported hyper-methylated in patients with acute leukemia; homeobox A9, forkhead box G1 and runt-related transcription factor 3, involved in TGF-b receptor-signaling; FGF8 and FGF6) [93].

HDAC1, 2, and 3 are overexpressed in 30–50 % of HBV-related HCCs and HDAC3 is an independent predictor of tumor recurrence following liver transplantation [94]. A significant upregulation of several HDACs (namely, HDAC1, 2, 3, 4, 5, and 11) was also described in HCV-related HCCs where DNA copy gains in *HDAC3 and HDAC5* correlated with their mRNA upregulation [95]. Importantly, combining the pan-HDAC inhibitor panobinostat and sorafenib strongly potentiated treatment efficacy and improved survival in HCC xenograft models [95]. Pathologic activation of *Ezh2 and PRC2*, either through Ezh2 overexpression or Ezh2-activating mutations, is among the most common alterations observed in a wide variety of cancerous tissue types, including non-Hodgkin's lymphoma, prostate, breast and HCCs [96–103]. Increased expression of EzH2 is frequently

detected in HCC tissues, correlate with the aggressiveness and/or poor prognosis of HCCs and may help to discriminate between pre-neoplastic/dysplastic lesions and cancer [104, 105]. Similarly, an increased expression of the G9a histone methyltransferase has also been reported in HCC [105]. Knockdown of EzH2 expression in HCC cells is sufficient to reverse tumorigenesis in a nude mouse model, thus suggesting a potential therapeutic value of EzH2 inhibition in HCC [106]. An HCC-specific long noncoding RNA (lcn) [lncRNA-HEIH] associates with EzH2 to repress EzH2 target genes and facilitate HCC tumor growth in HBV-related HCCs [107] and, in particular, EzH2-mediated repression of Wnt antagonists has been found to promote β-catenin-dependent hepato-carcinogenesis [108]. On the other hand, animal models of HBx- and HBV-mediated tumorigenesis downregulate the chromatin modifying proteins Suz12 [another PRC2 component] and ZnF198 [part of the LSD-Co-RESR-HDAC1 repressor complex] in liver tumors [109]. Suz12/Znf198 downregulation is accompanied, both in animal models and human HBV-related HCCs, by the overexpression of a number of Suz12/PRC2 direct target genes, including the hepatic cancer stem cell markers BAMBI and EpCAM [110].

Several studies also identified mutations in a group of chromatin regulators (*ARID1A*, *ARID1B*, *ARID2*, *MLL*, and *MLL3*) in approximately 20 % of all tumors, including virus- and alcohol-related HCCs [81, 111–113]. ARID1A and ARID1B are crucial and mutually exclusive subunits of the SWI/SNF ATPase-powered nucleosome re-modeling complex. ARID2 is a subunit of the poly-bromo- and BRG1-associated (PBAF) remodeling complex, which is implicated in the control of ligand-dependent transcription by nuclear receptors. Inactivating mutations in *ARID1A*, and its role as a tumor suppressor have been reported in several malignancies, including ovarian, colorectal, and gastric cancer [114–117].

Direct Oncogenic Roles of HBV

The long latency period between HBV infection and HCC and the strong relation between HCC incidence and age has often been used to support an indirect role of HBV in hepatocytes transformation. Increasing evidence suggests, however, that HBV contributes to HCC by directly promoting growth factor-independent proliferation, resistance to growth inhibition, tissue invasion and metastasis, angiogenesis, reprogramming of energy metabolism, and resistance to apoptosis, i.e., the host gene expression pathways and cellular phenotypes that are recognized as hallmarks of cancer [4, 118]. The ability of HBV-encoded proteins to blunt both the innate and adaptive immune responses favors the persistence virus replication and contributes to HCC by sustaining chronic inflammation without viral clearance. Notably, most virus-induced changes in host gene expression that promote HCC also support virus replication and/or protect virus-infected hepatocytes from cytokine- and cell-mediated damage or destruction.

HBV can promote carcinogenesis by three different mechanisms (Fig. 5.1). First, the integration of viral DNA into the host genome contributes to chromosome instability

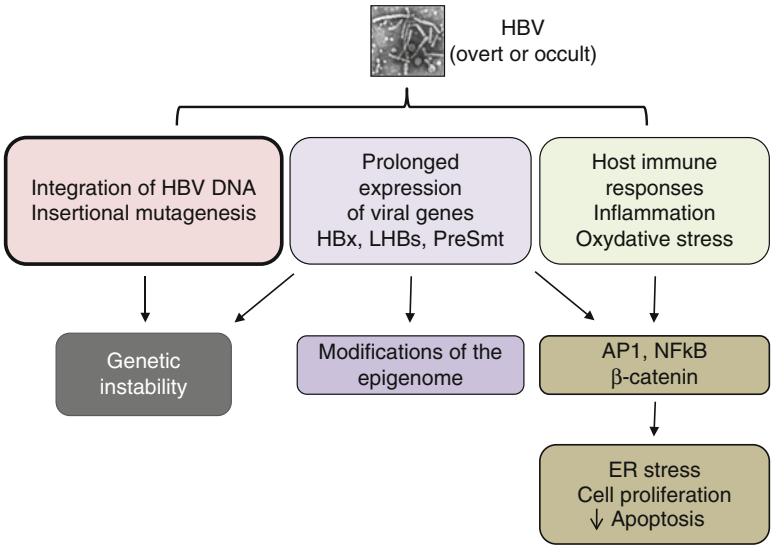

Fig. 5.1 HBV carcinogenesis. HBV contributes to HCC by (**a**) insertional mutagenesis due to integration of the viral DNA into host chromosomes; (**b**) increased genomic instability caused by both viral integration and the activity of the viral protein HBx; (**c**) modifications of the epigenome promoted by HBx and HBc; (**d**) modulation of cell death and proliferation pathways by the prolonged expression of viral proteins (wild-type and mutant HBx, LHB envelope proteins, truncated MHB proteins, HBc)

[119]. HBV DNA integration in host chromosomes, although dispensable for viral replication, is detected in about 80 % of HCCs [32]. Second, classic retrovirus-like insertional mutagenesis can occur. HBV integration at specific genomic sites provides a growth advantage to a clonal cell population that eventually accumulates additional mutations. HBV integrations within the retinoic acid receptor ß (RARß) and the cyclin A as target genes [32] provided the first evidence and additional genes were later found to be targeted by HBV integration in tumors, including recurrent HBV DNA integration into the hTERT gene encoding the catalytic subunit of telomerase [120–123]. More recently, next-generation sequencing (NGS) studies of ~400 HBV integration breakpoints from over 100 HBV-positive HCCs confirmed that HBV integration is more frequent in the tumors (86.4 %) than in adjacent liver tissues (30.7 %). Approximately 40 % of HBV breakpoints within the HBV genome are located near the viral enhancer and the X gene and core open reading frames and copy-number variations (CNVs) are increased at HBV breakpoint locations indicating that HBV integration likely induces chromosomal instability [113]. Multicentric tumor pairs develop from independent mutations [111]. Most HBV breakpoints fall near coding genes, mainly into exons or regulatory regions, including the TERT, MLL4 (mixed-lineage leukemia protein 4), CCNE1 (Cyclin 1), SENP5 (Sentrin-specific protease 5), ROCK1 (Rho-associated coiled-coil containing protein kinase 1) genes, whose expression was upregulated in tumors

versus the normal tissue [111, 113]. More recently, Lau and coworkers [124] have reported the integration of HBV sequences into the host long interspersed nuclear element (LINE) and the generation of a HBx-LINE1 chimeric transcript in 21 out of 90 HBV-related HCC patient tumors that is significantly associated with poor patient outcome [124]. Mechanistically, the HBx-LINE1 transcript acts as a long noncoding RNA by increasing the nuclear localization of β-catenin and by activating Wnt signaling and its oncogenic properties are independent from its protein product [124]. Notably, HBx-LINE1 fusion transcripts were not detected in 50 HBV-related HCCs from Europe [125]. The high frequency of this oncogenic transcript might be restricted to the Hong Kong population where HBV genotype C is predominant and remains to be validated in other independent series of HCC. The third direct mechanism of HBV carcinogenesis is based on the ability of viral proteins (HBx, HBc, and preS) to affect many cell functions, including cell proliferation and cell viability and to sensitize liver cells to mutagens. In transgenic mouse models, unregulated expression of the HBV X and large S proteins are associated with hepatocarcinogenesis [59, 126].

HBx Protein

HBx regulatory protein is both required for HBV cccDNA transcription/viral replication [29, 127], and thought to contribute to HBV oncogenicity. Although the mechanisms underlying these pleiotropic activities of HBx have not been fully elucidated, regulation of transcription, through direct nuclear (transcription and chromatin) and/or indirect cytoplasmic (cell signaling) mechanisms, is thought to play an important role (Fig. 5.2). In the following subsections we provide a rather comprehensive description of the vast scientific literature reporting on HBx activities over almost two decades. We have underlined wherever possible the relevance of those results that have been generated in the context of HBV replication/infection systems and/or conformed in either in vivo animal models or through the ex vivo evaluation of CHB- and HBV-related HCC patients samples.

HBx, Chromatin and Viral/Cellular Transcriptional Control

HBx is recruited to the cccDNA minichromosome in HBV-replicating cells to increase transcription of the nuclear cccDNA minichromosome [29, 127]. In the absence of HBx, cccDNA-bound histones are hypoacetylated, and the cccDNA transcribes significantly less pgRNA [29]. HBx also binds and blocks the inhibitory activity on HBV transcription exterted by the PRMT1 methyl-transferase [30] and the Tudor-domain protein Spindlin-1 [128]. Additional mechanisms by which HBx can potentiate HBV replication include the down-regulation of DNMT3A

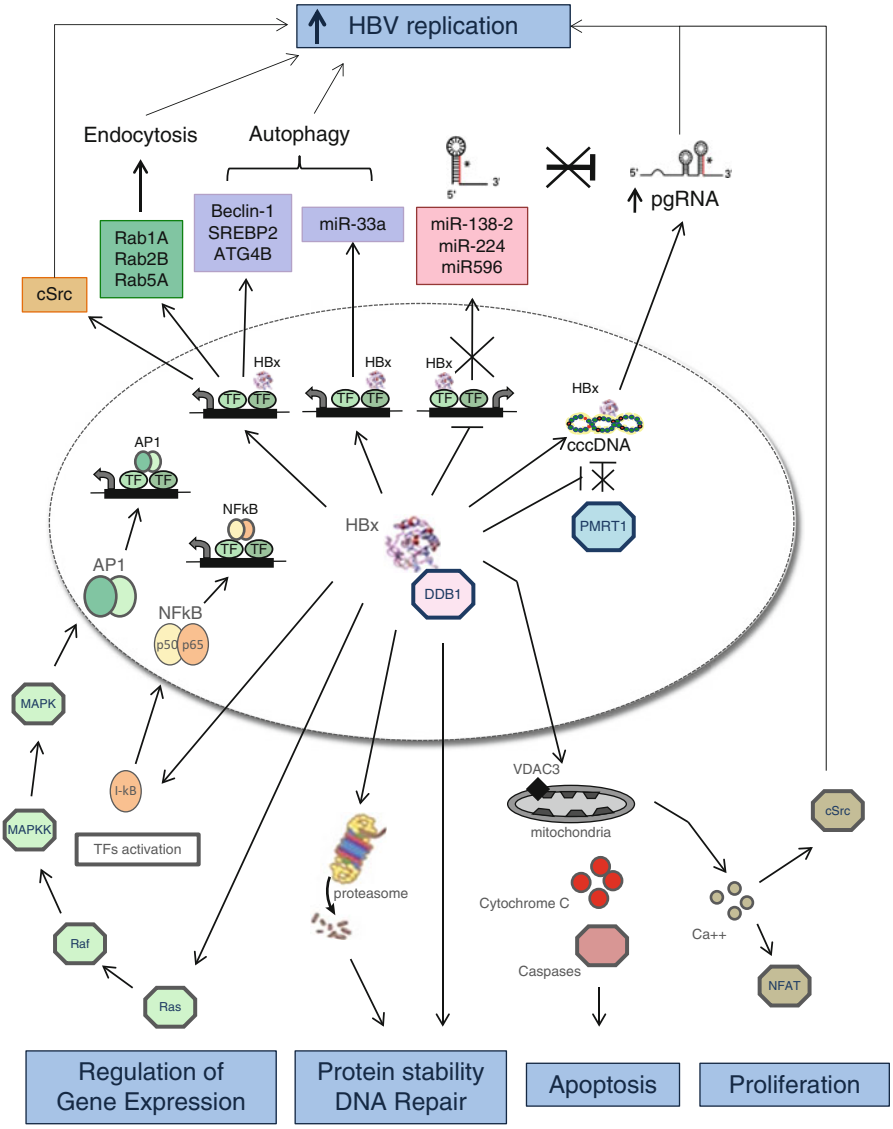

Fig. 5.2 Multiple cellular targets of the regulatory protein HBx. The regulatory protein HBx, in addition to be required for viral replication, contributes to hepatocytes transformation by multiple mechanisms, mediated by its interaction with a large number of cellular targets. HBx physically interacts with several cellular proteins that modulate cell proliferation, cell death, transcription, and DNA repair

expression through the induction of miR-101 [129], induction of endocytosis and autophagy that are required for viral replication [130–132], binding to the UV-DDB1 protein [133], and elevation of cytosolic calcium levels [134].

ChIP-Seq genome-wide analysis of HBx chromatin recruitment in HBV-replicating cells revealed a specific binding of HBx to a large number of new and known HBx target sequences [135], including protein-coding genes and ncRNAs [16 lncRNA promoters and 32 lncRNA intragenic regions, 44 snoRNA, 3 snRNA, and 75 microRNA promoter regions]. 39 out of the 75 HBx-targeted miRNAs are classified as intragenic and 15 of them display HBx peaks in the promoter region of their target genes. Multiple transcription factors seem to mediate the recruitment of HBx on the target chromatin (i.e., E2F1, CREB, β-catenin, NFkB) [135]. Pathway analysis of the protein-coding genes and miRNAs potentially regulated by HBx showed an enrichment in genes/ncRNAs involved in cell metabolism, chromatin dynamics and cancer but also in genes/ncRNAs that modulate HBV replication. (Ras, calcium transport, endocytosis, MAPK/WNT pathways, Src, the EGF/HGF family). Functional experiments identified new mechanisms by which HBx, in addition to its activity on the viral cccDNA, boosts HBV replication, mediated by direct transcriptional activation of genes and miRNAs that potentiate endocytosis (RAB family) and autophagy (ATGs, beclin-1, miR-33a) and the transcriptional repression of miRNAs (miR-138, miR-224, miR-596) that directly target the HBV pgRNA and would inhibit HBV replication [135].

Mechanistically, the activity of HBx on transcription of both cellular genes and the viral genome rely on the interaction with transcription factors and chromatin modifying enzymes and the modulation gene expression through epigenetic modifications (Fig. 5.3). Indeed, HBx binds several nuclear proteins involved in the regulation of transcription including component of the basal transcriptional machinery (RPB5, TFIIB, TBP, TFIIH), coactivators (CBP, p300, and PCAF) and transcription factors (ATF/CREB, ATF3, c/EBP, NF-IL-6, Ets, Egr, SMAD4, Oct1, RXR receptor, p53) [4]. HBx binds in vivo to the promoters of a number of CREB-regulated genes and increases the recruitment of CBP/p300 to these promoters leading to increased gene expression [136]. More recently, the same group has also shown that HBx prevents the inactivation of CREB by a PP1 (protein phosphatase 1)/HDAC1 complex [31].

HBx also increases total DNA methyltransferase (DNMT) activity by the upregulation of DNMT1, DNMT3A1, and DNMT3A2 [137] and, by relocating DNMT3a [138], selectively facilitates the regional hypermethylation of the promoters of certain tumor-suppressor genes, such as p16/INK4A (Fig. 5.3).

Animal models of HBx- and HBV-mediated tumorigenesis downregulate the chromatin-modifying proteins Suz12 [a component of the polycomb repressive complex 2—PRC2, that directs the (tri)methylation of lysine 27 on histone 3 9H3K27Me3) and gene silencing] and ZnF198 [that stabilizes the LSD-Co-RESR-HDAC1 repressor complex] in liver tumors [110]. SUZ12 and ZNF198 are targeted to poly-ubiquitibnation and proteasomal degradation by a Plk1-dependent phosphorylation that is enhanced by the long noncoding RNA HOTAIR that serves

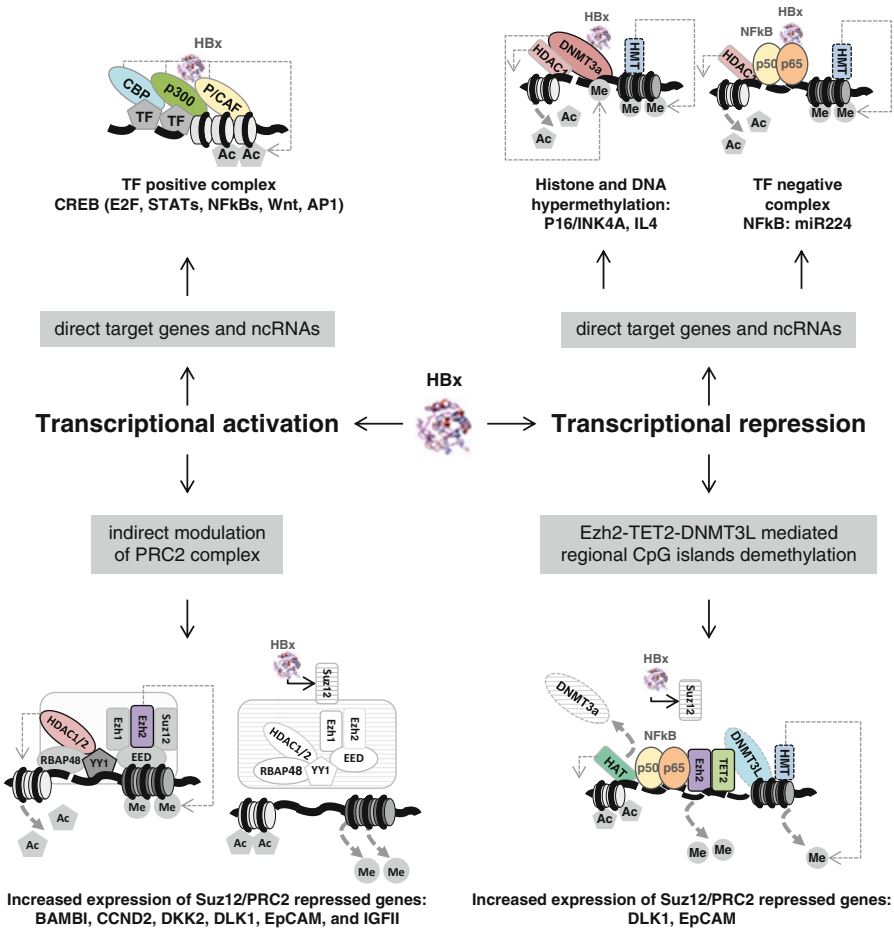

Fig. 5.3 HBx and chromatin dynamics. HBx binds several nuclear proteins involved in chromatin dynamics and the regulation of transcription: (**a**) HBx binds in vivo and increases the recruitment of CBP/p300 to the promoters of CREB-regulated genes (*upper left panel*); (**b**) HBx induces the PLK1- and proteasomal-dependent degradation of Suz12 [a component of the polycomb repressive complex 2—PRC2, that directs the (tri)methylation of lysine 27 on histone 3 (H3K27Me3) and gene silencing], leading to the overexpression of Suz12/PRC2 direct target genes, including the hepatic cancer stem cell markers BAMBI and EpCAM (*lower left panels*); (**c**) in Suz12-depleted cells expressing HBx a regional DNA de-methylation mediated by a complex containing Ezh2, TET2 and DNMT3L around half-NFkB sites releases the expression of EpCam and DLK1 expression (*lower right panel*) (**d**) HBx downregulates gene and ncRNAs transcription by (1) promoting the re-location of the DNMT3a DNA methyl-transferase to facilitate the regional hypermethylation of the promoters of certain tumor-suppressor genes, such as p16/INK4A and (2) by converting positive into negative transcription factors complexes (*upper right panels*)

as a bridge between the PRC2 and the LSD-Co-RESR-HDAC1 repressor complexes [139]. Suz12/Znf198 downregulation is accompanied, both in animal models and human HBV-related HCCs, by the overexpression of a number of Suz12/PRC2 direct target genes, including the hepatic cancer stem cell markers BAMBI and EpCAM [110] (Fig. 5.3). EpCAM over-expression in hepatic cells that have lost Suz12 is mediated by HBx and involves an active demethylation of CpG dinucleotides flanking NF-κB-binding sequences and the formation of a multiprotein complex containing the transcription factor RelA, the methyltransferase EZH2, the TET2 enzyme catalyzing the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, and the catalytically inactive DNMT3L [140] (Fig. 5.3).

HBx, Oxidative Stress, and Apoptosis

HBx has been shown to have both pro-apoptotic and anti-apoptotic properties, depending on its levels, the cell context (i.e., quiescent hepatocytes, neoplastic or preneoplastic liver cells with defective growth control, liver progenitor cells) and the experimental system used. In HBV replicating cells HBx promotes cytosolic calcium signaling, resulting in Ca2+ accumulation in mitochondria, increased levels of ROS [134] and the activation of the PYK2 and SRC kinases, which also promote HBV replication [32]. HBx also binds to mitochondrial voltage-dependent anion-selective channel protein 3 (VDAC3) [141], leading to membrane depolarization, reactive oxygen species (ROS) production [141] and eventually apoptosis [142]. Ca2+ signaling and increase ROS levels trigger ER stress and the unfolded protein response (UPR) [143]. On the other hand, high levels of HBx have been reported to block tumor necrosis factor-α (TNFα)- and FAS-mediated apoptosis by activation of NFκB [144], suggesting that infected hepatocytes may survive immune-mediated damage whereas uninfected hepatocytes undergo apoptosis in CLD.

HBx, Epithelial–Mesenchymal Transition, and Fibrogenesis

Epithelial–mesenchymal transition (EMT) plays multiple roles in the pathogenesis of CLD by promoting fibrogenesis, tumor progression, and metastasis. TGFβ is central to EMT by inducing collagen synthesis and promoting transcription factors that suppress epithelial markers [145–147]. HBx upregulates TGFβ1 [148] by SMAD-dependent (via stabilization of the SMAD4 complex) [149] and non-SMAD-dependent pathways (via activation of RAS–ERK and PI3K–AKT) [150]. HBx, similar to HCV core [151, 152], also seems to convert TGFβ1 signaling from negative to positive growth regulation and shift TGFβ responses from tumor suppression to EMT. Liver fibrogenesis (type 2 EMT) and HCC metastasis (type 3 EMT) are also mediated by miR 21, which is upregulated by NFκB in HBV-associated HCC [153]. As miR 21 activation usually occurs early during HBV CLD

progression, when ROS and HBx stimulates NF-κB and AP1, these observations link HBx, chronic inflammation, and hepatocytes transformation. HBx-stimulated SRC signaling promotes EMT [154] by destabilization of adherens junctions [155]. HBx also suppresses E cadherin by promoter DNA methylation and by upregulating SNAIL [156].

HBx, Hypoxia, and Angiogenesis

Cirrhotic nodules have a "relative" defect of vasculature that may generate local reductions in oxygen tension and hypoxia, upregulate HIF1α expression, and promote angiogenesis. HBx binds to and stabilizes HIF1α and stimulates HIF1α transcription [157], thus promoting angiogenesis and cell "stemness." HBx also promotes angiogenesis by upregulating the pro-angiogenic growth factor angiopoietin 2 (ANG2) [158]. HIF1α is also stabilized by increased insulin-like growth factor receptor 1 (IGFR1), epidermal growth factor receptor (EGFR) and PI3K–AKT signaling that are all activated by HBx [159].

HBx and Hepatic Stem/Progenitor cells (HSCs/HPCs)

Stemness markers [such as NANOG, OCT4, SOX2, and Krüppel-like factor 4 (KLF 4)] are reactivated and expressed in HCC [160]. About 20–40 % of HCCs display phenotypic markers of hepatic progenitor cells (HPCs) [161, 162] and share a common transcriptional signature with normal HPCs in cDNA microarray-based analysis [163]. HCCs expressing progenitor cell features have a worse prognosis and higher recurrence after treatment compared to HCCs, which are negative for these markers analysis [163]. Although a clinicopathological analysis of surgically resected HCC specimens suggested that $EpCAM^+$ CSCs were more frequently detected in HBV-related HCCs than in HCV-related HCCs [162] a validation on larger independent cohorts including HCCs from multiple etiologies is still lacking. HPCs (also called oval cells in rodent models of carcinogenesis) are small epithelial cells that can differentiate towards both hepatocytes and cholangiocytes and are located in the smallest branches of the biliary tree (canal of Herring and/or the ductular compartment). In animal models, liver cancers can originate from hepatocytes as well as from immature progenitor cells [164]. HBx promotes the expression of NANOG, KLF4, OCT4, and MYC as well as EpCAM (epithelial cell adhesion molecule) and β-catenin [160]. Stabilization of β-catenin transcriptionally upregulates EpCAM [160] and promotes the transcription of stemness genes in association with TCF/LEF1, OCT4, and NANOG. EpCAM+ cells display CSC-like properties and generate invasive tumours in HCC xenograft experiments [162]. HBx also promotes the expression of miR 181 family members, which upregulate EpCAM [165] and are highly expressed in embryonic livers, in HSC, and in patients with α-fetoprotein (AFP)-positive tumours [165].

HBx, Senescence, and Telomeres

Inflammation, oxidative, and oncogenic stress accelerate cellular senescence in chronic HBV (and HCV) infections. In cirrhotic livers, hepatocytes display decreased proliferation rates with a dominant replicative senescence phenotype, critically shortened telomeres and reduced regenerative potential [1]. Indeed, the length of telomeres progressively shortens from normal liver to chronic liver disease, and reaches the shortest levels in HCC [166, 167]. Senescence limits the proliferation of damaged cells and reduces the risk of malignancy by triggering the expression of tumor suppressors [168]. Transformed hepatocytes must bypass senescence and can survive despite critically shortened telomeres. Many studies have indeed showed that 80–90 % of HCCs display a high telomerase activity [169]. TERT promoter mutations activating telomerase expression represent the single most frequent genetic alteration in HCC [170, 171] but are less represented in HBV-related HCCs that re-activate TERT by other mechanisms including the integration of HBV DNA sequences into the TERT gene [111, 113, 121, 122] and the upregulation of TERT expression by HBx and PreS2 proteins [172]. Despite TERT activation telomers remain very short in HCC cells, predisposing to occasional telomere instability, chromosomal instability and polyploidy [172]. Indeed, the majority of HCC cells display a high incidence of chromosome instability that, similar to the accumulation of senescent cells [1], is already evident in cirrhotic liver tissues and increases during the hepato-carcinogenesis process [173]. LOH rate is higher in HBV-related HCCs [173] and HBx directly induces chromosomal instability by affecting the mitotic checkpoints [174]. HBx also binds and inactivates p53 and interacts with the DNA repair protein DDB1, which in turn affect repair functions and allow the accumulation of genetic changes [32]. RAS signaling and the AKT–ARF–p53–p21 and RAS–MEK–ERK–INK4A/p16–RB pathways, linked to oncogene-induced senescence (OIS) [175], are both active in HCC and are activated by HBx [176–178]. At the same time, HBx contributes to overcoming senescence by: a) upregulating DNA methyltransferases (DNMTs) [89]; b) inhibiting the p53 nucleotide excision repair and transcription-coupled repair functions [179]; and c) decreasing the expression of the p53 activators ASPP1 and ASPP2 [92]. HBx also suppresses the cyclin-dependent kinase (CDK) inhibitors INK4A and p21 via promoter methylation, resulting in the inactivation of the RB tumor suppressor [180]. miR 221, which is upregulated in HBV- (and HCV)-related HCCs, blocks the expression of the CDK inhibitor p27 and promotes tumor growth and progression by activation of the PI3K–AKT–mTOR pathway [181]. HBx also interacts with the peptidyl-prolyl cis/trans isomerase Pin1 and this interaction leads to HBx stabilization, enhanced HBx-mediated transactivation of target genes, and increased cellular proliferation [182].

HBx, Tumor Promotion, and Tumor Progression

Despite the large number of published studies, we still lack a unifying picture of HBx role in liver carcinogenesis that reconcile all HBx reported activities. Both wild-type HBx and truncated HBx proteins could demonstrate oncogenic functions and promote tumorigenesis [183–186]. However, it is not yet clear whether mutated HBx proteins "gain" oncogenic functions or rather "lose" activities that would restrain the oncogenic potential of wild-type HBx or that would not be no longer required for tumor progression. The recent demonstration in a large series of HBV-related HCCs that premature stop codon and large deletions leading to a complete inactivation of the HBx gene are selected and accumulate in the tumors in contrast to the surrounding non-tumor liver tissues in more than 70 % of the tumors suggests that HBx inactivation could have a role in liver carcinogenesis or tumor progression. The reported correlation between HBx inactivating mutations, the presence of TP53 mutations, a G1–G3 transcriptomic profile [83], an abnormal expression of onco-fetal genes (EPCAM, AFP and KRT19), and poorer prognosis [84] adds a further layer of complexity to the understanding of HBx contribution to HCC development.

HBc Protein

We and others have shown that the HBV capsid protein HBc not only binds the HBV minichromosome, i.e., the cccDNA nuclear replicative intermediate [25, 27] but also a subset of cellular genes involved in innate immunity, inflammatory responses, and the control of cell proliferation [187–189].

PreS/S Proteins

The potential pro-oncogenic role of mutated envelope proteins has been confirmed in many studies in transgenic mice and cell cultures [48, 61, 190–193]. PreS2 mutants may induce cyclin A and cyclooxygenase-2 overexpression leading to cell proliferation and anchorage-independent growth [65, 66]. PreS2 mutated proteins also directly interact with the Jun activation domain-binding protein 1 (JAB1), thus triggering cyclin-dependent kinase (Cdk) inhibitor p27 degradation, Retinoblastoma hyper-phosphorylation and cell cycle progression [64]. Cyclin A is located in the cytoplasm rather than in the nucleus in preS2 mutant-transgenic mice where favors centrosome over-duplication and consequently chromosome instability [61, 66]. Finally, the ER stress response induced by preS-mutated proteins increases vascular endothelial growth factor-A (VEGF-A) expression [193]. PreS/S sequences deleted at the 3′-end and producing functionally active MHBst are found in many viral

integrates from HBV-associated HCCs [50, 190, 194–196]. MHBst proteins retained in the ER trigger a PKC dependent activation of c-Raf-1/MEK/Erk2 signal transduction cascade, induction of AP-1 and NF-kB transcription factors, and an enhanced proliferative activity of hepatocytes [191, 197]. MHBst directly interact with a preS2-responsive DNA region in the hTERT promoter, resulting in the upregulation of telomerase activity and in the promotion of HCC development [192]. On the other hand, the inappropriate expression and accumulation of wild-type large envelope protein in ER membranes can be directly cytotoxic to the hepatocyte and initiate a cascade of events that ultimately progress to malignant transformation [59, 198].

Conclusions

HBV is a major risk factor worldwide for developing HCC. HBV contributes to hepatocellular carcinoma (HCC) development through direct and indirect mechanisms. Productive HBV infections triggers inflammation, continuous necrosis mediated by the immune response against infected hepatocytes, and cell regeneration favoring the accumulation of genetic and epigenetic lesions. HBV DNA integration into the host genome occurs at early steps of clonal tumor expansion and induces genomic instability and eventually direct insertional mutagenesis. Prolonged expression of the viral regulatory protein HBx and the large envelope protein deregulate the cellular transcription program and proliferation control and sensitize liver cells to carcinogenic factors. Epigenetic changes targeting the expression of tumor suppressor genes occur early in the development of HCC. A major role is played by HBx that is recruited on cellular chromatin and modulates chromatin dynamics at specific gene loci. Genome wide approaches begin to identify homogeneous subgroups of HBV-related tumors with defined genotypes and signaling pathways alterations.

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Chapter 6
Hepatitis B Virus: Persistence and Clearance

Christoph Seeger, Samuel Litwin, and William S. Mason

Introduction

Hepatitis B virus (HBV) has the ability to persistently infect hepatocytes, which constitute about 70 % of the cells in the normal liver. An adult liver contains about 5×10^{11} hepatocytes, of which the vast majority are HBV susceptible. Persistence is probably due to the fact that productive infection (Fig. 6.1), per se, is not cytopathic. That is, infection in the absence of an adaptive immune response does not perceptibly accelerate hepatocyte death. In addition, the hepatocyte population has a low rate of spontaneous turnover and covalently closed circular viral DNA (cccDNA), the viral transcriptional template, appears to be highly stable in nondividing hepatocytes [1], in addition to being present at up to 50 copies per cell. There is evidence that cccDNA may be lost during cell division, as well as evidence that it survives [2–4]; thus, the findings remain controversial.

In the first weeks after infection, HBV can spread through the entire hepatocyte population, often without an apparent induction of innate immunity or an inflammatory response (hepatitis). Despite a long delay, often of six or more weeks, the immune system may eventually activate and clear the virus, without destroying the liver in the process. About 95 % of adults recover following HBV infection. In infants and young children the anti-HBV immune response appears to be less vigorous; >90 % of infants under a year of age become chronically infected [5].

HBV clearance is generally associated with an acute inflammatory immune response that destroys large numbers of hepatocytes over a period of a few weeks, and then abates. Residual virus may remain, but its replication is inhibited by the immune system. Thus, subsequent immune suppression can lead to virus reactivation [6, 7]. Antibodies to the hepatitis B surface antigens (HBsAg) present in the

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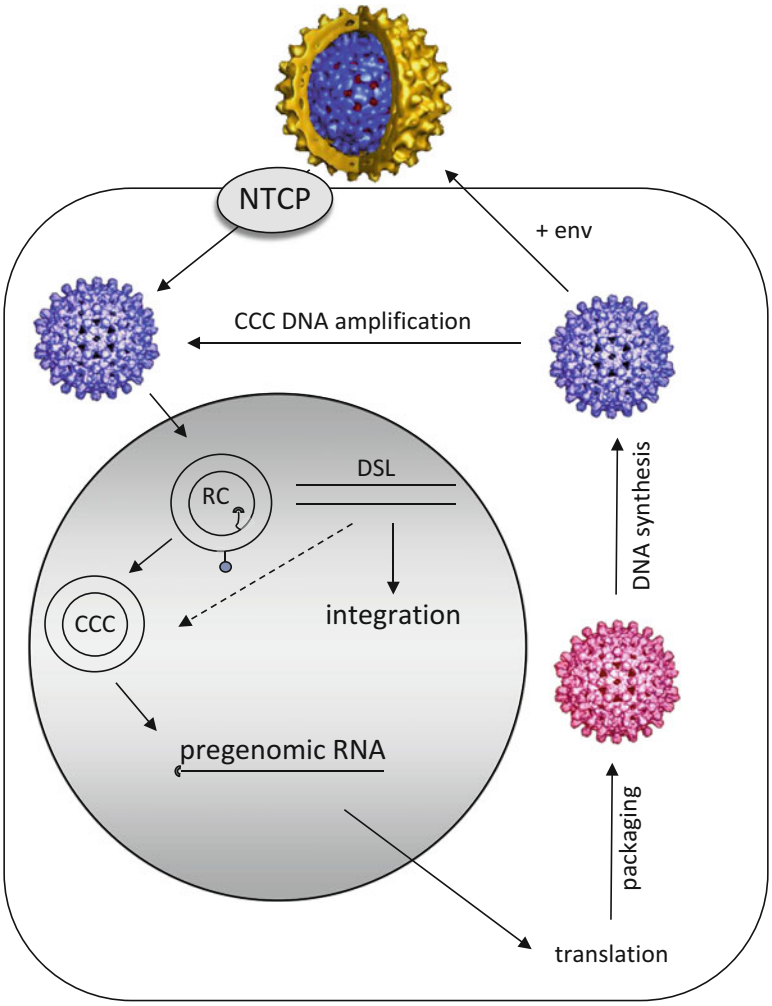

Fig. 6.1 Cellular life cycle of HBV. The figure shows a model for the life cycle of hepadnaviruses (adapted from ref. [72]; for a detailed description see ref. [72]). Briefly, RNA and DNA containing capsids are shown in *magenta* and *blue*, respectively. Envelope proteins are shown in *yellow*. Upon infection mediated by the entry receptor, NTCP, viral capsids are transported to the nucleus. About 90 % of virus has relaxed circular (rc) DNA, which is converted to covalently closed circular DNA (cccDNA). cccDNA does not undergo semiconservative DNA synthesis. Early in infection, when envelope protein concentrations are low, newly made nucleocapsids, with their enclosed viral DNA, are transported to the nucleus to amplify cccDNA copy. When sufficient envelope is present, nucleocapsids are directed to the secretory pathway and cccDNA amplification ceases. About 10 % of virus has a double stranded linear (dsl) DNA genome as a result of an error in viral DNA replication. These viruses can also infect hepatocytes. cccDNA is formed from dsl DNA by non-homologous recombination, resulting in a loss of sequences and, generally, rendering this cccDNA unable to support virus replication. Dsl DNA may also integrate into host DNA via nonhomologous recombination; this pathway does not appear to have a role in the virus life cycle, but occurs nonetheless. Even during transient infections, 0.01–0.1 % of hepatocytes may acquire integrated viral DNA

viral envelope proteins appear to be an important component of long-term protection against virus rebound, which is also associated with antiviral CTLs that persist in the circulation [8]. For those who do not resolve a productive infection, chronic liver disease often develops, driven to a major degree by the antiviral CTLs.

A hallmark of HBV infection of hepatocytes is that the target population may be entirely self-renewing. In addition, studies with animal models of HBV infection suggest that virus spreads into daughter cells as hepatocytes divide to compensate for death of other hepatocytes [3, 4]. Indeed, it is still unclear if there is a significant role for extracellular spread of virus to maintain infection once the liver is fully infected, or if persistence is maintained primarily by distribution of virus DNA to daughter cells.

In normal human liver, about 0.05 % of hepatocytes are in S phase, which provides a minimum estimate of liver turnover [9]. This rate may be much higher in chronic infection. For instance, in woodchucks chronically infected with woodchuck hepatitis virus (WHV), the hepatocyte turnover rate appears to be increased by ~tenfold, again by scoring S phase hepatocytes [10]. This enhanced turnover is thought to be immune mediated; for instance, it is not found during transient infections of woodchucks or chimpanzees, prior to the infiltration of inflammatory cells into the liver [11, 12]. The turnover rate during transient infections is elevated during the inflammatory response [12, 13].

Elevated turnover during decades of chronic infection has led to the idea that hepatocytes become increasingly senescent and, as a result, less able to contribute to liver maintenance as the disease progresses. Thus, it was thought that while hepatocyte replacement by division of mature hepatocytes declined due to senescence, it was compensated by emergence of oval cells, putative hepatocyte progenitor cells that differentiated into new hepatocytes [14]. This concept had been supported by studies of chemical carcinogenesis in the rat. These studies had suggested that under conditions of hepatocyte injury with agents that also inhibited hepatocyte proliferation, progenitor/stem cells present near the portal triad (e.g., Canals of Hering and bile ductules) gave rise to a proliferating population of oval cells that eventually differentiated into mature hepatocytes to maintain liver size and function [15, 16]. An important implication was that hepatocyte replacement during chronic HBV infection might become increasingly dependent upon a specialized progenitor/stem cell compartment.

However, recent studies suggest that the concepts of hepatocyte senescence and replacement from specialized hepatocyte stem/progenitor cells may be more complicated. First, in serial transplantation studies in mice, using either mouse or human hepatocytes, both normal hepatocytes, and hepatocytes with markers of senescence, were found to enter the cell cycle and contribute to maintenance of liver mass [17]. Second, studies using hepatocyte transplantation to distinguish resident cells from transplanted cells suggested that hepatocyte regeneration, during times of stress leading to oval cell proliferation, still depended, as in the healthy liver, upon the mature hepatocyte population [18, 19]. A recent tweak on this observation suggested that oval cells can contribute to hepatocyte replacement in times of stress, but that these oval cells are formed from the mature hepatocytes, not in a specialized

stem/progenitor cell compartment [20]. Dedifferentiation of mature hepatocytes to an oval cell phase may allow their temporary escape from an environment that inhibits proliferation of mature hepatocytes, providing an efficient pathway for hepatocyte replacement. Finally, subsequent findings failed to support the evidence that progenitor cells in the bone marrow contribute to hepatocyte replacement [21, 22]. This notion came from the observation that genetically tagged bone marrow cells appeared to migrate to the liver to form hepatocytes, but this now seems due to marker transfer as a result of cell fusion with hepatocytes [23].

In brief, it appears that virus persistence vs. clearance needs to be understood in the context of a target cell population that may be entirely self-renewing. This raises major questions about the mechanisms for immune clearance of HBV from the fully infected liver and the consequences of persistent immune pressure upon the hepatocyte population in the chronically infected liver. However, it may also rationalize a large body of data that suggest that HCC originates from hepatocytes and not a specialized progenitor cell compartment.

Transient Infection

As noted above, the persistence of HBV is believed to be aided by the stability of cccDNA in the nucleus of infected hepatocytes. Experiments with primary hepatocyte cultures prepared from woodchucks and infected with WHV indicated that cccDNA is stable in nondividing hepatocytes [1] as well as in hepatocytes that have been induced to divide [24]. In contrast, there is a lack of consensus on whether cccDNA survives mitosis in vivo [2–4], and how cccDNA responds to antiviral cytokines [25, 26].

During resolution of transient hepadnavirus infection, killing of infected hepatocytes is due to the cell-mediated adaptive immune response. This response is accompanied by the expression of cytokines including IFNγ and TNFα, that can change the physiological status of infected hepatocytes and lead to loss of virus DNA replication intermediates from the cytoplasm (Fig. 6.1), which appears to occur before loss of cccDNA [12]. The key question is what contributes to the subsequent loss of cccDNA? Is cccDNA loss entirely due to cell death? If so, how are uninfected hepatocytes generated? Is it that cccDNA does not survive mitosis, so that infected hepatocytes that divide, to compensate for the death of other infected hepatocytes, are cured? Or is elimination of cccDNA the result of dilution, requiring that hepatocytes pass though multiple rounds of mitosis to eliminate all the cccDNA copies that were present in the parent cell. Finally, does the inflammatory response activate cellular mechanisms that cause cccDNA destruction or contribute to the observed loss of cccDNA, perhaps during cell division or even in the absence of cell division?

The past 30 years have witnessed efforts to gain insights into the mechanisms that control clearance of HBV from infected livers, as it is observed during transient transfection in human patients, as well as in experimentally infected chimpanzees, woodchucks and ducks. The goal has been to establish a time line for the dynamics

of viral and immunological markers during the course of transient infections. While there is large variation between individual patients and experimental animals, the following pattern is common to most transient infections, as shown in mammalian hosts. The clearance phase can last several weeks and is accompanied by the activation of an adaptive immune response manifested by an influx of $CD4^+$ and $CD8^+$ T lymphocytes into the liver parenchyma, expression of IFNγ, TNFα and other cytokines, and by an increased rate of hepatocyte death. During the same time period, cytoplasmic rcDNA and nuclear cccDNA levels decline and become undetectable. Clearance of the virus from the infected liver will occur even when all hepatocytes have been infected, as determined by immunohistochemistry and/or in situ hybridization for viral antigens and nucleic acids, respectively [12, 27, 28]. Protective antibodies against the surface antigen become detectable during or following the clearance phase.

Observations in human patients are generally limited to analyses of blood samples for the presence of reactive T-cells, serological markers and enzymatic assays to determine hepatocyte cell death. These studies revealed the presence of IFNγ-producing $CD4^+$ and $CD8^+$ T cells that proliferate in vitro upon stimulation with HBV-specific peptides. Consistent with a role of $CD8^+$ CTLs and the presence of an inflammatory response against infected hepatocytes, serum ALT levels are elevated during the clearance phase. These observations provided the basis for a model where a combination of hepatocyte killing and regeneration and inflammatory cytokines are responsible for the natural cure of an HBV infection. However, due to the lack of available human liver biopsy tissue, these studies did not provide any direct information about the fraction of infected hepatocytes, the extent of liver damage or the exact sequence of events that leads to clearance.

The duck and woodchuck model for HBV infections permitted a more detailed study of transient infection, and collection of liver tissue biopsies at regular intervals during the recovery phase, to establish that recovery could occur even after all hepatocytes were infected [27–29]; that is, that full infection of the hepatocyte population did not of necessity lead to chronicity (for technical reasons, full infection means >95 %, beyond which uninfected cells would be virtually impossible to detect even if present). However, a major limitation of these models was the lack of commercially available reagents to follow the course of the immune response. This handicap was partially overcome with the cloning of woodchuck genes coding for the major T cell markers as well as IFNγ and TNFα.

The observation that recovery could occur after apparent infection of the entire hepatocyte population raised the question about the origin of hepatocytes in the recovered liver. Do they really arise from infected hepatocytes, as suggested above and consistent with the notion of a self-contained hepatocyte population, or do they arise from a progenitor/stem cell compartment? Studies of more than 20 woodchucks revealed that the time for recovery as well as the extent of liver damage varies among animals [11, 28–30], and can begin as early as 2–3 weeks post infection, though generally later. The fraction of apoptotic hepatocytes observed in liver biopsies ranged from less than 1 % up to 10 % of the hepatocyte population. While the results from these studies were consistent with a model where killing of

hepatocytes played a major role during clearance, they did not reveal the cumulative killing of hepatocyte during the resolution of an infection or identify the origin of virus-free hepatocytes.

An experimental strategy, taking advantage of the occasional integration of viral DNA into chromosomal DNA, provided a solution to both of these problems, the amount of hepatocyte destruction and the origin of uninfected hepatocytes [13]. Integration is a byproduct of hepadnavirus infection (Fig. 6.1) and, as such, not required for the viral life cycle. However, an analysis of the complexity and abundance of the viral-cell DNA junctions can be used to determine the fate of hepatocytes during recovery. The first observation was that the fraction of hepatocytes with integrated WHV DNA remains roughly constant during infection and in the recovered liver. This indicated that a large fraction of recovered hepatocytes were derived from infected hepatocytes, consistent with the idea that progenitor/stem cells do not play a major role in maintenance of the hepatocyte population.

These studies also provided an estimate of the amount of hepatocyte death during virus clearance. Since integration occurs at random sites in host DNA, the complexity of the collection of integration sites will remain at 1.0 unless these hepatocytes divide (complexity = (unique integration sites)/(total integration sites)). In fact, the complexity was much lower in recovered woodchucks, ~0.5 rather than 1.0, indicating that initially infected hepatocytes divided to fill in the space created by hepatocyte killing. Thus, the second major result of this experimental approach was an estimate that cumulative hepatocyte turnover equaled or exceeded 0.7 complete livers [11, 13].

These data, indicating that cured hepatocytes can be derived from infected ones, are again consistent with the more recent evidence that hepatocyte renewal occurs primarily from the hepatocyte population, not from a specialized stem/progenitor cell compartment [20, 31]. Thus, to explain recovery, there must be a mechanism for curing hepatocytes of HBV infection. A major gap in knowledge concerns, obviously, the mechanisms responsible for the loss of cccDNA. In particular, does a cure depend on the action of cytokines to destroy cccDNA or to increase loss of cccDNA during cell division? The notion that cytokines could play an important role in the clearance of HBV was derived from experiments with transgenic mice expressing HBV. In this system, IFNγ and TNFα, for instance, caused the rapid clearance of cytoplasmic DNA replication intermediates [32]. However, a major limitation of the HBV transgenic mouse model is that cccDNA is not formed.

Similar to those described in woodchucks, experiments were also conducted with a small number of HBV infected chimpanzees. The observations made with the primate model expanded upon results with the woodchuck model, especially as many more reagents were available to assess the host response to the infection [12]. Mathematical modeling of the declines in viral markers in the chimpanzee experiments suggested that non-cytolytic clearance of cccDNA due to action of antiviral cytokines (i.e., a mechanism in which cccDNA is eliminated even from nondividing hepatocytes) played a significant role in virus clearance [33].

In contrast, mathematical modeling of data from the woodchuck, this time focused on the amount of hepatocyte turnover during recovery, suggested that there

is enough hepatocyte turnover to explain cccDNA clearance, provided (a) new cccDNA formation from DNA replication intermediates is blocked (Fig. 6.1) and (b) existing cccDNA is lost during cell division [11]. This modeling did not invoke non-cytolytic clearance of cccDNA from nondividing hepatocytes.

While the concept of cccDNA loss during cell division seemed at odds with apparent cccDNA survival through cell division (e.g., during treatment of chronically WHV-infected woodchucks with an antiviral nucleoside analog (NA) [3], or in primary woodchuck hepatocyte cultures induced to divide [24]), a recent study with the duck model calls this conclusion into question. This study raised the possibility that NA inhibition of viral DNA synthesis may be incomplete when hepatocytes are dividing [4], allowing new cccDNA formation in dividing cells, a process presumably prevented during immune clearance.

However, interpretations of results from in vivo experiments to immune recovery have to be considered with caution, particularly because analysis of the results depended, ultimately, upon assumptions about the fate of cccDNA in nondividing hepatocytes in the presence of antiviral cytokines. Is it completely stable, or can it really be destabilized by antiviral cytokines? This is a particularly difficult question to approach in vivo because of the large amount of cell death during recovery from transient infections, as well as the inherent problem in obtaining adequate numbers of liver biopsy samples for precise analyses.

How can the information currently available from experimentally infected animals be interpreted and incorporated into a model for the mechanisms responsible for the cure of a transient HBV infection? In consideration of all the available data from woodchucks and chimpanzee, we envision the following model for the resolution of transient infections, which reflects our own bias that cccDNA is refractory to degradation in nondividing cells. Significant revision of this view will of course be necessary if support is obtained for recent evidence that cytokines destabilize cccDNA by APOBEC deamination of cytosine, followed by depurination and degradation by cellular nucleases [25]. To date, we have not found evidence that APOBEC editing of cccDNA is frequent enough to explain its non-cytolytic loss during resolution of transient infections (C. Seeger, unpublished observations).

In brief, infected hepatocytes are recognized by the immune system resulting in an influx of $CD4^+$ and $CD8^+$ T cells, activation of various other inflammatory cells, and production of IFNγ and TNFα. Second, the presence of cytokines in the liver parenchyma leads to an inhibition of HBV replication and elimination of viral DNA replication intermediates from infected cells [12], preventing new cccDNA synthesis. Third, $CD8^+$ CTLs kill infected hepatocytes. Fourth, infected hepatocytes that have lost replicative intermediates, due to cytokines exposure, divide to replace killed hepatocytes and in the process loose cccDNA, resulting in a new population of "cured" hepatocytes. How cured hepatocytes are protected from immediate reinfection cannot be predicted based on the experimental data. It is conceivable that a combination of events is required. They include, besides a lack of HBV production due to inhibition of viral replication, reduced susceptibility of hepatocytes to de novo infection. This might occur via induction of an antiviral state by cytokines expressed by monocytes and T cells. Whether antibodies against

viral envelope proteins play a role during these early steps of recovery is doubtful, because anti-HBsAg antibodies often appear weeks after viral DNA becomes undetectable [28, 29].

HBV Persistence During Chronic Infection

Chronic infection, typically acquired in early childhood, may proceed through a number of phases, including a prolonged immune tolerant phase, a clearance phase, a quiescent immune control phase, and sometimes, a virus rebound phase [34, 35].

The immune tolerant phase is so named because patients in this phase, which may last several decades, will, by definition, have high serum virus titers of 10^9 per ml or more, and normal ALTs. Where liver biopsies are available, these patients should also have little or no evidence of fibrosis or hepatic inflammation. It should be noted, however, that normal ATLs do not necessary indicate immune tolerance and, at least for some patients, this stage may be misdiagnosed in the absence of a liver biopsy [35–38].

The immune tolerant phase may progress to an immune clearance phase, which has many of the hallmarks of recovery from transient infections, except that it may be more prolonged and involve multiple cycles of partial virus clearance and rebound [39]. Optimally this leads to immune control of the infection, with virus titers often $<10^4$ per ml, and often at lower levels (e.g., $\leq 10^2$ per ml). HBsAg titers in the circulation may also be lowered, but not nearly as much as virus titers [40]. The reason HBsAg is more refractory to immune control mechanisms than virus production is unclear. One source of HBsAg production during the immune control phase may be integrated HBV DNA, which is probably present in 1–10 % or more of hepatocytes by this time. Importantly, liver disease may progress during the immune control phase, especially if virus titers exceed 10^5 per ml [35], probably because immune control of virus replication is incomplete, at least in this situation, so that elevated hepatocyte destruction persists. A more complete loss of control can lead to a severe exacerbation of liver disease [6, 34].

Unfortunately, progression through the immune clearance phase and into the immune control phase is often accompanied by a high incidence of cirrhosis. The risk of HCC in these patients is high, up to 20–30 % or more, especially in those that do not achieve full control of HBV replication [35]. Antiviral therapy with NAs during the immune clearance and immune control phase will reduce the short-term (e.g., 5-year) incidence of HCC ~fivefold, especially in patients with advanced liver disease, less so in patients with less advanced disease and, therefore, a lower short term HCC risk [41]. NA therapy can also lead to some reversal of fibrosis and cirrhosis [42, 43], probably by facilitating progression to and/or replacement of infected by uninfected cells, thereby causing a quantitative reduction in hepatocyte killing. It is not yet known if beginning NA therapy even earlier, during the immune tolerant phase, would have a greater impact on HCC incidence. The effect on HCC risk beyond that achieved by starting NA therapy during later stages of infection

remains to be determined [41]. It seems likely, based on existing data [43], that initiating therapy during the immune tolerance phase would prevent cirrhosis. Cirrhosis may increase the risk of HCC.

In the following sections, we focus on virus persistence, with emphasis on its effects on the hepatocyte population that may influence the progression of chronic hepatitis B (CHB), and contribute to the cancer risk and might, at least based on present knowledge, be mitigated by earlier initiation of antiviral therapy. The emphasis is on the effects of turnover in a closed population, in which dying hepatocytes are ultimately replaced by division of existing hepatocytes. Cirrhosis is not discussed explicitly, though it presumably exacerbates effects that occur in its absence, by providing strong pressure for clonal expansion of rare hepatocytes that are able to survive in the toxic environment of cirrhotic nodules, in which normal blood flow, lobular structure, etc. are impaired. More detailed information on immune control of virus replication, including the possible role of methylation of cccDNA, is discussed in Chap. 4.

Hepatocyte Evolution and Chronic Infection

If the hepatocyte population is closed, as indicated by recent data, even random death of some hepatocytes will lead to clonal expansion of others (including those with initiating mutations that may contribute, ultimately, to neoplasia). That is, in a closed population, some hepatocyte lineages will be lost over time due to random cell death and division, as others divide to maintain liver mass. The larger a lineages becomes (clone size), the lower its risk of being eliminated by random destruction. In comparison, lineages with only one or a few cells would be at greater risk of elimination. In effect, if a hepatocyte does not die early, there is a possibility that it will be able to clonally expand nonselectively, simply due to natural processes. Clonal expansion would be aided if the hepatocyte had a survival or growth advantage as a result of mutational or stable epigenetic changes. Subsequent changes could enhance its rate of clonal expansion even more, facilitating a progression towards neoplasia.

Cirrhotic nodules may show extensive clonality, as suggested above [44–50]. But what is the evidence that clonally expanded hepatocytes exist in the "non-cirrhotic liver"? And is clone size in the non-cirrhotic liver compatible with an origin via random death and division?

First, during chronic infection the emergence of foci of virus negative hepatocytes occurs in the non-cirrhotic liver, even in the presence of high titer viremia, as illustrated for example in a study of chronically infected chimpanzees [51] and, to a lesser extent, woodchucks [52]. Second, clonal expansion of hepatocytes has been detected by using randomly integrated viral DNA as a hepatocyte lineage marker. These assays have detected clones of up to 60,000 hepatocytes in older non-cirrhotic human carriers. This is much larger than expected from random death and regeneration (see below), implying that these cells had either a survival advantage,

growth advantage, or both [53, 54]. Even in immune tolerant carriers under the age of 30, unexpectedly large hepatocyte clones have been detected (Kennedy, Bertoletti, Mason, unpublished observations). In addition, early studies of HBV infection provide clear evidence that in long-term carriers many hepatocytes are not productively infected (e.g., [55, 56]). Second, Su and colleagues have reported on focal expansion of ground glass hepatocytes expressing variant HBsAg that has lost immunedominant epitopes due to deletion mutations [57]. Thus, one likely basis for clonal expansion is failure to support HBV replication, so that cells are no longer targeted by antiviral CTLs. Another is loss of virus epitopes by deletion or mutation. Another might be failure to present viral antigens to antiviral CTLs.

It should be noted, however, that while large numbers of virus negative hepatocytes that may also be HBV resistant arise during the course of chronic infections, this progression does not appear to lead to the immune control stage of chronic hepatitis. The data are more consistent with the current notion that the low level of infection during the immune control stage reflects, predominately, immune control of HBV replication, since immune suppression can lead to reemergence of the virus and acute liver disease [6]. Possible effectors of the immune control of HBV replication are discussed in Chap. 4.

In any case, an obvious concern is that extensive clonal expansion of hepatocytes, especially in an environment predisposing to DNA damage, may be an HCC risk factor. It is noteworthy that clonal hepatocyte expansion leading to liver repopulation and survival is seen in genetic diseases in response to host gene mutations that are toxic to hepatocytes (e.g., alpha-1-antitrypsin deficiency [58]). Repopulation involves hepatocytes with secondary mutations that bypass the toxicity. These patients have an elevated risk of HCC [59]. As in HBV patients, the mechanistic basis for the elevated HCC risk is unknown. It is interesting, though perhaps coincidental, that preneoplastic lesions and HCCs often appear to be resistant to HBV (or, in the woodchuck, WHV) infection [52, 60–64].

Liver cancer is thought to progress through stages of initiation, promotion, and progression [65]. Initiation would be a mutation or epigenetic change that is stably inherited by daughter hepatocytes and gives them a growth or survival advantage. Promotion involves the proliferation of the altered cell population (s). In the context of chemical carcinogenesis, for example, this is typically due to the ability of rare initiated hepatocytes to escape deleterious, growth inhibitory effects an initiating agent has on growth of the bulk of the hepatocyte population. Progression involves the subsequent changes that convert cells in initiated lineages to cancer cells. Possible initiation and progression events during chronic HBV infection would include viral DNA integration, excess mutations due to inhibition of DNA nucleotide excision repair by HBx [66, 67] and oxidative DNA damage, which may be due in part to the action of viral proteins including HBsAg and HBx [68]. Promotion in the context of HBV infection could, in theory, be either selective or nonselective, and likely coexists with progression. The analyses below suggest that promotion in HBV carriers is more likely selective than nonselective. Possible selective advantages could be due to hepatocyte mutations/epigenetic changes that either prevent HBV infection/replication or inhibit presentation of viral antigens to antiviral CTLs, in either case facilitating immune evasion.

Mathematical Modeling of Hepatocyte Dynamics

To illustrate the possible dynamics of clonal hepatocyte repopulation in an HBV patient, we have made some calculations based on estimated rates of hepatocyte turnover in the normal and chronically infected liver. We have based these simulations on a liver specimen size of one million hepatocytes, which reflected a practical computational limit using PCs. (Because the computer program (comp10, Ref. 51) employs a stochastic model, every run gives similar but not the exact same results. In what follows, we show results of single runs). Specifically, we first considered a scenario for expansion of hepatocytes with no survival or growth advantage. We then considered hepatocytes with survival advantages over the remainder of the hepatocyte population due to evasion from antiviral CTLs. Finally, we considered the effect on clonal hepatocyte repopulation if hepatocytes that can evade antiviral CTLs also have a slightly enhanced growth response; that is, they are more likely to divide to replace dying hepatocytes. For instance, studies with heterologous systems have suggested that HBV infection may retard progression of hepatocytes through the cell cycle (e.g., [69]). Initiated hepatocytes that did not support HBV expression would not be subject to this theoretical effect.

It has been reported that 0.05 % of hepatocytes are in S phase at any given time in the normal adult liver [9]. For the purpose of modeling, we assume that the rate of hepatocyte turnover is about three times higher (assuming S phase is about 8 h). This would give a daily turnover rate constant of 0.0015 (0.15 %) in normal liver. We also assume for the purpose of discussion that elevated hepatocyte turnover due to CTL killing usually begins in teenagers [36–38] and that, prior to this, the genetic complexity of the liver is 1; that is, all hepatocytes are uniquely tagged (for the purpose of modeling) but do not contain any deleterious mutations resulting, for instance, from oxidative DNA damage to hepatocytes due to antiviral immune responses. Thus, we only consider events that occur from this time on to expand individual hepatocyte lineages to reduce the complexity of the liver and, occasionally, lead to the emergence of clones with mutations that may predispose to subsequent neoplastic progression.

Effect of Random Death and Regeneration on Complexity and Clonal Expansion in the Hepatocyte Population

We first illustrate a scenario for immune-tolerant patients with neither immune pressure on the hepatocyte population, nor initiating events in hepatocytes that provide a survival or growth advantage. In this model, the daily rate constant for turnover of the hepatocyte population was 0.15 % (0.15 % of hepatocytes die every day), characteristic of healthy liver [9]. Under these conditions, about 0.55 livers worth of hepatocytes would die and be replaced by surviving hepatocytes every year. This rate of turnover, if due to random hepatocyte death and compensating division, would reduce the genetic complexity of the liver about ~12-fold after 20 years and

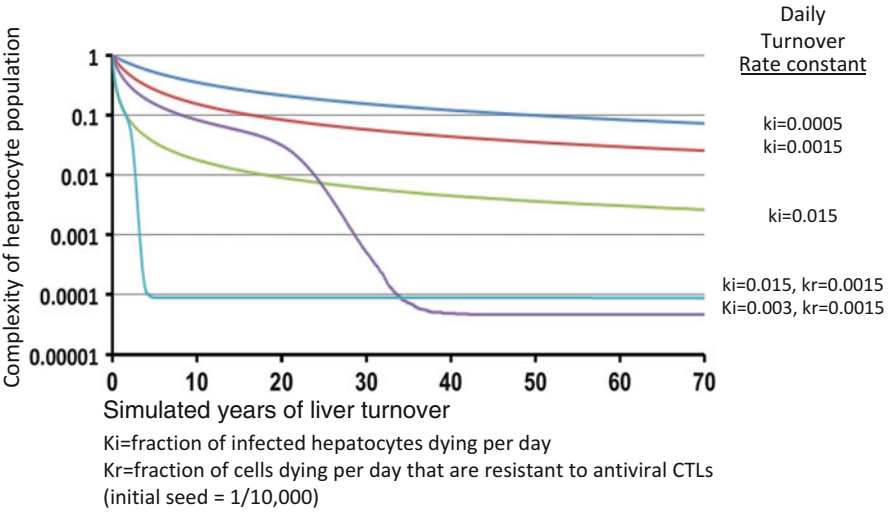

Fig. 6.2 Effects of death and division on the complexity of the hepatocyte population. Changes in complexity were calculated using a computer program, comp10 [51] (available upon request). This program tracks the individual fate of an array of one million hepatocytes during daily cycles of killing and division to maintain liver mass. At the start, hepatocytes are designated as either infected or uninfected. Uninfected and infected hepatocytes can have different daily death rates, which are considered here to be first order (i.e., of the form $dN/dt = kN(t)$, where N is the number of infected or uninfected hepatocytes at time t). Output includes the hepatocyte clone size distribution and complexity ((distinct hepatocyte lineages at time t)/(lineages at time zero (one million)) of the hepatocyte population (assuming a complexity of 1 at time 0). In the first three scenarios shown here, all hepatocytes are assumed to be infected and dying with a rate constant ki = 0.0005, 0.0015, or 0.015 (i.e., 0.05, 0.15, and 1.5 % per day). In the next two scenarios, the liver is assumed to contain 0.01 % hepatocytes at $t = 0$ that are resistant to CTL killing and die at with a background rate constant kr = 0.0015. Typical infected hepatocytes are assumed to die with ki = 0.015 or ki = 0.003

~30-fold after 50 years (Fig. 6.2). Thus, for immune tolerant HBV carriers as well as healthy individuals in their 30s, about the time the HCC incidence begins to rise, maximum hepatocyte clone sizes should still be relatively small, mostly less than 100 hepatocytes (Fig. 6.3a). If the daily turnover rate was tenfold higher in immune tolerant patients (i.e., if they were not truly immune tolerant), the complexity would be reduced ~100-fold fold after 20 years and ~300-fold after 50 years (Fig. 6.2), and the maximum clone size after 20 years would be larger, but <1500 (Fig. 6.3b). These clone sizes, predicted by random death and regeneration, do not explain the larger clone sizes observed in chronically infected woodchucks, chimpanzees and humans [51, 53, 70]. While it would be possible to achieve larger clones sizes by presuming extraordinarily high rates of random death and regeneration (e.g., see Ref. 51), in what follows, we point out that this is much more readily achieved even for low rates of turnover by assuming clones are composed of hepatocytes with a selective advantage.

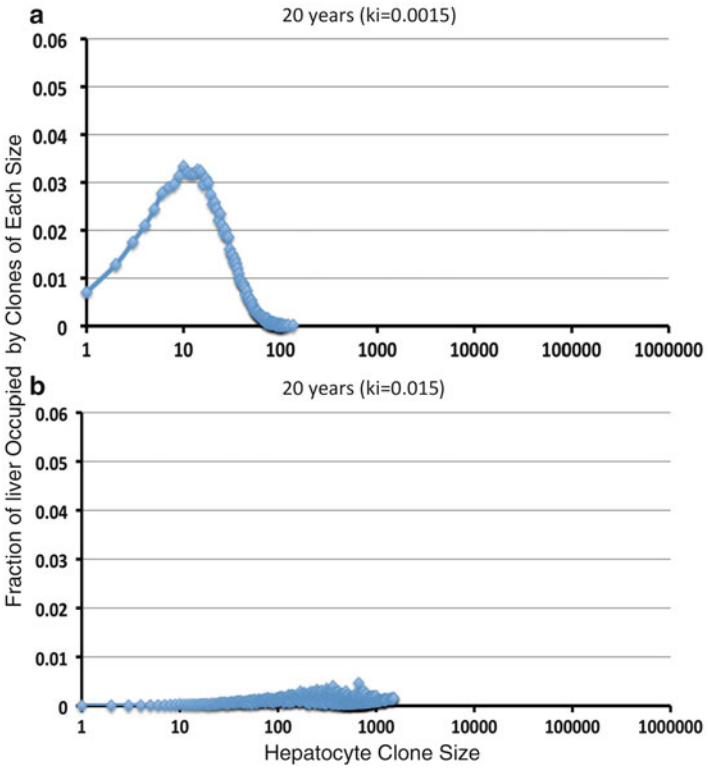

Fig. 6.3 Effects of random death and regeneration on clone size distribution of the hepatocyte population. All hepatocytes were assumed to be infected and die with a rate constant of ki = 0.0015 (panel a) or ki = 0.015 (panel **b**) (0.15 or 1.5 % per day). Clone size distribution is the fraction of the liver of one million cells that is comprised of clones of the sizes indicated on the abscissa (log scale). The effects on complexity are shown in Fig. 6.2

Clonal Expansion of Hepatocytes that are Resistant to CTL Killing

To illustrate the effect of even a small survival advantage on complexity and clonal hepatocyte expansion, we assumed that typical infected hepatocytes were killed at twice background (0.3 % per day), and that, initially, 1/10,000 hepatocytes had a survival advantage (i.e., due to evasion of CTL killing), dying at the background rate (0.15 % per day). (Note that we are referring to first order rate constants, so the number of cells killed every day will be the indicated percentage of cells in each population.) As illustrated in Fig. 6.2, between 20 and 30 years, a precipitous increase in the rate of complexity loss takes place; this occurs because starting at about 20 years, infected hepatocytes with the higher turnover rate constant are primarily replaced by hepatocytes which evade CTL killing. After either 20 or 30

years, the effect on clone size is dramatic. By 20 years, ~80–90 % of hepatocytes will have a resistant phenotype, dying at the background rate of 0.015 % per day, and these resistant hepatocytes will be in clones up to ~75,000 cells in size (Fig. 6.4b). The loss of complexity is mainly in the infected cell population; after 20 years, half of the original clones of resistant cells remain (clone size ~2,500–50,000), vs. only a few percent of the original infected clones (clone size ~1–50). This trend does not appreciably change after 30 years (Fig. 6.4c), with <1 % of the original infected cell clones remaining, again vs. half of the resistant clones. For contrast, very little happens in a short time of say, 2 years (Fig. 6.4a).

The effect on complexity and clonal expansion is even faster when typical infected hepatocytes die at ten times the background death rate (1.5 % per day) (Figs. 6.2 and 6.5). Again, a small fraction (1/10,000) of hepatocytes present, at the start, are able to evade CTL killing, dying instead at a background rate of 0.15 % per day. By 2 years, resistant cells will constitute 40 % of the hepatocyte population with clones of 10,000–100,000 hepatocytes, suggesting that even a short period of persistent hepatitis might have a dramatic impact on the hepatocyte population if there are cells present in the liver that can evade antiviral CTLs. In this situation, ~90 % of the resistant clones remain (clone size ~40–40,000).

While we assume that hepatocytes that can evade antiviral CTLs are present from time zero, they may, of course, arise later with the same result. We also realize that there may be anatomic restrictions on clonal expansion of hepatocytes with a normal growth response. These considerations point out that any stable heterogeneity in the hepatocyte population effecting death rates (e.g., resistance to antiviral CTLs) could lead to significant hepatocyte repopulation, especially within the hepatic lobule. This highlights the major implications of the reports that the hepatocyte population is self-renewing, even in hepatic disease [31], for selective expansion of rare hepatocytes with a survival advantage. Essentially the same picture is seen if the initial fraction of such cells is 1/100,000. These scenarios could explain the larger clone sizes observed in chronically infected woodchucks, chimpanzees and humans [51, 53, 70].

Effects of Enhanced Growth Rate on the Hepatocyte Population

We also considered the effect of an enhanced growth rate for clonal expansion of rare hepatocytes that are able to evade antiviral CTLs. As in Fig. 6.4, we assumed that typical infected cells were killed twice as fast as resistant cells. However, in contrast to that model, we added a proviso that resistant cells were twice as likely to respond to a signal to divide, to maintain liver mass. In the absence of this growth advantage, clone sizes remain <2000 hepatocytes even after 10 years (Fig. 6.6a). In contrast, if the resistant hepatocytes are also twice as likely to divide to maintain liver mass, clones of 10,000–100,000 hepatocytes appear (Fig. 6.6b), resistant cells constituting >90 % of the hepatocyte population. A selective growth advantage alone, without a survival advantage, had similar effects on clonal expansion (Fig. 6.6c)

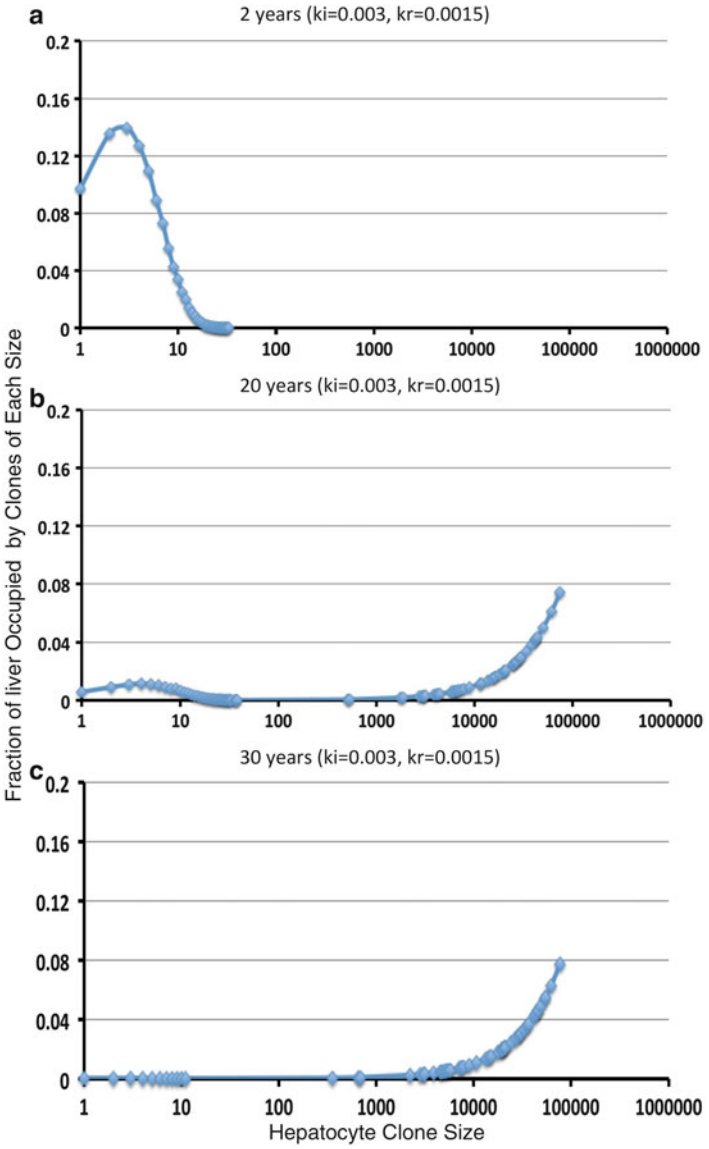

Fig. 6.4 Effects on clone size distribution when a minor population of hepatocytes is resistant to CTL killing and dies at a background rate. 99.99 % of infected hepatocytes at time zero were assumed to die with a rate constant of ki = 0.003 (0.3 % per day). 0.01 % of the hepatocytes at time zero were assumed to be resistant to CTL killing and to die with a background rate constant of kr = 0.0015. This lower turnover rate could be because the cells were resistant to infection or virus gene expression, and/or failed to present virus antigens to antiviral CTLs. Panels (**a**), (**b**), and (**c**) show the effects on clone size distribution after 2, 20, and 30 years, respectively. Effects on complexity are shown in Fig. 6.2

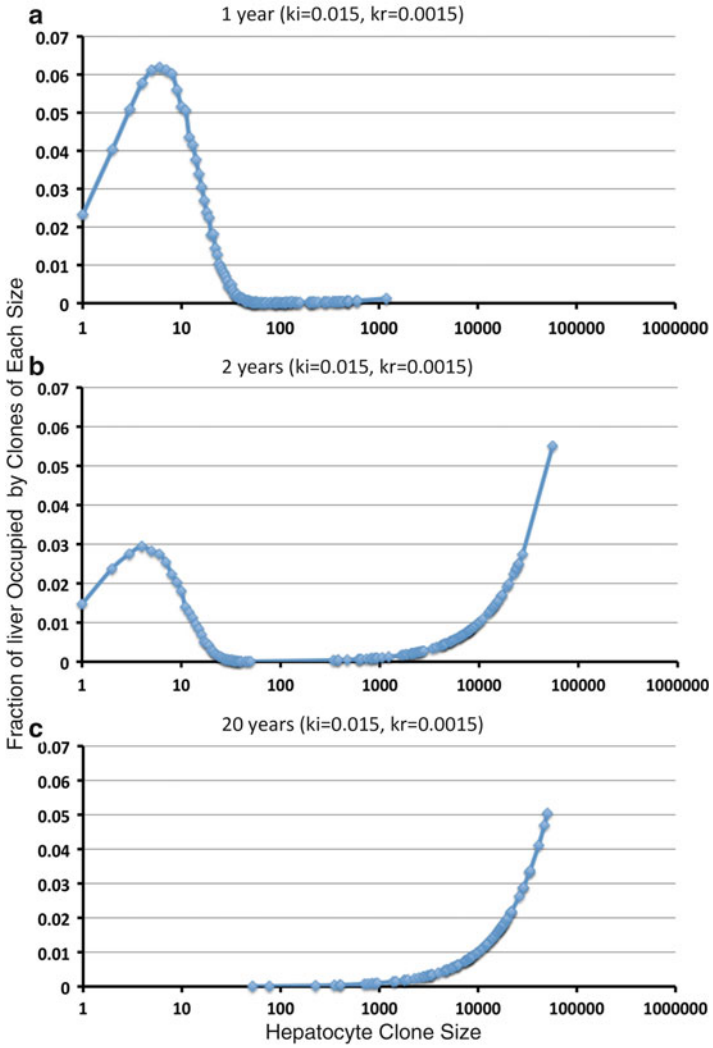

Fig. 6.5 Effects of a higher rate of killing of infected hepatocytes on the expansion of a minor population of resistant hepatocytes. 99.99 % of hepatocytes at time zero were assumed to die with a rate constant of ki = 0.015, five times higher than in Fig. 6.4. 0.01 % of hepatocytes at time zero were assumed to be resistant to CTL killing and to die at with a background rate constant kr = 0.0015. Panels (**a**), (**b**) and (**c**) show the hepatocyte clone size distribution after 1, 2 and 20 years, respectively. The effects on complexity are shown in Fig. 6.2

as a selective survival advantage with a growth advantage (Fig. 6.6b). Hepatocytes with a growth advantage (Fig. 6.6c) constitute ~70 % of the hepatocyte population after 10 years. (The effect on clone size with a growth advantage alone is similar to the scenario including a survival advantage because of the higher rate of hepatocyte turnover throughout (0.3 % per day)).

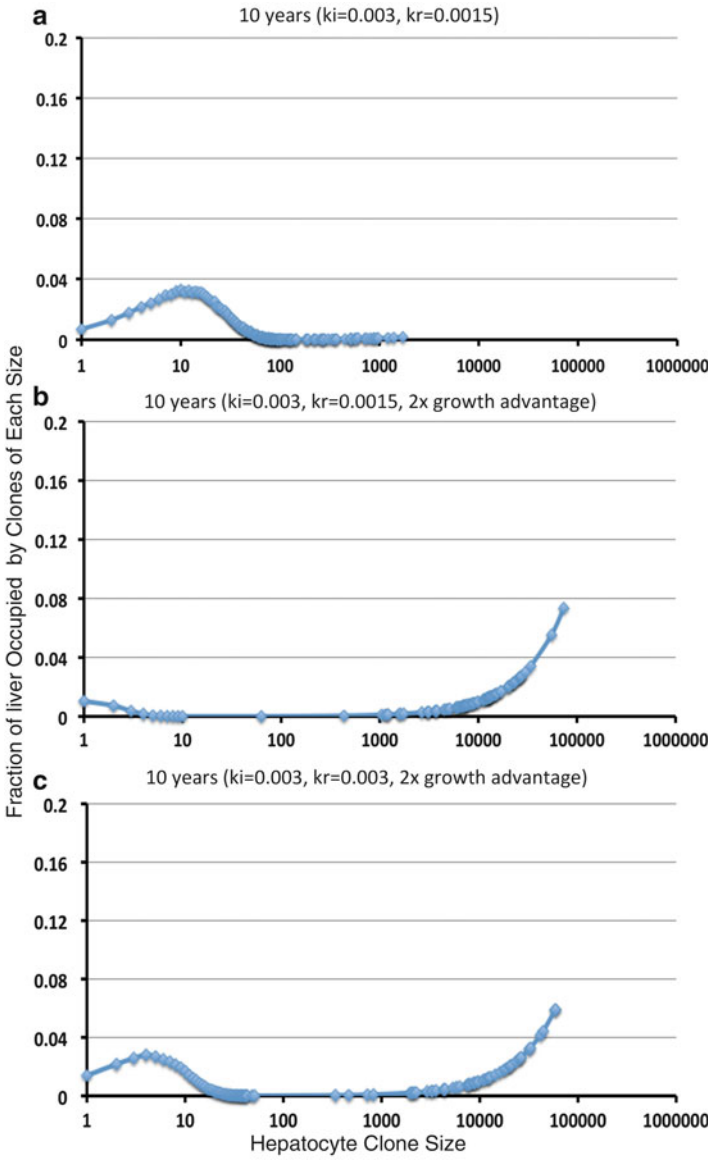

Fig. 6.6 Effects on clonal expansion of hepatocytes with growth and/or survival advantages. 99.99 % hepatocytes at time zero were assumed to die with a rate constant ki = 0.03, twice background. Panel (**a**): 0.01 % of hepatocytes at time zero were assumed to be resistant to CTL killing and to die with a rate constant of kr = 0.0015. All hepatocytes were assumed to have the same probability of dividing to maintain liver mass. Panel (**b**): As in panel (**a**), but resistant hepatocytes were assume to divide with twice the rate constant as the remaining hepatocytes. In this scenario, cells are selected individually and at random to divide, but on each call, a resistant hepatocyte will divide, whereas the remainder has only a 50 % chance of dividing. If not selected, another hepatocyte (either resistant or not) is selected at random and the process repeated until a cell divides. Panel (**c**): As in panel (**b**), except all hepatocytes die with the same rate constant, ki = kr = 0.003, twice the background rate of normal liver (see text)

In summary, the biology of the liver as currently understood is conducive to a scenario in which HBV infection promotes clonal expansion of a subset of hepatocytes which have resistance to immune killing, and therefore a greater risk of progressing to HCC, especially if additional changes are acquired that also facilitate neoplastic progression. Based on appearance of foci of virus negative hepatocytes during the course of infection, we assume many of these hepatocytes achieve CTL escape via resistance to HBV. It is important to note that damages that promote survival or growth will be cumulative in a closed population. It is also important to note, however, that the clonal hepatocyte expansion mentioned here largely involves normal appearing hepatocytes, not preneoplastic lesions [53, 54]. Thus, the idea that selective growth of hepatocytes that are resistant to antiviral immunity also reflects promotion of carcinogenesis remains an inference, though in our opinion a plausible one. As noted, studies of genetic diseases of the liver have led to similar conclusions about selective growth of disease resistant hepatocytes [59].

Persistence of HBV, Clonal Expansion, and Virus Evolution

The biological properties of large hepatocyte clones is not yet understood and it is conceivable that differences exist among clones in terms of their susceptibility to HBV infections as well as NA therapy. Indeed, differences among hepatocyte clones might also influence the dynamics of virus evolution because of continued selection of the fittest members within an existing viral population. Virus evolution during chronic infection is well documented, and has been attributed to immune selection against viral epitopes (Chap. XXX), i.e., immune evasion. This is thought to explain, for example, the HBeAg positive to HBeAg negative transition that sometimes occurs during the immune clearance phase, in which HBeAg negative variants of HBV emerge to become the predominant genotype and virus titers remain detectable and often, but not always, at levels many logs less than before their emergence (e.g., [71, 72]).

Evolution of the virus population to escape the antiviral immune response might occur at two levels, spread of resistant virus and selective clonal expansion, via immune escape, of hepatocytes that become infected by these variants. In the absence of selective pressure, as during the immune clearance phase, HBV variants probably exist in proportion to the estimated HBV mutation frequency of 10^{-4} to 10^{-5} per nt. However, hepatocytes infected by an immune escape mutant might be able to expand clonally at the expense of cells infected by wt HBV, if a low level immune selection occurs during the prolonged immune tolerant phase. It might also be that HBV variants with a higher replication rate expand in the liver even without a need for immune evasion. As noted, super-infection resistance is high [73]. However, there is currently no way to know if some leakiness could allow expansion of a mutant with a higher replication rate over the many decades that can characterize the immune tolerant phase of infection. Unfortunately, while there is evidence

that some HBeAg negative mutants replicate at a higher rate than wt [74] in cell culture, it is not known if this is true in vivo, especially during chronic infections.

In addition, during the immune clearance phase, at least two events might coexist, differential killing of hepatocytes infected with wt HBV and, if there are one or more rebound phases, spread of mutant and to a lesser extent, wt HBV, to hepatocytes which had been cured of their infection. Thus, elevated killing of hepatocytes infected with wt HBV, as compared to an HBeAg negative variant, might facilitate emergence of the mutant. Whether either of these scenarios actually occurs, in vivo, is unknown.

In summary, virus persistence in patients with active hepatitis seems facilitated by the expansion of immune escape variants of HBV, while wt HBV probably remains predominant in patients with low disease activity, as in the immune tolerance phase. Emergence of immune escape variants of HBV is probably associated with virus spread during the immune clearance phase, as well as clonal expansion of hepatocytes infected by these variants. Spread by super-infection does not, a priori, appear to be a helpful scenario for supplanting the wt unless the mutant has a higher replication rate; whether such variants occur naturally is unknown. To the extent that clonal expansion of hepatocytes leads to promotion and/or progression to HCC, immune escape variants of HBV might be intrinsically procarcinogenic, irrespective of any more direct role they may have in carcinogenesis.

Implications for Future Antiviral Therapies and Prevention of HCC

Investigations on the hepatocyte lineage in mouse models indicate that differentiated hepatocytes are the source for regenerated cells. Consistent with this concept, studies on WHV infections in woodchucks demonstrated that previously infected hepatocytes repopulate the liver after the resolution of transient infections. Moreover, the concept that the hepatocyte population is closed has potentially important implications for the progression to HCC during chronic hepatitis B. In essence, if the population is closed, hepatocyte turnover will inevitably lead to clonal expansion of some lineages at the expense of others. Clonal expansion can influence disease outcome in many ways, depending on properties of individual hepatocytes clones, in terms of their permissiveness for virus replication, their sensitivity to CTL killing and their growth rate. For example, HCC development might be influenced by early genetic lesions, or epigenetic events that are key to HCC development, but require additional changes to eventually lead to cancer.

Because non-cytocidal genetic damage to the hepatocyte population will be cumulative, it would seem useful to consider earlier NA treatment to restrict the accumulation of mutations and expansion of mutant cell populations. Similarly, expansion of clones could have an effect on the outcome of NA-based antiviral therapies, especially if the NAs have any negative impact on cell viability, even a

small one. This might explain “leakiness” observed with antiviral therapies. If so, antiviral therapies would benefit from new, non-NA drugs, such as the previously described capsid formation inhibitors.

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Chapter 7
Hepatitis Delta Virus: Virology and Replication

Camille Sureau

Introduction

When the hepatitis delta antigen (HDAg) was first identified in liver biopsies of Hepatitis B virus (HBV) chronic carriers [1], it was considered as a new HBV antigen. Similar to HBV core antigen (HBcAg), it was detected in abundance in liver cell nuclei, but HBcAg and HDAg stainings were mutually exclusive. HDAg was later found to be associated to a small RNA molecule within viral particles coated with the HBV envelope proteins, and it was then considered as a component of a new virus-like agent, whose propagation was dependent upon the presence of HBV [2–4]. The cloning and sequencing of the HDAg-associated RNA [5–7], revealed a single-stranded, circular genome, without sequence homology to that of HBV DNA, but with similarities of structure with plant viroid RNAs [8], including the ability to fold into a rod-like conformation stabilized by self-complementarities [9]. The HDV genome is larger in size than the average viroid RNA (1700 nt for HDV RNA versus 240–400 nt for viroid RNAs) and unlike viroids, it bears an open reading frame (ORF) coding for the HDAg protein.

The HDV genome replication in the hepatocyte nucleus is associated with HDAg protein expression and assembly of ribonucleoprotein (RNP) complexes by direct interaction of HDV RNA and HDAg proteins [10]. However, the neo-assembled RNPs are not exported from the cell unless the HBV envelope proteins are co-expressed [11, 12]. Packaging and export rely on both the ability of the RNP to interact with the HBV envelope proteins, and the intrinsic capacity of the latter for self-assembly and secretion [13, 14]. Once assembled as HDV virions, the HBV envelope proteins confer to the RNP an efficient mode of cell egress, a protection in the extracellular space and a means for entry in HBV-susceptible human hepato-

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cytes [15]. Because of its HBV-derived envelope, HDV infects only HBV-susceptible cells. Within human hepatocytes, HDV is autonomous, requiring helper functions only to cross the plasma membrane—outward and inward—and to survive in the extracellular space. HDV can thus be viewed as an obligate satellite of HBV, in that it cannot propagate in the absence of a helper virus (Fig. 7.1). It is currently classified as the unique member of the *Deltavirus genus* [16].

In humans, HDV infection occurs either as a coinfection when individuals are simultaneously infected with both HBV and HDV, or as a superinfection when HBV chronic carriers become infected with HDV. Acute HDV superinfection leads more often to fulminant hepatitis and liver failure than does acute infection with HBV alone. In all cases, HDV can cause severe liver diseases, and in most HBV carriers, superinfection with HDV becomes chronic. Liver damage also occurs more rapidly in chronically HBV/HDV infected patients as compared to HBV carriers (See Chap. 14 for details). It is estimated that 5–10 % of the more than 240 million HBV chronic carriers worldwide are coinfected with HDV [17]. HDV is present in many different countries worldwide, and it displays an extended genetic variability classified into eight genotypes [18]. Although HDV infection is declining in some endemic regions, it is not disappearing, in particular in Europe, as a result of immigration from high endemic countries [19, 20]. Unfortunately, the possibilities for treating chronic HDV infection are limited to interferon, a therapy characterized by a low rate of sustained virological response [21, 22]. Therefore, there is actually an increasing need for the development of novel antiviral strategies against HDV, which will hopefully emerge from studies conducted to better understand the molecular interplay between HDV and its helper HBV.

The HDV Genome and Its Replication

HDV displays many unique features, among which the obligate relationship with HBV and the structure of its genome. The HDV RNA is approximately 1700 nucleotides in size and the smallest genome of viruses known to infect humans [23]. Both genomic RNA, and its replication intermediate, the antigenomic species, are circular molecules that form an unbranched, quasi-double-stranded species, also referred to as rod-like structure due to a nucleotide base pairing of approximately 70 %. The circular conformation and self-annealing properties are thought to confer resistance to host nucleases. The rod-like structure includes alternates of base paired helices and small internal loops or bulges of single stranded sequences, which are required for efficient replication [24], interaction with the HDAg proteins, and assembly of RNP complexes [10, 25–28]. A single protein is encoded by an ORF present on the antigenomic RNA strand [29]. The viral protein bears HDAg and is detected under two isoforms referred to as small-HDAg (S-HDAg) of 195 amino acids in length, and large-HDAg (L-HDAg) that differ from S-HDAg by 19 additional amino acid residues at the C-terminus. These isoforms are the result of an RNA editing event that occurs on a replication intermediate of the viral genome and is copied onto the

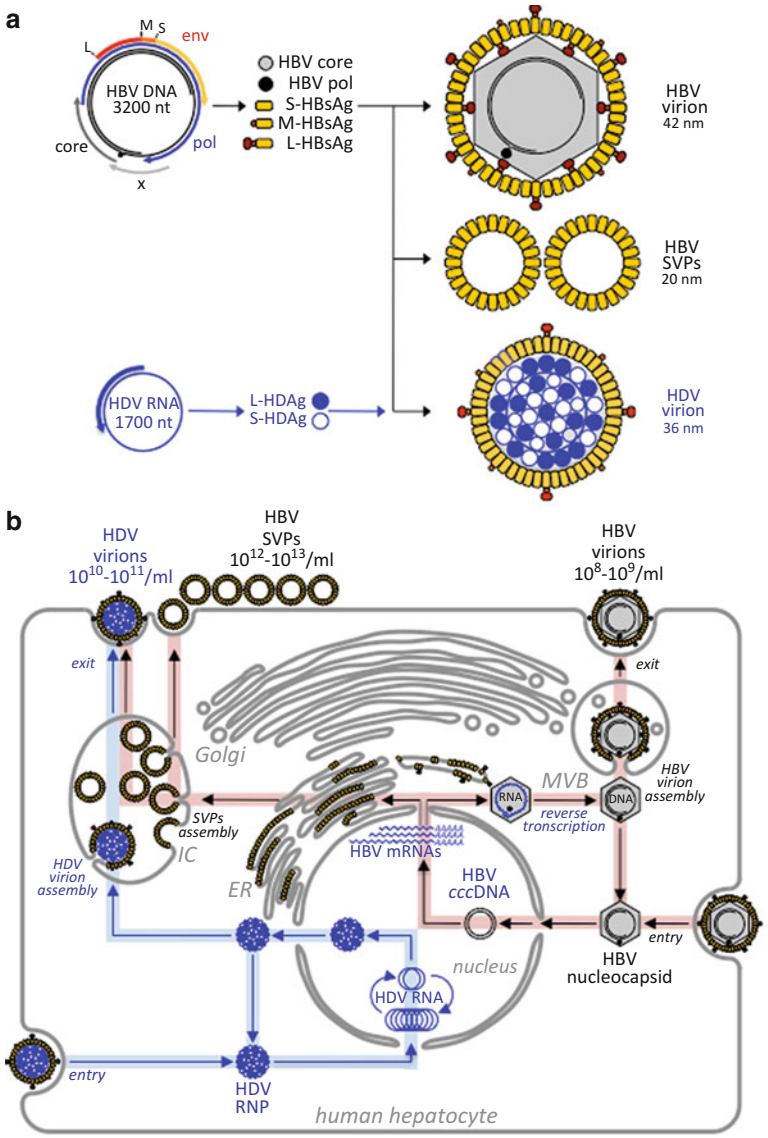

Fig. 7.1 (**a**) Schematic representation of the HBV virions, HDV virions, and HBV subviral particles (SVPs). The HBV DNA genome and the HDV RNA genomes are represented as *circular molecules*. *Open reading frames* for the envelope proteins (env), polymerase (pol), capsid (core), and x proteins are indicated on the HBV genome. HDAg ORF is indicated on the HDV genomic strand. *S-HBsAg* small HBV envelope protein, *M-HBsAg* middle HBV envelope protein, *L-HBsAg* large HBV envelope protein, *L-HDAg* large HDAg protein, *S-HDAg* small HDAg protein. The diameter of HBV virions, HDV virions, and SVPs is indicated in nanometers (nm). (**b**) Schematic representation of the HBV and HDV replication cycles. The HBV and HDV replication cycles are indicated in *pink* and *blue*, respectively. *IC* intermediate compartment, *ER* endoplasmic reticulum, *MVB* multivesicular bodies, *g* genomic HDV RNA, *ag* antigenomic HDV RNA

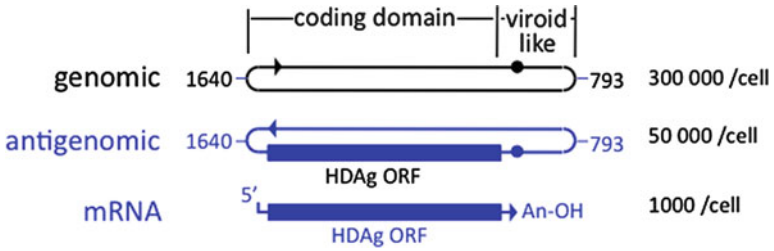

Fig. 7.2 The different forms of HDV RNAs in HDV-replicating cells. Genomic, antigenomic and messenger HDV RNAs are represented. The position of the tips of the rod-like structures is indicated. The HDAg ORF is indicated. *Arrows* on the RNA strands indicate the 5′–3′ direction

HDAg mRNA [30]. The rod-like structure of the HDV genome can be divided in two domains (Fig. 7.2), a viroid-like domain with a high degree of sequence conservation and a larger domain including the HDAg ORF and its complementary sequence in the rod structure [31].

After the initial sequencing of the HDV genotype-I (HDV-I) genome [23], seven additional genotypes have been identified [18], the nucleotide sequences of which display a surprisingly extensive divergence. HDV-III, a genotype specific to South America, is the most distant to other genotypes, nearing 40 % divergence with HDV-I. It is also associated to the most divergent helper HBV genotype (genotype-F), and to the most severe HDV-associated disease [32]. The majority of the molecular studies have been conducted with HDV-I, the prototype and ubiquitous genotype worldwide.

It was assumed that the replication mechanisms of HDV and plant viroids would be identical [33], since their RNAs displayed similarities [8, 23]. Experimental approaches to study HDV replication were therefore chosen based on the models already established for viroids [34, 35], including the initiation of HDV RNA replication by transfection of mammalian cells with cloned HDV cDNA. HDV RNA was observed to replicate in the nucleus, as efficiently as viroids in plant cell nuclei or chloroplasts, in the absence or presence of HBV [34, 36]. In subsequent studies, there were indications that the single-stranded circular genomic (or antigenomic) HDV RNA was used as a template for a rolling-circle mechanism of antigenomic (or genomic) RNA synthesis. In its principle, the rolling-circle mechanism should lead to synthesis of multimeric antigenomic RNA from the circular genomic template, but such a multimer does not accumulate because of its cleavage into monomers by an autocatalytic activity—defined as ribozyme—soon after synthesis. The linear monomer product is then ligated on itself to form a circular antigenomic molecule, which in turn serves as a template for the synthesis of genomic RNAs through the same rolling circle model [37]. Unlike viroids, the HDV RNA replication cycle includes the synthesis of a messenger RNA of antigenomic polarity for translation to HDAg proteins that fulfill multiple functions in the virus life cycle [38]. Although deprived of RNA polymerase activity, S-HDAg is required for HDV RNA replication, while L-HDAg inhibits replication and mediates assembly of HDV virions. Thus, HDV must use a host RNA polymerase to replicate its genome, as do viroids in plants.

HDV RNA Synthesis by Host RNA Polymerase

RNA-dependent RNA polymerase (RdRP) activity has long been considered absent in mammalian cells, but there is now clear evidence for its existence. For instance, the detection of intracellular mirror-spliced antisense transcripts that are reverse complement of spliced mRNAs was indicative of direct synthesis from an RNA template [39]. Many RNA viruses replicate their genome by encoding an RdRP for synthesis of both new genomes and viral mRNAs. However, in the case of *Retroviruses*, it is the host RNA Polymerase II (*Pol* II) that acts as a replicase to generate multiple viral genome copies from the provirus integrated into the host DNA. Pol II is also the enzyme that indirectly amplifies the HBV genome by transcribing the latter into multiple copies of pre-genomic RNA. For HDV RNA replication, several studies have shown that Pol II, which normally transcribes DNA templates into RNA, is somehow diverted to use the circular HDV RNA species as a template [40, 41]. Pol II would therefore act as a replicase for both HDV and HBV genomes.

The studies that identified Pol II as the enzyme responsible for HDV RNA synthesis, have been conducted in cell cultures in which there was no possibility of transcription of HDV RNA from DNA [42, 43]. In this setting, synthesis of genomic and antigenomic HDV RNAs, was prevented by the Pol II-specific inhibitor alpha-amanitin. HDV RNA replication was shown to colocalize with HDAg, Pol II, and the splicing factor SC35, in structures of the nucleoplasm referred to as speckles or transcription factories, whereas in the absence of HDV replication, HDAg would accumulate in the nucleoli [43]. When HDAg was artificially targeted to the nucleoli, initiation of genomic RNA synthesis was abolished, whereas antigenomic RNA synthesis was not affected. Forcing the release of S-HDAg from the nucleoli with actinomycin D would restore genomic RNA synthesis and the interaction between S-HDAg and Pol II, suggesting that synthesis of genomic RNA and HDAg mRNA would take place in the nucleoplasm, and antigenomic RNA in the nucleoli [44]. Yet, there is still controversy as to the implication of host RNA polymerases other than Pol II, in HDV RNA-directed synthesis, based on the observation that in cells transfected with genomic HDV RNA, synthesis of antigenomic species was recorded in the presence of 100 μg/ml of alpha-amanitin, whereas synthesis of genomic RNA was inhibited at 2.5 μg/ml [45]. It suggested to the authors that Pol I, an enzyme involved in the synthesis of ribosomal RNAs, rather than Pol II, could carry out antigenomic RNA synthesis [46].

Several attempts were made to reconstitute HDV RNA synthesis in vitro. For instance, it was shown that S-HDAg could bind directly to Pol II and stimulate transcription by displacing a negative elongation factor (NELF) [47]; S-HDAg could also interact with the clamp of Pol II, to ensure transcription fidelity [48]. Using purified Pol II, NTPs and an HDV antigenomic template scaffold, RNA-dependent Pol II activity appeared slower and less processive than its DNA-dependent activity [49]. Abrahem and colleagues [50], observed that an active Pol II pre-initiation complex could form on the HDV RNA with direct binding of the TATA-binding

protein. The promoter sequence for synthesis of antigenomic RNA was proposed to map to a 29-nucleotide region on the genomic strand (nucleotides 1650–1679), and the one for genomic synthesis, to a short region on the antigenomic species surrounding position 1679 [51, 52]. Pol II is assumed to recognize the quasi double-stranded structures of these hairpins as promoters. In cells undergoing active HDV RNA replication, two 5′-capped HDV RNA species of 18-25-nucleotides in length were identified, including one corresponding to the 5′ end of HDAg mRNA, which could interact with HDAg and Pol II. These small RNAs would act as initiators of RNA polymerization [53].

HDV Ribozymes

One characteristic of the rolling-circle mechanism used by viroids to replicate is the synthesis of longer-than-unit-length products from a circular template, which are processed to monomers by an autocatalytic, or ribozyme, activity. The linear monomers are then converted to circular molecules by self-ligation—or the action of a cellular ligase—to serve as templates for a second round of the rolling-circle process. HDV ribozymes consist in RNA sequences present on both genomic and antigenomic RNA species with self-cleavage activity and similar structural features, but they differ in size, sequence and secondary structure from hammerhead or hairpin ribozymes of viroids or satellites of plant viruses [54]. The reaction catalyzed by HDV ribozymes is a trans-esterification that converts a 3′–5′ phosphodiester bond to a 2′–3′ cyclic monophosphate group and a 5′ hydroxyl group. It is modulated by divalent metal ions, and easily observed in vitro in the absence of cellular proteins. The minimal size of the HDV ribozymes for self-cleavage in vitro was established at approximately 85 nucleotides and, a single nucleotide, 5′ to the cleavage site, is sufficient for cleavage. Considerable advances have been made recently, including X-ray structural data [55] providing details about the mechanism of HDV ribozyme activity (for review, see ref. [56]).

In an HDV-infected cell, self-cleavage and ligation are probably regulated to control the rate of genome replication and best accommodate the HDV life cycle. For instance, since antigenomic and messenger HDV RNAs are both synthesized from the same promoter on the genomic strand, it is expected that on the nascent antigenomic transcript, the ribozyme sequence, located just 3′ of the polyadenylation site, interferes with polyadenylation [57]. However, since mRNA and antigenomic RNA are transcribed in separate nuclear compartments, interference might be limited [45].

Interestingly, following the discovery of HDV, many HDV-like ribozymes have been identified in different branches of life and found to play a role in a variety of biological events, including retro-transposition in eukaryotes, raising the possibility of HDV RNA originating from the cell transcriptome [58]. Furthermore, recent studies have shown that circular RNA species are relatively abundant in cells [59], leading to the speculation that HDV RNA would have emerged in HBV infected

hepatocytes in which viral RNA species were processed to circular forms that became eventually replicative [60]. A cellular origin of HDV has been discussed previously [61], after the identification of the cellular homolog of HDAg, termed delta-interacting protein A (DIPA). DIPA was then proposed as a possible ancestral protein of HDAg. In this scenario, HDV would have evolved from capture of a cellular transcript by a self-replicating circular RNA. Since HDV is found only in humans, these findings might indicate a recent origin of HDV, with both ribozyme and coding sequence arising from the human transcriptome [62].

HDAg Proteins and RNA Editing

A single ORF on the antigenomic HDV RNA encodes the S- and L-HDAg proteins that differ from each other by 19 additional amino acid residues at the C-terminus of L-HDAg. S-HDAg is essential for RNA replication/accumulation, whereas L-HDAg acts as a potent dominant negative inhibitor of S-HDAg [63] while promoting virion assembly [64].

The origin of the two HDAg isoforms was initially revealed by analyzing the HDV RNA sequences that emerge during the natural course of infection: a heterogeneity at codon 196 in the HDAg ORF was observed, changing from a UAG amber codon to UGG for tryptophan, and respectively corresponding to S-HDAg (195 residues in length) and L-HDAg (214 residues) [65]. The sequence modification was found to occur during the course of infection, leading to the production of S-HDAg at the early phase of HDV RNA replication, and L-HDAg at the late phase of assembly. It was then found to result from an editing event on the full length antigenomic HDV RNA, catalyzed by a cellular adenosine deaminase that acts on RNA (ADAR). This enzyme converts adenosine to inosine, a nucleoside recognized as a guanosine [66]. The small form of ADAR-1 is in charge of editing HDV RNA [67, 68], and it plays a critical role in the regulation of the HDV life replication cycle by controlling the switch between genome amplification and virion assembly (for review, see ref. [30]). Interestingly, there are significant differences in the editing efficiency between HDV genotypes I and III [69].

The HDAg proteins undergo multiple posttranslational modifications to fulfill numerous functions in HDV RNA replication process, RNP assembly and interaction with the HBV envelope proteins [70, 71]. Several of these functions are achieved upon the ability of specific HDAg isoforms to interact with HDV RNA and to localize to particular subcellular compartments. In fact, the RNP and/or HDAg proteins can shuttle between nucleus and cytoplasm, and within the nucleus, between nucleoplasm and nucleoli.

HDAg proteins undergo methylation at Arg-13 [72], acetylation at Lys-72 [73], phosphorylation at Ser-177 [74] and sumoylation at multiple lysine residues [75]. All these posttranslational modifications are thought to alter the HDAg protein conformation, intracellular localization and function in RNA replication mechanism and RNP assembly (for review, see ref. [70]).

HDV Ribonucleoprotein

Replication of the HDV genome by RNA Pol II is very efficient, leading to the accumulation of up to 300,000 copies of genomic HDV RNA per cell, 50,000 copies of the antigenomic species and approximately 1000 copies of a 900-nt-in-length HDAg mRNA (Fig. 7.2). Since extracellular HDV virions contain exclusively genomic RNA, there must be a process for selection of this molecule to assemble RNPs that are released as enveloped virions. All forms of HDV RNA are synthesized in the nucleus, but soon after synthesis, a significant amount of newly synthesized genomic RNA is detected in the cytoplasm [76]. In fact, the majority of intracellular HDAg proteins and genomic RNA are detected in the cytoplasm, whereas most of the antigenomic RNA is retained in the nucleus. The proportion of genomic HDV RNA in the nucleus, compared to cytoplasm, remains relatively constant over time, indicating that genome export from the nucleus would occur continuously [26]. Since nuclear export of genomic RNA appeared resistant to leptomycin B, a cell region maintenance-1 (Crm1)-independent pathway is likely to be involved [76]. This pathway is used for the splicing-dependent export of cellular mRNAs synthesized by Pol II. This is in agreement with HDV RNA synthesis being carried out by Pol II.

HDAg proteins and HDV RNA form a stable RNP complex that adopts a spherical core-like structure with a diameter of 19 nm in HDV virions or within nuclei undergoing HDV replication. Unlike virion-associated RNPs, intracellular RNPs were found to contain both genomic and antigenomic RNAs. The molar ratio of HDAg proteins to HDV RNA was initially estimated at 70 in HDV virions and 30 in nuclear RNPs [10], then at 200 in both virions and intracellular RNPs [26]. More recently, a study based on RNP reconstruction assays estimated that a full-length HDV RNA molecule would associate to 32–40 HDAg proteins [28]. This ratio was in the same range as the early estimates of Ryu and colleagues [10].

To bind to HDAg proteins, the HDV RNA must be structured as an unbranched rod of at least 300 nucleotides that includes stretches of double stranded RNA interspersed with single stranded loops within the rod. According to Lin and colleagues [28], an HDAg multimer of fixed size, would assemble prior to RNA binding. The multimer would adopt the octameric organization that Zuccola and colleagues predicted using HDAg-specific synthetic peptide and X-ray crystallography [77]. These authors proposed that HDAg proteins would form dimers arranged as an antiparallel coiled coil (residues 13–48), and those dimers would further associate to form octamers. Combined with the data reported by Lin and colleagues [28], these results suggest that HDAg would bind HDV RNA as preformed octamers leading to an RPN consisting of one genome and 4–5 octamers.

Within S- and L-HDAg, two arginine-rich motifs (ARMs), I and II, located in the middle of the protein sequence, were initially thought to mediate binding to HDV RNA [78]. However a recent study demonstrated that ARMs I and II are not required for HDV RNA binding [79]. Instead, RNA binding would be established via multiple domains of the HDAg protein among which the N-terminal region of HDAg would play the prominent role [80, 81].

A functional nuclear export signal (NES) is present at the C-terminal 19-amino acid sequence of L-HDAg [82], however, it remains unclear whether it is required for RNP nuclear export and virion assembly. Since nuclear export of genomic RNA appeared independent of L-HDAg, it is conceivable that genome-containing RNPs would be assembled in the cytoplasm [76].

A CXXQ signal for farnesylation is also present at the L-HDAg C-terminus [83, 84]. This posttranslational modification is required for virion assembly [14, 85]. It is assumed that the farnesyl group serves to anchor the RNP to the membrane of the endoplasmic reticulum (ER), where the envelope proteins are synthesized. In tissue culture, the co-expression of L-HDAg and HBV envelope proteins leads to L-HDAg packaging within HBV subviral particles (SVPs), but such assembly of L-HDAg-containing SVPs would not occur during the natural course of HDV RNA replication [86].

The 19-amino acid C-terminus of L-HDAg also contains a clathrin box, and L-HDAg is found to colocalize with the HBV envelope proteins and clathrin heavy chain in clathrin-coated vesicles, suggesting the involvement of a clathrin-L-HDAg interaction in the process of virion assembly [87–89]. However, the L-HDAg C-terminal clathrin box is not conserved among HDV genotypes.

Finally, the 19-amino acid C-terminus of L-HDAg bears a motif responsible for binding to the HBV envelope proteins. It includes proline residues that are present at several positions, and important for both NES function and activity of the packaging signal [13, 14]. Surprisingly, beside the CXXQ farnesylation signal sequence and tryptophan at position 196, the 19-amino acid C-terminus sequence of L-HDAg is not well conserved among the different HDV genotypes, except for the presence of at least four proline residues that are dispersed at various positions between W-196 and C-211 [90].

HDV Virion Assembly

The HDV virions are heterogeneous in size with an average diameter of 36 nm. The envelope is similar to that of HBV particles, including cell-derived lipids associated to HBV envelope proteins surrounding the inner HDV RNP [26].

The HBV helper function solely consists in providing HDV the means for propagation. HBV is an enveloped virus of the *Hepadnaviridae* family, with an icosahedral nucleocapsid that contains a circular, partially double-stranded DNA. Its genome replicates through a mechanism that includes a step of reverse transcription (see Chap. 1 for details). But the distinctive features of the HBV life cycle, essential to HDV propagation, reside in its peculiar, envelope protein-driven budding mechanism. In HBV-infected cells, the three envelope proteins, designated small, middle, and large (S-HBsAg, M-HBsAg, and L-HBsAg, respectively), are produced in amounts exceeding by far the need for HBV virion assembly, and they have the capacity of self-assembly, leading to the secretion of SVPs [91]. S-HBsAg proteins can dimerize and form multimers at the ER membrane through lateral protein–protein interactions, and the resulting aggregates are thought to bud spontaneously into

the lumen of a pre-Golgi compartment as empty SVPs. Although the S-HBsAg protein provides the driving force to the budding process, it cannot direct HBV virion assembly because the recruitment of the HBV nucleocapsid is mediated by L-HBsAg. Owing to the overproduction of S-HBsAg and to its capacity for auto-assembly, HBV infectious virion formation is a rather rare event compared to the production of SVPs. As a result, an average infectious serum contains approximately $10^{12–13}$ SVPs and only $10^{8–9}$ virions per ml. In comparison, at the onset of an acute HDV infection, titers of $10^{9–10}$ HDV virions per ml and a tenfold excess of SVPs are observed. Therefore, HDV particles appear more efficiently assembled and secreted than HBV virions.

The HBV envelope proteins are membrane-spanning glycoproteins that differ from each other by the size of their N-terminal ectodomain [91]. L-HBsAg contains a N-terminal pre-S1, central pre-S2 and C-terminal S domains. M-HBsAg is shorter than L-HBsAg in lacking pre-S1. S-HBsAg only consists of the S domain. Envelope proteins are synthesized at the ER membrane; they form aggregates that bud at a pre-Golgi compartment before egress as SVPs [92]. Assembly of mature HBV virions requires, in addition to S-HBsAg, the presence of L-HBsAg as a matrix protein for nucleocapsid envelopment [93]. Recent findings indicate that HBV virions and SVPs follow distinct pathways for export: the late endosomal multivesicular bodies (MVBs) for budding of HBV virions at intracellular membranes, and an MVB-independent secretory pathway for the release of SVPs [94–96]. The HBV envelope proteins can also package the HDV RNP in the case of HBV/HDV coinfection [2, 12], leading to the assembly of HDV virions. Whether HDV uses the SVPs secretion pathway rather than an MVB-dependent route for export remains unclear.

HDV infection has only been found in humans and solely in association with HBV infection. However, among close members of HBV in the *Hepadnaviridae* family, the *Woodchuck hepatitis virus* (WHV) and the *Woolly monkey hepatitis B virus* (WMHBV) encode viral envelope proteins that are competent for HDV RNP envelopment [3, 97]. These findings have led to the use of woodchuck as a convenient small animal model for HDV, and to the possibility of using primary cultures of hepatocytes from woolly monkey or spider monkey to test for the infectivity of HDV particles pseudotyped with the WMHBV envelope proteins [97]. In contrast to WHV and WMHBV, the most distantly related *Hepadnavirus*, namely the *Duck hepatitis B virus* (DHBV), is unable to assist in HDV propagation. It is due in part to the failure of HDV RNA to replicate in avian cells [98], but also to the inability of the S-DHBsAg envelope protein to package the HDV RNP [99].

A crucial step in the HDV life cycle is the recruitment of the HBV envelope proteins by the RNP in the cytoplasm. L-HDAg exposed at the surface of the RNP must interact with a specific motif of the HBV envelope proteins. Early studies conducted by Chen et al. [100] had shown that a truncation of the C-terminal 50 residues of S-HBsAg was sufficient to abolish envelopment and secretion of co-expressed HDAg proteins. Then, O'Malley and Lazinski [99] demonstrated that S-HBsAg bearing a deletion in the AGL (aas 107–147) was competent for L-HDAg interaction, but defective for virion assembly and release. A deletion of residues 24–28 of the S-HBsAg protein, or the removal of the glycosylation site at position 146 could also impair the maturation of HDV particles while having no effect on

SVP secretion. In the two latter cases, the defect in HDV assembly was due to impairment of the mutant envelope to coat the RNP and not to a lack of interaction with L-HDAg. More recently, a major determinant of HDV assembly was identified in the C-terminus of S-HBsAg [13]. Within this domain, single substitutions of tryptophan (Trp) at positions 196, 199 and 201 with alanine or phenylalanine, were permissive to SVP secretion but detrimental to HDV assembly. This was proven to result from the inability of the S-HBsAg mutants to interact with L-HDAg. The Trp-rich domain in S-HBsAg was thus considered as a matrix domain for HDV assembly [13]. A recent study showed that in addition to the Trp residues, the HDAg binding site on S-HBsAg also includes a tyrosine residue at position 200 [101]. Intrestingly, the Trp-rich motif in S-HBsAg is strictly conserved in all HBV genotypes as well as in WHV and WMHBV, suggesting that it plays an essential function in the HBV life cycle. However, this assumption was contradicted by the observation that an alanine substitution for Trp-196, -199 and -201 had no effect on the HBV replication cycle [102]. The reason for the Trp-rich motif conservation in all *Orthohepadnaviruses* may just be a consequence of the overlap between the envelope proteins and polymerase (Pol) genes, a characteristic feature of the HBV genetic organization and a consequence of the small size of the HBV genome. In fact the ORF of HBV Env is entirely included in that of Pol, and the DNA sequence surrounding Trp-196 codon in S-HBsAg ORF also encodes the Tyr-Met-Asp-Asp motif of the Pol catalytic domain in the minus-one reading frame. Since there is a strict requirement for a Tyr-Met-Asp-Asp motif in Pol, there is no other possibility than Trp at position 196 in S-HBsAg. By extrapolation, a few S-HBsAg residues (i.e., Trp-199, Tyr-200 and Trp-201), in addition to Trp-196, might also be solely conserved because they share a DNA coding sequence with the Pol catalytic domain. Thus a conserved and essential Tyr-Met-Asp-Asp motif in Pol imposes a conserved, Trp-rich motif in S-HBsAg, which is fortuitously used by HDV as a matrix domain for assembly. Note that this HDV matrix domain could represent a target of choice for anti HDV therapy, because a drug competing with HDV RNP for binding to S-HBsAg would not lead to the occurrence of HBV escape mutants.

Clearly, HBV appears as the best equipped virus to help HDV overcome its propagation defect, for the following reasons: (1) in HBV-infected cells, there is always a huge overproduction of envelope proteins that self-assemble into empty lipoprotein transport vesicles—in other words, a guaranteed, free and reliable cell export machinery, (2) the HBV envelope proteins are very flexible, in that they can assemble into various types of particles, namely the spherical SVPs (22 nm in diameter), the empty filaments (22 nm in diameter, up to several hundred nanometers in length), and the 42-nm HBV virions, (3) the small size of the HBV genome, which imposes the *pol*/*env* genes overlap at the origin of the HDV matrix domain conservation in S-HBsAg. Stoichiometry also appears in favor of S-HBsAg/RNP interaction since an average infected cell contains an estimate of 6×10^6 copies of HDAg proteins and approximately 10^5 copies of genomic HDV RNA [26]. Noteworthy, HBV and HDV envelopes may differ from each other with regard to the relative amount of L-HBsAg, estimated at up to 25 % of the total surface proteins for HBV [103] and only 5 % or less for HDV [2, 104].

HDV Entry

Since HDV and HBV virions are coated with the same surface proteins, they are expected to use identical host factors for cell surface attachment and receptor binding. Based on this assumption, the HDV model has been used to study the HBV envelope protein function at viral entry. In fact, in vitro HDV infection assays offered several practical advantages: (1) HDV entry into susceptible cells leads to very high levels of replicating HDV RNA (up to 300,000 copies per cell) that is easily detectable by Northern blot hybridization as early as 6 days post inoculation, and (2) infections being nonproductive in the absence of the helper HBV, the level of intracellular viral RNA that accumulates in an infected cell is directly proportional to the viral titer of the inoculum [105]. These characteristics have been crucial to the recent identification of sodium taurocholate cotransporting polypeptide (NTCP) as a common HBV/HDV receptor [106].

The initial attachment of HBV/HDV particles is mediated by cell surface heparan sulfate proteoglycans [107–110]. Glycosaminoglycan (GAG) side chains of proteoglycans are used by various viruses as primary docking sites, and they are clearly implicated in HBV and HDV entry. HBV and HDV virions were shown to bind to GAGs at the surface of susceptible HepaRG cells, or to immobilized heparin. Furthermore, infection could be blocked by treatment of virions with heparin (or highly sulfated dextran sulfate), or upon treatment of HepaRG cells with heparinase prior to inoculation. The requirement for cell-surface GAGs as low-affinity receptor for HBV has also been demonstrated in primary cultures of Tupaia hepatocytes [108].

A ligand to cell-surface GAGs has been identified in the surface-exposed antigenic loop (AGL), of the HBV envelope proteins [111]. This infectivity determinant was shown to depend upon cysteine residues involved in structuring the AGL-associated a-determinant [111]. The a-determinant is a conserved immune-dominant determinant bearing most of the HBV neutralizing epitopes. The AGL-associated infectivity determinant was precisely mapped to a set of conserved residues—cysteines and non-cysteines—predicted to cluster together in a network of disulfide bridges that underlies the a-determinant [112]. The HBV envelope proteins would mediate electrostatic interactions with negatively charged GAGs through the positively charged residues R122 and K141 within the AGL. In addition, there might be a participation of the pre-S domain of L-HBsAg in HBV virion binding to GAGs because the pre-S domain of L-HBsAg is positively charged and, as compared to the HDV envelope, the HBV virion envelope is enriched in L-HBsAg [109]. Although the AGL determinant is clearly essential for HBV/HDV entry, it is still unclear to what extent its binding to GAGs at the surface of human hepatocytes participates to species specificity and tissue tropism.

After the reversible and low affinity attachment of HDV/HBV virions to host cell surface GAGs, the pre-S1 domain of L-HBsAg binds with high affinity to a specific receptor. This receptor has been identified recently as NTCP, a bile acid transporter highly expressed at the basolateral membrane of differentiated hepatocytes [106, 113]. The critical role of pre-S1 in infectivity was first demonstrated using HDV particles [15].

Then numerous studies based on neutralizing antibodies and mutagenesis approaches, demonstrated that both HBV and HDV were dependent on pre-S1 for infectivity. This was also demonstrated using pre-S1 specific peptides in in vitro infection assays. These experiments clearly established that the integrity of the N-terminal 75 amino acid residues of pre-S1, including N-terminal acylation with myristic acid, was essential. Furthermore, a synthetic myristoylated pre-S1 peptide (Myr-pre-S1) encompassing residues 2–48 could block infection [114–121]. This lipopeptide has since been used extensively to characterize the early steps of HBV/HDV infection, and it was also instrumental in the identification of NTCP as a receptor [106, 118, 122, 123]. It has been proven a potent HBV/HDV entry inhibitor, and it constitutes a promising antiviral drug (Myrcludex-B) currently in clinical trials. In tissue culture, Myr-pre-S1 blocks HBV/HDV infection at nanomolar concentrations, and it is active upon a short preincubation of cells with the lipopeptide, suggesting its rapid and efficient targeting to the NTCP receptor at the hepatocyte surface [118].

Because of their identical envelopes, HBV and HDV have in common the very early steps of binding and internalization, but once released in the cytoplasm, the HDV RNP and the HBV nucleocapsid most likely segregate in separate pathways to reach the nucleus [124]. In support of an endocytic route for HBV entry, a recent study demonstrated that extraction of cholesterol from HBV virions reduced infectivity without affecting particle integrity, antigenicity and the ability to bind hepatocytes [125]. The cholesterol content of the viral envelope was dispensable for viral binding, but likely involved at a post-binding step in the entry process [125]. HBV/HDV internalization is thought to be mediated by endocytosis, but there is actually no consensus on the exact pathway. Using different in vitro infection assays, HBV entry has been reported to use caveolae, clathrin or macropinocytosis [126–128]. Therefore the question of the entry pathway(s) for HBV or HDV remains unresolved.

HDV Inhibits HBV Replication

In vivo, superinfection of HBV chronic carriers with HDV leads to the inhibition of HBV replication during the acute phase of HDV replication [4]. This phenomenon has been described in both humans and experimentally infected chimpanzees, but it remains poorly understood [36, 129, 130]. In experiments conducted in tissue culture [130], S-HDAg exerted a strong inhibition of synthesis/stability of HBV mRNAs. In an independent study, HDAg proteins were shown to repress HBV enhancers I and II, and to activate the IFN-alpha-inducible MxA gene [131]. This repression of HBV transcription could by itself explain the HBV inhibition phenomenon observed in vivo [130]. But the suppressive effect could also be the consequence of an indirect, interfering mechanism driven by inflammatory cytokines, or result from a hijacking of the envelope proteins by the RNPs in host cells when HDV RNA replication reaches maximum levels. Considering that a coinfected cell

might contain as many as 6,000,000 copies of HDAg proteins and 300,000 copies of HDV RNA [26], it is likely that the HBV nucleocapsids be heavily outnumbered by the HDV RNPs in their access to the HBV budding machinery.

Conclusion

Because of its many unique features, HDV remains one of the most interesting human viruses. The nature of its genome, its origin and the mechanism used for replication of its RNA via a host DNA-dependent RNA polymerase remain enigmatic. Its ability to mainly rely on host functions and a helper virus for replication and propagation, is fascinating. With a single protein, HDV is able to accomplish multiple functions through a variety of posttranslational modifications, and to use ADAR-1 to switch between genome amplification at an early phase of replication and virion assembly at a later phase. Its relationship with HBV is also quite intriguing, considering that HDV could not have emerged, and could not survive, in the absence of HBV. Yet, HDV replication induces a strong repression of its helper HBV in coinfected cells. Although counterproductive in appearance, this inhibition is likely regulated by HDV to its advantage. More remarkable is the strategy used by HDV to exploit a conserved motif in the HBV envelope proteins as a matrix domain to assemble infectious particles, and thereby solve its propagation defect. This scenario suggests that HDV has emerged in HBV-infected cells. Then the recent observation that HDV RNA could persist in the liver of humanized mice for at least 6 weeks in the absence HBV, before a productive HDV infection could be rescued by superinfection with the helper suggests that HDV RNA eradication in chronically infected individuals may be as challenging a task as eliminating cccDNA in HBV carriers [132].

The range of HDV RNA sequence variability is somewhat surprising for such a small RNA, considering that the genome must conserve a precise degree of self annealing, two ribozyme sequences and an open reading frame. More than 30 years after its discovery, HDV is still the sole member of the *Deltavirus* genus, but the existence of HDV-like ribozymes, small circular RNAs and RdRP activity in animal cells suggest that self-replicating HDV-like RNAs may be more common than expected.

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Chapter 8
Translational Medicine in Hepatitis B Virus: What Can We Learn from Clinical Samples?

Antonio Bertoletti and Fabien Zoulim

Knowledge of the pathological mechanisms that are causing human diseases demands the use of in vitro and in vivo models, where different variables can be clearly controlled and where the impact of single genes, proteins or cells can be consistently measured. Such reductionist approach cannot be applied in clinical samples, and for this reason, experimental findings detected in clinical studies need to be defined in controlled models. On the other hand, it is questionable whether artificial experimental models can fully recapitulate the natural disease. This problem is present in the study of HBV related disease, and in this chapter we review the shortcomings of the current available in vivo and in vitro models of HBV infection and discuss how significant questions related to HBV pathogenesis can profit from a careful utilization of data derived from patients (Fig. 8.1).

In Vivo Models of HBV Infection: What Do They Mimic?

HBV infection causes acute and chronic liver diseases of variable severity. Since the virus is not directly cytopathic, the host immune system play an essential role in modulating the level of liver inflammation and in controlling the extent of virus

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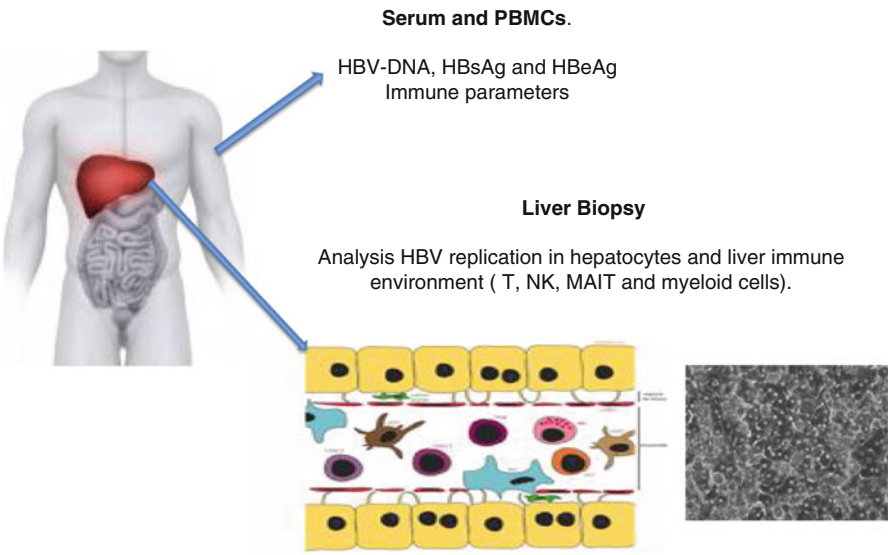

Fig. 8.1 Information derived from the analysis of human samples in viral hepatitis

spread and eventual resolution [1]. Distinct variables such as viral load, virus genotypes, route of the infection, age, sex and genetic makeup of the infected hosts are likely to influence HBV infection pathogenesis and their analyses will be greatly facilitated by reliable animal models. However, besides humans, HBV infects only chimpanzees and tree shrews but strong ethical constraints, handling difficulties as well as the 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].

Important insights on the effect of virus and host variables were obtained from studies of animal species (ducks and woodchucks) that are the natural hosts of other hepadnaviruses: woodchuck hepatitis B virus (WHBV) and duck hepatitis B virus (DHBV) [3, 4]. Nevertheless, there are shortcomings of such models. WHBV and DHBV are similar but not identical to HBV and cause a different spectrum of liver diseases. For example, woodchucks infected by WHBV develop liver tumors at a much higher rate than humans as a result of a "specific" mechanism *of insertional* mutagenesis [5, 6], but do not express the spectrum of liver disease (asymptomatic carrier, chronic active hepatitis, liver cirrhosis) observed in HBV-infected humans. More importantly, the analysis of pathogenic mechanisms related to viral control and liver damage is hampered both in ducks and woodchucks by the lack of appropriate reagents necessary to study the complexity of host–virus interaction.

The necessity of an easy to maintain, well-defined, inbred, small-animal model for studying immune control and immunopathogenesis during HBV infection led to

the production of different mouse models of HBV infection: HBV transgenic mouse, HBV hydrodynamically transfected mouse and chimeric mouse with humanized liver.

These different models have greatly contributed to our understandings of different aspects of HBV pathogenesis. HBV transgenic mice were instrumental to determine the role of HBV-specific T cells in viral control through non-cytopathic mechanisms and to analyze the importance of other cellular components of the immune system like macrophages, chemokines, and platelets in liver damage [7, 8]. Studies in HBV mouse model established by hydrodynamic transfection of replication competent HBV genome have also clarified the role of different cellular components of the immune system (CD8T, CD4T, NK cells) [9, 10], while HBV chimeric mouse reconstituted with human hepatocytes were instrumental in determining innate immune activation in HBV infected hepatocytes [11] and to test new therapeutic strategies [12].

These models, however, cannot completely recapitulate the virological and immunological events of the natural infection. 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 host genome) and subsequent implantation of the eggs into pseudo-pregnant female mice [13, 14]. The usage of the HBV endogenous or liver-specific promoter allows hepatocytes to express viral proteins, ensuring HBV transgenic mice to express either a particular viral antigen or the complete HBV genome within the hepatocytes. Nevertheless, HBV does not directly infect murine hepatocytes, thus a true infection does not occur in the HBV transgenic mice even with the presence of circulating virus [15]. Mice transgenic for hNTCP, the receptor of HBV entry, were engineered but these animals are still not susceptible to HBV infection as cccDNA formation seems to be a blocking step in this species [16]. Second, HBV transgenic mice are immunologically tolerant to viral antigens [17]. Hence, the original transgenic model does not display any classical induction of antiviral immunity and also sign of chronic liver disease. The immunological tolerance physiologically present in these mice is bypassed through adoptive transfer of syngeneic, unprimed splenocytes [18] or adoptive transfer of HBsAg-specific $CD8^+$ T cells into thymectomized, irradiated, and bone marrow-reconstituted HBV transgenic mice [19]. This adoptive transfer creates a model of immune mediated chronic hepatitis, but different from the natural chronic hepatitis B infection. The immune cells transferred in the HBV transgenic mice are acting in a completely normal liver environment, and as such the possible influence of the liver microenvironment affected by a chronic inflammation cannot be evaluated. Last, HBV covalently closed circular DNA (cccDNA), the primary viral transcriptional template during infection, is not found in the whole genome HBV-replicating transgenic mice [20] Hence, host attempts to clear HBV cccDNA or the role of HBV cccDNA in HBV persistence cannot be studied.

Similar limitations are also present in HBV hydrodynamic transfected mouse. Unlike transgenic mice where the hepatic expression of the HBV genome is controlled primarily by either endogenous (HBV) or liver-specific promoters, and is already present at birth, the systemic administration of the HBV plasmid under

hydrodynamic conditions preferentially, but not exclusively, delivers the HBV transgene into the hepatocytes [9]. Integration of the HBV genome is then mediated through the action of the inverted terminal repeats of adeno-associated virus or by the Sleeping Beauty transposon system, while expression is controlled by the endogenous or liver-specific promoters [21]. Hence, this technique provides a relatively simple and convenient method to produce mice with HBV-expressing hepatocytes for the analysis of viral dynamics and anti-HBV immune response. Indeed, since HBV-expression is not present at birth these mice are not tolerant to HBV antigens and they can mount an immune response against HBV.

Nevertheless, in addition to the fact that these mice are not permissive for HBV infection (virions are produced from the transfected hepatocytes but cannot reinfect the mouse hepatocytes), the technique is only partially specific for the liver, often resulting in off-target transfections. How these unintended transfections of other cells could affect the viral kinetics and antiviral immunity found in this model is difficult to predict. Furthermore, hydrodynamic transfection typically reaches peak transgene expression after approximately 8 h post injection of plasmid DNA and expression levels decreases thereafter [9, 10]. This transient transfection property of the technique precludes the generation of persistently HBV transfected mice without additional manipulations and is not mimicking the kinetics of viral replication of the natural infection.

Figure 8.2 depicts the differences in the kinetics of viral replication and antiviral immunity between natural infection in human (and chimpanzees), HBV-transgenic mice and hydrodynamically transfected mice. The limitation of using such models to study viral replication kinetics and immune response after primary infection is highlighted.

The human chimeric mouse represents a further advancement of HBV mouse models. In these mice, xenotransplantation of human hepatocytes progressively repopulate the mouse liver, with the goal of replacing the entire mouse organ with functional human hepatocytes. Different from the transfection mouse models where HBV virions were produced artificially without infection, these chimeric mice were fully permissive for HBV infection, creating a reproducible small animal model of true HBV infection and replication [22, 23]. 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 is restricted. The technical difficulties in standardizing the number of grafted human hepatocytes and the limited availability of human hepatocytes represent significant constraints for the production of this model. A further recent development is a human chimeric mice reconstituted with fetal human hepatocytes [24]. These chimeric mice are repopulated not only with human hepatocytes but also with human immune cells (monocytes, T and NK cells) with identical genetic background. Whether such immune cells fully reconstitute a normal immune system needs to be carefully evaluated but certainly this model might

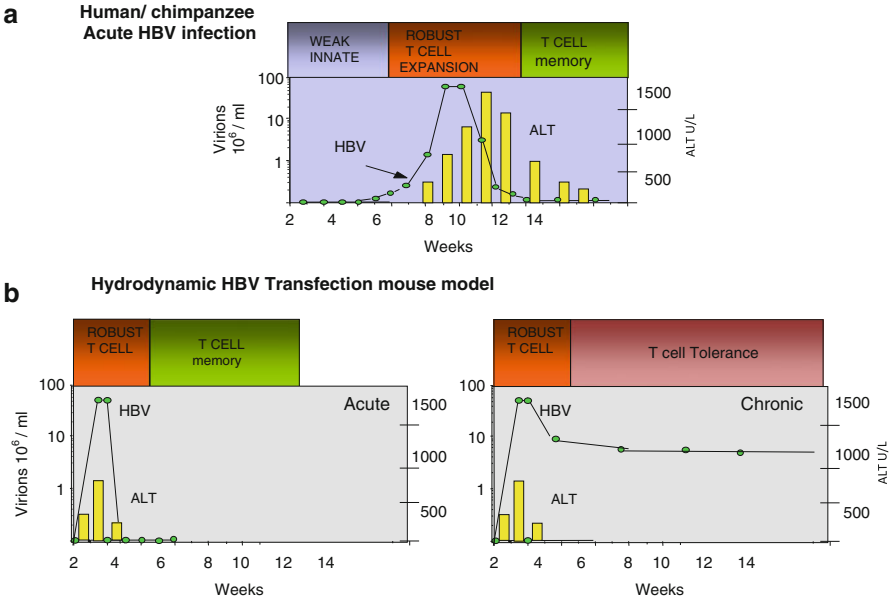

Fig. 8.2 Comparative HBV kinetic in human/chimpanzee (**a**) and hydrodynamic HBV Mouse models (**b**)

represent a great advantage for the study of the anti-HBV host immunity. Recent work using this model have shown that liver chronic inflammation can be induced after HBV infection while the ability to induce a HBV-specific adaptive immunity seems to be greatly compromised [24]. Improvements of this model are likely needed to fully recapitulate the immune features induced by HBV in infected patients.

In Vitro Models of HBV Infection: What Do They Mimic?

Human hepatocytes are the natural target cells of HBV and HDV. These cells can be isolated from liver resections and retain susceptibility to HBV infection for a short period in culture [25]. However, the accessibility to fresh human liver resections, the quality and the variability of the individual preparations limit their use. In the mid-1990s, several laboratories showed that primary hepatocytes of *Tupaia belangeri* were also susceptible to HBV infection [26, 27]. Although primary tupaia hepatocytes are valuable to study HBV infection, the difficulty to rear these animals, and the absence of tupaia-specific reagents for functional studies limit their use. To bypass the hurdles to using primary cell cultures, human hepatoma Huh7 and HepG2 cell lines were used for many years to perform in vitro experiments on HBV. Although these cells are permissive to HBV replication and viral particle assembly, they are not susceptible to infection due to the lack of expression of the

receptor (s) and thus only allow study of post-transcriptional steps of the HBV life cycle after plasmid transfection. Since the recent discovery of sodium taurocholate co-transporting polypeptide (NTCP) as a HBV/HDV receptor [28, 29], HepG2 and Huh7 cell lines (over)-expressing NTCP have been generated. These cells are susceptible to HBV and HDV infections but their capacity to allow virus propagation remains to be determined. Because of their transformed nature, their relevance for studies of virus–host cell interactions has to be considered with caution. Alternatively, the HepaRG cell line can be used for in vitro studies. HepaRG cells are liver progenitors that become susceptible to HBV and HDV infection after differentiation in culture [30]. However, infection rates are low and virus spread in cultured cells is not observed. Finally, a recent study showed that micropatterning and coculturing (MPCC format) of primary human hepatocytes or hepatocyte-like cells differentiated from induced-pluripotent stem cells (iPSC) with fibroblasts maintains prolonged HBV infection [31], a model eventually amenable to study virus–host interactions and antiviral drugs affecting early infection steps.

Several aspects should be discussed regarding the biological relevance of these study models. Besides the susceptibility to infection and replication, the propagation of the virus in the cell culture seems to be limited. Although these cells are overall susceptible to HBV infection, this limitation in the capacity to disseminate to other cultured cells might be linked to several factors including the low yield of HBV production from infected cells, the restriction of infection by innate responses of hepatocytes to HBV infection, and the fact that hepatocytes may de-differentiate with time in culture. Furthermore, these models are quite static as the most physiologic systems rely on nondividing differentiated cells limiting the possibility to generate a dynamic model in which daughter cells resulting from cell division could be infected.

Another important parameter is the capacity of these cells to support cccDNA formation, amplification and recycling. Indeed, a system allowing a dynamic formation of cccDNA is highly desirable for the identification of host cell factors involved in its formation, chromatinization, and stability, as well as for the study of the mechanism of selection of escape mutants which involves at least the spread of the mutants to susceptible cells and the establishment of a pool of mutant cccDNA. Unfortunately, the levels of HBV cccDNA in infected cultured hepatocytes remain low (approx. one copy per cell), and are consistently lower than that obtained with DHBV whether it is cultured in chicken or human hepatoma cells [32].

The host cell response to infection is also an important aspect that is amenable to experimental studies in cultured hepatocytes. Regarding innate responses of hepatocytes to HBV infection, primary human hepatocytes and HepaRG cells are capable of mounting interferon responses that can limit the rate of viral replication and the spread of the virus [31, 33]. In contrast, the transformed HepG2 cells exhibit a blunted activation of IFN signaling pathways allowing the persistence of high levels of viral replication [33]. These are important differences between these cell types that should be taken into consideration when performing studies of hepatocyte response to HBV infection (Table 8.1).

Table 8.1 Human cells for HBV study in vitro

	Immortalization	Transformation	Availability	Variability	Rate of infection	DMSO for infection	cccDNA levels[a]	HBV propagation	Innate immunity	Maintenance
Primary human hepatocytes	–	–	+	+++	20–100 %	1.8–2 %	1–2 copies per nuclei	–	+++	2–3 weeks
Differentiated HepaRG cell line	+	–	+++	++	5–20 %	1.8–2 %	0.2–0.5 copies per nuclei	–	+++	>6 months
HepG2/Huh7 cell lines	+	+	+++	+	0 %	0 %	–	–	–	–
NTCP-HepG2 cell line	+	+	+++	+	50–100 %	2.5–3.5 %	1–5 copies per nuclei	–	–	10 days

Adapted from Zeisel et al, [16] G

[a]After HBV infection with a multiplicity of infection of 1000 vge/mL (viral genome equivalent per mL)

The Importance of Clinical Samples in the Study of HBV Infection

We have summarized the features of in vivo and in vitro models of HBV infection. We will now discuss how analysis of clinical samples can help in addressing questions related to HBV infection and immunity.

Acute Versus Chronic Infection of Hepatocytes

The study of HBV biology in hepatocyte cultures is limited particularly in relation to the role of hepatocyte turnover. The access to liver samples of patients or from relevant animal models remains critical.

The outcomes of HBV infection are highly dependent on interactions between the virus and the host immune system. Indeed, whereas 95 % of immuno-competent adults clear the infection, only 5–10 % of children are able to do so. Infection of hepatocytes is thought to be non-cytopathic in the short-term. As hepatocytes are long lived (half-life of ~6 months) and have self-renewing properties, the liver loses genetic complexity over time. By increasing cell death and subsequent hepatocyte regeneration, hepatitis increases this loss of complexity and increases hepatocyte clonality. Chronic infection will thus have a major impact on complexity loss by raising the daily rate of hepatocyte turnover. The loss of hepatocyte complexity and/or the increase of DNA damage in proliferating hepatocytes could trigger hepatocyte transformation and tumor development. Transient infection causes one liver turnover, and does not have a significant impact in the long-term. It is also interesting to see that in chronically infected individuals, clonal expansion of hepatocytes not expressing viral antigens is observed. This may result from the survival advantage of these infected cells generated by liver turnover in the context of chronic immune killing. This clonal expansion may represent an important factor involved in liver tumor development [34–36] and this phenomenon cannot be mimicked in vitro or in an in vivo model. This is why the study of HBV pathobiology in liver samples from infected patients at different phases of the infection remains crucial.

The Quantity of HBV Infected Hepatocytes

Many clinically relevant questions regarding the number of infected hepatocytes remain unresolved. One important question is whether all hepatocytes are equally infectable. Observations from immunostaining of liver samples from chronically infected patients often show a high number of cells stained for HBsAg (usually >50 %), but a much lower number of hepatocytes stained for HBcAg (usually <50 %) [37]. It is not clear whether this discrepancy is due to a differential expression of these viral

proteins, to differences in trafficking in the infected cell or to methodological issues in the detection of these proteins.

The number of hepatocytes stained for HBV antigens depends also on the phase of the disease, with a much higher number in the so-called immune tolerant phase and a progressive decline through the immunoactive phase and the inactive carrier stage. In UPA-SCID mice or FRG mice with a humanized hepatocyte population, the number of hepatocytes stained for both viral antigens is usually consistent and >90 % [38]. This mainly reflects the situation of an acute infection in a host harboring defective immune responses. In chronically infected patients, the immune response (1) either by cell killing and hepatocyte turnover, or (2) by repressing the expression of viral proteins in a non-cytolytic manner, may contribute to a decreased number of cells expressing viral antigens. In the first scenario, the number of cells harboring cccDNA would be decreased, while in the second scenario the number of infected cells and copy number of cccDNA could be more or less stable. Only few studies have addressed the issue of the kinetics of cccDNA evolution over time during the natural or treated history of infection. Currently, the quantitative PCR based assays that have been developed to quantify cccDNA report results on a bulk of liver cells present in the liver samples (ranging from 0.01 copy/cell to approximately 1–5 copy/cell depending on the level of viral load and the phase of the infection) [35]. In the case of high HBV replication levels, it is not yet clear if all hepatocytes harbor cccDNA or if a small fraction of these cells is free of this viral DNA form. From studies performed in animal models (woodchuck and humanized mice), it seems that cccDNA is lost through cell division due to dilution by unequal transmission to daughter cells and that cell turnover is required for an active elimination of hepatocytes harboring cccDNA [38, 39].

The adult liver is thought to be composed of approx. 5×10^{11} hepatocytes; it is interesting to see that in the early non-inflammatory phase of the infection, viral titers can be as high as 10^{11} virus copies/mL suggesting that the multiplicity of infection is much lower in vivo than in cell culture experiments, an observation that was confirmed after experimental inoculation of animals (chimpanzee, woodchucks or ducks) [40]. This again highlights the lack of virus propagation in cell culture, whose reason remains unclear but may be related to additional factors present in the liver microenvironment.

Other clinically meaningful questions concern the infectivity and replication capacity of the different HBV genotypes and the main circulating mutants (pre-core mutants, antiviral drug resistant mutants, and vaccine escape mutants), i.e., the possible role of HBV genetic variability. The evaluation of viral load in patients infected with these different strains reflects many other factors including duration of infection, the host immune responses, the number of remaining hepatocytes susceptible to infection in a damaged liver. Only few studies have looked at the true infectivity of these viral strains either in cultured hepatocytes (HepaRG cells or primary human hepatocytes) or in animal models (chimpanzees or liver-humanized mice) [41, 42].

The study of the viral fitness of escape mutants or viral genotypes has also been hampered by the lack of easy in vitro or in vivo system to study competition between viral species and remains challenging compared to other virus models where the dynamic of infection is much higher (i.e., HCV or HIV).

Assessment of cccDNA in View of New HBV Cure Strategies

Methodological Issues

Clearance of cccDNA will be the major treatment endpoint for a HBV cure, either using silencing or degradation strategies [43]. Thus, it will be critical to develop new sensitive and robust methodologies to assess in a quantitative manner (1) the number of infected cells harboring episomal viral DNA versus hepatocytes harboring only integrated viral sequences (witness of a past infection of the parental cells or of cccDNA cure in a non-cytolytic manner), (2) the number of cells expressing viral antigens, and (3) the quantitative determination of the epigenetic status of cccDNA in infected cells. These new technologies may require the establishment of in situ hybridization/PCR methods to detect and visualize cccDNA in hepatocytes, as well as methods to analyze chromatin-bound cccDNA in cultured or fresh liver samples. However, the access to human liver biopsies is getting more difficult due to ethical considerations and implementation of noninvasive methods of liver fibrosis assessment. Thus, the development of fine needle aspiration technologies linked to highly sensitive methods to analyze HBV in a few hepatocytes with single cell PCR methods is warranted.

Anti-cccDNA Strategies: Experimental Issues

Physical elimination of cccDNA harboring cells can occur by specific cytotoxic T cell responses, as demonstrated by resolution of acute infections in the chimpanzee [44]. Nevertheless, this immune response is impaired in chronically infected patients [45, 46], hence putting little immune pressure on infected hepatocytes.

When viral eradication is not achievable, lowering of liver cccDNA levels and/or inactivation of cccDNA directed transcription to prevent viral replication and remission of liver disease could be a realistic endpoint. Identification of cccDNA-free woodchuck hepatocytes containing traces of the infection in form of viral integrations indicated that cccDNA clearance may occur without killing the infected cells. This could be achieved mainly by two mechanisms: (1) “dilution effect”: since cccDNA is not replicated along with the host genome, cccDNA-free cells could arise through multiple rounds of cell division and unequal partitioning of cccDNA molecules into daughter cells [47, 48]. Notably, studies in the duck model showed that antiviral therapy with polymerase inhibitors induced a greater cccDNA reduction in animals displaying higher hepatocyte proliferation rates [49]. Inducing hepatocyte death and division would not be an easily controllable phenomenon in view of clinical application. Moreover, it has been demonstrated that very low levels of cccDNA can persist indefinitely in few liver cells even after the resolution of an acute infection [50]; (2) “targeted cccDNA degradation”: the recent discovery that IFN-α and lymphotoxin-β are capable of inducing a partial non-cytolytic degradation of the cccDNA pool through cytidine deamination in vitro invokes the possibility to cure HBV infected cells via pharmacological activation or triggering of host

antiviral pathway [51]. It remains debated whether this APOBEC3A/B mediated degradation of viral DNA occurred on a single stranded DNA or on true cccDNA [52]. Similarly, DNA cleavage enzymes, including homing endonucleases or meganucleases, zinc-finger nucleases (ZFNs), TAL effector nucleases (TALENs), and CRISPR-associated system 9 (Cas9) proteins [53], specifically targeting the cccDNA are currently being engineered. These enzymes are clearly working in vitro [53, 54], *but* need further demonstration of their efficacy in vivo.

Interfering with cccDNA-associated chromatin proteins might be another exciting approach to achieve HBsAg loss. Indeed, the acetylation and/or methylation status of the histones bound to cccDNA affect its transcriptional activity and, consequently, HBV replicative capacity [55]. In cell culture and in humanized mice, IFN-α administration induces cccDNA-bound histone hypoacetylation, as well as active recruitment of transcriptional co-repressors on the viral minichromosome [55]. This may represent a molecular mechanism whereby IFN-α mediates epigenetic repression of cccDNA transcriptional activity, which may assist in the discovery of novel therapeutics.

The Role of the Liver Microenvironment in HBV Infection and Immune Pathogenesis

Another important issue that affect in vitro and in vivo models of HBV infection is the role of the liver microenvironment during the early phase of infection to control the outcome of infection, i.e., resolution versus chronicity, as well as its role in the pathogenesis of chronic infection [56, 57]. This type of questions is difficult to address both in tissue culture and with liver samples from infected patients or animals. Experiments performed in tissue culture can identify the potential role of individual cell types of the microenvironment, but the reconstitution of the liver lobule architecture with all the immune cells residing in the liver remains a challenge. Studies performed with liver samples from infected patients or animals can benefit from improved cell sorting technologies to isolate liver cells and perform functional studies. These studies remain limited by the difficulty to study sequential events in the same patient, while in animal models the impact of human/animal chimera in the liver or the host specificity is difficult to handle.

When HBV enters the liver, it is confronted with many different cell types. Indeed, the liver is a complex and structured organ that contains hepatocytes (parenchymal cells), non-parenchymal cells such as liver sinusoidal endothelial cells (LSEC), stellate cells and numerous resident immune cells, including Kupffer cells (KC), dendritic cells (DCs), NK/NKT, CD4+ T cells, CD8+ T cells, regulatory T cells (Treg), B cells [56, 58] These cells are organized according to a very particular and unique architecture. The importance of the liver microenvironment is often underestimated, and one should be cautious with in vitro experiments using hepatocytes that may behave differently when studied outside this microenvironment. Similar consideration can be done for anti-HBV immunity study (Fig. 8.2).

For example, we have already discussed that most of the detailed knowledge of intrahepatic HBV-specific T cell function derived from studies performed in mice with a normal liver environment, However, IL-10 [59], TGF-β [60], and arginase [61] are elevated in chronic HBV infections and such inhibitory cytokines impair T and NK functions. In addition, the composition of immune cells in pathological and normal livers is also altered in patients [62]. Thus, the function of immune cells in normal or pathological liver environment can differ.

The ability of cytokines to inhibit HBV replication in infected hepatocytes can also be influenced by the pathological liver microenvironment. IFN-alpha and IFN-gamma inhibit HBV replication in cell culture and in vivo models, but again most of the experiments performed to measure the ability of cytokines to inhibit HBV replication have been performed in experimental systems devoid of chronic inflammatory events [55, 63]. However, intrahepatic levels of SOCS3, a negative regulator of cytokine signaling and a predictor of poor IFN-alpha therapy response in HCV patients [64] are increased in patients and woodchucks with chronic hepadnavirus infections [65, 66].

Age and HBV Infection

The development of chronic hepatitis B (CHB) is inextricably linked to the patient's age at the time of infection. HBV is thought to exploit the immaturity of the neonatal immune system to establish a persistent infection, reflected in the 90 % of neonates who develop chronicity following perinatal transmission. On the contrary, acute hepatitis B infection in adults is almost invariably associated with control of HBV infection through the induction of an efficient HBV-specific T and B cell response [1]. HBV infection in infants or young children rarely causes acute hepatitis and results in the asymptomatic disease phase characterized by high levels of HBV replication and a low incidence of liver inflammation defined as immune tolerant [67]. To explain this dichotomy, data from experimental animal models (i.e., HBV transgenic animals) have described the presence of immunological defects which impair HBV-specific T- and B-cell priming in neonatal animals [68–70] that could cause HBV persistence. However, it is important again to consider that since HBV does not infect murine hepatocytes, these animal studies can only partially imitate the events occurring during natural vertical HBV infection.

Indeed, a better analysis of data generated in clinical samples reveals that a proportion of neonates exposed to HBV at birth, mount a HBV-specific T cell response. Studies performed in HBsAg-negative children born to HBV-positive mothers [71, 72] have shown the presence of core and polymerase-specific T cells. Neonates of HBV positive mothers have also minimal alterations or normal dendritic cell functions [73, 74] and the efficacy of HBV vaccination within the first year of life in HBV positive children [75–77] raises considerable doubts that the HBV immune tolerance and T–B cell interaction defects, detected in murine models [46, 78], are the inevitable consequence of HBV-exposure in neonates and children.

A direct demonstration that HBV immune response is not completely absent in young patients with chronic hepatitis B labeled as "immunotolerant" has been also recently detected in two separate clinical studies. In one, analysis of the immune response in CHB-infected adolescents with ostensible immunotolerant profile (normal ALT and high HBV replication) of hepatitis B disease, demonstrated that these patients did not display any tolerogenic T cell features and they could mount a perfectly normal Th1 T cell response and harbor HBV-specific T cells. These HBV-specific T cells, though weak and functionally impaired as one would expect in CHB patients, were in fact quantitatively and functionally superior to those found in CHB-infected adults in the "immune clearance" phase of disease [79]. In a second study, analysis of HBV quasispecies in children with an immunotolerant clinical profile showed high HBV diversity [80], a virological profile compatible with the presence of an active immune pressure against HBV. Taken together, these clinical data challenge the concept that HBV infection at birth is inevitably associated with immunological deficiencies. This prevailing belief was supported by the idea that ALT levels can act as a surrogate of the anti-HBV immune activity and by technically impeccable data in animal models that however cannot fully recapitulate the immunological and virological events secondary to HBV exposure in utero [81]. Indeed, the recent direct observation that HBV exposed neonates possess a more mature innate immune system than healthy ones [82] confirm the complete dissociation between animal and human studies in the early stage of HBV infection. Therefore, we think that a better understanding of the influence of vertical HBV infection in the development of chronic HBV infection should mainly derive from a direct characterization of immune and virological profile of children vertically infected by HBV.

Immunomodulatory Roles of HBV Antigens

A hallmark of HBV infection is the persistent production of the soluble form of HBV surface antigen (HBsAg) and e antigen derived from the core protein (HBeAg) in excessive amounts over whole virions. Persistent exposure to circulating HBsAg or HBeAg has been suggested to impair the frequency and function of myeloid [83] and plasmacytoid [84–86] dendritic cells, modulate TLR-2 surface expression [87] or interfere with TLR-mediated cytokine production [88]. It is also believed that soluble viral antigens can inhibit antigen-presenting function, altering their ability to produce cytokines, and inhibit the induction of HBV-specific T cells [89]. These data have generated some controversy, since it is somehow difficult to understand why these diffuse immune defects should only impair anti-HBV immunity. In fact, we would expect that CHB patients with such reported alteration of immune systems produced by HBV antigens would be highly susceptible to bacterial and other opportunistic infections. However, to our knowledge, epidemiological studies conducted in HBV chronically infected subjects have not reported an increased incidence of bacterial infections or vaccine unresponsiveness in HBsAg + subjects with

normal liver function. In contrast, other clinical studies have shown that in patients with malaria, HBsAg positivity is associated with lower parasitemia [90] or to episodes of cerebral malaria, that is a pathological manifestation indicative of a heightened Th1 response against the parasite [91].

A caveat of many studies that have suggested an immunomodulatory role of HBV antigens is that they have been often performed in vitro with proteins expressed in *Escherichia coli* or yeast, or purified from the sera of CHB patients. Despite a high level of purity of these preparations, contaminants from bacteria or enzymes cannot be completely ruled out and the phenomena of LPS-induced tolerance of antigen presenting cells may have influenced the outcome of some experiments [92]. The ability of HBeAg to induce an HBV-specific T helper cell tolerance has been formally demonstrated in HBV-transgenic mice [68], but a clear distinction in the quantity of HBV-specific T cells have not been detected in HBeAg+or anti-HBe+CHB patients [78]. Overall, direct characterization of HBV-specific helper T cells in CHB patients showed that such response is defective in all patients, irrespective of their HBeAg status. In this regard, the main limitations of the analysis of the immunomodulatory effect of HBV proteins in vivo models (HBV-transgenic mouse), is that we excluded, aprioristically that HBV infection would not affect activation or maturation of the immune system, a concept that has been recently challenged after analysis of the immune maturation of cord blood of HBV+ neonates [93]. At the same time, however, we want to call attention to the fact that observations directly derived from clinical studies can also be influenced by variables that are different from HBV.

The presence of high doses of circulating antigens in CHB patients with chronic liver inflammation is often linked with immunosuppressive cytokines (IL-10) [59] or liver enzymes (i.e., arginase) known to alter the function of different components of cellular immunity [61, 93]. In this respect, a study performed in CHB patients with mild or absent liver inflammation but high HBsAg levels, the frequency and T cell stimulatory activity of circulating professional antigen presenting cells (monocytes, dendritic cells and B cells) were not altered [94]. In contrast, another study reported alteration of DC function ex vivo in CHB infection corresponding with HBsAg and HBeAg levels but also with high levels of CXCL-10, a chemokine associated with liver inflammatory events causing increased arginase/IL-10 levels [89]. It is therefore plausible that these different results are due to the difference in suppressive cytokines or enzymes in the circulation of patients with liver inflammatory diseases and not to differences in HBsAg levels.

Conclusions

The field of HBV research is evolving towards a better understanding of the virus biology and its immunopathogenesis. It will be critical to work with the most relevant study models to reach the ambitious goal to define new successful therapies. However, we think it will be also mandatory to have access to patient's samples to

rule out non relevant observations due to differences in the host innate and adaptive immune responses and in viral isolates discussed in this chapter. This will allow us to identify the aspects of the pathobiology of chronic HBV infection that are unique to the interaction between HBV and its human host.

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Chapter 9
Global Epidemiology of Hepatitis B Virus Infection

Daniel Lavanchy and Mark Kane

Introduction

Hepatitis B is one of the major diseases of mankind, estimated to cause about 800,000 deaths per year mostly from liver cancer and cirrhosis. Primarily an asymptomatic infection that occurs in childhood in areas of the world highly endemic for the disease, hepatitis B related mortality and morbidity occurs decades after infection, allowing chronic carriers to spread the infection perinatally, from child to child, and to susceptible subjects via sexual activity and unsafe medical procedures such as unsafe injections. In areas of lower endemicity the virus spreads mainly among young adults as a result of lifestyle or occupational exposures. Most significantly, hepatitis B is now a vaccine preventable disease and efforts to control it through routine immunization have been highly successful.

Hepatitis (inflammation of the liver) has been recognized as an illness causing jaundice since antiquity. However, it was not until World War II that it was realized, on the basis of epidemiologic data, that there were two distinct types of hepatitis: *epidemic or infectious hepatitis* and *serum hepatitis*. *Serum hepatitis*, with a long incubation period (50–180 days), was sometimes linked to blood transfusions, vaccination or other unsafe parenteral procedures. In 1965, Blumberg, Alter, and Visnich [1] discovered the Australia antigen found in the blood of some Australian aboriginal people. This antigen was later shown to be the hepatitis B surface antigen (HBsAg), the envelope protein of the hepatitis B virus (HBV), subsequently shown to be responsible for serum hepatitis [2]. Prof. Baruch Blumberg received the Nobel

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Prize in Physiology or Medicine in 1976 for these achievements [3]. *Epidemic hepatitis* with a short incubation period (15–45 days) contracted by oral exposure, was shown to be caused by the hepatitis A virus (HAV) in 1973 by Feinstone, Kapikian, and Purcell [4]. However, hepatitis A and B did not cover all cases of acute and chronic hepatitis, leading to an intense search for other agents. The discovery of hepatitis delta (HDV) in 1979 by Rizzetto, Canese, Arico, Crivelli, Trepo, Bonino, and Verme [5], of the hepatitis C (HCV) in 1989 by Choo, Kuo, Weiner, Overby, Bradley, and Houghton and of the hepatitis E virus (HEV) in 1990 by Reyes, Purdy, Luk, Young, Fry, and Bradley [6] closed the missing gap almost completely and allowed for a precise epidemiological analysis of the hepatitis A–E viruses globally.

The history of the epidemiology of hepatitis B represents a landmark in the general understanding of viral infections, their distributions and their outcomes. It all began, as mentioned, in 1965 with the discovery of immune-precipitating bands in Ouchterlony gels [1]. These, surprisingly, were linked to the Australia antigen and later to the hepatitis B virus, a serendipitous finding that opened the way to the development of diagnostic tests, the prevention of post-transfusion hepatitis, the development of vaccines, the prevention of end-stage liver disease and hepatocellular carcinoma through medication and surgical interventions [7], and ultimately opening the path to the discovery of the other viruses also causing diseases of the liver (hepatitis A–E).

In 1992, and several times subsequently, the World Health Assembly (WHA), the World Health Organization's (WHO) governing body called for all countries to add hepatitis B into their National Immunization Programs. Finally, in 2010 and 2014 resolutions of the World Health Assembly, have acknowledged at the political level that viral hepatitis is a global health challenge comparable to HIV/AIDS, tuberculosis and malaria. This underscores the need for global and national policy development to realize primary and secondary prevention through access to immunization, screening, prevention and treatment. Time wise this has been a very long road, but is an inspiring example that patience and perseverance pays off. We must, however, always remain cognizant that in 2015 the work is far from completed.

Global Prevalence of HBV Infection

HBV infection is an extremely important global public health problem because the infection is extremely common in much of the world and often leads to chronic infection, cirrhosis and liver cancer. The global prevalence of chronic carriage varies between 0.1 and more than 20 % [8]. Approximately 15–40 % of chronically infected patients will develop liver cirrhosis, liver failure, or hepatocellular carcinoma (HCC) and 15–25 % will ultimately die [8–12]. In 2010, the total number of annual HBV related deaths globally was estimated to be about 800,000, ranking HBV as the 15th cause of death in all cause global mortality [12, 13].

HBV is spread predominantly by percutaneous or mucosal exposure to infected blood and other body fluids from an infected person. Because HBV is highly transmissible, about one third of the world's population has been infected: most recover but primarily depending on the age at infection many become chronic carriers. The most common routes of exposure are maternofetal (vertical) and transmission between children (horizontal), as well as drug use, institutionalization, sexual transmission, occupational exposure, blood products and organ transfusions, unsafe injection practices, and cosmetic and cultural practices. As of today (2014), approximately 2 billion people have been infected worldwide, about 30 % of the total of 7.2 billion people living on earth [14].

After an acute infection episode, the probability of becoming chronically infected is inversely correlated to age. Eighty to 90 % of newborns and children less than 1 year old and 25–30 % of children infected at the age of 1–6 years will develop a chronic infection [15], where HBV replicates in the liver usually for life. Immune-competent adults have about a 95 % chance of eliminating the virus and remaining protected for life in case of reexposure [8, 16, 17]. Most infant and childhood infections are asymptomatic while adults have about a 30 % chance of developing symptomatic acute hepatitis B [17].

The prevalence of chronic HBV infection varies strikingly in different geographic areas and in different populations, with national prevalence ranging from 0.1 to 35 % [18–22] (see Fig. 9.1). The prevalence of HBV defined as hepatitis B surface antigen (HBsAg) positive is classified into four (formerly three) levels of low (<2 %), lower intermediate (2–4.9 %), higher intermediate (5–7.9 %), and high (≥8 %) endemicity [18]. Generally, the prevalence is significantly higher among males [23].

About 60 % of the global population lives in areas of high chronic HBV prevalence [24–27]. The areas where HBV is highly endemic include: Asia, sub-Saharan Africa, the Pacific, parts of the Amazon Basin, parts of the Middle East, the central Asian Republics, the Indian subcontinent, and some countries of Central and Eastern Europe [18, 19, 28]. In these parts of the world as many as 70–90 % of the population has been infected at one time or another and often infections occur during childhood either from an infected mother to her baby (perinatal transmission) or from one child to another (horizontal transmission).

Intermediate endemic zones of HBV infection exist in the Middle East, Eastern and Southern Europe, South America and Japan. Among these populations the infection rate is approximately 10–60 % and the chronic carrier rate is 2–7 %. The epidemiologic pattern is a mixture of childhood and adult infection. The rate of chronic infections is higher in infants due to early childhood exposure to viral infection [29].

A low prevalence is found in western and northern European countries, North America, Central America, and the Caribbean, where chronic HBV infection is relatively rare (below 2 %) and acquired primarily in adulthood [30].

Notably, regions with a high prevalence of chronic HBV also have high rates of HCC and HCC is one of the three major causes of cancer death in Asia, the Pacific

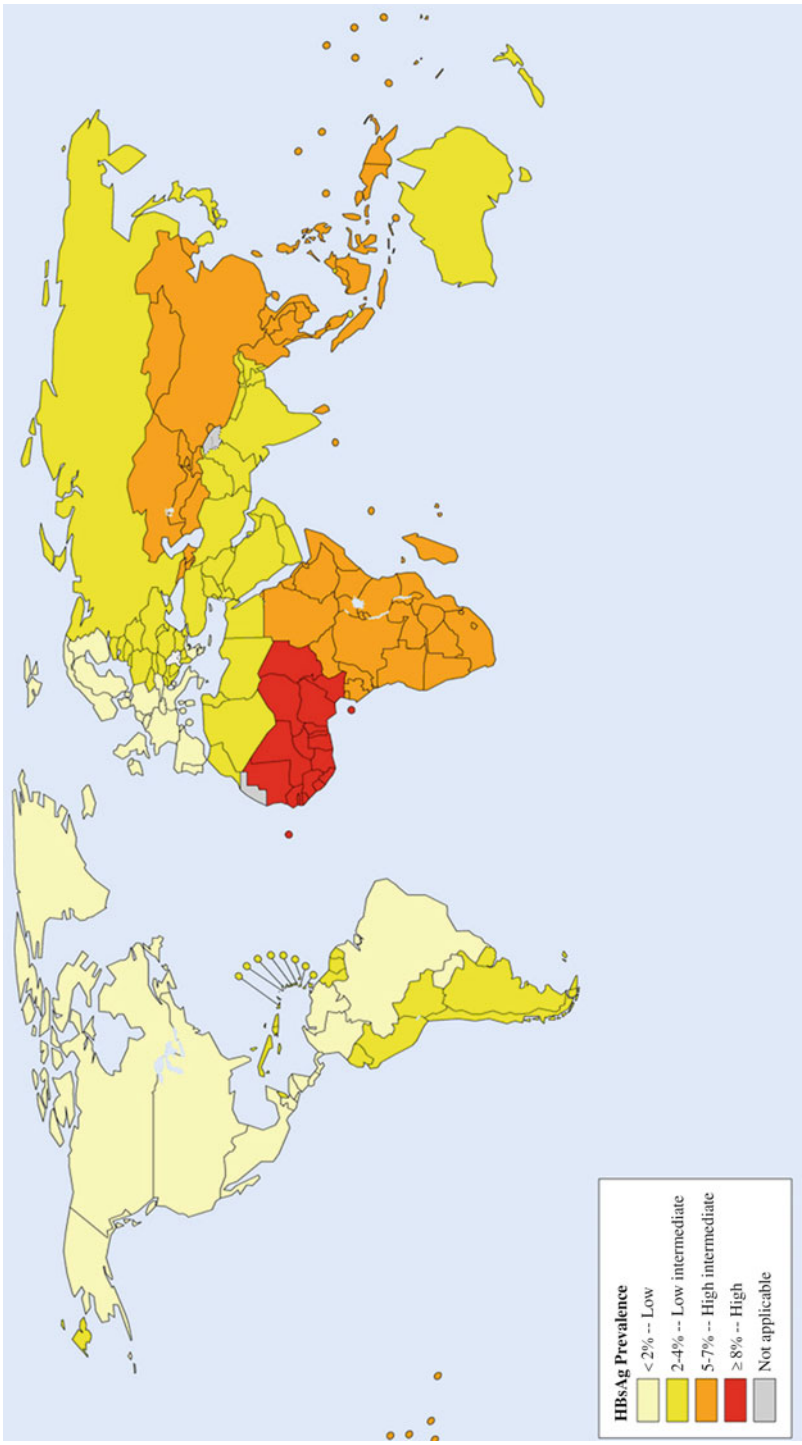

Fig. 9.1 GlobalHepatitis B Prevalence 2005 (adults 19–45 years old). Source: J.J. Ott et al./Vaccine 30 (2012) 2212–2219

Rim and sub-Saharan Africa [11, 21, 31, 32]. Because of increasing and aging populations, its incidence has increased worldwide [33].

Occasionally, the presence and the importance of HBV mutants are discussed. As of today, there is no evidence that HBV mutants, especially vaccine escape mutants, have had a public health impact globally [34].

Acute Hepatitis B

The World Health Organization (WHO) estimated that five million cases of acute hepatitis B occur each year [35]. Adult subjects developing acute hepatitis B usually have clinical symptoms ranging from an asymptomatic or mild anicteric acute illness, to severe disease. Recovery is usually complete. Fulminant hepatitis occurs in 1–2 % of acute infections, with a fatal outcome in the majority of cases, causing about 40,000 deaths annually [36, 37].

Acute hepatitis B is a reportable disease in most countries but laboratory testing is often unavailable in many developing countries and differentiation from chronic carriage can be problematic for many reporting systems. Acute hepatitis B surveillance can be useful for detecting outbreaks and trends such as increases in drug use or occupational related infections. Acute hepatitis B incidence does decline with successful hepatitis B immunization of children, but this may take several years.

Chronic Hepatitis B

Of the 2 billion individuals infected worldwide, 240 million are chronic carriers of HBV [18]. This represents a decrease of 31 % in HBsAg prevalence as compared to the former published figures of 350–400 million [2, 15, 38, 39]. The decrease is mainly observed in younger age groups and it is likely due to the availability of better population based data, population wide vaccination against hepatitis B in newborns, young children, and adolescents [40], improved screening of blood products [41, 42], and improved safe injection procedures [35, 43, 44] in a timeframe where the total world population increased from about 5.5 billion to more than 7 billion [14]. However, globally an increase in both genders was observed between the 1990s and 2005 causing a change from low to a low-intermediate endemicity level in young men [18].

A decrease in the prevalence of hepatitis B has been found in North America and Europe, linked to increased hepatitis B vaccine coverage, improved screening of blood products, increased availability of safe injection materials [18, 43, 45] and behavior changes secondary to HIV/AIDS in drug users and sexually active persons [46]. The generally low HBsAg endemicity levels in these countries are paralleled by a steady decline in reported cases of acute hepatitis B [45, 47]. While the incidence of acute infection may be falling, relatively large cohorts of chronically infected

Table 9.1 Vaccination coverage (%) in 2009

Region	2009
Africa	70 %
Americas	86 %
Eastern Mediterranean	84 %
Europe	77 %
South-East Asia	41 %
Western Pacific	90 %
Global	70 %
Total number of countries	154

Source: WHO vaccine-preventable diseases monitoring system, 2010 global summary [110]

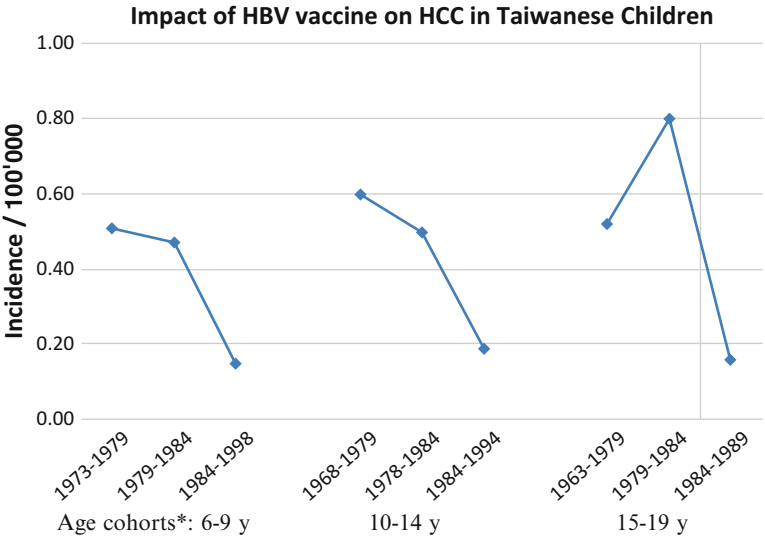

Fig. 9.2 Impact of HBV immunization on the incidence of HCC in children in Taiwan. Source: Extracted from: Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study [111]. * age at diagnosis (year)

adults continue to progress to cirrhosis and liver cancer. This highlights a need to carefully consider the benefits and risks of screening and surveillance programs to identify chronically infected individuals to potentially reduce further transmission as well as to provide opportunities for secondary and tertiary prevention [48, 49].

It is important to note that recent HBV prevalence data needs to take into account the great impact of routine infant and childhood hepatitis B immunization programs, which more than 90 % of countries now have. A successful immunization program will cause a dramatic reduction of HBsAg carriage (often to 1–2 %) in immunized cohorts of children (see Table 9.1) while the prevalence in older unimmunized cohorts will change little, as has been shown in studies conducted in Alaska, the Gambia, Hawaii, Italy, Malaysia, and Taiwan [50] (see Fig. 9.2).

Africa

Sub-Saharan regions of Africa have very high levels of HBV prevalence particularly in western sub-Saharan African countries with up to 12 % of children and adolescents below 19 years of age being infected [18]. Since the first evaluations conducted in the late 1980s and 1990s, significant decreases in the HBsAg prevalence have been documented in 2005 in African countries such as the Gambia and Senegal [18]. In Eastern and Southern sub-Saharan Africa, the situation is more confusing showing an increase in chronic HBV infection among younger age groups (0–14 years), with age-specific prevalence rates of 7–9 % among young females and a prevalence peaking at about 7 % in 0–4 years old children, a decline in older age groups and almost no change in other age groups [18]. This situation may be related to the inadequate implementation of immunization programs in some countries [51]. A decrease in prevalence was evident in Central sub-Saharan Africa which transitioned from high endemicity among younger individuals (age-groups up to 34 years) in 1990 into intermediate endemicity across all ages in 2005 [18].

North Africa showed a lower intermediate HBsAg endemicity across all age groups in 2005 with a decrease in prevalence from 1990 to 2005, particularly among males up to 34 years.

Americas

The prevalence in the high-income countries of North America (Canada and the USA) was low and declined in both sexes and across all ages between the 1990s and 2005. Males had higher HBsAg positivity than females in both periods, peaking in the male 0–4 years age group at 2.7 % and 2.1 % in the 1990s and 2005, respectively. The oldest ages (65+ years) showed the lowest prevalence of approximately 1 % in 2005. However, one should note that special population groups might have a high prevalence [52–57].

Both Tropical Latin America and Central Latin America demonstrated a strong decrease in HBsAg prevalence between the 1990s and 2005. Tropical Latin America changed from an intermediate into a low endemicity region. In 1990, 0–9 year aged boys had a higher intermediate HBsAg prevalence endemicity of over 5 %, in 2005 it was only 1.6 %. Similarly, in Central Latin America prevalence has halved in this period and most adult age groups shifted to low endemicity levels in 2005. A slight decline in prevalence from the 1990s to 2005 among Andean Latin Americans was paralleled by an increase in HBsAg prevalence in Southern Latin America. In Caribbean countries children and adolescents aged 0–19 years HBsAg prevalence was rather constant over time ranging from 4.3 to 5.4 % [18, 58, 59].

Asia

In Asia, East Asia has the highest prevalence of HBV infection, with a minor decrease in children and an increase in all age groups above 25 years reported in the 1990s to 2005 comparison, and the highest prevalence of >8 % found in males aged over 35 years. An intermediate HBsAg endemicity was reported in Central Asian children and younger adults with a HBsAg prevalence of ~5 %, where a small decrease was observed between the 1990s and 2005. In South Asia, approximately 3 % of the population younger than 45 years of age was HBsAg positive, while in older individuals a decrease was demonstrated and the prevalence was low in 2005 [18]. One should note, that Asian countries put tremendous efforts to increase the routine coverage of infant hepatitis B immunizations leading to the reported reductions in HBsAg prevalence. The impact of the decrease in HBsAg prevalence was also linked to a substantial reduction in the HBV-related disease burden in China [60], Japan [61], Malaysia [62] and Taiwan [63, 64]. The seroprevalence of HBV infection reported in India is >5 %, but population based data are scarce and therefore the information may be misleading in this country of >1 billion people, with figures varying from 2 to 11 % [65], thus having potentially a major impact on the HBV global figures [66, 67]. In South East Asia, a reduction was also reported in the age group 0–14 years with prevalence of ~1.4 % in 2005. Adults continued to have higher-intermediate HBsAg prevalence of 5 to >6 % [18].

The global burden of disease from hepatitis B is largely driven by the situation in China because of its massive population and high endemicity. Remarkable progress has been made in China with hepatitis B immunization of children, lower horizontal child-to-child transmission because of their single child policy, and safer injections. These factors have reduced the HBsAg prevalence in immunized cohorts of children from approximately 10 to 1 %. It is estimated that China has about 9.8 % chronic carriers and 300,000 deaths from cirrhosis and liver cancer [68–70].

In Japan, the Republic of Korea, and Singapore, endemicity remained at a lower intermediate level around 4 % in 2005 with subjects aged 25–54 being the most affected [18].

Australia and Oceania

In Australia the prevalence of chronic HBV is estimated around 1 %, including a majority of subjects born abroad, or belonging to Aboriginal and Torres Strait Islander communities. However, the disparity between indigenous and nonindigenous people has decreased since the implementation of the HBV vaccination program in 2000 [71–73], but the need for HBV screening remains in order to identify people who would benefit from vaccination or treatment [74].

For the islands of the Pacific and Indian Oceans (Oceania), a geographically, culturally, and socioeconomically highly heterogeneous region, data are again limited and often of questionable quality. A moderate to high endemicity for HBsAg of 5–7 % is assumed [75].

Europe

A wide variation in the prevalence of HBsAg between countries in Europe is reported [76], a finding that has not changed over time. In Western Europe, seroprevalence of HBsAg was consistently low and consistently lower in females [76, 77]. Central and Eastern European children had a higher intermediate HBsAg endemicity with a decrease observed in 2005, especially among elder Central European females. Prevalence in infant and young girls declined from 6 % in the 1990s to 3 % in 2005. In contrast, the youngest age groups of Eastern European countries showed a limited reduction in HBsAg prevalence. In Central and Eastern Europe the age group 0–9 years remains the most affected [77].

Middle East

The prevalence of hepatitis B infection in the Middle East varies among its geographic areas significantly from 0.3 to 7 % [22, 78]. The prevalence of HBsAg has declined significantly in most Middle East countries, especially Saudi Arabia, Egypt and Iran during the last two decades [79–81]. There can be considerable differences in the prevalence of HBV infection within individual countries. Such a variability has been observed in Turkey (<2–8 %), Pakistan, and the Yemen, with higher prevalences in rural and/or poorer communities [22].

Vulnerable Populations

Mobile Populations

Mobile populations present unique health care concerns for society and for HBV in particular. Although travel and immigration is an old phenomenon, today an increasing number of people are on the move, and the demography of these mobile populations is changing and becoming more heterogeneous, dynamic and complex. Forced and voluntary mass migration involves hundreds of millions of people each year and it is estimated that two to four million people migrate permanently each year. Generally, populations flow from developing to developed countries, from east to west, from south to north, and from high to low HBV endemicity regions. Metropolitan areas are the main recipients of migrants in all countries concerned [82].

While HBsAg prevalence is low inWestern European countries, the epidemiological situation is changing because of high rates of immigration from high and intermediate endemicity areas. The low fertility in many Western European populations has led to a situation where a large proportion of births in large cities are to immigrant mothers from endemic areas. Several Nordic countries and the UK do not do routine childhood hepatitis B immunization and most of their children and young adults are susceptible to hepatitis B infection.

Persons from high-risk populations, especially immigrants from nations where hepatitis B is highly endemic, should be tested for HBV and should be vaccinated if they are found to be negative. Equitable access to and availability of quality, effective, affordable, and safe diagnostics and treatment regimens for HBV are lacking in many countries from which migrants move out. Country-specific HBV data to target these most vulnerable population groups will be crucial for implementing national HBV prevention and control programs. The elimination of stigmatization and discrimination against people living with HBV are warranted and policies for equitable access to prevention, diagnosis and treatment for HBV must be applied to indigenous people, migrants and vulnerable groups.

Europe and North Americaface great geographical variation with higher prevalence and higher hepatitis-related mortality among migrants [82, 83]. Many countries have only in the last decades received migrants on a mass scale and are therefore not prepared to deal with these important populations moves. Europe is a primary destination for many spontaneous or undocumented illegal workers, refugees, asylum seekers, and people who are victims of trafficking. These people face poverty, limited access to health care, and low sympathy and acceptance from the host population.

In addition to migration, international adoption has become increasingly common due to the decline in children offered for adoption in the USA and Europe. This relatively new phenomenon became more pronounced after World War II. As an example, in the USA international adoptions increased from 4864 to 16,000 between 1979 and 1998 [84]. Many adopted children come from high endemic areas such as Eastern Asia (e.g., China, Vietnam), sub-Saharan Africa, South America and from countries of the former Soviet Union, where HBsAg prevalences range from 2 to 15 %. These adopted children have a much higher HBsAg carrier rate than the general population in the countries of adoption [84–87]. Families considering intercountry adoption should be made aware that the child may need to be immunized before it arrives in its new family. The child should also be tested for HBV upon arrival in the country of adoption.

Drug Users

Although great progress in hepatitis B control is being made in many areas, the infection in drug users is still a major public health problem in both the industrial and developing worlds and transitional economies such as the former Soviet Union where the problem of drug use and resultant viral infections is increasing in many areas. HBV is highly transmitted through needle and paraphernalia sharing. 1.2 million of the 16 million people injecting drugs are chronically infected with HBV [88]. The WHO recommends that injecting drug users (IDUs) need to be specifically targeted for prevention and treatment of viral hepatitis [89]. Reduction of risky behaviors and risk reduction strategies such as needle exchanges secondary to HIV/AIDS control efforts have reduced the prevalence of HBV infections

in some drug using populations. Efforts to immunize drug users with HBV vaccine have been problematic since drug users are often already infected by the time they are reached by immunization programs and often have little motivation to participate in HBV immunization programs.

High-Risk Sexual Behavior and Sex Workers

BecauseHBV is highly transmissible sexually, transmission between sexual partners is both an important cause of HBV infection, and is one of the most frequent sexually transmitted infections [90–93]. However, most persons with chronic HBV infection are not aware that they are infected and these silent carriers are an important and difficult to identify source of infection: this is particularly true for sex workers [94–98]. Men who have sex with men (MSM) are one of the groups at highest risk for HBV infection, especially if they engage in high-risk sexual practices [90]. It is mandatory that these populations are targeted for prevention activities and that susceptible individuals receiveHB vaccination.

People Coinfected with HIV

Globally, there are more than three million people coinfected with HBV and HIV. Coinfection is associated with more rapid progression of liver disease and consequential high morbidity and mortality [99, 100]. People at risk of HIV infection, should be counseled about HVB (and also HCV) coinfection probabilities and dangers and should be considered for receiving effective antiviral therapies for both HIV and HBV.

Additional Vulnerable Groups

Organ transplant recipients, hemophiliacs and patients on hemodialysis disserve special care and attention. Although these groups, due to their limited population size, play a lesser role from a global perspective, the high incidence and prevalence of HBV in some of these groups make it mandatory to offer them special consideration.

Health Care Workers

Health-care workers (HCWs)are at risk of many infections at their workplace and HBV infection is the most important one. It is estimated that 8–21 million HBV infections are transmitted by unsafe injections each year worldwide [35, 101] and

about more than 65,000 HCWs get infected annually at their work place [44, 102]. Transmission occurs most commonly through needle stick or sharps injuries subsequent to percutaneous and mucocutaneous exposures, and sometimes, through exposure to other body fluids. The consensus is that most of these infections are preventable through vaccination of HCWs against HBV, implementation of general precautionary measures, improving equipment and use of safety engineered devices [103]. HBV transmission in HCWs should be eliminated.

Occult Hepatitis B Virus Infection

Occult HBV infectionis defined as the presence of HBV DNA in the liver (with detectable or undetectable serum HBV DNA) in HBsAg-negative individuals tested by currently diagnostic available assays. In rare cases, HBV DNA may only be detectable in peripheral blood mononuclear cells (PBMC). Occult HBV infection occurs often when HBV viruses are strongly suppressed in their replication activity, rarely in HBV variant viruses (S-escape mutants) not detected by HBsAg commercial diagnostic kits. HBV infection is associated with the persistence of the highly stable cccDNA (covalently closed circular HBV DNA) in the liver cell nucleus, which correlates with intracellular and serum HBV DNA, these levels being lowest in patients with occult hepatitis B [104, 105]. Occult HBV infection poses problems due to the transmission HBV infection through blood transfusions and organ transplantations, particularly in liver transplantations. Of particular concern is acute HBV reactivation in an immunosuppressive status. Occult HBV infection may contribute to the development of cirrhosis and may have a role in the genesis of HCC [106, 107]. The prevalence of occult HBV infection varies significantly between geographical regions as well as among various populations with or without liver disease [108, 109].

Summary

HBV infection remains in 2015 a most serious global public health problem with 800,000 annual deaths globally, ranking HBV as the 15th cause of death in all cause global mortality. The HBV prevalence varies widely (0.1–more than 20 %) between geographical areas and populations. Tremendous efforts have been and still are undertaken by many scientists, medical professionals and public health persons to maintain and increase the fight against HBV. The World Health Organization has acknowledged HBV (and HCV) as a global health challenge. Successful vaccination strategies have been implemented and have led to significant decreases in the HBV prevalence, the transmission of HBV and the occurrence of HBV related cirrhosis and liver cancer in various populations. However, the work is not finished and a lot remains to be done to prevent the infection in susceptible persons and to cure the ones already

chronically infected. An improved awareness about the risk factors associated with the transmission of HBV infection and its consequences is still warranted and adequate strategies for the prevention and control are still needed in many settings.

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Chapter 10
Clinical Virology: Diagnosis and Virologic Monitoring

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Introduction

The clinical course of an HBV infection is highly variable. The course may either be asymptomatic or may manifest clinically as an inflammatory liver disease with accompanying liver cell damage. Furthermore, the course of HBV infection is dynamic and transition between various phases of the disease is possible at any time. The assessment of several serological parameters allows the correct diagnosis of HBV infection as well as virological monitoring and differentiation of distinct phases of HBV infection. In addition, vaccine-induced immunity can be confirmed. The confirmation of an HBV infection and the correct classification is an indispensible prerequisite for appropriate management. The most common virological laboratory tests for the assessment of HBV infection are (quantitative) HBsAg, anti-HBc, HBeAg, anti-HBe and HBV DNA.

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Diagnosis

Indications for Performing Hepatitis B Virus Diagnostic Testing

The first step of the diagnosis consists of the identification of individuals with an indication for performing HBV diagnostic testing. Although local recommendations may vary, especially with consideration to varying prevalence of HBV infection, national and international guidelines [1, 2] endorse testing in the following patient groups:

1. Persons with elevated liver enzymes and/or clinical signs of hepatitis.
2. Patients with fibrosis of the liver or liver cirrhosis
3. Patients with hepatocellular carcinoma.
4. Persons born in regions with an intermediate or high prevalence rates (or who have emigrated from such regions).
5. Families, household members, or sexual partners of persons infected with HBV.
6. Medical personnel.
7. Homosexual/bisexual men and/or persons with frequently changing sexual contacts.
8. Active i.v. drug users or persons with a history of i.v. drug use
9. Dialysis patients.
10. HIV and/or HCV infected persons.
11. Recipients of organ transplants before and after transplantation.
12. Blood and organ donors.
13. Patients before or during immunosuppressive treatment or chemotherapy.
14. Pregnant women (HBsAg only).
15. Newborns of HBsAg and/or isolated anti-HBc positive mothers.

These recommendations reflect the currently known main risk factors for infection. HBV is transmitted through percutaneous or parenteral contact with infected blood or body fluids. Thus, most infections are acquired either perinatally, percutaneously by blood-to-blood contact, or by sexual contact. Very small amounts of the virus are sufficient to infect an individual, therefore it may also be transmitted by close contact, e.g., within friends and/or family. Consequently, special considerations should be given the prevalence of HBV infection. Although distributed worldwide, some areas are hyperendemic with more than 50 % of the population being anti-HBc positive and more than 5 % are HBsAg positive (Asia, the South Pacific, in Africa south of the Sahara Desert, and in the Middle East). Areas with intermediate prevalence are the Mediterranean Region and Eastern Europe. Under these circumstances it might be worthwhile to perform testing for HBV infection on a routine basis in persons who themselves, or their mothers, are born in these regions [3]. This is insofar very important as HBV infection is asymptomatic. Regions with a lower prevalence of HBV infection are North America, Western and Northern Europe, Australia, and parts of South America, in which less than 2 %, in most areas less than 1 % of the population are HBsAg positive.

Although it is very obvious that individuals with elevated liver enzymes should be tested for viral hepatitis, including HBV, physicians often implicate alcohol abuse as reason for liver damage and therefore neglect other causes.

A prevalent way of transmission is perinatal transmission. Therefore it is important to test pregnant woman for HBV infection and in case of positive findings, active and passive immunization of the newborn can prevent infection. Importantly, HBV DNA should also be quantified, because HBV DNA >10^6–10^7 IU/mL is associated with an increasing risk of vertical transmission despite active immunization and treatment of the pregnant woman prior to delivery can reduce this risk [2, 4].

In immunosuppressed patients, reactivation of HBV replication and also severe hepatitis can occur [5]. Thus, patients who receive immunosuppressive therapy, i.e., chemotherapy, should be tested for HBsAg and anti-HBc before commencing therapy [6]. Reactivation can also occur, albeit at a lower rate in HBsAg negative but anti-HBc positive individuals (occult HBV infection). This is for example observed in patients treated with rituximab as well as in patients receiving stem cell transplantation. In general, HBsAg positive individuals should receive preemptive treatment to prevent reactivation of HBV replication and HBsAg negative/anti-HBc positive individuals require at least close monitoring of HBV DNA [2, 6].

Serological and Molecular Diagnosis of HBV Infection

Diagnosis of HBV infection is confirmed by the demonstration of the presence of specific antigens and/or antibodies as well as direct measurement of HBV DNA. Measurement of HBsAg, anti-HBc, HBeAg, and HBV DNA allow definite confirmation of HBV infection as is used worldwide. Testing can be performed on a step-by-step approach, to avoid unnecessary testing.

If a HBV infection is suspected, initial testing is done for HBsAg and anti-HBc. HBsAg is the hallmark of HBV infection and reflects the transcriptional activity of the covalently closed circular HBV DNA (cccDNA). Anti-HBc is positive if the person had contact with HBV. In case of positive findings for HBsAg and anti-HBc further test should be performed, which are HBeAg, anti-HBe, HBV DNA, and anti-HDV. Additional testing for anti-HBc-IgM helps to differentiate acute from chronic HBV infection and should be tested especially if acute HBV infection is suspected.

If both, HBsAg and anti-HBc, are negative and a high risk for acute HBV infection is suspected, testing for HBV DNA should be strongly considered. In the early phase of HBV infection, the amount of HBsAg can be so low that detection can be missed even by sensitive tests. This phase may last for several weeks. If acute HBV infection cannot be confirmed but is strongly suspected repeated measurements may be necessary as well as monitoring of liver transaminases.

In case of isolated positive findings for anti-HBc, anti-HBs should be analyzed. If anti-HBs is >10 IU/ml, the HBV infection has resolved and an immune response against HBV has been established. If anti-HBs is negative, occult HBV infection

can be excluded by HBV DNA PCR [2]. If HBsAg is isolated positive, the test should be repeated and if a high probability for HBV infection is suspected, HBV DNA should be tested additionally.

Hepatitis Delta Virus (HDV) coinfection must always be excluded after confirming HBV infection by means of testing for anti-HDV and/or HDV-RNA. The anti-HDV antibodies are detected with an enzyme immunoassay [7]. HDV-RNA should be identified using RT-PCR [8]. Quantification allows to monitor therapy [9].

Table 10.1 provides an overview for various states of HBV infection and the corresponding biomarkers.

HBsAg is determined with great sensitivity and specificity using an enzyme immunoassay [10–12]. Quantitative testing is possible and becomes increasingly popular in recent years as a tool to further differentiate the various states of HBV infection [13, 14]. HBsAg reflects the transcriptional activity of the cccDNA and is a useful surrogate marker to predict the natural course of HBV-infection and for treatment guidance [15, 16]. Escape variations of HBsAg epitopes, which do not bind to the antibodies that are used for detection, may cause a false-negative test [17, 18]. Two assays for HBsAg quantification have become commercially available, the Architect HBsAg assay (Abbott Diagnostics, Abbott Park, IL, USA) and the Elecsys HBsAg II quant assay (Roche Diagnostics, Indianapolis, IN, USA). Results from both assays are strongly correlated ($r>0.96$) [19, 20].

HBeAg is linked to the activity and the clinical course of HBV infection and may aid as a prognostic marker [21]. Therefore, regular testing for HBeAg/anti-HBe is useful in the evaluation of the clinical course of the disease and in monitoring the therapy to assess HBeAg to anti-HBe seroconversion. It should be noted that HBV variants with mutations in the precore or core promoter region exists [22]. In these cases, HBeAg is commonly not detected and hepatitis is the result. In this so-called HBeAg negative chronic hepatitis B, the patients are infected either with a variant virus, bearing nucleotide substitutions at position 1896 (G1896A) in the precore region of the preC/C gene, which prevents the HBe protein from being synthesized. The second group of polymorphisms is located within the core promoter region at positions 1762 (A1762T) and 1764 (G1764A), which down-regulate HBe protein production up to 70 %. In addition other mutations have also been described [23]

The presence of anti-HBc antibodies is associated with ongoing or resolved HBV infection and the detection of the IgM and IgG immunoglobulin classes can help to distinguish between acute or chronic HBV infection. Anti-HBc-IgM is found in high concentrations during acute hepatitis B and drops subsequently [24]. However, during acute exacerbation of chronic HBV infection, anti-HBc-IgM may increase [25]. Anti-HBc persists also in patients with resolved HBV infection and immunization, indicated by anti-HBs levels >10 IU/ml.

Detection of HBV DNA may help tremendously in the diagnosis of HBV infection and plays an important one for the prognosis of the disease [26, 27]. In case of unclear results for HBsAg and anti-HBc, detection of HBV DNA can confirm HBV infection. Furthermore HBV DNA is a major tool for the monitoring of HBV treatment. Both copies/ml and IU/ml are used as units in viremia. Five copies

Table 10.1 Phases of HBV infection and their biomarkers

Phase	Lab characteristics
Acute HBV infection	– HBsAg positive <6 months, anti-HBc IgM positive – HBeAg positive, HBV DNA positive – Normal ALT or elevated ALT (symptomatic patients have usually >10× ULN)
Chronic "Immuntolerant" HBeAg positive HBV infection (high replicative HBsAg carrier)	– HBsAg positive >6 months, anti HBc positive – HBeAg positive, anti-HBe negative, HBV DNA >2,000 IU/ml, usually >10^6 IU/ml – Normal ALT or ALT <2× ULN – Histology: mild or no liver necroinflammation and no or slow progression of fibrosis
Chronic "immune reactive" HBeAg positive hepatitis B	– HBsAg positive >6 months, HBeAg positive – HBeAg positive, anti-HBe negative, HBV DNA >2,000 IU/ml – Elevation of ALT ≥2-fold ULN (persistent high or fluctuating levels) – Histology: moderate or severe liver necroinflammation and more rapid progression of fibrosis
"Inactive" HBeAg negative HBsAg carrier (Low replicative HBsAg carrier)	– HBsAg positive >6 months, HBeAg negative, anti-HBe positive – HBV DNA <20,000 IU/ml, ideally <2,000 and HBsAg <1000 IU/ml – Normal ALT or <2-fold ULN (multiple measurements required) – Histology: mild or no liver necroinflammation and no or mild fibrosis
Chronic HBeAg negative Hepatitis B	– HBsAg positive >6 months, anti-HBc positive – HBeAg negative, anti-HBe positive, HBV DNA >2,000 IU/ml – Elevation of ALT ≥2-fold ULN (persistent high or fluctuating levels) – Histology: moderate or severe liver necroinflammation and more rapid progression of fibrosis
Hepatitis delta coinfection	– HBsAg positive, anti-HBc positive – Anti-HDV positive – If HDV-RNA positive: active infection – Often HBeAg negative and low HBV DNA levels
Occult HBV infection	– HBsAg negative, anti-HBs negative – Anti-HBc positive (confirmed) – HBV DNA detectable – Normal/elevated ALT serum activity
Anti-HBc only	– Anti-HBc positive (confirmed) – HBsAg negative, anti-HBs negative or <10 IU/l – Normal ALT serum activity
Clinical resolved HBV infection	– Anti-HBc positive and anti-HBs ≥10 IU/l – HBsAg negative – HBV DNA negative – Normal ALT serum activity
Vaccinated	– HBsAg negative – Anti-HBc negative – Anti-HBs >10 IU/ml

ULN upper limit of normal, *IU* international units, *ALT* alanine aminotransferase

of HBV DNA correspond to about 1 IU. An international standard for HBV DNA concentrations has been defined by the WHO [28] and it is recommended to use IU/ml. The lower limit of detection is around 6–20 IU/ml with modern real-time PCR assays. To maintain comparability, the same test should be used in longitudinal assessment of a patient preferentially.

HBV Genotypes

There are eight know HBV genotypes (A–H) which have distinct geographical distributions. In Europe, genotypes A and D are most prevalent while in Asia genotypes B and C dominate. Genotypes E and F may have originated in aboriginal populations of Africa and the New World. In addition there are at least 24 subtypes. For example, genotype A has two subtypes: Aa (A1) in Africa and Asia and Ae (A2) in Europe and North America. There are also different subtypes for other genotypes with regional differences. Details see Chap. 3. The different genotypes have some clinical relevant differences. Genotype C has been associated with faster disease progression and HCC development [29]. Patients with genotypes A and B respond better to interferon alpha treatment compared to genotypes C and D [30]. However, HBV genotyping is not strongly recommended by current guidelines [2] because of the limited relevance. Although genotypes have been associated with response to IFN, the positive or negative predictive value is not high and other predictors (HBV DNA $<10^8$ IU/ml, ALT >2–5× ULN, HBsAg kinetics) exist [2, 4].

Non-serologic/Molecular Assessments for HBV Infection

Although the diagnosis of HBV infection is a serologic diagnosis, further assessments are important to provide adequate care for the infected persons and prevent spreading of the disease. A thorough medical history should be taken, specifically asking for risk factors (e.g., blood transfusions, i.v. drug use) and family and partner history, which may facilitate further testing. Family members or partners of persons infected with HBV must be offered diagnostic testing for HBV infection and vaccinations.

A physical examination helps to identify persons with advanced liver disease. Laboratory testing for possible coinfections (HDV, HIV, HCV) is mandatory. Clinical chemistry laboratory tests for liver inflammation, blood count, as well as blood coagulation status help to assess liver function and the degree of liver injury. If advanced liver disease is suspected, additional tests (albumin, bilirubin and parameters for renal function) should be done to determine the synthesis output of the liver and detect possible complications. A liver ultrasound examination also aids in the assessment of the degree of liver injury and is indispensable in the diagnosis of hepatocellular carcinoma.

Liver biopsy is important for the diagnosis, staging and prognosis of HBV infection. Histological evaluation of a liver biopsy helps to clarify the following issues:

1. Assessment of the inflammatory activity (grading);
2. Assessment of extent of fibrosis (staging);
3. Etiologic statements (especially comorbidity).

Several scoring systems exists for the staging and grading of liver diseases (e.g., ISHAK, Desmet) [31, 32]. The histologic workup should be done by an experienced pathologist, especially in regard of potential concomitant liver diseases if the etiology is unclear and no firm diagnosis can be made by the assessment of seromarkers.

Presently, liver biopsy is still considered as gold standard with respect to fibrosis evaluation. However, if no doubt regarding the etiology exists, noninvasive tests like transient elastography can also be used to reliably assess the extent of fibrosis and even complications of liver cirrhosis (e.g., portal hypertension, hepatocellular carcinoma) [33–37]. However, influencing factors such as high ALT values need to considered [38].

Virologic Monitoring

Virologic Monitoring During Acute HBV Infection

Acute HBV infection should be monitored until anti-HBs seroconversion has occurred. Testing for HBsAg and HBV DNA as well as the liver enzymes help to monitor the clinical course.

Initially serum transaminase activity should be monitored closely until normalization to recognize a potential fulminant liver failure. Once serum transaminases have normalized, testing for HBsAg and anti-HBs every 3–6 months until HBsAg is negative and anti-HBs >10 IU/ml should be done. If HBsAg is negative and anti-HBs <10 IU/ml, follow-up every 12 months is required and HBV DNA should be added to the test regimen to test for occult hepatitis B.

Virologic Monitoring During Chronic HBV Infection

Follow-up for chronic HBV infection should be done initially every 3 months for the first year to assess the clinical activity of the disease over time. The definition of the phase of chronic hepatitis B (Table 10.1) can be difficult because ALT and HBV DNA can fluctuate [4] (see Fig. 10.1). Testing should include clinical chemistry (liver inflammation and synthesis parameters, blood count, blood coagulation parameters), HBsAg, HBeAg (if initially positive) and quantitative HBV DNA.

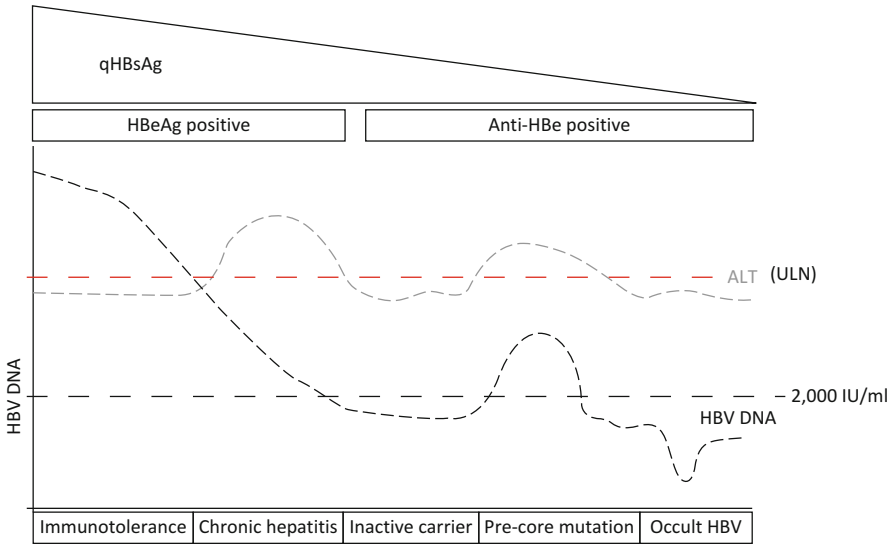

Fig. 10.1 Virological markers during the different phases of HBV infection

After the initial year, it is recommended to perform follow-up visits every 6 months. In case of cirrhosis, visits should include ultrasound evaluation for HCC [2, 4].

In dependence of the clinical course of the disease and the clinical activity follow-up visits can be adjusted. In patients with fluctuating ALT and HBV DNA follow-up visits every 3 months should be considered. In patients without cirrhosis showing normal results for ALT and a stable HBV DNA <2,000 IU/ml in longitudinal monitoring, follow-up visits may be scheduled every 12 months [2].

Virologic Monitoring During Antiviral Therapy

Before treatment initiation clinical chemistry laboratory tests, quantitative HBV DNA, quantitative HBsAg, HBeAg should be performed. HBV genotyping can be performed if interferon therapy may be an option but is not reimbursed in many countries (see above).

If patients are treated with interferon alpha, it is recommended to test HBV DNA and HBsAg after 12 weeks of treatment. HBsAg kinetics have a good negative predictive value. Different stopping rules for HBeAg positive and HBeAg negative patients exist [16].

During treatment with nucleoside or nucleotide analogues (NUC), measurement of HBV DNA is important to assess the treatment response and should be performed every 3–6 month. In addition, HBeAg, anti-HBe, and HBsAg should be monitored every 6 month preferentially. Twelve months after anti-HBe seroconversion, treatment with NUC may be stopped [4]. Follow-up visits should be scheduled as in patients without treatment; however, closer monitoring (i.e., every 4 weeks) should be strongly considered directly after treatment withdrawal to detect an early relapse. An increase in HBV viremia is a sign of therapy failure, which might be caused by resistance to the antiviral agents or low adherence [4, 39]. To detect all possible resistance mutations, sequencing of the polymerase gene is the preferred method of detection. However, before further resistance testing is done, it should be assessed whether the antiviral medications was taken on a regular basis. Furthermore, it should be noted, that recent discoveries have identified the immune system and not viral resistance as the cause for a slow treatment response [40].

Treatment with modern NUC such as entecavir or tenofovir are highly potent and resistance has become a minor issue. HBV DNA suppression will be achieved in >95 % after 5–6 years of therapy [4]. However HBsAg loss, the ultimate goal of treatment, is a rare event [41]. It has been shown, that HBsAg quantification can also be a tool to determine the chance of HBsAg loss during NUC therapy [42, 43]. In the future there may be further biomarkers that help to manage patients during NA therapy, such as IP-10 [43], HBV-RNA [44], HBcrAg [45], or HBsAg fraction (LHBs, MHBs, SHBs) [46].

Conclusion and Perspective

Several biomarkers exist that allow the reliable diagnosis of HBV infection. If the diagnosis of HBV infection has been made, lifelong virological monitoring should be established. Based on the longitudinal virological monitoring potential hazardous events can be prevented in patients who do not require treatment. In patients treated with interferon alpha, virological monitoring allows to assess the success of the treatment as well as help in identifying patients who will not respond to interferon alpha and thus prevent unnecessary treatment. In patients on NUC treatment, virological monitoring is mandatory to assess the treatment response and to identify treatment failures.

However, several unmet needs exist for the virological monitoring. The positive predictive value to guide interferon alpha therapy is rather low and more precise markers to identify patients most likely responding to interferon based therapy are strongly needed. In addition, markers that predict successful NUC discontinuation, especially in HBeAg negative patients are needed.

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Chapter 11
Natural History of Hepatitis B Virus Infection

Chia-Ming Chu and Yun-Fan Liaw

Introduction

Hepatitis B virus (HBV) infection is a global public health problem. Despite the development of highly effective vaccines against the disease since the early 1980s and the implementation of universal newborn vaccination programs in more than 168 countries, there is still a huge burden of liver disease due to chronic hepatitis B [1]. An estimated 240 million people in the world, representing over 3.7 % of the global population, are chronically infected with HBV and 75 % of them reside in Asia-Pacific region [2]. Between one-quarter and one-third are expected to develop progressive liver disease, including cirrhosis and hepatocellular carcinoma (HCC) and 15–25 % will die from HBV-related liver disease. Worldwide, HBV infection accounts for 30 % of patients with cirrhosis and 53 % of those with HCC, and over 200,000 and 300,000 hepatitis B surface antigen (HBsAg) carriers die each year from cirrhosis and HCC, respectively [3]. In Taiwan, HBsAg carriers are at 5.4- and 25.4-fold increased risk of mortality from cirrhosis and HCC, respectively [4].

The natural course of HBV infection is complex and variable. Substantial improvement during the past decades in the understanding of HBV virology and host immune response to HBV, combined with the recent availability of highly sensitive HBV DNA assays and quantitative HBsAg assays, has led to new insights into the natural history of HBV infection.

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Epidemiology

Worldwide, an estimated two billion people have been infected with HBV, and some patients with acute HBV infection develop chronic HBV infection. The global prevalence of chronic HBV infection varies greatly among different geographical areas, and was classified into high-prevalence (Southeast Asia, China, sub-Saharan Africa, and Alaska), intermediate-prevalence (Mediterranean countries, Eastern Europe, Central Asia, Japan, Latin, and South America), and low-prevalence areas (USA, Western Europe, Australia, and New Zealand) based on the prevalence of HBsAg carriers of >8, 2–8, and <2 %, respectively [1]. The corresponding lifetime risk of being exposed to HBV infection is approximately 60–80, 20–60, and 10–20 %, respectively.

Associated with a wide range in prevalence of chronic HBV infection are differences in the predominant mode of transmission and age at infection. In high-prevalence areas, the mode of HBV transmission differs remarkably between Southeast Asia and sub-Saharan Africa. In Southeast Asia, perinatal transmission is common and accounts for 40–50 % of chronic infection. In contrast, in sub-Saharan Africa, perinatal transmission does not play a major role and inapparent horizontal transmission by HBsAg positive family members and playmates or by unsafe therapeutic injections is the major route of HBV transmission, with most children being infected by the age of 5 years. This difference is related to the higher prevalence of hepatitis B e antigen (HBeAg) in Southeast Asia female carriers of childbearing age (40–50 %) than in sub-Saharan Africa (10–18 %) [5, 6], as 80–90 % of HBeAg positive mothers will transmit the disease to their off springs, compared with only 15–20 % of those seronegative for HBeAg [7]. In low-prevalence areas, hepatitis B is a disease of young adults, typically those who have high-risk behavior such as sexual promiscuity or drug abuse or are in high-risk occupations.

The worldwide incidence of HBV infection is decreasing as a result of vaccination and public health education. For instance, in Taiwan, after the implementation of universal vaccination programs in newborns in 1984, the HBsAg carrier rate among children <15 years of age decreases from 10 % in pre-vaccination era to 0.6 % in 2004 [8] and 0.5 % in 2009 [9]. The prevalence of HBsAg in the population ≤25 years of age born after the vaccination program is 0.9 % [9]. In the USA, the incidence of reported acute hepatitis B declines by 81 % from 8.5 to 1.6 cases/100,000 during the period 1990–2006. However, immigrants from high-prevalence areas are now responsible for an increasing burden of chronic HBV infection in many developed countries.

Acute HBV Infection

Clinical Manifestations

Acute HBV infection in neonates is clinically asymptomatic in most cases. Symptomatic hepatitis occurs in only 10 % of children less than 4 years old [10]. In rare instances, mothers seropositve for antibody against HBeAg (anti-HBe) may

transmit HBV to their babies, resulting in severe or fulminant hepatitis within the first year of life [11]. HBV with mutations of basal core promoter (BCP) and precore regions, which reduce or abrogate HBeAg production, may be one of the factors in the pathogenesis of fulminant hepatitis in children [12].

On the contrary, approximately 30 % of immunocompetent adults with acute HBV infection develop icteric hepatitis [10], and 0.1–1 % develop fulminant hepatitis [13]. Among patients hospitalized for acute hepatitis B, the fatality rate is 1 %. HBV infection accounts for >50 % of fulminant cases of viral hepatitis. The reason that HBV has a fulminant course in some patients remains unclear. Both viral and host factors may be involved in the pathogenesis of fulminant hepatitis B. However, the association of precore and BCP mutations with fulminant hepatitis B in adults is controversial [14, 15].

Chronic Evolution

The risk of chronicity after primary HBV infection varies and depends on the age and immune status at the time of infection. Among infants born to HBeAg positive mothers, hence infected in the perinatal period, the probability of chronic infection approaches 90 %. When infected at 1–5 years of age, 20–30 % of the children become chronically infected, while among older children the probability falls to 5–10 % [10]. The extremely high chronicity after perinatally acquired infection is presumably related to the immature immune system of the neonates. Another possible mechanism is that the fetus is tolerated in utero to HBV following transplacental passage of viral proteins [16]. The risk of chronicity among normal, healthy, immunocompetent adults is ≤5 %, but varies considerably (<1–12 %) among diverse populations [17], being extremely low (0.2 %) in Greece [18] and appreciably high (12.1 %) in Germany [19]. The risk of chronicity is greatly increased in immune compromised patients, such as patients on chronic hemodialysis, those on immunosuppression following solid organ transplantation, and those receive cancer chemotherapy. Patients with concomitant human immunodeficiency virus (HIV) infection are also at significant risk of developing chronic infection, with 20–30 % remain HBsAg positive after acute infection.

Classically, persistence of serum HBsAg for more than 6 months is considered to represent a progression to chronic infection. However, a recent study from Japan showed that 90.2 % of patients cleared serum HBsAg within 6 months, 7 % between 7 and 12 months after the onset of acute hepatitis B and 2.8 % had persistence of HBsAg for more than 12 months [20]. Another study showed that the rate of persistence of HBsAg was 23.4 % at 6 months and 7.5 % at 12 months in genotype A HBV infection; while the corresponding figure for genotype non-A HBV infection was 8.6 and 0.9 %, respectively [21]. These results further indicate that genotype A HBV is an independent risk factor for progression to chronic infection. The different risk of chronicity in adults from diverse geographical areas may be in part attributed to HBV genotype difference. It is also possible that the longer persistence of

serum HBsAg reflects the higher sensitivity of the most up-to-date assays for HBsAg as compared to previous assays. Persistence of HBsAg for more than 12 months, as measured with a highly sensitive method, may be suitable for redefining the progression of acute hepatitis B to chronicity.

Chronic HBV Infection

Clinical Presentation

In low- or intermediate-prevalence areas, approximately 30–50 % of patients with chronic HBV infection have a history of classical acute hepatitis that progressed to chronic infection. In patients from high-prevalence areas, most patients are incidentally identified to be HBsAg carriers, almost none had evidence of progression from overt acute hepatitis.

Patients with chronic HBV infection may experience acute hepatitis flare that may be asymptomatic or mimic acute hepatitis with fatigue, anorexia, nausea and, in rare instances, jaundice or even hepatic decompensation. In Taiwan, as many as 40 % of HBsAg positive patients with clinical diagnosis of acute hepatitis are actually chronic HBsAg carriers who remained unrecognized until they present the episode of overt acute hepatitis. They are positive for HBsAg but negative for immunoglobulin class M antibody against hepatitis B core antigen (IgM anti-HBc), so-called "previously unrecognized HBsAg carriers with acute hepatitis flares or superimposed other forms of acute hepatitis" [22]. In these high-prevalence areas, an episode of acute hepatitis in HBsAg positive patients is more likely an acute hepatitis flare of chronic HBV infection rather than acute hepatitis B.

Phases of Chronic HBV Infection

The patients with chronic HBV infection may present one of the following four biochemical and serological profiles: (1) HBeAg positive with normal alanine aminotransferase (ALT) levels; (2) HBeAg positive with abnormal ALT levels; (3) HBeAg negative with normal ALT levels; and (4) HBeAg negative with abnormal ALT levels. These four patterns of presentation actually represent different phases of chronic HBV infection.

As a result of the dynamic interplay of complex interactions involving HBV, the hepatocyte and the host immune response, the natural course of chronic HBV infection consists of distinct phases, characterized and diagnosed on the basis of HBeAg/anti-HBe serology, serum HBV DNA levels, ALT levels and liver histology. Typically, chronic infection acquired perinatally or during infancy consists of three chronological phases: the initial immune tolerance phase, followed by immune clearance phase, and finally the low replicative inactive phase [23–25]. In a subset

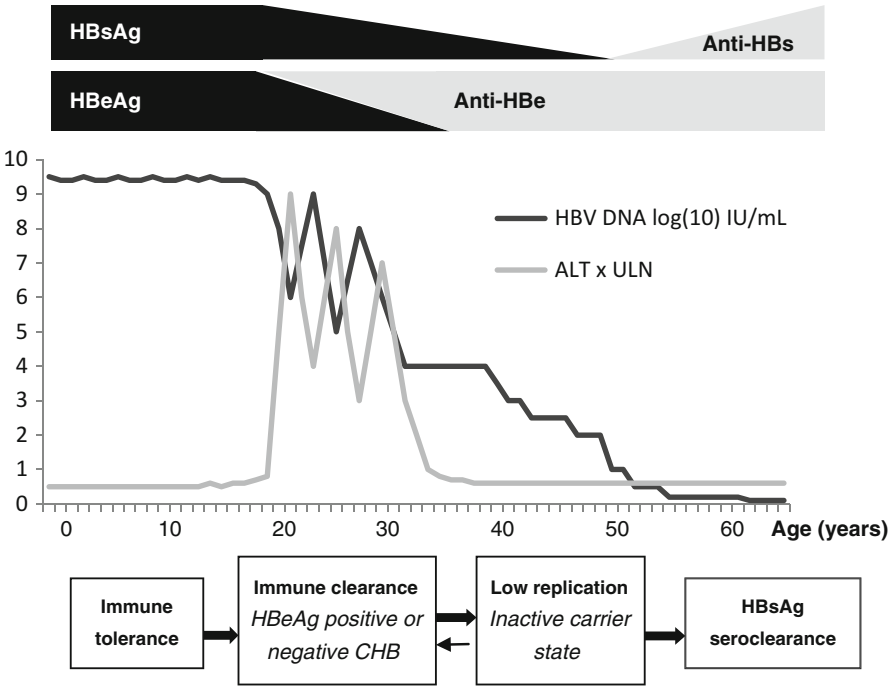

Fig. 11.1 Serological course of chronic hepatitis B virus infection. The initial immune tolerant phase is characterized by a high level of HBV replication, HBeAg positivity, and a normal ALT. This period can last up to 20–25 years following perinatal infection, but is short or absent in adult acquired infection. During the immune clearance phase, there is a reduction in HBV DNA levels associated with raised ALT levels (HBeAg positive chronic hepatitis B). This phase can last for years until HBeAg seroconversion. After HBeAg seroconversion, the patient enters the low replicative phase, characterized by low or undetectable HBV replication and a normal ALT (inactive carrier state). Some inactive carriers can develop HBV reactivation with either wild-type HBV and reversion to HBeAg positivity or more frequently with HBV variants with mutations limiting HBeAg production (HBeAg negative chronic hepatitis). Reactivation of HBV can be viewed as a variant of immune clearance phase. Serum levels of HBsAg decrease gradually during the natural course of chronic HBV infection. In a subset of inactive carriers, serum HBsAg even disappears spontaneously, followed by anti-HBs seroconversion in up to 75 % at 10 years following HBsAg seroclearance. *ULN* upper limit of normal

of inactive carriers, HBV may reactivate and trigger immune mediated liver injuries. This reactivation phase can be viewed as a variant of immune clearance phase [1]. Among the minority of adult patients who progress to chronic hepatitis B, there is usually no or very short initial immune tolerance phase. Successful immune clearance occurs more readily. Otherwise, the clinical course is the same as seen in perinatally acquired infection.

The serological course of chronic HBV infection is shown in Fig. 11.1. The clinical, serological, histopathological and virological characteristics of immune tolerance phase, immune clearance phase, inactive carrier state and reactivation of HBV are summarized in Table 11.1.

Table 11.1 Phases of chronic HBV infection: clinical, serological, histopathological and virological characteristics

Characteristics	Immune tolerance phase	Immune clearance phase	Inactive carrier state	Reactivation of hepatitis B
Age (years)	<20–25	20–40	>35-–40	>35–40
Serology				
HBsAg (log_{10} IU/mL)	4.5–5.0	3.5–4.5	2.0–2.5	3.0–3.5
HBeAg	Positive	Positive	Negative	Negative
HBV DNA levels (log_{10} copies/mL)	Very high 8–12	High 6–10	Low or undetectable <4	Moderate, fluctuating 4–8
ALT	Normal	Elevated	Normal	Elevated
Precore/basal core promoter	Wild type	Mixed (Wild type > mutant)	Mixed (mutant > Wild type)	Mutant
Histopathology				
Inflammation	Absence	presence	Absence	Presence
Hepatocyte HBsAg	Positive (membrane/ cytoplasm)	Positive (membrane/ cytoplasm)	Positive (cytoplasm)	Positive (membrane/ cytoplasm)
Hepatocyte HBcAg	Positive (nucleus)	Positive (nucleus/ cytoplasm)	Negative	Positive (cytoplasm)
Disease progression	No/minimal	Yes	No	Yes
HBsAg seroclearance	No	No	1–2 %/year	No

HBV hepatitis B virus, *HBsAg* hepatitis B surface antigen, *HBeAg* hepatitis B e antigen, *ALT* alanine aminotransferase, *HBcAg* hepatitis B core antigen

Immune Tolerance Phase

The immune tolerance phase is characterized by the presence of HBeAg, very high serum level of HBV DNA (>2×10^7 IU/mL) and HBsAg (4.5–5.0 log_{10}IU/mL) [26–28], normal ALT level, normal liver or only minimal necroinflammatory activity and scant fibrosis. Immunostaining of HBV antigens in liver shows that HBsAg is distributed diffusely on the hepatocyte membrane and focally in the cytoplasm, and hepatitis B core antigen (HBcAg) is distributed predominantly in nuclei [29]. There is usually little or no disease progression as long as serum ALT levels remain normal and the immune tolerance is maintained [30].

The exact mechanisms for immune tolerance are unknown. Even though HBV virus does not cross the placenta, 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 to HBeAg 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-Iymphocyte (CTL) response

to HBcAg, the major target of the immune response [16]. The viral population identified during the immune tolerance phase usually consists of exclusively wild type HBeAg-positive HBV with little or no mutant type HBeAg-negative HBV [15, 16].

Immune Clearance Phase

The transition from immune tolerance to immune clearance phase usually occurs between age 20 and 40, but may sometimes start earlier and even occur in pediatric patients. During this phase, serum HBeAg is still positive but ALT levels become abnormal, accompanied by declining levels of serum HBV DNA and HBsAg. Serum HBV DNA levels generally exceed 20,000 IU/mL and HBsAg levels are usually in the range of 3.5–4.5 $\log_{10}$ IU/mL [26–28]. There is a positive correlation between serum HBsAg levels and serum HBV DNA or intrahepatic covalently closed circular DNA (cccDNA) levels. Liver biopsy demonstrates moderate or severe necroinflammation with variable amounts of fibrosis. HBsAg is distributed diffusely on the hepatocyte membrane and focally in the cytoplasm, as seen in immune tolerance phase, but intrahepatic nuclear HBcAg expression decreases with concomitant increase in cytoplasmic/membranous HBcAg expression [29]. These results suggest that membranous expression of HBsAg is closely related to active viral replication but is probably not responsible for liver cell damage, and that hepatocytes with cytoplasmic/membranous HBcAg expression might be possible targets for immune hepatocytolysis [29].

Little is known about the mechanisms that regulate the loss of immune tolerance in chronic HBV infection. The finding that 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. However, a more recent study suggests that the shift of hepatocyte HBcAg from nucleus to cytoplasm during the immune clearance phase may be secondary to liver cell damage and regeneration [31].

Hepatitis Activity and Acute Hepatitis Flare During Immune Clearance Phase (HBeAg Positive Chronic Hepatitis)

Most patients in the immune clearance phase are asymptomatic and have mild to moderate elevation in ALT levels and hepatitis activity, so called HBeAg positive chronic hepatitis B (CHB). However, the clinical course may be punctuated by spontaneous acute hepatitis flare, defined as an abrupt elevation of ALT >5 times the upper limit of normal (ULN). These acute hepatitis flares are considered to be the results of HLA-class I antigen-restricted, CTL mediated immune response against HBV antigen(s) and its downstream apoptotic mechanisms [32]. The reasons for spontaneous acute hepatitis flares are not clear but are likely explained by subtle changes in immunological controls of viral replication. Several studies have found that acute hepatitis flares are often preceded by a sudden increase in serum levels of

HBV DNA [33, 34], HBeAg and HBeAg-specific immune complexes [34], and enhanced T-cell response to HBcAg and HBeAg [35]. These results suggest that increases in viral replication, accumulation of nucleocapsid proteins in serum and hepatocytes, and the subsequent immune response play an important role in initiating acute hepatitis flares in chronic HBV infection [34]. Histological evidence of lobular hepatitis superimposed upon the changes of chronic viral hepatitis is frequently observed [36]. IgM anti-HBc is positive in 14.4 % of patients during acute flares, but generally in lower titers than in acute HBV infection [37]. As HBeAg seroconversion is often preceded or accompanied by a transient ALT flare, it is believed that hepatitis flares are the results of the host attempt to clear the virus by the immune response. However, not all acute hepatitis flares lead to HBeAg seroconversion and HBV DNA clearance from serum, a phenomenon termed as "ineffective or abortive immune clearance" [32]. In this context, the patients may experience repeated episodes of acute hepatitis flares, which can account for increased risk of HBV-related cirrhosis.

The annual rate of acute hepatitis flare in patients with HBeAg positive CHB was as high as 28.6 % in an early hospital-based study from Taiwan [38]. However, in another study that followed up asymptomatic patients beginning at the immune tolerance phase through HBeAg seroconversion, the overall incidence of acute hepatitis flare was 28.8 % during immune clearance phase (mean 3.7 years), with an annual rate of 7.8 % only [39]. Most acute hepatitis flares are asymptomatic, but around 20 % of patients present with symptoms of overt acute hepatitis [37], and approximately 2–3 % may be complicated with hepatic decompensation [40]. One recent report from Taiwan found that a serum HBV DNA level $\geq 1.55 \times 10^9$ copies/mL at the onset of acute flare can predict hepatic decompensation [41]. In HBV high-prevalence areas, acute hepatitis flares of chronic HBV infection is the most important etiology of acute hepatitis and fulminant hepatitis in adults [42, 43].

HBeAg to Anti-HBe Seroconversion

The immune clearance phase has a variable duration and often lasts for many years until HBeAg seroconversion occurs. HBeAg seroconversion is frequently preceded by ALT elevation, followed by a marked reduction of serum HBV DNA levels that can only be detected by sensitive polymerase chain reaction (PCR) assay, decline of serum HBsAg level, ALT normalization and resolution of liver necroinflammation [32, 36]. However, abnormal ALT levels and high-level HBV DNA persist at the time of HBeAg seroconversion in about 5 % of patients [44]. These patients progress directly from HBeAg positive chronic hepatitis to HBeAg negative chronic hepatitis.

The average annual incidence of HBeAg seroconversion is 10 % (range, 2–15 %), depending on factors such as ethnicity, mode of transmission, age, ALT levels, histological activities and HBV genotype. HBeAg seroconversion is much more delayed in children with HBeAg positive carrier mothers than in children with HBeAg negative carrier mothers or children with non-carrier mothers [45]. Different

mode of HBV transmission accounts for the much lower HBeAg positivity rates in black Africans of childbearing age than in women in the Far East [5, 6]. A higher HBeAg seroconversion rate has been reported in non-Asian children with horizontal transmission than Asian children with vertical transmission [46]. In Taiwan, the annual rate of HBeAg seroconversion is <2 % in children ≤3 years of age and 4–5 % in older children, so that around 85 % of children still remain HBeAg positive by age 15 [47]. The likelihood of HBeAg seroclearance correlates positively with ALT levels: HBeAg seroclearance rates at 18-months of follow-up are 0, 3–8, 17, and 59–70 %, respectively, if baseline ALT levels increase over <1, 1–2.5, 2.5–5, and >5 times ULN [32]. In patients with acute hepatitis flare, 72 % undergo HBeAg seroclearance within 3 months if serum α-fetoprotein (AFP) levels >100 ng/mL, compared to only 18 % of those with AFP <100 ng/ml [48]. Serum HBV DNA levels ≤7 $\log_{10}$ copies/mL during acute hepatitis flare also can predict HBeAg seroconversion within 6 months [49]. The likelihood of HBeAg seroconversion also correlates with histological activities: the 5-year cumulative probabilities of HBeAg seroconversion is >65 % in patients with high necroinflammatory (interface or lobular) activities, compared to <25 % in those with low necroinflammatory activities [48]. HBeAg seroclearance may occur within 3 months in two-thirds of the patients with bridging hepatic necrosis [48]. In Eastern countries, patients infected with genotype B HBV seroconvert earlier and more frequently than those with genotype C HBV [50–52]. In Western countries, HBeAg seroconversion is similar in genotypes A, B, D, and F HBV infection but much slower in genotype C HBV infection [53, 54]. In Alaska native carriers, the median age of HBeAg seroclearance is <20 years in patients with genotypes A, B, D, and F HBV, but >40 years in patients with genotype C HBV [53]. Interestingly, HBeAg seroconversion is more frequently preceded by ALT flares >5 times ULN in genotype C HBV infection 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 [52].

In Taiwan, HBeAg seroconversion occurs at a median (interquartile range) age of 32 (26–36) years, with 90 % before age of 40 [55]. In accordance with these data, the prevalence of serum HBeAg declines remarkably from 85 % in children (age < 15 years) [46] to 5–10 % in adults over 40 years of age [5]. These findings suggest that HBeAg seroconversion most often 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 [55].

HBeAg Persistence and Its Outcome

In some patients, the immune clearance phase may last for many years without HBeAg seroconversion. A prolonged HBeAg positive phase is associated with increased risk of disease progression. A recent cohort study from Taiwan demonstrated that the risk of progression to cirrhosis increased with increasing age of HBeAg seroconversion, with a hazard ratio of 3.8 per decade increase in age of HBeAg seroconversion [39]. In particular, patients with HBeAg seroconversion

after 40 years of ages were associated with a remarkably high risk of progression to cirrhosis [55, 56].

Several other studies also showed that persistence of serum HBeAg was associated with an increased risk for progression to cirrhosis, HCC development and liver related mortality [57–59]. For instance, in one study from Taiwan that followed up 233 untreated patients with HBeAg positive CHB for a median of 6.8 years, the annual incidence of cirrhosis and HCC development was significantly higher in 147 patients with persistent HBeAg (3.7 and 1.6 %, respectively) than in 86 patients who underwent HBeAg seroconversion (1.8 and 0.4 %, respectively) [58].

Low Replicative Inactive Phase

After successful immune clearance, serum HBeAg is seroconverted to anti-HBe. The patients are still positive for HBsAg, but there is usually a >1 $\log_{10}$ IU/mL reduction in HBsAg levels, compared to preceding immune clearance phase, and HBsAg levels rarely exceed 1000 IU/mL during this phase [26–28]. The hallmark event of HBeAg seroconversion usually signals a transition from CHB to an inactive carrier state. HBV DNA is usually undetectable by hybridization techniques but often detectable by PCR assays. The patients are asymptomatic and have normal ALT. Liver biopsy shows no or mild necroinflammatory activity with variable degrees of fibrosis, including inactive cirrhosis. HBsAg is distributed exclusively in hepatocyte cytoplasm and intrahepatic HBcAg is absent [29].

The majority of inactive carriers had levels of HBV DNA less than 2000 IU/mL, a level that has been used to discriminate inactive carrier state from HBeAg negative chronic hepatitis [60]. However, a recent study from Taiwan in 250 inactive carriers with persistently normal ALT for more than 10 years showed that only 64 % had levels of HBV DNA $<10^4$ copies/mL, and 26 and 10 % had levels of HBV DNA in the range of 10^4–10^5 and 10^5–10^6 copies/mL, respectively [61]. It thus should be more appropriate to adopt HBV DNA levels of 20,000 IU/mL, instead of 2000 IU/mL, as a cut-off value to discriminate active from inactive HBV infection [62].

Most inactive carriers remain in this phase with sustained remission and a lifelong inactive state, particularly if this phase is reached early in the disease course. In a Taiwan study of 283 HBeAg seroconverters, 189 (67 %) remained HBeAg negative with persistently normal ALT levels over a 9-year follow-up. Of these, only one progressed to cirrhosis and two developed HCC, with estimated annual rate of cirrhosis and HCC being 0.1 and 0.2 %, respectively [44]. The prognosis of inactive carriers from intermediate- or low-prevalence areas is even better, possibly due to the shorter duration of the infection [63, 64].

Reactivation of Hepatitis B

Following HBeAg seroconversion, a subset of patients ultimately undergo spontaneous reactivation of HBV replication, with reappearance of high levels of HBV DNA (>2000 or 20,000 IU/mL) and a rise in ALT levels. Only a small proportion of

carriers with HBV reactivation is associated with reappearance of serum HBeAg (HBeAg reversion) and the remainders are persistently HBeAg negative [39, 44], suggesting that reactivation of hepatitis B usually results from HBV variants with precore or BCP mutations. In addition, HBV replication can reactivate as a result of immunosuppression or cancer chemotherapy [65].

HBeAg Reversion

In a study from Alaska, 109 (20 %) of 541 seroconverters developed HBeAg reversion, which was frequently accompanied by hepatitis flare, and HBeAg tended to fluctuate between seroconversion and reversion [66]. HBeAg reversion, however, is much uncommon in other studies. In two studies from Taiwan, one involving 283 patients with HBeAg positive hepatitis and another involving 240 HBeAg positive carriers with normal baseline ALT, HBeAg reversion following initial HBeAg seroconversion occurred in 12 (4.2 %) and 7 (2.9 %) patients during a mean follow-up of 8.6 years and 6.8 years, respectively [39, 44]. In another study from Italy, only one (1.6 %) of 61 seroconverters had HBeAg reversion during a mean follow-up of 22.8 years [59]. Despite the low frequency, HBeAg reversion is significantly associated with increased risk of progression to cirrhosis as well as development of HCC [44, 66].

HBeAg Negative Chronic Hepatitis

The majority of patients with reactivation of hepatitis B are negative for HBeAg [39, 44] and have "HBeAg negative CHB." Patients with HBeAg negative CHB are usually older than patients with HBeAg positive CHB and are more likely to have advanced fibrosis and cirrhosis at the time of their first presentation. Serum levels of HBsAg are lower in HBeAg negative CHB than in HBeAg positive CHB by about 0.5–1 $\log_{10}$ IU/mL (3.0–3.5 vs. 3.5–4.5) [26–28]. Serum HBV DNA levels also tend to be lower (4–8 $\log_{10}$ copies/mL), compared to HBeAg positive CHB (6–10 $\log_{10}$ copies/mL). However, many patients with HBeAg negative CHB have wide fluctuations in both HBV DNA and serum ALT levels. Episodes of hepatitis flare are frequently seen, with a rate of about 1/3–1/2 of that in HBeAg positive counterparts [38]. Spontaneous sustained remission of disease activity is rare [67].

The prevalence of HBeAg negative case in patients with CHB varies widely in different geographical areas: 80–90 % in the Mediterranean basin, 30–50 % in Taiwan and Hong Kong, but less than 10 % in the USA and Northern Europe [68]. This difference may be in part attributed to the different HBV genotype distribution: precore mutant is frequently detected in genotype D (the main genotype in the Mediterranean basin) and genotypes B and C infection (the predominant genotypes in East Asia), but rarely detected in genotype A infection (the main genotype in the USA and Northern Europe). The prevalence of HBeAg negative CHB has been increasing over the last few decades as a result of aging of the HBV-infected population

and the effective prevention measures restricting new HBV infections. HBeAg negative CHB has become much more common than HBeAg positive CHB in many countries of the world nowadays.

However, the incidence of HBeAg negative CHB among HBeAg negative carriers remains largely unknown. This issue has been addressed in a few prospective studies that followed up the natural course following spontaneous HBeAg seroconversion. In two studies from Taiwan, the annual rate of HBeAg negative CHB was 2–3 % with a cumulative incidence of 25 % at 16 years, but hepatitis B reactivation typically occurred within the first 5–10 years [39, 44]. However, in another study from Italy, only 9 (14.8 %) of 61 seroconverters developed HBeAg negative CHB during a mean follow-up of 22.8 years (annual rate of 0.6 %) [59]. In another Italian study involving pediatric patients, the rate is even lower: only 4 (6.3 %) of 64 patients developed HBeAg negative CHB during a mean period of 15 years [69]. These differences can be explained by the finding that age of HBeAg seroconversion is an important factor for HBV reactivation [56].

The incidence of hepatitis B reactivation among incidentally identified inactive carriers also varies in different geographical areas. In a study of 1241 inactive carriers from Taiwan, 211 (17.0 %) developed HBeAg negative CHB during a mean follow-up of 12.3 years, with the annual incidence of 1.4 % and the cumulative incidence of 20.2 % at 20 years [70]. Reactivation of hepatitis B occurred much more commonly during the first 5–10 years and became extremely rare after 20 years [70]. In other studies that enrolled a relatively small number of inactive carriers, the annual incidence of reactivation of hepatitis B varied from 0.4 % in Italy [71] and Greece [72] to 2.1 % in Japan [73]. However, in a more recent study of 85 inactive carriers from Greece, the cumulative incidence of HBeAg negative CHB was 24 % at 4 years [74]. The reason for such a high rate of HBV reactivation remains unclear.

Factors predictive for hepatitis B reactivation following HBeAg seroconversion include male gender [75], genotype C HBV (>genotype B) [75], genotype D HBV (>genotype A) [54], HBV-DNA levels >2000 IU/mL [76] or $\geq 10^5$ copies/mL [73] and HBV DNA $>10^4$ copies/mL at 1 year after HBeAg seroconversion [77]. Age of HBeAg seroconversion <30 years is associated with a particularly low incidence of HBV reactivation [75]. In addition, ALT levels >5 × ULN during the immune clearance phase and age of HBeAg seroconversion >40 years are also associated with increased risk of hepatitis B reactivation [56, 75]. The latter findings suggest that HBV is more likely to reactivate if more vigorous immune-mediated hepatocytolysis or a more prolonged immune clearance phase is needed to clear the virus.

Recent studies have shown improved diagnostic accuracy by combined HBsAg and HBV DNA measurements to predict hepatitis B reactivation in inactive carriers: HBsAg >1000 IU/mL and HBV-DNA >200 IU/mL [78], HBsAg levels >850 IU/mL and HBV DNA >850 IU/mL [79], or HBsAg levels >1000 IU/mL in HBeAg negative carriers with HBV DNA <2000 IU/mL [80]. The latter finding reported from Taiwan of genotypes B and C patients is in keeping with the results of an earlier study from Italy of genotype D patients, in which the combined single point quantification of HBsAg <1000 IU/mL and HBV-DNA ≤2000 IU/mL allows the identification of inactive carriers with a very high diagnostic accuracy (94.3 %) [81].

Patients with hepatitis B reactivation have a 20-fold increased risk of progression to cirrhosis as compared with those without [82]. The annual rates of progression to cirrhosis and HCC were 2–3 % and 0.5 %, respectively, in patients with hepatitis B reactivation, significantly higher than 0.1 % and 0.2 %, respectively, in those with sustained remission of hepatitis [44, 82]. Notably, among patients with hepatitis B reactivation, the incidence of cirrhosis is significantly higher in males and in those with age of reactivation older than 40 years [82]

Spontaneous HBsAg Seroclearance

Rates and Predictive Factors

During the low replicative inactive phase, serum HBsAg may disappear (HBsAg seroclearance) spontaneously. Short-term studies showed that the annual incidence of HBsAg seroclearance was 1–2 % in Caucasian carriers, and even lower (0.1–0.8 %) in carriers from the high-prevalence areas [83, 84]. However, a recent long-term follow-up study from Taiwan showed that the incidence of HBsAg seroclearance was appreciably high with an overall annual incidence of 1.2 %, being higher (1.8 %) in those >50 years than in those <30 years (0.8 %), and a cumulative incidence of 8 % at 10 years, increasing disproportionately to 25 % at 20 years, and 45 % at 25 years of follow-up [85].

Factors significantly associated with HBsAg seroclearance include older age [54, 64, 66, 83, 85–87], normal ALT levels [85, 86], HBeAg negativity [66, 83, 86], low viral load (<300 copies/mL) [87], genotype A HBV (> genotype D) [54], or genotype B HBV (> genotype C) infection [88], sustained remission of hepatitis [85], presence of cirrhosis [83] and HCV superinfection [89]. Among these, advanced age is the most constant and important predictor for HBsAg seroclearance [90]. The annual incidence of HBsAg seroclearance varies among different series, but correlates significantly with the mean or median age of patients at enrollment of each cohort [90]. The median age of HBsAg seroclearce in three large cohorts from Taiwan [85], Hong Kong [91] and Japan [92] is approximately 50 years (range, 48–51). Given that the mean or median age of HBeAg seroconversion in Asian adult carriers ranges from 30 to 35 years, it can be expected that sustained remission of hepatitis for a mean of 15 years after HBeAg seroconversion is required to achieve subsequent HBsAg seroclearance. Of note, HBsAg seroclearance can occur sometimes in carrier children, albeit at a relative low rate (0.58 % per year during a mean follow-up of 20.6 years), usually after age 15 (mean, 17.7 ± 7.8; range, 4.1–33.0) and is more common in those with non-carrier mother [93]. Interestingly, in one case–control study, carriers with HBsAg seroclearance had significantly higher body mass index and higher degrees of fatty liver than those without [94]. Furthermore, the mean age of HBsAg seroclearance is significantly younger in patients with fatty liver than in those without (48.7 years vs. 53.4 years) [95]. Notably, in two large cohort studies, fatty liver [96] and obesity [87] were independent factors significantly associated with HBsAg seroclearance. The underlying mechanism by which fatty liver enhances HBsAg seroclearance remains unclear.

Table 11.2 HBsAg quantitation in predicting HBsAg seroclearance

A single point HBsAg level <100 IU/mL in HBeAg negative patients predict HBsAg seroclearance over time [97]
HBsAg level <100 IU/mL and HBV DNA <200 IU/mL at 1 year after HBeAg seroconversion correlate HBsAg seroclearance within 6 years [98]
HBsAg level <10 IU/mL in HBeAg negative carriers with HBV DNA <2000 IU/mL correlates both 5–year and 10–year HBsAg seroclearance [99]
HBsAg levels <200 IU/mL plus >1 $\log_{10}$ IU/mL decrease in preceding 2 years predicts HBsAg seroclearance at 1 and 3 years [100]
HBsAg levels <200 IU/mL or annual decrease of >0.5 $\log_{10}$ IU/mL predict HBsAg seroclearance within 3 years [101]
HBsAg <1000 IU/mL and annual decrease of ≥ 0.3 $\log_{10}$ IU/mL predict HBsAg seroclearance over time [78]

HBeAg hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBV* hepatitis B virus

More recent studies have investigated both absolute and serial changes of serum HBsAg levels in predicting HBsAg seroclearance [78, 97–101], as summarized in Table 11.2. Of these, two Asian studies used an HBsAg level <100 IU/mL as a remote (6–10 years) predictor of HBsAg seroclearance [97, 98]. For short-term prediction, a study from Taiwan has shown that HBsAg level <200 IU/mL plus >1 $\log_{10}$ IU/mL reduction in preceding 2 years can predict HBsAg seroclearance at 1 and 3 years [100]. Another study from Hong Kong has also shown that HBsAg <200 IU/mL or an annual reduction of >0.5 $\log_{10}$IU/mL is predictive for HBsAg seroclearance within 3 years [101].

Virological, Clinical and Histological Profiles After HBsAg Seroclearance

Only 17 % have detectable antibody against HBsAg (anti-HBs) within 1 year after HBsAg seroclearance, but the rate of anti-HBs seroconversion increases to 56 % after 5 years and 76 % after 10 years. Virtually all test negative for HBV DNA by hybridization assays after HBsAg seroclearance, but in some HBV DNA still can be detected by PCR-based assays. The persistence of low-level viremia after HBsAg seroclearance might be a potential source of HBV transmission through blood transfusion or transplantation and account for HBV reactivation with chemotherapy or immunosuppression. In a recent investigation using commercially available, ultrasensitive real-time PCR assay, HBV viremia was detectable in 24 % within 1 year after HBsAg seroclearance, and low-level HBV viremia persisted in ~15 % up to >10 years after HBsAg seroclearance [102]. Serum levels of HBV DNA all are below the sensitivity of hybridization assays (<100 IU/mL in 86 % and 121–2770 IU/mL in 14 %).

Despite the extremely low viremic states, 5–18 % of patients have abnormal ALT levels after HBsAg seroclearance. Non-HBV-related etiologies of abnormal ALT levels can be identified in 75–100 % of such cases, with fatty liver, alcoholism

and herbal medicine administration being the most common [90]. In addition, HCV might have displaced HBV to cause continuing ALT elevation and hepatitis activities [103].

Most patients with liver histological assessment after HBsAg seroclearance have only mild necroinflammation and no significant fibrosis. Immunostaining for HBsAg and HBcAg in liver is negative in all patients; however, all patients tested still harbor HBV inside the liver, mainly in the form of cccDNA, up to 4 years after HBsAg seroclearance [88], albeit at a very low replicative level and in a transcriptionally inactive phase.

Long-term Outcome After HBsAg Seroclearance

In an early study in 55 patients with spontaneous HBsAg seroclearance from Taiwan by Huo et al. [104], 32.7 % developed serious complications, including HCC, cirrhosis, and hepatic failure during a mean follow-up of 23 months. This study probably overestimated the frequency with which complication occurs, as it included 20 patients who had hepatitis C virus (HCV) or hepatitis D virus (HDV) coinfection. In subsequent studies that enrolled a large series of patients from Taiwan and Japan, virtually none of non-cirrhotic patients without HCV or HDV superinfection developed HCC, hepatic decompensation, or liver related death during a mean follow-up of 5 years [92, 105], as summarized in Table 11.3. HBsAg seroclearance usually confers excellent long-term prognosis, provided that HBsAg loss occurred in the absence of concurrent viral infections, and preceded the development of cirrhosis. However, in patients who have preexisting cirrhosis or HCV or HDV superinfection, clinical outcomes of disease progression may still occur [92, 105–108]. A recent report from Hong Kong suggested that cumulative risk for HCC was higher in patients with HBsAg seroclearance at age ≥50 years compared with those with HBsAg seroclearance at age <50 [91]. However, the majority of their patients who developed HCC (6 out of 7) after HBsAg seroclearance had ultrasonographic evidence of cirrhosis before or at the time of HBsAg seroclearance. Of note, the mean age of HBsAg seroclearance in the series of Huo et al. [104] is also appreciably high (see Table 11.3). It is highly suspected that patients who achieved HBsAg seroclearance at older age may be more likely to have undiagnosed cirrhosis and hence remain at risk for HCC. Older age of HBsAg seroclearance per se cannot be considered as an independent risk factor for HCC development after HBsAg seroclearance.

A more recent prospective population-based cohort study in 1271 Alaska native persons with chronic HBV infection followed for an average of 19.6 years showed that the incidence of HCC after HBsAg seroclearance was 36.8 per 100,000 per year (95 % CI 13.5–80.0), which was significantly lower than that in those who remained HBsAg positive (195.7 per 100,000 per year [95 % CI 141.1–264.5]; $P<0.001$) [109]. This study is the first to show a significant reduction in the risk of developing HCC after HBsAg seroclearance.

Table 11.3 Long-term outcome following HBsAg seroclearance in chronic HBV infection

Status at seroclearance	Authors/countries	No. of patients	Mean age (years)	Follow-up (months)	Outcome		
					Cirrhosis	Decompensation	HCC
Non-cirrhosis	Huo [104]/Taiwan	55 (20)[a]	55	23	6 (?)[a]	1 (?)[a]	11 (?)[a]
Non-cirrhosis	Chen [105]/Taiwan	189 (43)[a]	43	65	3 (3)[a]	2 (2)[a]	1 (1)[a]
Non-cirrhosis	Arase [92]/Japan	164 (0)[a]	51	61	0	0	0
Non-cirrhosis	Ahn [107]/Korea	32 (0)[a]	NA	NA	0	NA	1
Non-cirrhosis	Tong [108]/USA	22 (?)[a]	NA	NA	NA	NA	0
Cirrhosis	Fattovich [106]/Italy	32 (5)[a]	NA	55		2 (?)[a]	1 (1)[a]
Cirrhosis	Chen [105]/Taiwan	29 (12)[a]	54	51		4 (2)[a]	1 (1)[a]
Cirrhosis	Arase [92]/Japan	67 (0)[a]	53	74		0	2
Cirrhosis	Ahn [107]/Korea	17 (0)[a]	NA	NA		NA	4
Cirrhosis	Tong [108]/USA	13 (?)[a]	NA	NA		NA	4
Non-cirrhosis and cirrhosis	Yuen [91]/Hong Kong	298 (0)[a]	50	36	NA	5	7 (6 had cirrhosis)
Non-cirrhosis and cirrhosis	Simonnetti [109]/USA	158 (0)[a]	NA	109	NA	NA	6 (2 had cirrhosis)

HBsAg hepatitis B surface antigen, *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma, *NA* not available

[a]Number in ( ) indicates number of patients with HCV or HDV superinfection

Concurrent Viral Infection as Part of Natural Course

In high-prevalence areas such as Taiwan, 50–60 % of adult patients hospitalized for overt acute hepatitis are previously unrecognized HBsAg carriers with reactivation of hepatitis B or non-B viral superinfection, as they are HBsAg positive but IgM anti-HBc negative [22]. Viral superinfection is demonstrated in as high as 30–40 % of these patients, with HCV and HDV being the most common [43]. Non-B viral superinfection in HBsAg carriers tends to increase the severity and case fatality rate during the acute phase. The incidence of fulminant hepatitis ranges from 10 to 20 %, without difference between HCV and HDV superinfection [43, 110].

Hepatitic C Virus

Worldwide, approximately 5–20 % of HBsAg carriers are found to be anti-HCV positive. In HBV endemic areas, HCV superinfection in the setting of chronic HBV infection is the most common scenario of HBV and HCV dual infection. Acute HCV infection in HBsAg carriers with serum HBeAg and HBV DNA may result in only transient HCV infection. In contrast, most acute HCV superinfection in HBsAg carriers without serum HBeAg and HBV DNA progress to persistent HCV infection [111]. These findings suggest that the presence of underlying active HBV replication may interfere with HCV replication and thereby inhibit the persistence of HCV infection.

Two studies from Taiwan showed that a substantial proportion of fulminant hepatitis in HBsAg carriers could be attributed to HCV superinfection [112, 113]. Another study showed that in patients admitted with acute HCV infection, the incidence of fulminant hepatitis was significantly higher among those with underlying HBV infection than those without (23 % versus 3 %, $P<0.01$) [114].

Most patients with HBV and HCV dual infection have detectable serum HCV RNA but not HBeAg or HBV DNA, suggesting that HCV is the predominant cause of liver disease in such cases [115]. More importantly, HCV superinfection is associated with earlier and more frequent progression to cirrhosis. In long-term follow-up analyses from the onset of acute HCV infection, patients with HCV superinfection had higher cumulative rates of cirrhosis (29 % at 5 years, 48 % at 10 years) and HCC (14 % at 10 years, 32 % at 20 years) than those with acute HDV superinfection or HBV mono-infection [110].

Finally, de novo HCV superinfection in HBsAg carriers may lead to a decrease in serum and liver HBV DNA levels and can result in HBeAg seroconversion and, in some cases, HBsAg seroclearance [103, 116]. Such patients had persistence of chronic hepatitis C after successful clearance of HBV [103].

Hepatitis D Virus

Hepatitis HDV is highly prevalent in the Mediterranean countries. The prevalence of HDV infection has significantly declined in some endemic areas, largely because of the HBV vaccination campaigns and the increased awareness on bloodborne infections following the HIV scare.

The clinical features of acute HDV coinfection are indistinguishable from acute hepatitis B [117], although it may be more severe and biphasic ALT peaks may be observed. The rate of progression to chronicity is the same as that of acute hepatitis B. On the contrary, HDV superinfection in HBsAg carriers more likely causes severe acute hepatitis, which progresses to chronicity in up to 80 % [117]. HBV replication is usually suppressed to low levels during acute HDV superinfection and this suppression becomes persistent when progresses to chronicity [118, 119]. Once chronic HDV infection is established, it usually exacerbates the preexisting liver disease due to HBV [120]. In Western studies as many as 70–80 % of chronic hepatitis D patients may develop cirrhosis within 5–10 years [121] and 15 % within 1–2 years [122]. Overall, the relative risk of developing cirrhosis in patients with chronic HDV infection is twofold that in patients with chronic HBV mono-infection [123]. In addition, among patients suffering from compensated HBV-related cirrhosis, there is a three- and twofold increase, respectively, of developing HCC and of death, compared with those with HBV mono-infection [124].

In Taiwan, the prevalence HDV infection is also decreasing [125]. HDV superinfection is associated with relatively milder disease, compared to the Western studies. In one longitudinal study, although HDV superinfection tended to accelerate the progression to cirrhosis relatively shortly after the onset of acute HDV superinfection (21 % at 5 years), the overall incidence of cirrhosis (21 % at 10 years) and HCC (7 % at 10 years) was similar to those with HBV mono-infection [110]. These apparent differences are probably related to the different geographic distribution of HDV genotypes, with genotype II being dominant in Taiwan and genotype I in the Western countries [126].

Human Immunodeficiency Virus

In HBV low-prevalence areas, the majority of the population is not protected by antibodies to natural HBV infection by the age of sexual maturity. Thus, HBV and HIV infections are confined to specific adolescent and adult risk groups, and exposure to both these viruses may occur at more or less the same time. About 10 % of HIV infected patients are coinfected with HBV. HBV tends to be more aggressive in HIV-positive individuals, with higher HBV carrier rates following acute exposure, higher levels of HBV viremia in chronic carriers, and diminished incidence of spontaneous seroclearance of HBeAg and/or HBsAg, more frequent episodes of activation, and faster progression to cirrhosis [127, 128]. HCC occurs more often, its onset is earlier, and its course is more aggressive in HBV and HIV coinfection than HBV mono-infection [129]. In a multicenter study involving 5293 homosexual men,

liver-related mortality was significantly higher in HIV and HBV coinfection (14.2 per 1000 person-years) than in HBV (0.8 per 1000 person-years) or HIV mono-infection (1.7 per 1000 person-years). In coinfected individuals, the liver related mortality rate was highest in those with lower nadir CD4+ cell counts and was twice as high after 1996, when highly active antiretroviral therapy (HAART) was introduced [130]. The development of effective antiretroviral regimen has led to immune reconstitution in many HIV-infected patients. The so-called immune reconstitution flare of hepatitis B has been observed in HBV and HIV coinfected patients following the initiation of HAART [131].

Conversely, most adolescents and adults in HBV high-prevalence areas are already protected from HBV infection or are chronic HBsAg carriers by the time of their first exposure to HIV infection. The rate of HBV coinfection in HIV positive individuals in Taiwan is 21.7 % [132], a little higher than the background HBsAg carrier rate (15–20 %) in the general population. The great majority of patients with HBV and HIV coinfection are presumed to be chronic HBsAg carriers with HIV superinfection. Interestingly, acute HIV superinfection in HBsAg carriers can suppress HBV replication and result in HBeAg seroclearance and, in some instances, HBsAg seroclearance [133]. It remained unclear whether this suppression is transient or persistent. The natural course difference between HBsAg carriers with and without HIV superinfection has rarely been addressed before. However, a higher risk of hepatitis flare, hepatic decompensation and liver-related death in HBV and HIV coinfection than in HIV mono-infection in the era of HAART was also reported [132].

Sequelae and Mortality

The long-term outcomes of chronic HBV infection vary considerably from an inactive carrier state to the development of cirrhosis, hepatic decompensation, and HCC. Contrary to patients in the immune tolerance phase and those in inactive carrier state, patients with active hepatitis either in the immune clearance phase or the reactivation phase are at high risk of disease progression. The estimated 5-year cumulative rates of progression from chronic hepatitis to cirrhosis, compensated cirrhosis to hepatic decompensation, and compensated cirrhosis to HCC are 8–20 %, 15–20 %, and 6–15 %, respectively (Fig. 11.2).

Cirrhosis and Contributing Factors

It is estimated that cirrhosis develops in approximately 20 % of patients with chronic HBV infection [1]. Whether this rate is higher in perinatally acquired infection because of the longer duration of infection than adult-acquired infection remains unknown. In one clinicopathologic study from Taiwan, cirrhosis was noted in 21 % of asymptomatic HBsAg carriers with age over 40 [134].

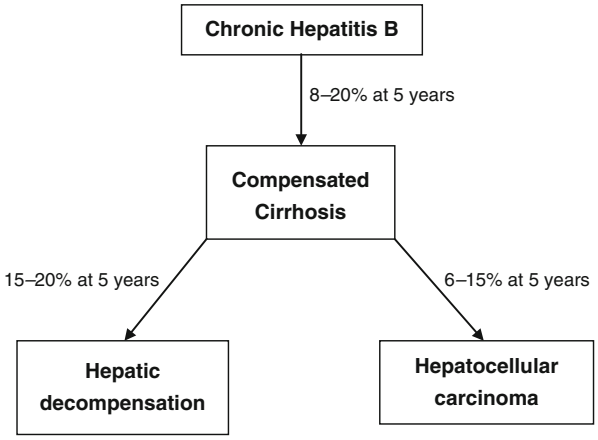

Fig. 11.2 Long-term sequelae of chronic hepatitis B virus infection

The annual incidence and cumulative probability of cirrhosis 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 the variable severity of liver injury in each phase. In two representative studies that enrolled 684 and 105 patients with chronic hepatitis B from Taiwan and Italy, the annual incidence of cirrhosis ranged from 2 to 6 % and the 5-year cumulative incidence ranged from 8 to 20 % [135, 136]. Factors identified to contribute to the development of cirrhosis include older age [135, 136], HBeAg positivity at recruitment [137, 138], persistent HBeAg seropositivity [57–59], persistence of HBV DNA by non-PCR assays [136, 139], HBeAg reversion [44, 66], delayed HBeAg seroconversion over age 40 [39, 55, 56], hepatitis B reactivation [39, 44], especially if reactivation at age over 40 [82], severe chronic active hepatitis with bridging hepatic necrosis [136], and ALT flares complicated with hepatic decompensation or recurrent AL T flares with high AFP or bridging hepatic necrosis [135]. HBV genotype is also a contributing factor [51, 52]. 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 cirrhosis [140] These data implies that the ultimate outcome of chronic HBV infection appears to depend on the duration of the immune clearance phase and reactivation phase, as well as on the severity of liver damage during these phases. Other factors significantly correlated with progression to cirrhosis include advanced age, longer duration of infection, male gender, and concurrent HCV, HDV or HIV superinfection, alcoholism and superimposed non-alcoholic fatty liver disease.

A recent population-based cohort study (the REVEAL-HBV) of more than 3500 untreated HBsAg carriers (median 45 years of age at enrolment, 85 % HBeAg negative, 94 % with normal ALT) in Taiwan found that the risk of cirrhosis increased significantly with increasing baseline serum HBV DNA levels at a dose-dependent manner [141]. The adjusted relative risks (RR) of progression to cirrhosis was 2.5,

5.6, and 6.5 when baseline HBV DNA levels were $\geq 10^4$, 10^5, and 10^6 copies/mL, respectively and HBV DNA levels $\geq 10^4$ copies/mL was the strongest predictor of future cirrhosis, regardless of HBeAg and ALT levels at baseline [141]. It seems likely that these patients are prone to have hepatitis B reactivation and progress to HBeAg negative CHB prior to cirrhosis development. A more recent study from Taiwan suggested that HBsAg levels greater than 1000 IU/mL in HBeAg negative carriers with low viral load (<2000 IU/mL) also tended to increase the risk of HBeAg negative CHB and cirrhosis [77].

Hepatic Decompensation

HBV replication and necroinflammation may have subsided at the onset of cirrhosis. However, at least 1/3–1/2 of patients with HBV-related cirrhosis still have a high level of HBV replication (positive HBeAg or HBV DNA by non-PCR assays, or HBV DNA $>10^5$ copies/mL) at presentation [140, 142]. About 3–4 % of the patients with compensated HBV-related cirrhosis developed decompensation (jaundice, ascites, hepatic encephalopathy) and/or gastroesophageal varices each year, with a 5-year cumulative incidence of 15–20 % [123, 142]. The risk of hepatic decompensation is fourfold higher in HBeAg or HBV DNA positive patients (4 % per year) than in HBeAg and HBV DNA negative patients (1 % per year) [142] . The average annual incidence of hepatic decompensation after the onset of cirrhosis is 1.5 %, but hepatic decompensation tends to occur later in the course, with the cumulative incidence of 5, 18 and 31 %, respectively, at 5, 10 and 20 years [140]. As the mean age at the onset of cirrhosis ranges from 41 to 44 years [140, 143] and that at the onset of decompensation ranges from 55 to 60 years, it is estimated that hepatic decompensation usually occurs 10–15 years after the onset of cirrhosis.

One form of hepatic decompensation in HBV-related cirrhosis is secondary to acute hepatitis flares. In two early studies from Taiwan, the annual incidence of acute hepatitis flare was 15–25 % in HBeAg positive patients and 5–10 % in HBeAg negative patients. Some 10–15 % of acute hepatitis flares were complicated with jaundice and 3–5 % with ascites [143, 144].

Hepatocellular Carcinoma and Contributing Factors

The incidence of HCC in chronic HBV infection correlates closely with the severity of the underlying liver diseases, as summarized by Fattovich et al. [123]. In the East Asian countries, the summary annual incidence of HCC ranges from 0.2 % among inactive carriers to 0.8 % in patients with CHB and 3.7 % in subjects with compensated cirrhosis; the corresponding 5-year cumulative incidences is 1, 3, and 17 %, respectively. In the Western countries, the summary annual incidence of HCC is 0.02 % in inactive carriers, 0.3 % in patients with CHB and 2.2 % in patients with compensated cirrhosis; the corresponding 5-year cumulative incidences is 0.1, 1, and 10 %, respectively. These data confirm that cirrhosis is a well documented risk

factor for HCC development and also suggest that perinatally acquired HBV infection is associated with a greater risk of HCC than infection acquired in adults, possibly because of the longer duration of infection.

Most cases of HCC are likely to have concomitant cirrhosis. Factors significantly predictive for progression to cirrhosis therefore also contribute to HCC development. Other factors significantly associated with HCC development in chronic HBV infection include race (Asians and Africans), a family history of HCC, HBV genotype, BCP mutations and pre-S deletion mutations, aflatoxin exposure and alcohol drinking [145–147]. In HBeAg negative carriers with low viral load, HBsAg levels >1000 IU/mL also is an independent risk factor for HCC development [148].

In the REVEAL-HBV study, the risk of HCC increased significantly starting at the level of 10^4 copies/mL and was highest for patients with the highest baseline HBV DNA level (>10^6 copies/mL) with a hazard risk of 2.3 and 6.6, respectively [149]. Unfortunately, this study did not report the prevalence of cirrhosis among HCC patients, so it is difficult to determine whether increased viral replication, known to encourage the development of cirrhosis, may have any additional impact on HCC development. The prognostic value of HBV replication for the development of HCC in patients with HBV-related cirrhosis remains controversial [140, 143, 150–152]. A recent case–control study did not show significant difference in serum levels of HBV DNA between HBV-related cirrhosis with and without HCC [153].

Finally, although inactive carriers with HBV DNA <10^4 copies/mL and normal ALT are at lowest risk for HCC among chronic HBV infected individuals, they still have a substantial risk of HCC as compared with HBV and HCV negative controls. The multivariate-adjusted hazard ratio is 4.6 (95 % CI: 2.5–8.3). Older age and alcoholism are independent predictors of risk for inactive carriers [154].

Survival

The 5-year survival of compensated, Child-Pugh class A HBV-related 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 non-PCR assays but only 60–72 % in HBeAg or HBV DNA positive patients [142]. Among the latter, HBeAg seroclearance is associated with a 2.2-fold decrease in mortality [155] and ALT normalization is a better predictor of improved survival than HBeAg seroclearance [156].

Once hepatic decompensation has developed, survival probability decreases remarkably. The reported 5-year survival rates of decompensated, Child-Pugh class B or C HBV-related cirrhosis vary considerably from 14 to 88 % (average, 30–50 %) [142]. In one study from Hong Kong, the 5-year survival rate was significantly lower in patients with serum HBeAg at presentation (57 %) than in HBeAg negative patients (88 %) [157]. In contrast, in another study from the Netherlands, the 5-year survival rate was extremely low (14 %) independent of serum HBeAg at enrollment

[155]. These data may suggest that survival probability correlates significantly with the status of HBV replication in patients with less severe hepatic decompensation but not in patients with more severe hepatic decompensation.

Conclusion

The natural history of HBV infection and the estimated overall and annual incidence of each event are summarized in Fig. 11.3. HBV replication with subsequent interactions between HBV, hepatocytes and immune cells during the immune clearance or reactivation phase may lead to hepatitis activity and disease progression. High HBV DNA levels and hepatitis activity at enrollment or during follow-up are the best predictors of adverse clinical outcomes. Sustained reduction of HBV replication before the onset of cirrhosis confers a favorable outcome. Sustained reduction of HBV replication in cirrhotic patients also reduces the risk of hepatic decompensation, HCC development and improves survival. The improvements in the knowledge of the natural history of HBV infection and a detailed understanding of predictors for disease progression will help in the management of patients with chronic HBV infection.

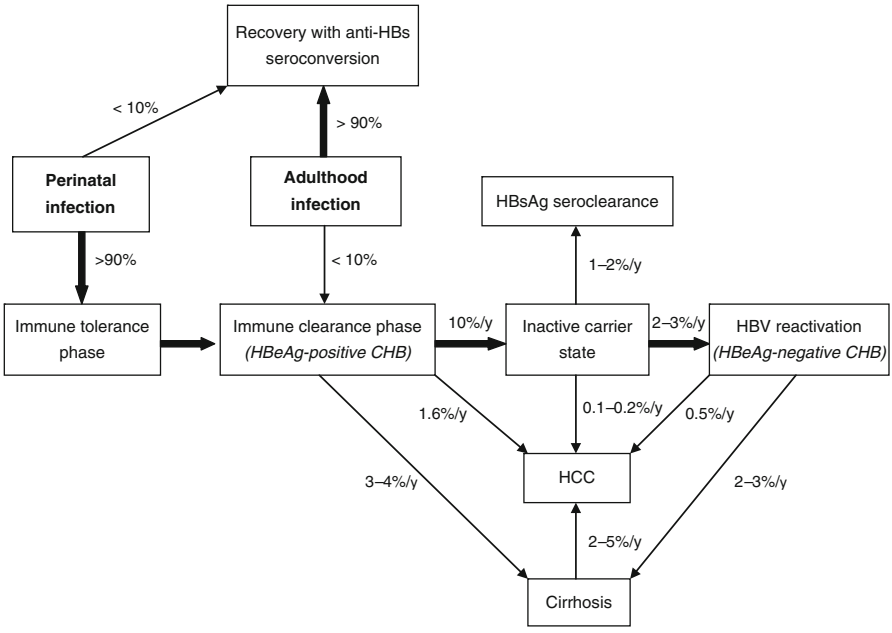

Fig. 11.3 Natural history of chronic hepatitis B virus infection. The overall rates as well as the annual rates of each event are included

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Chapter 12
Natural History of HBV Infection in the Community

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Introduction

The natural history of a disease is the course a disease takes in individual people, from its pathological onset, until its eventual resolution through complete recovery or death [1]. As the pathological onset of a disease is not easy to define, the natural history of a disease may also be considered to start at the moment of exposure to causal agents [1, 2]. Knowledge of the natural history of disease is important for disease prevention and control. The natural history of a disease is composed of multiple steps that ultimately lead to the occurrence of end-stage disease and or death. In the multistage pathogenesis of a disease, there exists various driving (pushing or pulling) factors that work at different stages to promote either disease progression or regression.

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More importantly, there is significant individual variation in the driving factors and speed of disease progression/regression. Among victims exposed to causal agents in the environment, there is often a wide spectrum of disease severity, ranging from subclinical, to mild, moderate, severe, and even lethal. For many infectious diseases, there is a pyramid-like distribution of disease severity, with most infections being subclinical or mild, and severe and lethal cases only comprising the tip of the iceberg of all infected persons. The time interval for the transition from one disease stage to another also varies individually. Some patients will develop disease more rapidly and more severely than others, and such individual variation in disease severity and progression reflects a saying by Dr. William Osler, in which he stated that "Medicine is a science of uncertainty and an art of probability."

Findings of natural history studies may vary according to study design (cross-sectional vs. longitudinal), study setting (community vs. hospital), follow-up scheme (regularly or irregularly), health events (different stages in disease progression), examination methods (physical checkup, laboratory medicine, imaging, etc.) and repeated measurements of driving factors (initiators, promoters, progressors, and regressors) [3, 4]. It is not surprising to observe a wide variation in disease incidence rates or stage transition rates, or differences in the identification of risk predictors, and the derivation and validation of prediction models. Therefore, a community-based long-term prospective study of a large cohort with a wide range of disease severity that includes comprehensive repeated follow-up examinations is considered the best for elucidating the natural history of a disease.

The natural history of hepatitis B virus (HBV) infection has been extensively studied [5–10]. Despite significant differences in transition rates, determinants of disease progression, and risk prediction models between these studies, the consensus is that the majority of infections are acute and self-limited with persistent infection rates ranging from <5 to 90 % depending on age at infection, maternal hepatitis B surface antigen (HBsAg) and e antigen (HBeAg) serostatus, and immune competence of the infected individual. Among chronically infected persons, chronic hepatitis (indicated by elevated serum levels of alanine aminotransferase), fibrosis, cirrhosis, and liver cancer may progressively develop after a long period of subclinical infection.

This chapter will review community-based studies of the natural history of HBV infection. Compared to individuals recruited from hospitals or clinics, community-based study participants are usually asymptomatic or only affected with mild chronic hepatitis B at enrollment. It will therefore be feasible to estimate transition rates and identify major driving factors of chronic hepatitis B progression starting from early stages in its natural history. The majority of data in this chapter is derived from the REVEAL-HBV study because of its large sample size, long-term follow-up, comprehensive questionnaire interview on lifestyle demographics, medical history and family history, regular examinations with serological tests and abdominal ultrasonography, repeated collection of biospecimens, and computerized data linkages with national cancer and death registries [11–14].

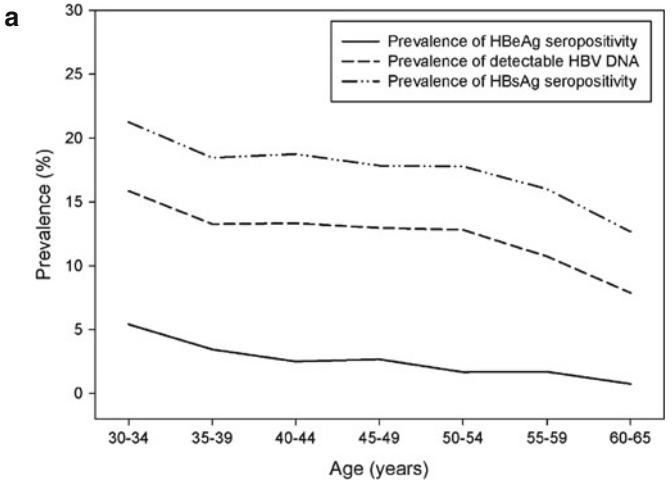
An Overview of the Natural History of HBV Infection: Probabilities and Determinants

In an early epidemiological study from Taiwan, where HBV infection was hyperendemic in the general population before 1980, the probability of becoming a chronic carrier in neonates born to mothers who were seropositive for HBsAg and HBeAg was as high as 30 and 90 %, respectively [15]. Vertical (perinatal) transmission plays an important role in maintaining a high prevalence of HBV infection in infants. Although early childhood horizontal transmission of HBV through iatrogenic exposure also results in chronic HBV infection, the probability of chronicity is lower than that of vertical transmission. Acute and chronic HBV infections in infants and preschool children are usually asymptomatic and self-limited. However, a very small proportion of them may develop fulminant hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Recent studies have shown that the national hepatitis B vaccination program launched in 1984 has significantly reduced the risk of fulminant hepatitis and chronic liver diseases including cirrhosis and hepatocellular carcinoma in Taiwan [16–18].

In an early survey in Taiwan [19], an unvaccinated population had an HBV infection prevalence as high as 80 % in young children, that plateaued at 90 % in early adulthood, with a peak HBsAg seroprevalence of around 20 % at ages 10–14 years old. In other words, more than 80 % of adults infected in childhood recovered from acute HBV infection, while 20 % became chronic HBV carriers. Most preschool children with chronic HBV infection were seropositive for HBeAg with high serum levels of HBV DNA and HBsAg immediately after infection. The prevalence of HBeAg among children with chronic HBV infection was around 85, by age 15 [20]. In a study of the natural history of childhood chronic HBV infection in Taiwan, the annual HBeAg seroclearance rate was as low as <2 % during the first 3 years of life, and later increased with age [20]. The seroclearance of HBeAg in children increased significantly for those who had elevated serum levels of alanine aminotransferase (ALT) and HBsAg-seropositive mothers. Information on the incidence and determinants of HBV DNA seroclearance in children or adolescents is still lacking.

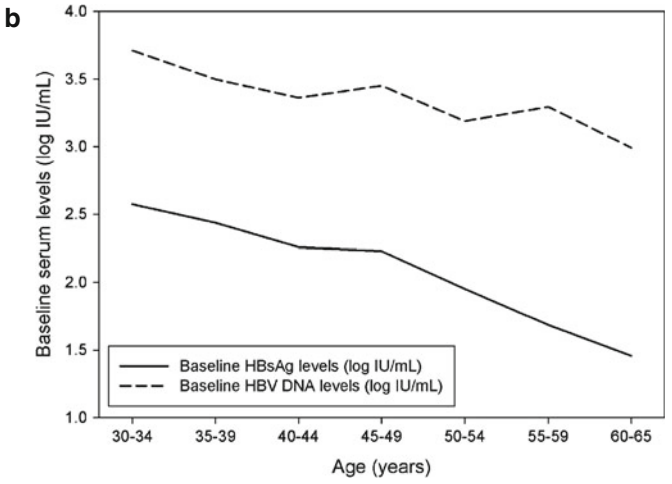
Based on age-specific baseline data from the REVEAL-HBV study in 1991, the seroprevalence of HBsAg declined steadily from 21 % at ages 30–34 years, to 13 % at ages 60–64 years, as shown in Fig. 12.1a. In addition, the HBeAg-seropositivity rate among chronic HBsAg carriers declined from 26 % (5.42 %/21.25 %) at ages 30–34 years, to 6 % (0.76 %/12.67 %) at ages 60–64 years. The detectability of serum HBV DNA levels (>60 IU/mL) also decreased with increasing age, with a prevalence of 15 % at ages 30–34 years, to 8 % at ages 60–64 years. In addition, mean serum levels of HBV DNA and HBsAg at study entry also decreased significantly with increasing age, as shown in Fig. 12.1b.

Lifetime cumulative risks of active hepatitis (as indicated by elevated serum ALT levels), cirrhosis, and HCC from 30 to 75 years old were also estimated in the REVEAL-HBV study. The cumulative lifetime risk was 67, 41, and 19 % for active



Prevalence (%) by Age

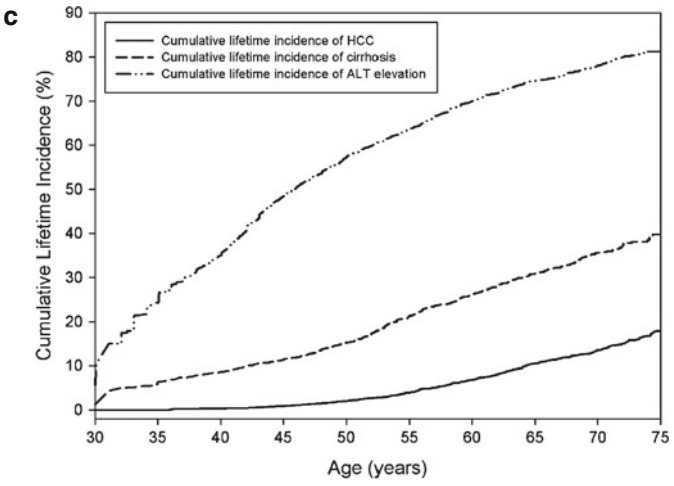
	30-34	35-39	40-44	45-49	50-54	55-59	60-65
HBsAg	21.25	18.46	18.75	17.84	17.78	16.01	12.67
Detectable HBV DNA	15.86	13.27	13.33	12.97	12.82	10.74	7.88
HBeAg	5.42	3.46	2.51	2.68	1.68	1.71	0.76



Mean (standard deviation) Serum Levels by Age

	30-34	35-39	40-44	45-49	50-54	55-59	60-65
HBV DNA (log IU/mL)	2.57(1.88)	2.43(1.76)	2.26(1.81)	2.23(1.76)	1.95(1.74)	1.69(1.94)	1.46(1.84)
HBsAg (log IU/mL)	3.71(2.04)	3.49(1.88)	3.36(1.77)	3.45(1.76)	3.19(1.59)	3.29(1.69)	2.99(1.54)

Fig. 12.1 (**a**) Prevalence of HBsAg, detectable HBV DNA, and HBeAg in the REVEAL-HBV cohort by age. (**b**) Mean serum levels of HBV DNA and HBsAg among HBsAg seropositives in the REVEAL-HBV cohort by age. (**c**) Cumulative lifetime incidence of abnormal serum ALT level, liver cirrhosis, and hepatocellular carcinoma among HBsAg seropositives in the REVEAL-HBV cohort by age



Cumulative Lifetime Incidence by Age (%)

	35	45	55	65	75
ALT Elevation	24.3	48.4	63.6	74.6	81.2
Cirrhosis	5.8	11.3	21.2	30.8	39.8
HCC	0	0.9	3.9	10.5	17.9

Fig. 12.1 (continued)

hepatitis, cirrhosis, and HCC, respectively, as shown in Fig. 12.1c. Milestones of chronic hepatitis B progression as well as the transition rates, determinants, and prediction models for the seroclearance of HBeAg, HBV DNA and HBsAg and the occurrence of active hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) will be reviewed below.

Milestones of Chronic Hepatitis B Progression

Chronic HBV infection (CHB) is comprised of dynamic interactions between HBV, hepatocytes, and the host immune system. CHB patients may achieve several milestones during the natural history of chronic HBV infection, as shown in Fig. 12.2. These milestones of CHB progression can be divided into two groups; one involves clinical phases that patients may experience, and the other involves parenchymal disease progressions such as the occurrence of cirrhosis and hepatocellular carcinoma (HCC).

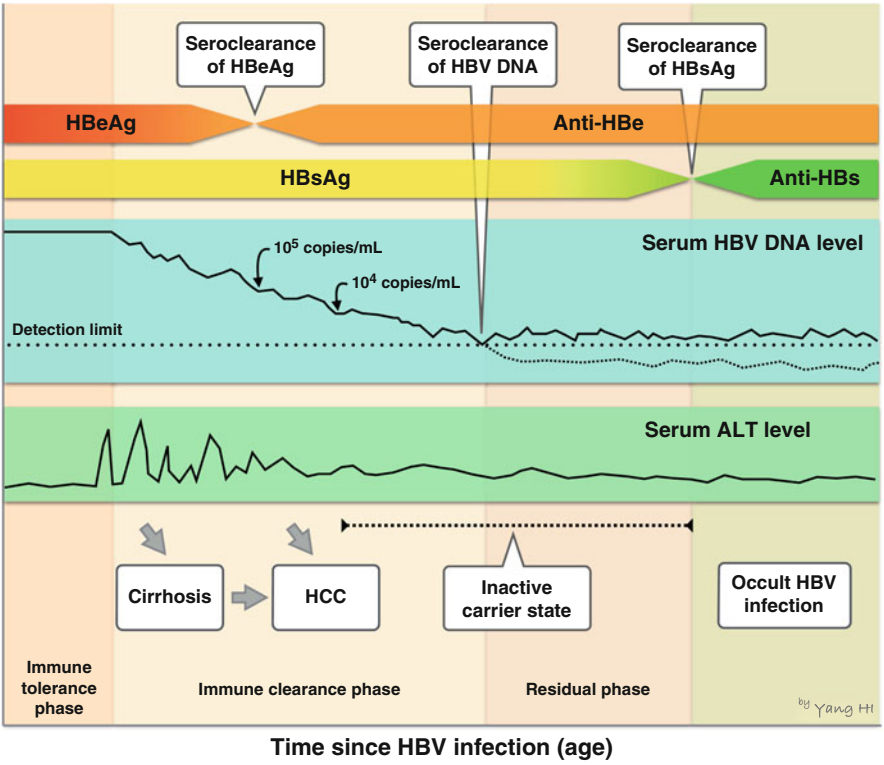


Fig. 12.2 Milestones of chronic hepatitis B infection

Traditionally, the natural course of perinatally acquired chronic HBV infection involves three chronological phases; the immune tolerance phase, the immune clearance phase, and the low-replicative residual integrated phase [8]. These phases can be defined based on a combination of serum markers such as HBeAg and its antibody, serum HBV DNA levels, HBsAg, and serum ALT levels. The immune tolerance phase is characterized by HBeAg-seropositivity, high HBV DNA levels, normal-ALT levels and no evidence of liver injury. Most of the liver injury occurs during the immune clearance phase as the host immune system tries to clear infected hepatocytes, which may result in the development of cirrhosis and HCC. This phase features inflammation of the liver, elevation of serum ALT levels, gradual reduction of circulating HBV DNA levels, and hopefully, seroconversion of HBeAg to its antibody (anti-HBe).

Finally, a proportion of infected persons are able to inactivate the replication of HBV and enter the residual phase. This phase is characterized by the continued presence of HBsAg, the presence of anti-HBe antibody, low/undetectable levels of serum HBV DNA, and normal ALT. A very small proportion of infected persons are then able to spontaneously clear HBsAg and resolve the infection. An inactive carrier

state can be identified during the late stages of the immune clearance phase and in the residual phase, where HBeAg is seronegative, serum HBsAg remains detectable, but serum HBV DNA levels are lower than 2000 IU/mL with repeatedly normal (or minimally raised) alanine aminotransferase levels. After the seroclearance of HBsAg, a small proportion of patients may also be identified as occult HBV infection, in whom low levels of HBV DNA can be detected by sensitive PCR assays in the serum and/or liver samples, despite HBsAg seronegativity.

The idea of these classifications is based on the reciprocal relationship between age, viral replication, and histological activity [21, 22], with the host immune response further underlying the segmentation. However, the boundaries between phases are poorly defined. For example, it is difficult to identify the point at which a given patient enters the immune clearance phase, or the point at which the patient enters the residual phase. Unless there are robust immunologic markers that can reflect the interaction between the host immune response and the virus, it would be difficult to detect transitions between phases.

Another approach to classifying the clinical phases in the natural history of CHB is based on seromarker changes, including the seroclearance of HBeAg, HBV DNA, and HBsAg [23–26]. The advantage of this approach is that these milestones can be detected through repeated measurements of these seromarkers. These seromarkers are highly associated with the risk of subsequent disease progression, and are important indicators of the efficacy of antiviral treatment.

Serological milestones and traditional clinical phases of CHB may be correlated. HBeAg seroclearance/seroconversion mostly occurs during the immune clearance phase, while the seroclearance of HBV DNA might be regarded as the end of the immune clearance phase and the beginning of the residual phase. The seroclearance of HBsAg can be the end of the residual phase and also the beginning of occult HBV infection. The development of end-stage liver disease such as cirrhosis and HCC mostly occurs during the immune clearance phase, but may also be associated with the length of time spent in certain clinical phases.

One important question in the natural history of CHB is the chronological order of the serological milestones. The REVEAL-HBV study has demonstrated that HBeAg seroclearance occurs first, followed by the seroclearance of HBV DNA, and then the seroclearance of HBsAg [23]. Through repeated measurements of HBeAg, HBV DNA levels, and HBsAg serostatus, it has been shown that at HBeAg seroclearance, only 11 % of REVEAL participants had undetectable serum HBV DNA levels, while most participants still had high levels of HBV DNA (median, 10^5 copies/mL). On the contrary, at HBV DNA seroclearance, almost all (98 %) participants had already cleared HBeAg prior to clearing HBV DNA from the serum. In addition, 96 % of participants had undetectable serum HBV DNA levels at the point of HBsAg seroclearance, leaving only 4 % patients with newly incident occult HBV infection [25]. This order of events is quite different than what occurs during antiviral therapy, where patients' HBV DNA levels are quickly suppressed to undetectable levels in HBeAg seropositive patients.

The courses of CHB that patients may experience are heterogeneous. The time spans of clinical phases and ages where milestone transitions occur can be very distinct

among individual patients. For example, some patients are still HBeAg-seropositive at age 70, while others may develop HCC at only 30 years of age. Therefore, it is important to predict what will happen and when in individual infected persons.

Transition Rates, Determinants, and Predictors of HBeAg Seroclearance

The presence of hepatitis B e antigen (HBeAg) in the serum usually indicates active viral replication of HBV in hepatocytes. Loss of detectable HBeAg, together with the emergence of antibodies against HBeAg, has been a key end point in the development of new antiviral treatment. It has been shown that among chronic hepatitis B (CHB) carriers, the incidence of HCC was 3.6-fold higher in carriers who were HBeAg seropositive (1169.4 per 100,000 person-years) than in those who were HBeAg seronegative (324.3 per 100,000 person-years). The cumulative incidence of HCC was significantly higher among those who were HBeAg seropositive than those who were HBeAg seronegative ($P<0.001$), and the relative risk of HCC was also higher in those with HBeAg seropositivity (60.2, 95 % CI = 35.5–102.1) than in those with HBeAg seronegativity (9.6, 95 % CI = 6.0–15.2) [11]. Moreover, in the natural history of CHB infection, the spontaneous or interferon alpha induced development of antibodies against HBeAg leads to improvement in clinical outcomes, such as lower risk for major liver complications [27] and a lower frequency of HCC development [28], suggesting that HBeAg is a useful marker for end stage liver diseases.

In children infected with HBV, the HBeAg seropositive rate in children under 15 years of age is 80–85 % [29]. Spontaneous HBeAg seroclearance rarely occurs before the age of 3. In most instances, HBeAg seroclearance occurs during adolescence and early adulthood [29]. In a long-term follow-up study from Taiwan, the annual HBeAg seroclearance rate was 4–5 % in children older than 3 years of age, and was <2 % in those under 3 years of age [30]. HBeAg seroclearance in children is determined by age and maternal HBsAg status. In another study, HBeAg seroclearance occurred in only 9.7 % of carrier infants under 3 years of age, and the HBeAg seroclearance rate was lower in infants whose mothers were seropositive for HBsAg than in those whose mothers had undetectable HBsAg (14.3 % vs. 35.3 %) [20]. Additionally, higher HBeAg seroclearance rates have been reported in children infected horizontally (44 %) than in those infected perinatally (24 %) [31].

The REVEAL-HBV study has shown an annual incidence rate of 61.6 per 1000 person-years for spontaneous HBeAg seroclearance (Table 12.1) [24]. Among individuals with high serum HBV DNA levels ($\geq 10^4$ copies/mL) at study entry, the cumulative lifetime incidence of spontaneous HBeAg seroclearance at 40, 50, 60, 70, and 74 years of age was 38.8, 69.4, 81.9, 89.1, and 95.5 %, respectively (Table 12.1) [23].

In multivariate analyses adjusted for age, gender, serum ALT levels, smoking, alcohol consumption, precore mutation, basal core promoter, HBV genotype, serum HBsAg levels, and serum HBV DNA levels, serum HBV DNA levels remained as a

Table 12.1 Transition rate and determinants of HBeAg seroclearance in the community

Annual incidence rate (per 1000)	61.6
Cumulative lifetime incidence from 30 to 74 years old	95.5 %
Hazard ratio for predictors[a]	Multivariate adjusted rare ratio [95 % CI]
Gender (Female vs. Male)	1.92 [1.29–2.85]
Serum ALT (≥45 vs. <45 U/L)	2.11 [1.40–3.18]
Serum HBV DNA level (copies/mL)	
10^6 to $<10^8$ vs. $\geq 10^8$	1.89 [1.28–2.78]
10^4–10^6 vs. $\geq 10^8$	3.27 [2.01–5.32]
HBV Genotype (B or B+C vs. C)	3.06 [2.11–4.44]
Precore mutant (1896G/A + mixed type vs. wild type)	1.66 [1.03–2.68]

[a]Multivariate analysis from Liu et al. [26]

significant determinant of HBeAg seroclearance. Compared to individuals with HBV DNA levels $\geq 10^8$ copies/mL, the multivariate-adjusted rate ratio (95 % CI) of HBeAg seroclearance was 1.89 (1.28–2.78) and 3.27 (2.01–5.32) for those with HBV DNA levels of 10^6 to $<10^8$, and 10^4 to $<10^6$ copies/mL, respectively (Table 12.1) [26]. In addition to serum HBV DNA levels, gender, serum ALT levels, precore mutation, and HBV genotype were also significantly associated with HBeAg seroclearance after multivariate adjustment [23, 26]. The multivariate-adjusted rate ratio (95 % CI) of spontaneous HBeAg seroclearance was 1.92 (1.29–2.85) for women compared to men; 2.11 (1.40–3.18) for baseline serum ALT levels of 45 or more compared to less than 45 U/L (45 U/L is the ULN for the REVEAL study); 1.66 (1.03–2.68) for the precore 1896 G/A mutant compared to wild type; and 3.06 (2.11–4.44) for HBV genotype B or B and C compared to genotype C (Table 12.1) [26].

A score-based prediction model and nomogram for HBeAg seroclearance was created by integrating the previously mentioned significant determinants. The prediction model's total score ranged from 0 to 7 as shown in Fig. 12.3a [26]. The AUROCs (95 % CI) for predicting the 5- and 10-year probability of HBeAg seroclearance were 0.85 (0.80–0.90) and 0.78 (0.73–0.83), respectively. The 5- and 10-year probabilities of HBeAg seroclearance ranged from 0.08 to 0.72 and from 0.23 to 0.98, respectively, providing a well-performing and clinically applicable tool for clinicians.

Transition Rates, Determinants, and Predictors of HBV DNA Seroclearance

Serum HBV DNA level is a marker of viral replication and antiviral treatment efficacy in CHB patients [32]. Previous studies have reported the association of serum HBV DNA levels with the development of liver cirrhosis and HCC. Follow-up studies have shown that among CHB carriers, the incidence of HCC

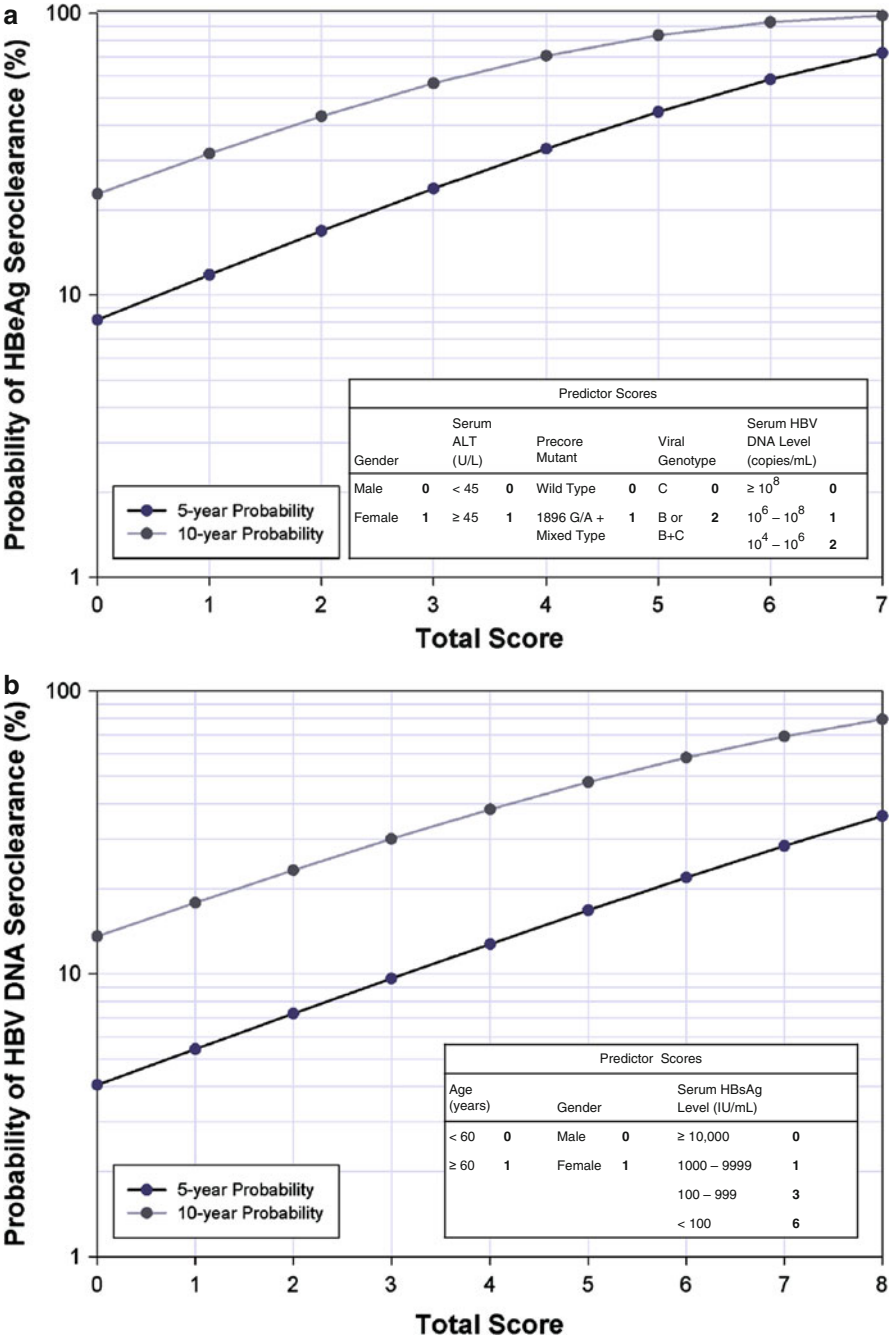
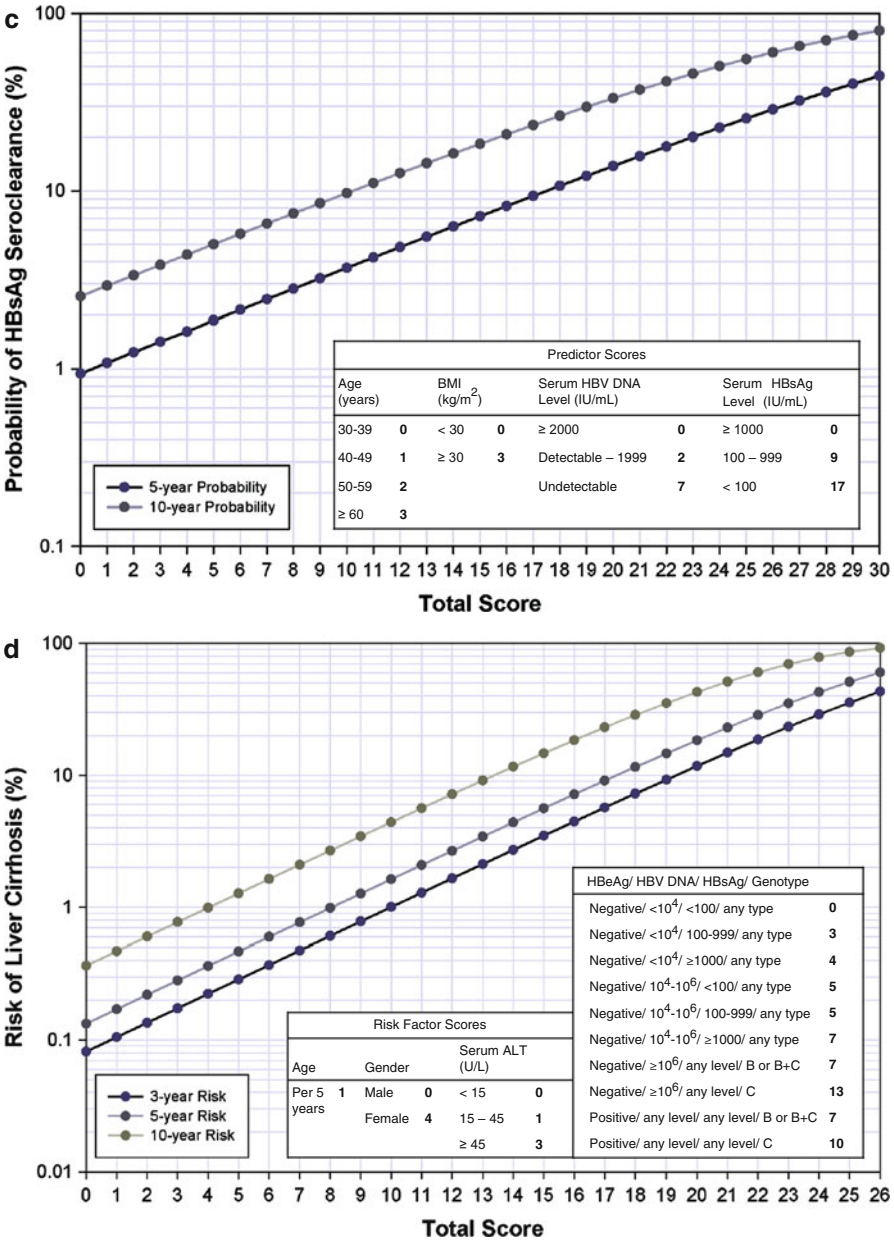


Fig. 12.3 (**a**) Nomogram for the prediction of 5- and 10-year probability of spontaneous HBeAg seroclearance in the REVEAL-HBV cohort. (**b**) Nomogram for the prediction of 5- and 10-year probability of spontaneous HBV DNA seroclearance in the REVEAL-HBV cohort. (**c**) Nomogram for the prediction of 5- and 10-year probability of spontaneous HBsAg seroclearance in the REVEAL-HBV cohort. (**d**) Nomogram for the prediction of 3-, 5-, and 10-year cumulative risk of liver cirrhosis in the REVEAL-HBV cohort. (**e**): Nomogram for the prediction of 3-, 5-, and 10-year cumulative risk of hepatocellular carcinoma in the REVEAL-HBV cohort



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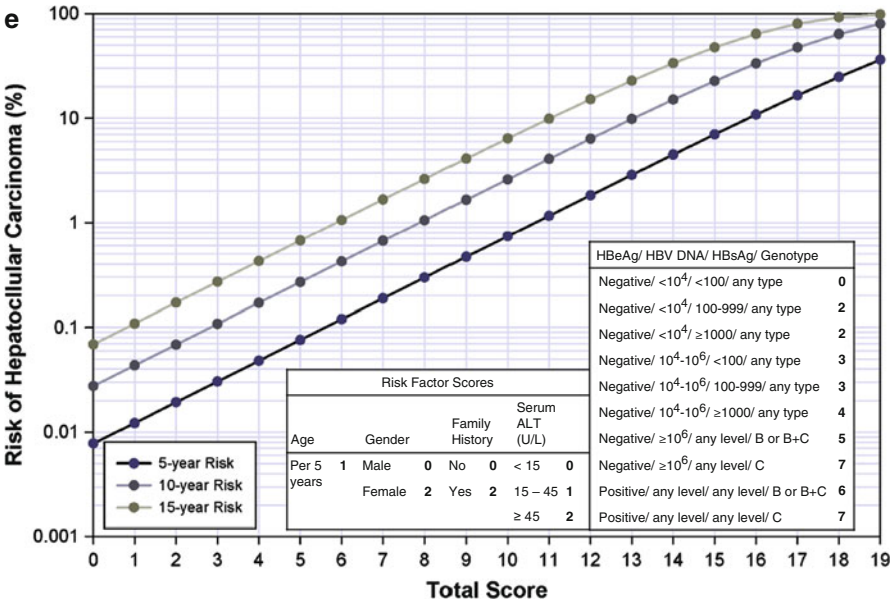

Fig. 12.3 (continued)

increased with serum HBV DNA levels at study entry in a dose–response relationship, with rates ranging from 108 per 100,000 person-years for serum HBV DNA levels of less than 300 copies/mL to 1152 per 100,000 person-years for serum HBV DNA levels of 10^6 copies/mL or greater [12]. The same dose–response relationship was also observed in the cumulative incidence of HCC, ranging from 1.3 % for serum HBV DNA levels of less than 300 copies/mL to 14.9 % for serum HBV DNA levels of 10^6 copies/mL or greater. The same pattern was observed in liver cirrhosis, with cumulative incidence ranging from 4.5 % for carriers with serum HBV DNA levels of less than 300 copies/mL to 36.2 % for carriers with serum HBV DNA levels of 10^6 copies/mL or more [13]. Compared to carriers with serum HBV DNA levels of <300 copies/mL, hazard ratios of developing HCC (adjusted for HBeAg, serum ALT levels, and liver cirrhosis) increased with serum HBV DNA levels [12]. A similar situation was also observed for the relative risk of liver cirrhosis progression, after adjustment for HBeAg and serum ALT levels [13]. These findings suggest that elevated serum HBV DNA levels are a prominent risk predictor of HCC independent of HBeAg status, serum ALT levels, and the presence of liver cirrhosis, and that progression to liver cirrhosis is strongly correlated with increasing serum HBV DNA levels, independent of HBeAg status and serum ALT level. Thus, the seroclearance of HBV DNA is an important milestone that signals an improved prognosis and lower rates of end-stage liver disease.

The REVEAL-HBV study showed an annual incidence rate of 30.1 per 1000 person-years for spontaneous HBV DNA seroclearance (Table 12.2) [26]. Among individuals with high serum HBV DNA levels ($\geq 10^4$ copies/mL) at study entry,

Table 12.2 Transition rate and determinants of HBV DNA seroclearance in the community

Annual incidence rate (per 1000)	30.1
Cumulative lifetime incidence from 30 to 77 years old	82.8 %
Hazard ratio for predictors	Multivariate adjusted rate ratio [95 % CI]
Age (≥60 vs. <60 years)[a]	1.35 [1.00–1.82]
Gender (Female vs. Male)[a]	1.37 [1.10–1.72]
Serum HBV DNA level (copies/mL)[b]	
10^4 to $<10^5$ vs. $\geq 10^6$	3.45 [1.73–6.91]
Serum HBsAg level (IU/mL)[a]	
10^3 to $<10^4$ vs. $\geq 10^4$	1.18 [0.61–2.27]
10^2 to $<10^3$ vs. $\geq 10^4$	2.49 [1.31–4.74]
$<10^2$ vs. $\geq 10^4$	6.18 [3.24–11.79]
Precore mutant (1896G/A + mixed type vs. wild type)[b]	0.55 [0.36–0.85]
HBV genotype (C vs. B or B + C)[c]	1.52 [1.21–1.91]

[a]Multivariate analysis from Liu et al. [26]
[b]Multivariate analysis from Yang et al. [23]
[c]Univariate analysis from Liu et al. [26]

the cumulative lifetime incidence of spontaneous HBV DNA seroclearance at 40, 50, 60, 70, and 77 years of age was 10.1, 25.0, 38.8, 54.2, and 82.8 %, respectively (Table 12.2) [23].

In multivariate analyses adjusted for age, gender, serum ALT levels, smoking, alcohol consumption, precore mutation, basal core promoter, HBV genotype, serum HBsAg levels, and serum HBV DNA levels, serum HBsAg level was a significant determinant of HBV DNA seroclearance. Compared to individuals with serum HBsAg levels $\geq 10^4$ IU/mL at study entry, the multivariate-adjusted rate ratio (95 % CI) of HBV DNA seroclearance was 1.18 (0.61–2.27), 2.49 (1.31–4.74), and 6.18 (3.24–11.79) for carriers with serum HBsAg levels of 10^3 to $<10^4$, 10^2 to $<10^3$, and $<10^2$ IU/mL, respectively (Table 12.2) [26]. In addition to serum HBsAg levels, age and gender were also significantly associated with HBV DNA seroclearance after multivariate adjustment. The multivariate-adjusted rate ratio (95 % CI) for HBV DNA seroclearance was 1.35 (1.00–1.82) for those ≥60 years compared to <60 years, and 1.37 (1.10–1.72) for women compared to men (Table 12.2) [26]. In a subset of carriers with high serum HBV DNA levels ($\geq 10^4$ copies/mL) at study entry regardless serum HBsAg level, serum HBV DNA level and precore mutation were also significant determinants of HBV DNA seroclearance. Multivariate-adjusted rate ratios (95 % CI) were 3.45 (1.73–6.91) for carriers with serum HBV DNA levels of 10^4 to $<10^5$ compared to those with serum HBV DNA levels $\geq 10^6$ copies/mL, and 0.55 (0.36–0.85) for precore 1896 G/A mutant compared to wild type (Table 12.2) [23]. HBV genotype was associated with HBV DNA seroclearance in univariate analyses with a rate ratio (95 % CI) of 1.52 (1.21–1.91) for genotype C compared to HBV genotype B or B and C (Table 12.2) [26].

According to the multivariate analyses from previous study including serum HBsAg levels, the addition of HBV genotype, serum HBV DNA level, and precore mutation did not improve the predictability of HBV DNA seroclearance [26]. Therefore, a score-based prediction model and nomogram for HBV DNA seroclearance was created by integrating only age, gender, and serum HBsAg levels, as shown in Fig. 12.3b. The total score of the prediction model for HBV DNA seroclearance ranged from 0 to 8. Predictive accuracy of the model was measured by the AUROC. The AUROCs (95 % CI) for predicting the 5- and 10-year probability of HBV DNA seroclearance were 0.77 (0.72–0.82) and 0.73 (0.70–0.76), respectively. The 5- and 10-year probabilities of HBV DNA seroclearance ranged from 0.04 to 0.36 and from 0.14 to 0.80, respectively [26].

Serum HBV DNA levels play a critical role during the transition between milestones of CHB progression. Although previous studies identified HBV DNA as the most important predictor of HBeAg, HBV DNA, and HBsAg seroclearance, recent studies have shown that serum HBV DNA level was no longer a significant predictor of HBV DNA seroclearance after taking serum HBsAg levels into consideration [26], indicating that serum HBsAg levels are the strongest predictor of HBV DNA seroclearance.

Transition Rates, Determinants, and Predictors of HBsAg Seroclearance

Previous studies have shown that HBeAg-seropositive patients are at increased risk for clinical endpoints such as hepatocellular carcinoma, and during clinical management of these patients, HBeAg seroconversion is an important milestone [11, 33]. For HBeAg-seronegative patients, HBsAg seroclearance has been well-documented as the most important clinical and treatment end point, as it leads to an improved prognosis, and confers lower rates of HCC and other clinical consequences [24, 33–35]. Previous community-based studies in Taiwan and Alaska examined prognoses of those who spontaneously cleared HBsAg. In a study among Alaskan natives, the incidence rates of HCC were significantly decreased in those with HBsAg seroclearance (36.8 per 100,000 person-years), when compared to those who remained HBsAg-positive (195.7 per 100,000 person-years) [35]. In another study from Taiwan using repeated measurements of seromarkers, reaching HBsAg seroclearance during follow-up was indicative of significantly decreased risk for developing HCC in the future [24]. Therefore, elucidating the determinants of HBsAg seroclearance is crucial to the clinical management of individuals with chronic hepatitis B infection.

However, the spontaneous seroclearance of HBsAg is quite rare. In highly endemic Taiwan, the annual incidence rate of spontaneous HBsAg seroclearance among untreated individuals in the community was 2.26 % per year, or 22.6 per 1000 person-years [25]. In a study of 3087 community-based individuals from the REVEAL-HBV cohort, HBsAg seroclearance was associated with female gender,

increasing age, increasing body mass index (BMI), ethnicity of mainland Chinese (versus Fukkienese), and decreasing serum HBV DNA levels [25]. After the introduction of quantitative HBsAg (qHBsAg) as a potential marker for immune response, the determinants of HBsAg seroclearance were reanalyzed [36].

In this study, which was further limited to 2491 HBeAg-seronegative individuals >30 years old, the cumulative lifetime incidence of spontaneous HBsAg seroclearance at ages 40, 50, 60, 70, and 77 among those with detectable serum HBV DNA (≥57 IU/mL) was 3.0, 14.4, 26.5, 42.6, and 62.1 %, respectively. On the other hand, the cumulative lifetime incidence of HBsAg seroclearance at ages 40, 50, 60, 70, and 77 among those with undetectable (<57 IU/mL) HBV DNA was 31.5, 56.7, 74.2, 89.1, and 98.8, respectively. In multivariate analyses, serum HBsAg levels were the strongest predictor of spontaneous HBsAg seroclearance. While serum HBV DNA level was still significant, its rate ratios decreased after adjustment for serum HBsAg levels. Compared to baseline serum HBsAg levels ≥1000 IU/mL, the multivariate-adjusted rate ratio (95 % CI) of spontaneous HBsAg seroclearance was 3.55 (2.51–5.02) and 10.96 (7.92–15.16), respectively, for those with serum HBsAg levels of 100–999 and <100 IU/mL. These results suggested that both serum HBsAg and HBVDNA levels should be considered during monitoring of chronic hepatitis B, as they provide complementary information. Other significant predictors included increasing age and BMI (Table 12.3) [36].

Using this model, a score-based prediction model and nomogram were developed, assigning integer scores to each predictor as shown in Fig. 12.3c. Using each individual's combined score, a 5 and 10 year probability of HBsAg seroclearance was calculated using equations derived from Cox Proportional Hazards models. This 30-point scale combining age, BMI, HBV DNA levels, and HBsAg levels, was able to predict 5 and 10 year probabilities of spontaneous seroclearance with AUROC's of 0.89 and 0.84, respectively. Therefore, this model was able to accu-

Table 12.3 Factors Determining Spontaneous HBsAg Seroclearance in the Community

Annual incidence rate (per 1000 person years)	22.6
Cumulative lifetime incidence from 30 to 77 years old	
Among those with detectable HBV DNA	62.1 %
Among those with undetectable HBV DNA	98.8 %
Determinants[a]	Multivariate adjusted rate ratio [95 % CI]
Age (every 1-year increase)	1.01 [1.01–1.02]
Body Mass Index (≥30 vs. <30 kg/m^2)	1.46 [1.02–2.08]
Serum HBV DNA levels (IU/mL)	
Detectable—1999 vs. ≥2000	1.31 [0.98–1.74]
Undetectable vs. ≥2000	2.57 [1.95–3.40]
Serum HBsAg levels (IU/mL)	
100–999 vs. ≥1000	3.55 [2.52–5.02]
<100 vs. ≥1000	10.96 [7.92–15.16]

[a]Multivariate analysis from Liu et al. [36]

rately estimate the probability of HBsAg seroclearance for different clinical profiles in the community, and showed that the addition of serum HBsAg levels to current HBV DNA-based models significantly improves the predictability of HBsAg seroclearance among genotype B and C HBeAg-seronegative individuals (Table 12.3) [36]. These results have also recently been externally validated among a hospital based cohort of 1934 untreated patients, in which the model still performed adequately well, and was well calibrated, even among patients with more severe disease [37].

Additional studies among a cohort of children followed-up into adolescence also found that children with serum HBsAg levels <1000 IU/mL had a much greater chance of clearing HBsAg (HR [95 % CI] = 5.23 [2.77–9.85]) [38]. In addition, there was a significant association between HBsAg seroclearance and maternal serostatus of HBsAg and HBeAg. In conclusion, the determinants of HBsAg seroclearance have been well established in the community, and will provide important direction and information for the clarification of prediction of HBsAg seroclearance among treated individuals.

Transition Rates, Determinants, and Predictors of Active Hepatitis

In the natural course of chronic hepatitis B infection, HBeAg-seronegative carriers represent a large majority of infected individuals. The severity of disease among HBeAg-seronegative individuals varies widely, and can include those who are either inactive, or active carriers [6, 33]. Inactive carriers are defined as HBeAg-seronegative individuals with serum HBV DNA levels <10,000 copies/mL (2000 IU/mL) and persistently normal ALT for 1 year, while active carriers are individuals with serum HBV DNA levels ≥10,000 copies/mL (2000 IU/mL) with persistently or intermittently abnormal ALT [33]. Previous studies have shown inactive carriers to have significantly improved survival, which is comparable with that of noninfected individuals [39]. In addition, in previous studies from the REVEAL-HBV cohort, inactive carriers also have significantly decreased risk for hard outcomes such as liver cirrhosis and hepatocellular carcinoma [12, 13, 40]. Thus, differentiating between active chronic hepatitis and inactive carriers is clinically meaningful, as it would allow the identification of a lower-risk population in need of less stringent follow-up, while, on the other hand, an earlier diagnosis of active hepatitis could lead to earlier initiation of antiviral therapy. However, accurate identification of active carriers is difficult, as ALT levels fluctuate and can often be affected by environmental exposures or nonviral factors. Moreover, studies examining inactive or active hepatitis among the community are rare, and the factors that can accurately differentiate the two are still relatively unknown.

Tohme et al. examined determinants and risk factors for reactivation of hepatitis B among a chronically infected community-based cohort of 414 Alaskan Native Persons who already had inactive hepatitis, with viral loads <2000 IU/mL, and persistently normal ALT for 1 year [41]. This study included individuals with viral

Table 12.4 Determinants of Active Hepatitis

Authors (year)	Tohme et al. (2013) [41]	Chu et al. (2007) [42]
Number of patients	414	113
HBV genotypes included	A, B, C, D, F	B, C
Age at study entry (years)		
30–39 (vs. 18–29)	0.34 (0.12–0.90)	–
40–49 (vs. 18–29)	0.20 (0.05–0.70)	–
>50 (vs. 18–29)	0.77 (0.33–1.77)	–
Gender (male vs. female)	2.41 (1.17–4.96)	2.99 (1.08–8.22)
Genotype	6.08 (1.32–28.0) [B vs. non-B]	3.75 (1.56–9.01) [C vs. B]
Maximal ALT during HBeAg positive phase (>5 ULN vs. <2 ULN)	–	3.57 (1.22–10.46)
Age at HBeAg seroconversion (≥40 vs. <40)	–	4.40 (1.69–11.36)
HBV DNA levels at study entry (IU/mL)		
29–199 (vs. <29)	2.51 (0.75–8.36)	–
200–999 (vs. <29)	2.28 (0.80–6.46)	–
1000–1999 (vs. <29)	7.61 (2.81–20.6)	–

genotypes A, B, C, D, and F, and reactivation was defined as HBV DNA ≥2000 IU/mL and ALT ≥40 U/L (Table 12.4). Over 2984 person-years of follow-up, 36 cases of reactivation occurred, for an annual incidence rate of 12 per 1000 person-years. In multivariate analyses, compared to individuals between 18 and 29 years old, those who were 30–39 or 40–49 years old had adjusted hazard ratios (HR [95 % CI]) of reactivation of 0.34 (0.12–90) and 0.20 (0.05–0.70), respectively. In addition, males, those with HBV DNA levels of 1000–1999 IU/mL (compared to HBV DNA <29 IU/mL), and genotype (compared to genotype) were significant predictors of hepatitis B reactivation, with adjusted hazard ratios (95 % CI) of 2.41 (1.17–4.96), 7.61 (2.81–20.6), and 6.08 (1.32–28.0), respectively [41].

In another community-based study in Taiwan, Chu et al. followed 113 asymptomatic HBeAg-seronegative individuals to elucidate factors predicting reactivation of hepatitis B (Table 12.1) [42]. In this cohort consisting of genotype B and C individuals, reactivation occurred with an annual incidence rate of 33 per 1000 person-years. In this study, compared to females, males also had higher risk of reactivation (HR [95 % CI] = 2.99 [1.08–8.22], while genotype C also had higher rates of reactivation than genotype B (HR [95 % CI] = 3.75 [1.56–9.01]). Moreover, compared to those with ALT levels <2 × ULN, those with ALT levels >5 × ULN had adjusted hazard ratios (95 % CI) of 3.57 (1.22–10.46), while individuals with HBeAg seroconversion at ≥40 years old also had higher rates of reactivation (HR [95 % CI] = 4.40 [1.69–11.36]) (Table 12.4) [42].

Recent studies have investigated the role of quantitative hepatitis B surface antigen (qHBsAg) in the natural history of chronic hepatitis B infection, including its role in differentiating inactive and active hepatitis B carriers. Two studies conducted among clinic-based patients attempted to use single-point measurements of qHBsAg and HBV DNA to differentiate inactive from active carriers [43, 44]. In a study

among 209 genotype D carriers in Italy, a one-time measurement of HBV DNA <2000 IU/mL and qHBsAg <1000 IU/mL could accurately differentiate inactive from active carriers with a sensitivity, specificity, and diagnostic accuracy of 91.1, 95.4, and 94.3, respectively [43]. In a second study among 129 patients with genotypes A–E, a one-time measurement of HBV DNA >200 IU/mL and qHBsAg >1000 IU/mL differentiated inactive from active carriers with a sensitivity and specificity of 92 and 51 %, respectively [44]. However, these results have not yet been validated in the community.

From the limited studies available among community-based populations, age, gender, ALT, and HBV DNA levels are significant factors that can predict the risk of hepatitis B reactivation. However, the role of qHBsAg in defining inactive carriers is still unclear, and remains to be confirmed among untreated community-based populations. Moreover, studies among younger individuals are still lacking. Further studies among larger cohorts that incorporate all of these factors, or among cohorts with a wider age range are still needed, and will significantly impact clinical management of patients with chronic hepatitis B.

Transition Rates, Determinants, and Predictors of Cirrhosis

Chronic HBV infection is well known to cause progressive liver disease. Globally, at least one third of liver cirrhosis cases can be attributed to HBV infection [45]. The annual incidence of cirrhosis among asymptomatic HBV carriers was 0.7 % [46]. In a study of 1400 Alaskan natives seropositive for HBsAg, 824 men and 576 women of all ages were followed prospectively. After liver biopsy confirmation, 8 cases of cirrhosis were found, with an incidence rate of 107 per 100,000 person-years in men and 95 per 100,000 person-years in women [47]. However, community-based studies evaluating the risk factors for cirrhosis among HBV carriers are limited. Previously reported risk factors for liver cirrhosis among HBV carriers include advanced age, male gender, HBeAg serostatus, elevated ALT levels, serum HBV DNA levels, serum quantitative HBsAg levels, and HBV genotype [6, 13, 46, 48].

Over an average of 11 years of follow-up, the REVEAL-HBV study identified 365 individuals with newly diagnosed liver cirrhosis, giving an incidence rate of 912 per 100,000 person-years. The incidence of cirrhosis increased with elevated serum HBV DNA levels at study entry; HBV carriers with serum HBV DNA levels <300, 300–9999, 10,000–99,999, 100,000–999,999, and ≥1,000,000 copies/mL had cirrhosis incidence rates of 339, 430, 774, 1879, and 2498 per 100,000 person-years, respectfully [13]. After adjustment for age, gender, cigarette smoking and alcohol drinking, HBeAg serostatus, and serum ALT levels at study entry, the relative risks (95 % CI) for cirrhosis were 1.4 (0.9–2.2), 2.5 (1.6–3.8), 5.6 (3.7–8.5), and 6.5 (4.1–10.2) for individuals with serum HBV DNA levels 300–9999, 10,000–99,999, 100,000–999,999, and ≥1,000,000 copies/mL respectively, using serum HBV DNA levels <300 copies/mL as a comparison group. The dose–response relationship between serum HBV DNA levels and liver cirrhosis risk was still seen

even after stratification by sex (female or male), age (≤50 or >50 years old), cigarette smoking (no or yes), and alcohol consumption (no or yes). In addition to the incidence of liver cirrhosis, serum HBV DNA levels were also an important determinant of death from chronic liver disease and cirrhosis [49, 50]. The relative risks (95 % CI) for mortality from chronic liver diseases and liver cirrhosis was 1.0 (referent), 5.3 (0.7–43.5), 7.6 (0.9–63.1), 11.1 (1.3–94.4), and 15.6 (1.8–134.7) for serum HBV DNA levels of <300, 300–9999, 10,000–99,999, 100,000–999,999, and ≥1,000,000 copies/mL, respectively. In addition, quantitative serum HBsAg levels and associated risks for liver cirrhosis were also evaluated in the REVEAL-HBV study [48]. After long-term follow-up, the cumulative lifetime risk (30–75 years of age) for liver cirrhosis was 11.4, 23.3, and 36.8 % for individuals with serum HBsAg levels of <100, 100–999, and ≥1000 IU/mL, respectively. The multivariate adjusted relative risks (95 % CI) for liver cirrhosis were 1.68 (1.12–2.54) and 2.20 (1.48–3.27) for serum HBsAg levels of 100–999 and ≥1000 IU/mL, when compared to those with serum HBsAg levels <100 IU/mL (p for trend <0.001). To further evaluate quantitative HBsAg levels, serum HBV DNA levels, and risk for liver cirrhosis, the relative risks for cirrhosis for individuals with various combinations of HBV DNA and HBsAg levels were estimated. The multivariate-adjusted relative risks (95 % CI) were 1.0 (referent), 2.21 (1.28–3.80), 2.52 (1.48–4.29), 1.96 (0.80–4.79), 3.52 (2.08–5.94), 5.71 (3.56–9.16), and 7.84 (4.62–13.30) for the combinations of HBV DNA (copies/mL) and HBsAg (IU/mL) of $<10^4$/<100, $<10^4$/100–999, $<10^4$/≥1000, 10^4 to $<10^6$/<100, 10^4 to $<10^6$/100–999, 10^4 to $<10^6$/≥1000, and HBV DNA $\geq 10^6$ copies/mL, respectively. Particularly for individuals with serum HBV DNA levels lower than 10^6 copies/mL, serum levels of HBsAg could further predict long-term incidence of liver cirrhosis. In addition to serum HBsAg and HBV DNA levels, both HBV genotype and mutant types were also significantly associated with the risk of cirrhosis. The multivariate-adjusted relative risk (95 % CI) of cirrhosis was 1.9 (1.5–2.3) for HBV genotype C (vs. genotype B), 0.5 (0.3–0.6) for precore G1896A mutant (vs. wild type), and 1.9 (1.4–2.5) for basal core promoter A1762T/G1764A double mutant (vs. wild type) [6].

A noninvasive score-based risk prediction model for 3-, 5-, and 10-year risk of liver cirrhosis was developed by incorporating host and virus profiles as shown in Fig. 12.3d [48]. This risk prediction model included age, gender, serum ALT levels, HBeAg serostatus, serum HBV DNA and HBsAg levels, and HBV genotype, and risk scores were assigned to each category of relevant risk factors associated with liver cirrhosis. To use this risk prediction model in a clinical setting, patients should first be tested for HBeAg serostatus. For HBeAg seronegatives, serum HBV DNA levels should be further examined. If the patients have serum HBV DNA levels $<10^6$ copies/mL, then quantitative HBsAg levels should be tested to further stratify their risk for liver cirrhosis. On the other hand, for patients seropositive for HBeAg or with serum HBV DNA levels $\geq 10^6$ copies/mL, HBV genotype may be helpful for further risk stratification. This scoring system has total risk scores ranging from 0 to 26, and predictive accuracy for 3-, 5-, and 10-year risk was 0.86, 0.86, and 0.83, respectively. When this risk prediction model was validated internally, the predictive accuracy was 0.79, 0.80, and 0.82 for the prediction of 3-, 5-, and 10-year liver cirrhosis.

Transition Rates, Determinants, and Predictors of Hepatocellular Carcinoma

A previous large cohort study of 22,707 Taiwanese men who were followed up for 3.3 years (75,000 person-years) found that individuals seropositive for HBsAg had a 223-fold increased risk of developing hepatocellular carcinoma (HCC) [5]. The risk and presence of HCC in hepatitis B carriers may depend on multiple risk factors [51, 52]. Older age, male gender, alcohol consumption, the presence of cirrhosis, elevated serum ALT levels, family history, metabolic factors, and obesity were associated with increased risk for HCC [12, 53–60]. Those coinfected with hepatitis C virus are at higher risk of developing HCC than those who are infected by HBV only [61].

Hepatitis B viral factors play important roles in hepatocarcinogenesis. A previous study found that compared with HBV carriers negative for HBeAg, those with HBeAg seropositivity had increased risk of developing HCC [11]. The increased HCC risk associated with HBeAg seropositivity remained significant even after stratification analyses by age, gender, cigarette smoking and alcohol consumption, serum ALT levels and liver cirrhosis status [11], implying that active viral replication was a relevant determinant for HCC. A further nested case–control study showed a significant dose–response relationship between serum HBV DNA levels and the risk of HCC [11]. By using serum HBV DNA levels <2.5 pg/mL as a reference group, the odds ratios of HCC risk were 2.3 and 6.0, respectively, for serum HBV DNA levels of 2.5–13.0 and >13.0 pg/mL. The quantitative HBV DNA levels have also been found to contribute to HCC risk in disparate populations as well [62]. The REVEAL-HBV study further examined the serum HBV DNA levels of 3653 individuals who were seropositive for HBsAg and seronegative for antibodies against hepatitis C virus at study entry, following them for incidence of HCC [12]. A strong biological gradient of HCC risk was observed across serum HBV DNA levels. The corresponding relative risks with 95 % confidence intervals were 1.0 (referent), 1.1 (0.5–2.3), 2.3 (1.1–4.9), 6.6 (3.3–13.1) and 6.1 (2.9–12.7), respectively, for serum HBV DNA levels of <300, 300–9999, 10,000–99,999, 100,000–999,999, and ≥1,000,000 copies/mL. Serum HBV DNA viral load may also increase the mortality from hepatocellular carcinoma among HBV carriers as well [50]. In addition to a one shot measurement of HBV DNA levels, the risk for HCC can also be accurately determined by long-term tracking of repeated serum HBV DNA measurements [4, 63]. Recently, quantitative HBV surface antigen levels were found to be an independent predictor of HCC, even after adjustment for serum HBV DNA levels [48]. The cumulative lifetime risk (30–75 years of age) for HCC was 3.3, 12.0, and 28.3 % for those with baseline serum HBsAg levels of <100, 100–999, and ≥1000 IU/mL. Multivariate-adjusted hazard ratios (95 % CI) were 2.83 (1.55–5.18) and 4.06 (2.24–7.36), respectively, for serum HBsAg levels of 100–999 and ≥1000 IU/mL, when compared to HBsAg levels <100 IU/mL (p for trend <0.001).

To date, at least ten HBV genotypes (A–J) have been identified according to differences in genome sequence, and there are large geographical variations in the

distributions of various HBV genotypes [64]. One study conducted in Alaska, where HBV genotype D and F are predominant, found that native Alaskans with HBV genotype F had higher risk of developing HCC than other genotypes [65]. On the other hand, a nested case–control study conducted in Taiwan, where HBV genotype B and C are predominant, found that genotype C was associated with increased risk for HCC, with an adjusted odds ratio (95 % CI) of 5.11 (3.20–8.18) [66]. The REVEAL-HBV study has indicated that incidence rates for HCC in participants with HBV genotype B and genotype C were 306 and 786 per 100,000 person-years [55]. Individuals with HBV genotype C had 2.4 times higher risk of developing HCC than individuals with genotype B. In addition to HBV genotype, mutations at the precore and basal core promoter (BCP) regions were also associated with HCC risk. Individuals with the precore G1896A mutation had a relative risk (95 % CI) 0.34 (0.21–0.57) for HCC, compared to wild type individuals. In addition, those with the A1762T/G1764A double mutation at the BCP region also had increased risk for HCC, with an adjusted relative risk (95 % CI) of 1.73 (1.13–2.67).

In the past few years, risk calculators to predict HCC risk have been developed [48, 67, 68]. These easy-to-use risk scores are based on noninvasive clinical characteristics, and have helped HBV carriers to stratify their HCC risks according to their personal profiles, including age, sex, family history, alcohol consumption, serum ALT levels, HBeAg serostatus, serum HBV DNA and HBsAg levels, and HBV genotypes, as shown in Fig. 12.3e. The HCC risk calculator was developed in the community-based REVEAL-HBV cohort using reliable and easily accessible clinical parameters, and has been externally validated in clinical settings, showing satisfactory accuracy [69]. The well-known REACH-B score (Risk Estimation for Hepatocellular Carcinoma in Chronic Hepatitis B) allows clinicians to identify individuals at high risk who need intensive care. In the future, other immunomarkers and host genetic markers may also be incorporated into the risk prediction models for further risk stratification.

Extrahepatic Diseases Associated with HBV Infection

In addition to liver-related morbidity and mortality, HBV infection seems to increase the risk for extrahepatic deaths [70–72]. A Previous population-based study found that HBV infection may be associated with the risk for intrahepatic cholangiocarcinoma and non-Hodgkin lymphoma [73]. The cohort consisted of 1,782,401 pregnant Taiwanese women whose HBV serostatus was obtained from the National Hepatitis B Vaccination Registry, who were then followed for newly diagnosed cholangiocarcinoma or non-Hodgkin lymphoma through computerized data linkage with National Cancer Registration Profiles. The investigators found that the age-adjusted hazard ratio for women seropositive for HBsAg was 4.80 (1.88–12.20) and 2.63 (1.95–3.54) for the development of cholangiocarcinoma and non-Hodgkin lymphoma, respectively, when compared to women seronegative for HBsAg. Among non-Hodgkin lymphoma cases, most were diffuse large B-cell lymphoma

(99 of 192, 52 %). HBsAg seropositive women had an increased risk of developing diffuse large B-cell lymphoma with an age-adjusted relative risk of 3.09 (2.06–4.64). In the REVEAL-HBV cohort, the associations between HBV infection and pancreatic cancer have also been assessed [74]. Chronic carriers of HBsAg had an increased risk of pancreatic cancer, with an adjusted relative risk of 1.95 (1.01–3.78). Compared to HBsAg seronegative noncarriers, the adjusted relative risks of developing pancreatic cancer were 1.64 (0.79–3.42) for HBeAg seronegative carriers, and 5.73 (1.73–19.05) for HBeAg seropositive carriers, respectively (p for trend = 0.004). The relationship between HBV infection and the development of diabetes is still unclear. There is limited population-based data to elucidate the association. However, a study that enrolled women in Hong Kong found that HBsAg carrier status may explain gestational diabetes [75]. One study conducted in Alaska with over 20 years of follow-up found that there was no association between HBV infection and diabetes [76].

The Natural History of Occult Hepatitis B

It is very difficult to eradicate HBV from the body, as persistent covalently closed circular DNA (cccDNA) can still be detected in the liver of patients with resolved HBV infection, despite active maintenance of robust antiviral T-cell immunity [77–79]. Of note, the remaining cccDNA in the liver seems to be replication competent, as HBV reactivation may occur in patients who receive immunosuppressive agents [80–83]. After the emergence of highly sensitive HBV DNA PCR assays, some patients can be further identified having occult HBV infection (OBI). Occult hepatitis B infection is indicated by the presence of HBV in the blood or liver without the detection of HBsAg.

OBI has been documented in a number of clinical patient groups, such as HCC patients with chronic HCV infection, liver transplant recipients from hepatitis B core antibody (anti-HBc)-seropositive donors, anti-HBc-seropositive patients coinfected with HCV, patients with cryptogenic cirrhosis or advanced fibrosis, intravenous drug users, and routine blood donors [84]. It has been proposed that a population-based long-term follow-up study in a randomly selected cohort that includes repeated measurements of HBV infection markers would be the best approach to studying OBI [4]. Although data on the natural history of occult hepatitis B in the community is still lacking, clinic-based studies of occult HBV infection measured at the point of spontaneous HBsAg seroclearance can provide important preliminary clues.

One study from Taiwan followed 218 patients (mean age, 44.8 years) who had undergone spontaneous HBsAg seroclearance for 12–179 months [85]. Serum HBV DNA measured by PCR was detectable in 6 of 106 (5.7 %), 4 of 128 (3.1 %), and 2 of 158 (1.3 %) available serum samples collected at the time of HBsAg seroclearance, 6 months, and 1 year after HBsAg seroclearance, respectively. Notably, of the samples with undetectable HBV DNA 1 year after HBsAg seroclearance, more than half (56 %) were anti-HBs seronegative. For patients with OBI, half or less were anti-HBs seropositive at different time points (3 at the time of HBsAg seroclearance,

2 at 6 months after, and 0 at 1 year after HBsAg seroclearance). This study provided important information on the occurrence of occult HBV infection in CHB patients with spontaneously cleared HBsAg. However, this study did not directly evaluate intrahepatic HBV DNA, and also did not address the association between OBI and HCC risk, most likely due to the small sample of OBI patients.

Another study from Hong Kong investigated 298 patients (median age, 49.6 years) with HBsAg seroclearance, most of whom (96 %) did not receive any treatment [86]. Half of the patients (52 %) had detectable anti-HBs after HBsAg seroclearance until the end of follow-up, and cumulative rates of developing HCC were not different between patients with and without anti-HBs. Within 1 year after HBsAg seroclearance, 13.4 % (19 patients) had detectable HBV DNA with a median level of 7.0 IU/mL. In the subsequent follow-up, 6.1 % of samples ($n=6$) taken between 5 and 10 years after HBsAg seroclearance had detectable HBV DNA with a median level of 13.9 IU/mL; and 3.7 % of samples taken more than 10 years after HBsAg seroclearance (only 1 OBI sample at 53.6 IU/mL) had detectable HBV DNA. One of the major achievements of this study was that it assessed HBV DNA and messenger RNA within livers of a small portion of patients ($n=29$). The results showed that all patients had detectable intrahepatic HBV DNA, and 79.3 % (23 patients) had detectable cccDNA, with median levels of 1.7 copies/cell and 0.031 copies/cell, respectively. However, of the 11 samples evaluated for mRNA, all had undetectable mRNA expression of the surface and precore/pregenomic genomes, and only 1 patient had detectable X gene mRNA expression. This study did not analyze the association between OBI that occurred at HBsAg seroclearance and subsequent HCC risk. However, it did find that at the time of HCC diagnosis, two out of five patients had low levels of HBV DNA (23.3 and 169.5 copies/mL, respectively).

From the aforementioned natural history studies conducted in clinical patients, a substantial number of OBI subjects were discovered by measuring serum HBV DNA at or shortly after HBsAg seroclearance, and the proportion of OBI declined gradually during follow-up. However, by measuring HBV DNA in liver tissues, it was demonstrated that patients still harbored HBV inside the liver after HBsAg seroclearance, although the virus was low replicative and transcriptionally inactive [86]. As the numbers of OBI patients were quite small, it was difficult to investigate the association between OBI and long-term HCC development. As the natural history of OBI in terms of molecular mechanisms, dynamic fluctuations, and health risk cannot be accurately delineated through cross-sectional, case-series, or case–control studies, a large-scale longitudinal population-based study is urgently needed. The REVEAL-HBV cohort may be able provide important data in the future [4].

Conclusion and Perspectives

This chapter described the natural history of HBV infection in the general population of several communities. Both the incidence of seroclearance of HBV seromarkers and the risk of end-stage liver diseases are quite different from those observed in

clinical patients from clinics and hospitals. The predictors for entering three milestones of HBV infection, i.e., HBeAg seroclearance, HBV DNA seroclearance, and HBsAg seroclearance, have been incorporated to derive prediction models, respectively. The long-term risk predictors of cirrhosis and HCC have also been combined to develop several risk calculators which have been externally validated. OBI remains a challenging research and clinical topics for the management of chronic hepatitis B using immune moderators and antivirals.

In the epoch of genomics, transcriptomics, proteomics, metabolomics, glycomics and lipidomics, more and more biomarkers dynamically changed in various stages of the natural history of HBV infection will be identified. They may be combined with clinical data to form the health data cloud of each person. Through the big health data analysis more detailed natural history of HBV will be further elucidated in near future. It is expected that personalized preventive and therapeutic medicine for chronic HBV infection will come true soon.

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Chapter 13
Occult HBV Infection

Giovanni Raimondo and Teresa Pollicino

Introduction

Hepatitis B virus (HBV) occult infection is defined as the presence and long-lasting persistence of viral DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing negative for the HBV surface antigen (HBsAg) [1]. Apart from some cases in which the lack of HBsAg detection is attributable to the HBV genetic heterogeneity, i.e., infection with S-escape mutants producing a modified HBsAg that is not recognized by the commercially available diagnostic assays [2, 3], in most cases the occult HBV infection (OBI) is due to replication-competent viruses with degrees and relevance of genetic heterogeneity comparable with those of the HBV isolates from individuals with HBsAg positive (namely "overt") infection [4]. In OBI cases, however, the viruses are subjected by the host's defense mechanisms to a potent suppression of the replication activity and gene expression, leading to the lack of both HBsAg synthesis and production/secretion of virions and thus to the absence (or presence in minute traces) of HBV DNA in the serum [5, 6].

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The molecular basis of the occult infection is strictly related to the peculiar life cycle of the HBV, and in particular to the high stability and long-term persistence of viral cccDNA molecules in the nuclei of the hepatocytes that—together with the long half-life of the liver cells—imply that, once the HBV infection has occurred, it may possibly continue for life [7]. Indeed, according to the European guidelines on HBV management, OBI is recognized as one of the five phases of the natural history of chronic hepatitis B [8]. In this context, it is important to stress that HBV DNA may be found integrated into the host's genome in each of these five phases regardless of the HBsAg positive/negative status. Viral DNA integrants have no role in the HBV life cycle, and their possible presence in HBsAg-negative subjects does not per se have to be identified as an occult infection since OBI is essentially related to the intrahepatic persistence of entire, episomal, replication-competent HBV genomes.

Suspected for several decades of existing (reviewed in ref. [5]), the occult phase of the HBV infection was better identified in the late 1990s when some important clinical-virological studies (based both on the analysis of well-selected and characterized human liver samples and on the application of highly sensitive molecular biology techniques) made it possible to start revealing its potential implication in various clinical contexts, to show its worldwide diffusion, and to disclose its virological aspects [9]. Indeed, in recent years there has been a continuous increase of the number of studies in this field published by journals covering different areas of biomedical interest (reviewed in ref. [5]), thus making OBI one of the most challenging and fascinating issues of the research into viral hepatitis.

Mechanisms Leading to Occult HBV Infection Development

Major advances have been made in the last few years in understanding the molecular mechanisms potentially involved in the induction and maintenance of the HBV infection in an occult status. Although viral factors may be implicated in some cases, host factors (immune response and epigenetics) likely play a preeminent role (Fig. 13.1), and there is evidence that coinfection with other viral and nonviral agents might also be involved in some circumstances [5, 6, 10].

Viral Factors

The lack of detectable HBsAg in spite of the presence of episomal, free HBV genomes at intrahepatic level is attributable in some cases to the HBV genetic variability. In fact, a fairly large number of studies have linked OBI occurrence to specific HBV variants. Indeed, it has been reported that OBI individuals are infected with HBV variants showing (a) mutations clustering in major hydrophilic region (MHR) of the small (S) protein, (b) mutations in the pre-S1/S2 genomic region, (c) specific structural alterations in virus regulatory elements, (d) mutations affecting posttranslational production of virus envelope proteins, and (e) mutations selected under antiviral treatment with nucleos(t)ide analogs (NUCs) that may cause amino acid changes both in

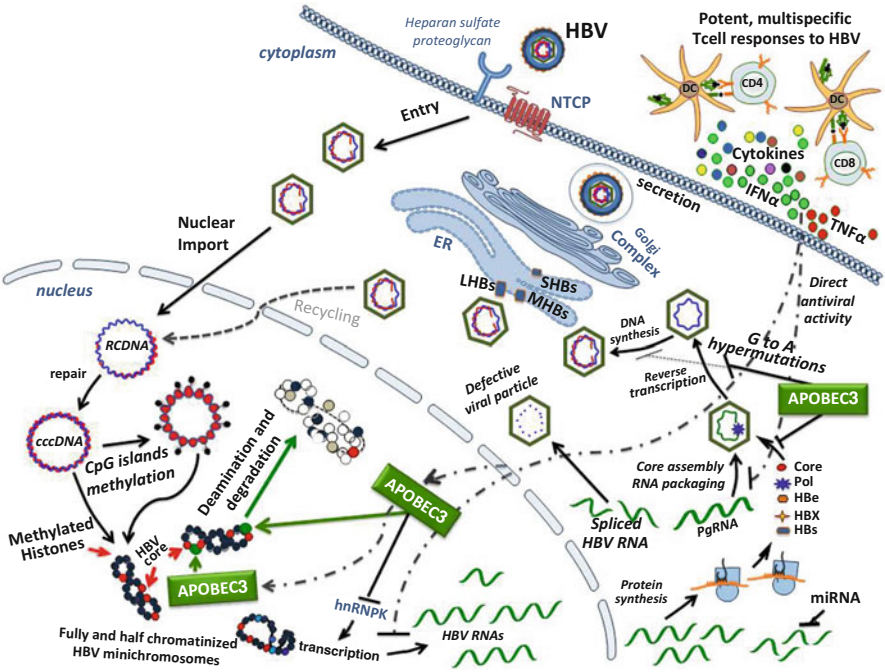

Fig. 13.1 Schematic representation of the main mechanisms leading in the control of HBV activities and potentially involved in OBI occurrence. In particular, mechanisms related to host's adaptive (functionally efficient memory HBV-specific T cell response) and innate immune response (cytokines like IFN-α and TNF-α) as well as genetic (APOBEC3 hyperediting resulting in HBV genomic hypermutation, apurinic/apyrimidinic site formation, and cccDNA degradation), epigenetic (methylation of HBV CpG-islands and cccDNA-bound histones tails, full nucleosomal packaging of HBV minichromosome) and co-/posttranscriptional (cellular miRNAs- and/or APOBEC3s [editing-independent]-induced inhibition of HBV replication, and HBV RNA splicing) mechanisms are summarized. *NTCP* sodium taurocholate cotransporting polypeptide, *DC* dendritic cell, *CD4* CD4+ T cell, *CD8* CD8+ T cell, *RC DNA* relaxed circular DNA, *cccDNA* covalently closed circular DNA, *pgRNA* pregenomic RNA, *LHBs* large hepatitis surface protein, *MHBs* middle hepatitis surface protein, *SHBs* small hepatitis surface protein, *APOBEC3* apo B mRNA editing enzyme catalytic polypeptide, *hnRNPK* heterogeneous nuclear ribonucleoprotein K, *miRNAs* microRNAs

viral polymerase and S protein [5, 10–12]. A high frequency of mutations has been found particularly within the MHR of HBsAg in HBV strains isolated from OBI individuals [4, 11, 13–24]. These mutations have been functionally associated with S protein structural changes that may lead to an impaired detection by commercially available HBsAg assays. In addition, there is evidence that occult HBV of specific genotypes may show not only the mutations in the MHR, but also a very high frequency of mutations in the T-cell epitopes, thus further supporting the hypothesis that the selection of these HBV variants may represent a mechanism of immune escape, as also suggested by the inability of anti-HBs antibodies from individual patients to recognize their own circulating viruses [11, 19–21, 25, 26]. Some recent studies have strengthened these data by applying ultra-deep pyro-sequencing. Indeed, a higher

degree of genetic variability was found in the S gene of occult HBV compared to viruses from HBsAg-positive patients, and it has been postulated that the complex HBV quasi-species with mutations in HBsAg immune-active regions may help HBV to escape both from neutralizing and diagnostic antibodies [24, 27]. However, this evidence has been challenged by a very recent study showing that the genetic heterogeneity of reactivated HBV is significantly lower in patients with reactivation from OBI carrier status than that from HBsAg-positive carriers, suggesting that OBI individuals are infected with HBV populations of low genomic heterogeneity in their liver [28]. The very low or absent viral load characterizing OBI carriers has suggested that viral genomic mutations could also negatively impact any step of HBV life cycle [21, 23, 29, 30]. Indeed, there are data showing that occult HBV with specific amino acid substitutions in the MHR displays an impaired virion and/or S protein secretion when transfected in hepatoma cells or hydrodynamically injected in mice [21, 23, 30]. In this context, it is worth mentioning that also mutations at the level of the pre-S2/S splice donor site have been detected in occult HBV strains. Pre-S2/S splicing occurs during HBV replication, and mutations that interfere with pre-S2/S mRNA splicing may cause a marked reduction of functional unspliced pre-S2/S transcripts and of HBsAg synthesis, thus leading to OBI development. There is evidence that RNA secondary structure at the 5′ splice site can regulate the splicing efficiency of transcripts and modulate the binding of RNA-splicing factors as well as the recognition of splice site consensus elements [31]. Thus, it has been postulated that mutations at the pre-S/S 5′ splice donor site may affect the interaction of RNA with components of the spliceosome, hence impairing posttranscriptional RNA processing and/or nuclear export via the posttranscriptional regulatory element [25, 32, 33]. Pre-S mutations have also been associated with OBI occurrence. In particular, it has been shown that deletions in the pre-S1/S2 genomic region correlate with an impaired expression of envelope proteins, and that some of these deletions may contribute to persistence of the virus in the occult state by implying the elimination of HLA-restricted B-cell and T-cell epitopes [34–36]. The association of mutations and deletions in the pre-S gene with a lack of secreted HBsAg and low levels of HBeAg and HBV DNA was demonstrated using functional analysis by transfection into hepatocyte cell lines [36].

Despite all these lines of evidence, however, it is proved that the great majority of OBI individuals are not infected with specific HBV mutants. Moreover, important data have demonstrated that pre-S/S variants can frequently be found also in patients with overt HBV infection, including subjects with high viral loads [4, 12, 22, 35, 37]. Furthermore, strong evidence from different studies indicates that "occult" HBV genomes are usually replication-competent and that their genetic heterogeneity is comparable with those from HBsAg-positive individuals [4, 28, 37]. In vitro functional analysis showed that occult viral isolates "re-acquire" normal replication, transcription, and protein synthesis abilities once taken out from the host's liver microenvironment. These viruses appear to normally replicate when transfected in hepatoma cells and to be competent in HBsAg production [4]. Therefore, according to these findings genomic variability does not usually appear to play a fundamental role in inducing the OBI status, which rather seems to be dependent on a strong suppression of the virus replication and transcriptional capabilities in the majority of the cases.

Host Factors

Immunological Factors

Many clinical studies have provided strong evidence indicating that all the conditions inducing immunosuppression expose patients to risk of OBI reactivation with the reappearance of the typical serological profile of the overt, active HBV infection [5, 38–40]. Though indirect, this is strong evidence of the role played by the host's immune surveillance in OBI induction. The importance of the immune system in OBI occurrence has also been demonstrated by the findings showing that HBV DNA along with a functional memory HBV-specific T cell response can be readily detectable several years after recovery from an acute hepatitis B event [41, 42]. Thus, it is plausible to hypothesize that during the occult phase of the infection, HBV is still able to synthesize very small amounts of antigens that, however, are sufficient to maintain an HBV-specific T cell response. This assumption is confirmed by the findings showing that, apart from HBV covalently closed circular DNA (cccDNA) molecules [43–46], all viral HBV transcripts (including the pregenomic RNA, pgRNA) can also be detected and quantified in the liver of OBI individuals [44, 46]. Importantly, some recent studies have shown that OBI individuals can display a potent HBV-specific T cell response [22, 47]. In particular, it has been demonstrated that OBI patients with or without antibodies to HBV core antigen (anti-HBc) display different profiles of HBV-specific T cell responses. Indeed, although in anti-HBc negative (namely, seronegative) OBI patients circulating HBV-specific T cells can be detected at frequencies comparable with that found in anti-HBc positive (namely, seropositive) OBI subjects, in vitro expansion and IFN-γ production by HBV-specific T cells from seronegative cases are much weaker than those from OBI seropositive individuals [47]. On the basis of the data obtained in the woodchuck animal model infected with the corresponding hepadnavirus (woodchuck hepatitis virus, WHV), it has been hypothesized that these distinct behaviors of cell-mediated immune responses in seropositive and seronegative OBIs might reflect different modalities of HBV transmission. Indeed, exposure to low WHV doses (less than 10^3 virions) may lead to a persistent infection without appearance of viral serum markers. Interestingly, this so-called woodchuck "primary" occult infection does not confer protective immunity, indicating that only infection with a higher dose of inoculum can elicit an efficient memory T cell response [48]. Potent, HBV-specific T cell responses were also observed in blood donors with seropositive OBI [22]. Of interest, it was observed that HBV-specific T-cell responses could be quantitatively stronger in OBI than in inactive carriers, and similar or even higher than those in subjects with previously resolved hepatitis B [22].

Many relevant data have suggested that the innate immune response also may play a role in the control of HBV activities. Experiments in transgenic mice and chimpanzees have shown that inflammatory cytokines, such as type I interferons (IFN-I) and tumor necrosis factor-α (TNF-α), can efficiently suppress viral replication through noncytolytic immune response [49]. In accordance, it has been recently demonstrated that liver cells can mount an effective innate immune response to HBV infection with

the expression of IFN-stimulated genes, which in turn limit HBV replication via inhibition of cccDNA transcription and encapsidation of pgRNA [50]. Moreover, it has been shown that activation of the retinoic acid-inducible gene 1 (RIG-I) like receptors in infected hepatocytes induces the production of IFNs and different proinflammatory cytokines, and also activates intracellular antiviral pathways to disrupt HBV replication by targeting multiple steps of the viral life cycle [51].

Interestingly, recent studies have proved that the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3) cytidine deaminases represent a major strategy of innate immunity to retroviruses as well as to the pararetrovirus HBV [52]. It has been shown that the expression of APOBEC3G in HBV-replicating cells results in more than a 50-fold decrease in HBV DNA release in the cell culture medium [53]. Both deamination-dependent and deamination-independent mechanisms have been implicated in APOBECs-induced inhibition of HBV replication [52]. Very recently it has been shown that IFN-alpha can up-regulate APOBEC3A in HBV-infected cells and that HBV core protein mediates the interaction of APOBEC3A with HBV cccDNA, resulting in cytidine deamination, apurinic/apyrimidinic site formation, and finally in cccDNA degradation [54]. Interestingly, APOBEC hyperedited sequences have also been detected in OBI individuals [35, 55]. Altogether, these findings indicate that the innate immune response may have a leading part in the control of HBV activities in OBI, and particularly in seronegative OBI patients in whom poor in vitro T cell expansion has been observed.

Epigenetic Factors

Recently, studies on the role of viral chromatin organization have revealed the importance of dynamic viral-host chromatin interactions in modulating the control of essential viral processes including gene expression and replication [56]. Many different chromatin-organizing factors have been associated with the epigenetic configuration of the viral chromosome. For DNA viruses like Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) known to establish latent infection, the contribution of chromatin remodeling to the latent state has been investigated in depth. During latency, both EBV and KSHV genomes are maintained as minichromosome molecules that adopt a chromatin conformation similar to that of the host cell chromosome, and many data indicate that both viruses make use of chromatin binding factors and histone tail epigenetic modifications as mechanisms to maintain unchanged gene programs during latent infection [56–58]. Many recent studies have shown that epigenetic mechanisms play a relevant role also in controlling HBV transcription/replication [59, 60].

HBV cccDNA molecules are harbored in the nucleus of infected hepatocytes as stable minichromosomes displaying the typical "beads-on-a-string" structure at electron microscopy, and showing the DNA packed into the full or half complement of nucleosomes, which can reflect dynamic changes related to transcriptional activity [61–63]. HBV cccDNA minichromosomes associate with both histone and non-histone proteins [59]. Indeed, H1, H2A, H2B, H3, and H4 histones as well as the

viral core protein have been shown to be a structural component of the HBV minichromosome [61]. Data from transfected hepatoma cells and liver tissues have shown that HBV replication is regulated by the acetylation status of viral cccDNA-bound H3 and H4 histones, and that recruitment of histone deacetylase 1 (HDAC1) onto the cccDNA correlates with low HBV replication [64]. In addition, treatment with inhibitors of class I or class III HDACs induces a significant increase of the acetylation status of cccDNA-bound histones and HBV replication in HBV-replicating cells [64]. Interestingly, there is evidence demonstrating that IFNα is able to inhibit cccDNA-driven transcription of viral RNAs, both in HBV-replicating cells and in HBV-infected humanized uPA/SCID mice [65, 66]. In particular, it has been found that cccDNA-bound histones become hypoacetylated, and components of the transcriptional repressor complex PRC2 are actively recruited on the cccDNA after IFNα treatment [66]. Therefore, IFN-α appears to be capable of inducing a condition of "active epigenetic control" of HBV cccDNA minichromosome activity, which may have a part in the persistent (although reversible) "off therapy" inhibition of HBV replication. Of note, it has also been shown that the HBX regulatory protein produced in hepatoma cells replicating HBV is recruited onto the cccDNA minichromosome, and that HBx-defective HBV mutants are impaired in their replication [67, 68]. There is evidence that, in addition to chromatin dynamics, CpG site-specific DNA methylation levels in the HBV genome may also contribute in modulating viral gene expression and replication [5, 10, 59, 60]. Interestingly, DNA methylation analysis of a certain number of OBI cases revealed that specific CpG sites in the HBV genome are frequently hypermethylated [35]. However, more recent results have argued that in normal hepatocytes—unlike in hepatocellular carcinoma (HCC) cells—DNA methylation could be a major epigenetic mechanism responsible for chronic silencing of HBV gene expression [69]. Therefore, the exact impact of the observed CpG islands methylation on the function of HBV genome and occult infection remains to be established.

The contribution of cellular and viral micro-RNAs in regulating viral replication and chromatin is also under intense investigation. To examine cellular micro-RNAs affecting HBV replication, Zhang et al. applied a loss-of-function approach by transfecting antagomirs targeting many different human micro-RNAs in hepatoma cells [70]. Both miR-199a-3p and miR-210 have been found to suppress HBsAg expression. In addition, another study showed that also miR-125a-5p may interfere with HBsAg expression and release in the cell culture medium [71]. Recently, many cancer-related micro-RNAs, including miR-15a/miR-16-1, the miR-17-92 cluster, and miR-224, have been shown to target HBV mRNAs, thus inhibiting HBV replication [72–74]. Besides directly targeting HBV, some cellular micro-RNAs have been shown to inhibit HBV replication by indirectly regulating different cellular transcription factors. In particular, miR-141 has been shown to significantly suppress HBV expression and replication in HepG2 cells by targeting the peroxisome proliferator-activated receptor alpha [75], and miR-155 may impair HBV replication in hepatoma cells through targeting the suppressor of cytokine signaling proteins (SOCS1), and promoting the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway [76].

Coinfection

Several studies have shown that HBV replication is frequently impaired in individuals coinfected with other infectious agents. In particular, it has been shown that hepatitis C virus (HCV) infection can strongly suppress HBV replication, and this has led to hypothesize that the inhibitory activity exerted by HCV on HBV might ultimately result in OBI occurrence. This assumption is supported by the large body of evidence showing that OBI has the highest prevalence precisely in HCV-infected patients [5, 39], and by the in vitro data demonstrating that the HCV "core" protein can strongly inhibit HBV replication and gene expression [77–80]. However, more recent evidence has challenged the existence of interplay between HCV and HBV. Indeed, studies performed in animal models coinfected with HCV and HBV or in hepatoma cells transfected with HCV replicon (instead of single viral proteins) and full-length HBV genome have found no interference between the two viruses [81–85]. Thus, the available data cannot allow any definitive conclusion to be drawn for a role of HCV in the induction of the OBI status. It is known that also individuals positive for human immunodeficiency virus (HIV) frequently show either overt or occult HBV coinfection, but there is no evidence of possible direct effects of HIV on HBV activity or of the existence of any peculiar specific mechanisms leading to OBI occurrence in HIV-infected individuals [86–88]. Other infectious agents potentially capable of interfering with HBV activity include *Schistosoma mansoni*, a parasite that affects more than 200 million people worldwide [89]. Coinfection with HBV and Schistosoma occurs frequently in geographic areas where both agents are endemic [90, 91], and it has been demonstrated that infection with Schistosoma mansoni in HBV transgenic mice induces a strong suppression of HBV replication [92].

Prevalence

The peculiar life cycle of the HBV with its long-term persistence at intrahepatic level regardless of the HBsAg status represents scientific support of the large body of evidence indicating that OBI is a common, worldwide occurrence. Nevertheless, a reliable evaluation of the general prevalence of OBI is at present a very difficult objective to achieve mainly because of the lack of standardized, valid and commercially available assays for its detection, and because the present gold standard for OBI identification (i.e., to test liver DNA extracts by highly sensitive and specific molecular biology approaches such as nested-PCR or real-time PCR) is of course applicable only in the little minority of cases in which a liver specimen is available [1]. In addition, the positivity of circulating anti-HBc antibody—often used as a surrogate marker for OBI identification in HBsAg negative subjects—may be misleading since anti-HBc tests may provide false positive results [1, 93, 94], and also because about 20 % of OBI cases are negative for all HBV serum markers (namely, OBI

seronegative individuals) [1, 5, 39]. Despite the above-mentioned limitations and some discrepancies in the available epidemiological data mainly due to the differences in sensitivity and/or specificity of the methods used in the various studies (reviewed in refs. [5, 95]), there is more than one solid piece of evidence that OBI is a largely world-wide diffused entity with a distribution that may reflect the diffusion of the HBV in the various geographic areas and in the various populations [96–99], and thus with a prevalence that appears to be higher in countries where HBV is endemic and among subjects at high risk of parenterally transmitted infections such as drug addicts and hemophiliacs [100, 101]. Of importance, OBI appears to be highly prevalent in chronically HCV infected individuals, and generally in patients with chronic liver diseases (i.e., alcoholic, cryptogenic, etc.) or with hepatocellular carcinoma [5, 39, 46, 78, 102–105]. In fact, HBV DNA is detectable in about one third of HBsAg-negative HCV carriers in the Mediterranean area, in more than 50 % in Far East Asian countries and in 50 % of US patients of Caucasian origin undergoing liver transplantation for end-stage HCV-related liver disease [39, 95, 106]. This last observation is particularly important also considering that the HBV general prevalence in the Caucasian American population is one of the lowest in the world [107].

Clinical Implications

The vast majority of individuals with OBI will never suffer from any clinical event related to the small amounts of viral genomes segregated in the liver cells. Nevertheless, in some particular circumstances and contexts OBI may acquire a pathogenic role and may become a (co)factor implicated in different clinical conditions that may also have severe sequels (Fig. 13.2). Indeed, since the suppression of viral replication and gene expression typical of the OBI status is a reversible condition, there is no doubt about the possibility that OBI, once transmitted by blood transfusion or liver transplantation from an "occult carrier," may induce a typical, overt hepatitis B in a recipient naive for HBV infection. In analogy, an occult HBV infection may be reverted in an overt infection and reactivated with development of hepatitis B—often acute and severe—in patients undergoing therapeutic immunosuppression. Moreover, growing evidence exists on the possible contribution of OBI to the progression of liver fibrosis and establishment of cirrhosis as well as to the development of hepatocellular carcinoma, this last effect being related to the maintenance in the OBI phase of the mechanisms responsible for the pro-oncogenic properties of the overt, active HBV infection. In this context, however, it has to be taken into account that OBI appears to shape up as a complex scenario, which includes several different clinical/virological conditions quite different from one another. In fact, it is possible to distinguish *seropositive* (anti-HBc and/or anti-HBs positive) and *seronegative* (both anti-HBc and anti-HBs negative) OBI individuals (Fig. 13.3). In seropositive OBI, the HBsAg may have disappeared either very early after the resolution of an acute hepatitis event or after many years of overt carriage, whereas the seronegative OBI cases might have either progressively lost all HBV serum markers or

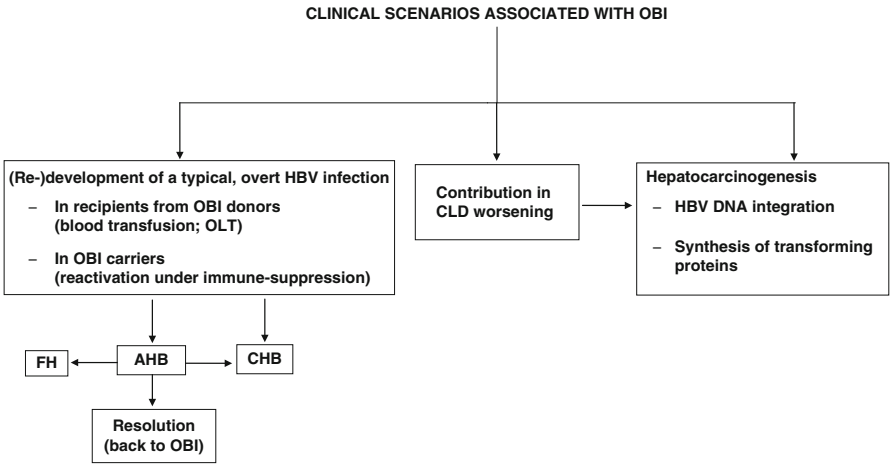

Fig. 13.2 Schematic representation of different conditions associated with the lack of detectable HBsAg in individuals with occult HBV infection (OBI)

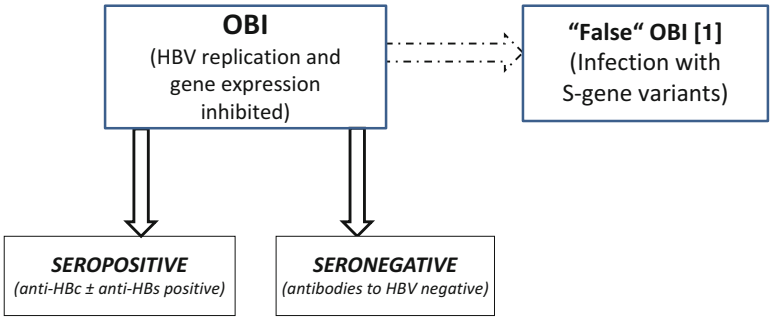

Fig. 13.3 Schematic representation of the possible clinical implications of the occult HBV infection. *OBI* occult HBV infection, *OLT* orthotopic liver transplantation, *CLD* chronic liver disease, *FH* fulminant hepatitis, *AHB* acute hepatitis B, *CHB* chronic hepatitis B

might have been HBV negative since the beginning of the infection. Indeed, one cannot rule out the possibility that each of these conditions may have different roles and/or impacts on the occurrence or outcome of the liver disease.

HBV Transmission from OBI Patients

Transmission Through Blood Transfusion

All blood donations containing HBV DNA are potentially infectious also in the absence of HBsAg. Thus, carriers of occult infection with residual circulation of viral genomes may be a source of HBV transmission in the case of blood

transfusion with the consequent, possible development of typical hepatitis B in the recipients [11, 93, 94, 108]. This possible occurrence was first reported in the late 1970s and then experimentally confirmed in chimpanzees [109–111]. Thus, the high level of alert still maintained in blood banks for identification of OBI positive donors is more than justified. Thanks to this alert and the implementation of progressively more sensitive and specific diagnostic tests, the risk of HBV infection after blood transfusion has dramatically decreased in the last decades, and in fact post-transfusional hepatitis B is now a rare event in the western world. In this context, however, it is important to consider that epidemiological studies based on the most sensitive screening tests for HBV detection [i.e., Nucleic Acid Testing (NAT)] have shown that the frequency of HBV DNA positive cases among HBsAg negative blood donors varies considerably according to the prevalence of the infection in the different geographical areas. Since HBV is highly endemic in many developing countries that have not yet adopted the expensive NAT techniques for blood screening, the persistence of a not negligible risk of HBV transmission by transfusion in the less rich areas of the world is understandable. Schematically, the transfusional transmission of HBV may occur when the donor is an "OBI carrier" in two different situations:

1. The donor has a typical occult HBV infection with wild-type HBV populations suppressed in their replication and gene expression capabilities. In this context, it has to be considered that chronic occult infection is frequently characterized by periods of transient HBV viremia alternating with periods in which the viral DNA is undetectable in the serum [112, 113]. Thus, an "occult HBV carrier" may have a profile of blood infectivity fluctuating over time, although it has to be taken into account that the amount of circulating viruses is usually very low and the amount sufficient to induce an acute hepatitis B event in the recipient remains questionable. Moreover, apart from the viral load of the donor, the possibility of inducing acute hepatitis likely depends on a sum of factors including the amount of plasma transfused and the immuno-competence of the recipient. Nevertheless, the lack of acute hepatitis development does not exclude the possibility that the HBV has been transmitted and infection has occurred, with the consequent theoretical and intriguing possibility that the recipient might in turn become an occult HBV carrier.
2. The donor is infected with S-escape HBV mutant strains. Infection with these genetic variants has also been named "false OBI" since the virus may normally replicate but it synthesizes modified surface proteins that are not identified by the HBsAg diagnostic kits (Fig. 13.3) [1]. This condition appears to be a major cause of the very few, residual cases of HBV transmission by blood transfusion in the most developed countries [93, 94].

Transmission Through Liver Transplantation

OBI transmission may also occur in cases of orthotopic liver transplantation (OLT) and—much less frequently—in cases of kidney, heart and bone marrow transplantation (reviewed in ref. [5]). De novo hepatitis B in OLT HBV naïve recipients

receiving the organ from an OBI donor is a frequent and well-recognized occurrence. It is the clear consequence of the fact that the liver cells are the reservoir of the viral populations, and it largely explains and justifies the anti-HBV prophylaxis [with high doses of anti-HBs immunoglobulin and NUCs inhibiting the HBV reverse-transcriptase] that is generally performed in HBsAg-negative transplanted patients who receive livers from anti-HBc positive donors (HBV transmission from OBI seronegative donors is uncertain and, in any case, very difficult to diagnose). This prophylaxis appears to be very effective in preventing HBV hepatitis in the recipients [114] but it is insufficient to avoid HBV reinfection and the establishment of a new occult infection [115]. In fact, there is clear evidence of OBI occurrence in transplanted individuals who were occulting infected prior to OLT and/or received the new organ from an OBI carrier. In the transplanted liver, viral DNA (including HBV cccDNA) is present and may derive from occult viruses previously infecting the recipient, the donor or even both [116]. An important topic of debate is whether OBI might have any clinical impact in the long-term outcome of OLT patients. In this context, some preliminary evidence suggests a possible involvement of OBI in a faster progression toward cirrhosis of the post-OLT liver disease in patients with HCV infection [117, 118]. Finally, it is appropriate to point out that occult infection also develops in all HBsAg-positive transplanted patients who receive anti-HBV prophylaxis and become HBsAg-negative in the post-OLT period but invariably show the reinfection of the liver [116].

HBV Reactivation in Cases with Occult Infection

As stressed above, an HBV infection enters in the occult phase when the host's defense mechanisms (essentially the mechanisms of immune surveillance) succeed in determining a potent inhibition of viruses that are per se competent in their replication and gene expression capabilities. Thus, all conditions inducing profound changes of the host's immunological status and the interruption of the efficient control of the HBV activities might lead to OBI reactivation with the consequent possible development of a typical acute hepatitis B showing (re-)appearance of HBsAg and even of HBeAg, and with a clinical behavior that is often severe and sometimes fatal for the patient (reviewed in refs. [40, 119–121]. In this context, it is worth mentioning some interesting although anecdotic reports indicating that OBI reactivation might also occur under treatment with histone deacetylase inhibitors, thus suggesting the possibility that also drugs potentially influencing the epigenetic control of the HBV cccDNA minichromosome might cause viral reactivation [122, 123].

HBV reactivation is almost the rule in inactive HBsAg-positive patients undergoing immune-suppression, whereas the frequency with which it occurs in OBI carriers is still undefined. In this context, it has to be considered that OBI individuals may frequently change their HBV serological profile if immuno-compromised. In fact, the anti-HBs antibody—when present—may progressively disappear during immune-suppressive therapy and this occurrence may be followed by HBsAg reappearance that, however, is accompanied by development of clinically evident

acute hepatitis in only a few cases [124–126]. Consequently, OBI reactivation appears to be an event occurring more frequently than usually believed, but it is often clinically silent and the diagnosis might be missed in many cases. Nevertheless, although the incidence of reactivation in individuals with OBI is much lower than in overt HBV carriers, it has considerable importance and represents an every-day challenge in clinical practice because of both the huge number of potential "OBI carriers" (namely, anti-HBc positive individuals) worldwide and the availability of new, potent and efficacious immunological drugs and complex chemotherapy schedules longitudinally administered over several subsequent cycles in different clinical contexts. Indeed, this topic has been discussed in all international guide lines for the management of HBV infection published in the last few years, and it is also included in a recent alert by the FDA directed to physicians of various specialties and concerning the risk of HBV reactivation in patients undergoing anti-CD20 therapies [127]. At present, no reliable marker that helps in predicting HBV reactivation in OBI patients is available. In fact, there are contrasting data on the possibility that patients positive for anti-HBc alone have different risks of OBI reactivation compared to those positive for both anti-HBc and anti-HBs [124, 128, 129] and whether detectable serum HBV DNA at basal time before starting immune-suppressive therapy has any value in predicting the reactivation is also debated [128]. However, on the basis of the literature data, clinical/therapeutic conditions at higher or at lower risk for the occurrence of reactivation have been identified (Table 13.1). Indeed, patients with hematological malignancies (in particular, non-Hodgkin lymphoma, multiple myeloma, myelo-monoblastic acute leukemia, chronic lymphocytic leukemia) have the highest risk of OBI reactivation, especially when treated with schedules including anti-CD20 monoclonal antibody (i.e., Rituximab, Ofatumumab) and, in particular, combinations of Rituximab with Cyclophosphamide, Hydroxydaunorubicin, Oncovin and Predniso(lo)ne, R-CHOP [40, 119, 124, 130–133]. Another category of individuals showing a quite high incidence of OBI reactivation are patients undergoing hematopoietic stem cell transplantation (HSCT) [125, 126]. OBI reactivation appears to be an infrequent—but existing—event in individuals with rheumatologic diseases undergoing treatments including biologics (mainly, anti-CD20 but also anti-TNFα drugs) or with schedules containing high doses of corticosteroids [40, 132–136]. Anecdotic cases of OBI reactivation in patients with HCC undergoing trans-arterial-chemo-embolization as well as in patients with inflammatory bowel diseases under treatment with biological agents have been reported [5], whereas several doubts exist on the real risk of OBI reactivation in patients with solid tumors undergoing chemotherapy [137], and no report exists about OBI reactivation in other categories of patients undergoing treatments with biological drugs (i.e., individuals with psoriasis).

Apart from anti-CD20, a number of other drugs have been reported to be associated with some cases of OBI reactivation (reviewed in refs. [5, 40, 120, 124, 130]): in particular, the anti-CD52 monoclonal antibody Alemtuzumab that is used in onco-hematology therapeutic schedules [138], and the anti-TNFα drugs that are largely utilized for treatment of autoimmune, inflammatory diseases (of note, TNF-alpha is a chemokine able to inhibit HBV replication [139]). Finally, also corticosteroids administered at high doses and for long periods may be involved in OBI

Table 13.1 Schematic representation of conditions exposing at different risk of HBV virological/clinical reactivation in OBI carriers or in recipient from OBI donors

Higher risk
For OBI carriers
– Onco-hematological malignancies under treatment
– R-CHOP (rituximab–cyclophosphamide, adriamycin/doxorubicin, vincristine, prednisone) treatments
For recipients
– Liver transplantation
– Hematopoietic stem cell transplantation
Lower risk
For OBI carriers
– Rheumatological diseases treated with biological agents or high dosage of steroids for prolonged time
– HIV infection
– Inflammatory bowel diseases treated with biologics
– Trans-arterial chemoembolization for treatment of hepatocellular carcinoma
For recipients
– Kidney transplantation
– Bone marrow transplantation
Uncertain risk
For OBI carriers
– Dermatological diseases treated with biologics
– Solid tumors treated with chemotherapy
For recipients
– Organ transplantation different from liver and kidney

reactivation as a possible consequence of both their immune-suppressive effects and their capacity to directly stimulate the HBV replication through the glucocorticoid responsive element present in the viral genome [140].

While prophylactic anti-HBV therapy with NUC inhibitors is a generally adopted practice for the prevention of reactivation in inactive HBsAg-positive carriers undergoing immunosuppressive therapies, the prophylactic antiviral treatment of patients suspected to be OBI positive is still a matter of debate. Indeed, NUCs treatment of onco-hematologic HBsAg negative/anti-HBc positive patients before starting R-CHOP therapy is now quite widely adopted in clinical practice [40]. In all other clinical/therapeutic contexts in which the risk of OBI reactivation is lower, strict surveillance is nevertheless recommended (see also the guidelines for the management of Chronic Hepatitis B licensed by the European Association for the Study of the Liver) and these patients should be followed by alanine-aminotransferase (ALT) and HBV DNA testing and treated with a NUC upon confirmation of HBV reactivation before ALT elevation to prevent hepatitis development [8]. Finally, an additional point worthy of discussion concerns the question of whether HBV reactivation may also occur in patients with seronegative OBI. Of course, this subset of patients is very difficult to identify because of the lack of any marker that helps when the infection is suspected. Indeed, convincing data are available showing that the HBV-specific

T cell response is much weaker in OBI sero-negative than in OBI sero-positive individuals, thus likely insufficient to provoke immune-mediated liver injury [47]. According to this observation one may suppose that OBI reactivation is a phenomenon only occurring in anti-HBV antibody-positive subjects.

Occult HBV Infection and Chronic Liver Disease

An important and widely debated topic is whether occult HBV may favor the progression toward cirrhosis of chronic liver disease (CLD) in HCV-infected patients (as well as in individuals with liver disease of other etiology), as suggested by a quite large body of evidence and confirmed by a recent meta-analysis [5, 39, 141]. Indeed, how OBI may induce (or contribute to) liver injury despite the profound suppression of its replication and gene expression is difficult to explain, and one can only postulate some hypotheses. In this context, it seems important to consider that individuals who have recovered from self-limited acute hepatitis usually show no clinical or biochemical sign of liver damage but, when their liver tissue is examined even several decades after the resolution of the acute hepatitis, HBV genomes are invariably detected together with histological patterns of a mild necroinflammation [142–145]. Moreover, these individuals maintain a very high level of specific anti-HBV cytotoxic T lymphocyte (CTL)-response even many years after clinical recovery and anti-HBs seroconversion, as a possible consequence of the continuous stimulus exerted by the minute amounts of viral proteins that OBI produces [41, 42]. In addition, studies performed on the woodchuck model analyzing liver tissues of these rodents showed that animals that have recovered from acute hepatitis due to WHV show a life-long persistence of an occult infection associated with a mild but persistent liver necroinflammation [146]. Long-term studies evaluating HCV patients with contemporary occult HBV infection have shown that phases with a rise of ALT levels correspond to the reappearance of circulating HBV DNA [112, 113]. Summarizing, all these observations might suggest that patients with OBI show transient phases of viral reactivation over time that is promptly controlled by the CTL-response, although a modest but histologically evident degree of liver damage persists.

A recent long-term observational cohort study evaluating the clinical outcome of a large series of chronic hepatitis C patients tested for OBI by liver DNA analysis in the 1990s and followed up for a median time of 11 years showed that OBI is significantly associated with both development of HCC (see below) and the most severe evolution of the CLD (i.e., decompensated cirrhosis), and finally that chronic HCV patients with OBI have a significantly increased risk of liver-related death compared to OBI-negative patients. Notably, the negative effects of OBI disappeared in patients therapeutically cured from hepatitis C [147].

Altogether, these data seem to confirm the hypothesis that—at least in immune-competent individuals—the occult infection is in itself innocuous, being unable to provoke a clinically significant liver injury, but if other causative agents of liver injury co-exist (i.e., HCV infection, alcohol abuse, etc.) it might be a factor making the course of the liver disease worse [148].

A further point that has to be considered is that part of the patients with productive HBV infection and classic chronic hepatitis B, after years or decades of HBsAg carriage, may show a progressive reduction of viral replication and amount of serum HBsAg that may even disappear over time with consequent development of OBI. However, if cirrhosis had already been established during the overt phase of the infection it obviously persists also in the occult phase, and, importantly, the risk of HCC development persists although reduced in comparison with cases of long-lasting HBsAg positive status [149–152].

Occult HBV Infection and HCC

HBV is a well-recognized oncogenic virus and one of the main etiologic agents of HCC worldwide. Much evidence indicates that HBV may maintain its pro-oncogenic propensity also when it is in the occult phase of the infection [153, 154]. Subjects with chronic hepatitis C appear to be particularly prone to HCC development in cases with concomitant OBI [155–157], as also confirmed by the above-mentioned long-term observational cohort study that evaluated the clinical outcome of chronic HCV patients according to their OBI status [147]. Moreover, a recent meta-analysis subsequently confirmed that OBI is an important risk factor for HCC development not only in HCV-infected individuals but also in patients with CLD unrelated to viral infection [158]. Indeed, among HCV-negative patients OBI seems to exert its tumorigenic effect in individuals with genetic and alcoholic diseases as well as in individuals with cryptogenic CLD [44, 159–161]. In this context, a recent population-based cohort study, conducted for more than two decades on Taiwanese mothers screened for HBV infection at each delivery, should be mentioned. This study showed that HCC occurrence was significantly associated with the persistence of the HBsAg-positive status, but among the HBsAg-negative mothers those who underwent HBsAg seroclearance during the follow-up had a significantly higher risk of HCC development compared to women never exposed to HBV [162]. Thus, this study shows that HBV maintains its hepatocarcinogenetic role after becoming occult even in the women that are known to be much less prone to develop liver cancer than men. Interestingly, a further recent study indicates that individuals undergone HBsAg seroclearence have a risk of HCC development comparable to that of subjects with persisting HBsAg positivity but with undetectable serum HBV DNA [163]. A final important note concerns the studies performed in woodchucks and in ground squirrels. Both these rodents are susceptible to hepadnavirus infections and have a high risk of developing HCC also after the apparent clearance of the hepatitis virus with disappearance of the viral surface antigen and seroconversion to the corresponding antibody but, invariably, with the long-term persistence of viral DNA at intrahepatic level [164, 165].

Summarizing, large parts of the available data indicate that OBI is a potential pro-oncogenic condition. Although the pathogenesis of the OBI-induced hepatocarcinogenesis still has to be mostly elucidated, evidence exists that helps to delineate the mechanisms through which the occult HBV might contribute to hepatocyte

transformation. Indeed, it is generally accepted that an overt and active HBV infection may exert its pro-oncogenic role both indirectly (by inducing a chronic state of necroinflammatory liver injury that may progress through cirrhosis to HCC) and directly [by the synthesis of viral proteins (i.e., X protein, truncated preS/S proteins) provided with pro-oncogenic properties and by the propensity of the viral DNA to integrate into the host's genome] [153, 154]. OBI might maintain both indirect and direct tumorigenic potentialities. As reported above, in fact, it may induce a very mild but persistent necro-inflammation of the liver that—when another concomitant cause of liver injury is present—may contribute to the development of cirrhosis that is the most important predisposing factor of liver cancer. In addition, HBV DNA integration may be present in the occult infection, and low levels synthesis of viral proteins—including X and mutated preS/S proteins—may persist in the OBI phase.

Conclusion and Perspective

OBI is a fascinating and intriguing topic of viral hepatitis field, and learning about it appears to be of great importance for an overall understanding of HBV infection. In recent years a large number of studies have made it possible to disclose several of its virological aspects, to show its worldwide diffusion and to reveal its possible implication in various clinical contexts. The molecular basis of OBI is related to the long-term persistence of HBVcccDNA in the nuclei of the liver cells despite the absence of viremia and the HBsAg negativity, and indeed OBI appears to be a phase in the natural history of chronic HBV infection. The mechanisms determining OBI status have still to be mostly clarified, but it is evident that host defense mechanisms play an essential role in its induction by suppressing the viral replication and gene expression. Occult HBV infection is a well-known danger for human health in terms of risk of viral reactivation in conditions of immunosuppression as well as of transmission of the infection during liver transplantation. Increasing evidence also indicates that it may favor the progression toward cirrhosis of chronic liver diseases related to different etiologies and above all that it maintains most of the pro-oncogenic properties of overt HBV infection. Diagnosis of OBI currently relies on non-standardized techniques and can be performed only in highly specialized laboratories. Thus, the development in the near future of valid and commercially available assays allowing the detection of OBI in all cases in which its presence might be a clinical risk appears to be of great importance and the true challenge in this field of research.

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Chapter 14
The Basis for Antiviral Therapy: Drug Targets, Cross-Resistance, and Novel Small Molecule Inhibitors

Peter Revill and Stephen Locarnini

There is currently no cure for chronic hepatitis B (CHB), with current treatments limited to a small number of direct acting antivirals (Chap. 17), or interferon (Chap. 16), the latter being poorly efficacious against most HBV genotypes. There is an urgent need for new treatments that augment existing therapies, as well as development of cure strategies for the 300 million people living with CHB worldwide. In this Chapter we discuss the basis and limitations of current antiviral therapy, as well as recent advances leading to new approaches which may form the basis of future treatment and cure regimens.

The HBV Life Cycle

Genomic Replication Pathway

Hepatitis B virus (HBV) is a pararetrovirus with a partially double-stranded relaxed circular DNA genome which replicates via reverse transcription of an RNA intermediate (pregenomic RNA, pgRNA) (Fig. 14.1). Viral entry is mediated first via attachment of the hepatitis B surface antigen (HBsAg) to cell surface heparan sulfate (HS) proteoglycans (HSPGs) [1, 2], followed by viral entry through engagement of the preS1 domain of the large envelope protein to the sodium-taurocholate co-transporting polypeptide (NTCP) receptor [3]. Following this, the viral envelope is removed and viral nucleocapsids comprising the relaxed circular DNA (RC DNA) genome, the endogenous HBV DNA polymerase, and a host protein kinase, all

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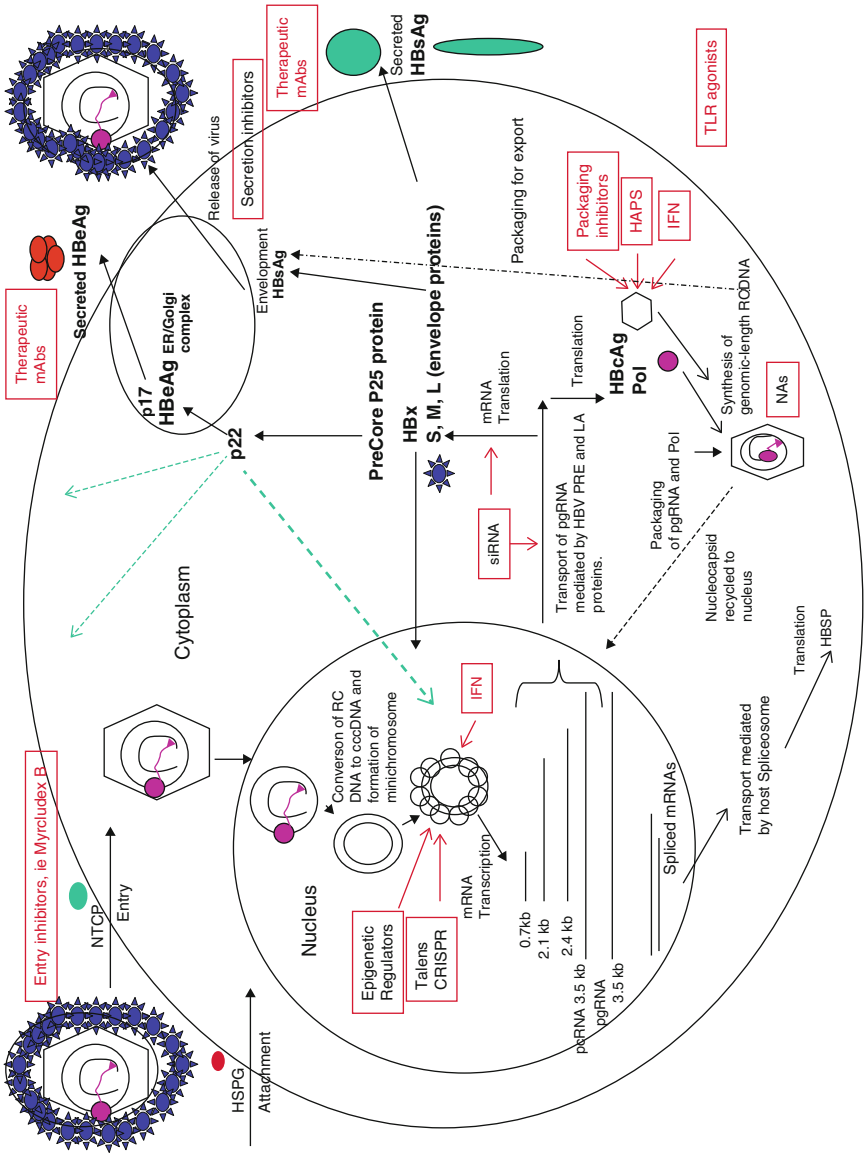

Fig. 14.1 Hepatitis B life cycle, showing nuclear and cytoplasmic stages of HBV infection and sites of action for current and future HBV treatments

enclosed by the hepatitis B core protein, are released into the cytoplasm. The capsids are transported to the hepatocyte nucleus [4], where the capsid disassembles and releases the viral genome [5]. The partially double-stranded RC DNA is "repaired" by the endogenous HBV DNA polymerase and host cell DNA polymerase II to be fully double-stranded forming a covalently closed circular (ccc) DNA structure [6]. This highly stable nuclear-localized cccDNA is the major transcriptional template for pgRNA and viral mRNA production, which complexes with host cell histones, the viral core protein (HBcAg) and other nuclear proteins and transcriptional factors to form viral minichromosomes [7].

Two forms of RNA molecule are transcribed from cccDNA, namely the greater than genome length 3.5 kb pg RNA which also serves as mRNA for the HBV core and polymerase proteins, as well as viral mRNAs encoding the HBV precore (pcRNA, 3.5 kb), envelope (2.4 and 2.1 kb), and HBx (0.7 kb) proteins.

Following the export of pgRNA from the nucleus and translation of the viral core and polymerase proteins, the pgRNA and the polymerase protein are packaged within newly forming viral nucleocapsids. The reverse transcriptase domain within the viral polymerase promotes synthesis of minus sense DNA, which is then copied to plus sense DNA, producing the relaxed circular DNA genome. Two fates await the nucleocapsid-localized RC DNA—it may either be recycled back to the nucleus to continue the cycle of cccDNA generation and formation, mRNA synthesis and RC DNA formation, or it may bud into the Golgi (microvesicular body) where it is enveloped by viral surface proteins, and secreted from the cell. The mature virion is then secreted into the blood, enabling the infection cycle to continue.

Generation of HBV Proteins

mRNAs transcribed from cccDNA encode the core protein (nucleocapsid) and polymerase (translated from the 3.5 kb pgRNA), the precore protein which is processed to form the secreted hepatitis B e antigen (HBeAg, translated from the pcRNA), envelope proteins and the HBx protein. The pcRNA is initially translated as an intracellular 25 kDa protein (p25), which is subsequently processed to a 22 kDa molecule (p22) and finally the 17 kDa HBeAg which is secreted from the cell [8]. The HBeAg in serum exists as dimeric and multimeric forms and delineation of both the HBeAg [9] and HBcAg [10] crystal structures, sheds light on structural differences between these molecules that may facilitate identification of future therapeutic targets for both molecules. Cysteine molecules at positions −7 and 61 are critical for HBeAg stability, whilst core dimers are formed via the core dimer interface of two monomeric core proteins via the cysteines at position 61 [10]. In addition to replication of unspliced RNA molecules, up to 15 different splice derived transcripts have been identified, some of which encode novel proteins, including the hepatitis B splice protein, and have been associated with disease progression [11–15], although they do not appear to be critical for HBV replication.

HBV Genotypes

HBV exists as ten different genotypes (A–J) and multiple sub-genotype with geographically distinct distributions which are becoming increasingly blurred through human migration [16]. CHB should not be thought of as a single disease, but rather as a "disease state" or spectrum with marked diversity in CHB natural history including differences in age of acquisition (neonate vs. adult), modes of transmission, disease progression, replication phenotype, response to therapy, and disease resolution across HBV genotypes [16]. For example, persons infected with genotype A respond better to treatment with IFN-α than do all other genotypes and patients infected with genotype B respond much better than genotype C. However interferon is virtually ineffective against patients infected with genotype D. Genotype C is generally associated with more severe liver disease and a higher propensity for liver cancer than most other genotypes, although there are exceptions with subgenotype A1 associated with rapid progression to cancer without prior cirrhosis in young African males [17, 18] via mechanisms that have not been fully identified. HBV sequences differ by at least 8 % across the complete HBV genome between genotypes, and by between 4 and 8 % between sub-genotypes, suggesting that new treatments targeting specific areas of the HBV genome (such as the HBeAg) may need to be tailored to individual genotypes or subtypes.

Molecular Targets: Current and Future HBV Treatments

Current Direct Acting Antiviral Therapies

Current direct acting antiviral therapies (DAAs) inhibit the reverse transcription of pgRNA to DNA, i.e., the replication stage of HBV that takes place within the viral nucleocapsid (Fig. 14.1). They include the class of nucleos(t)ide analogs (NA) and act as competitive inhibitors of the HBV pol, inhibiting synthesis of viral DNA from the pgRNA template through chain termination. The clinical activity of these molecules is explained in detail in Chap. 16.

DAAs have no direct impact on the initial formation of cccDNA, although in reducing the amount of RC DNA they should indirectly impact cccDNA that is formed following the recycling of RC DNA to the nucleus. However, since this recycling happens early in the HBV infection cycle and DAA treatment routinely commences following the establishment of persistent infection long after initiation of HBV infection, the impact of DAAs on cccDNA levels is essentially minimal. This is important, as the continual presence of cccDNA in the nucleus, even in the absence of productive viral replication, is one of the main impediments to curing HBV infection. In turn, continual production of hepatitis B surface antigen (HBsAg) originating from cccDNA or integrated DNA is associated with disease progression and liver cancer, meaning that eradication of HBV-associated disease

Table 14.1 Cumulative annual incidence of resistance for lamivudine, telbivudine, adefovir, entecavir, and tenofovir

	Year 1	Year 2	Year 3	Year 4	Year 5	References
Lamivudine	23	46	55	71	80	[21]
Telbivudine	4.4	21	–	–	–	[24, 25]
Adefovir	0	3	6	18	29	[26, 27]
Entecavir	0.2	0.5	1.2	1.2	1.2	[28]
Tenofovir	0	0	0	0	0	[22, 23]
LMV resistant Adefovir	Up to 20 %	–	–	–	–	[29]
LMV resistant Entecavir	6	15	36	46	51	[19]

Shows percentage of HBeAg-positive patients encoding resistant strains of HBV, at each year of treatment

may only be achieved when strategies are developed that eliminate or suppress cccDNA.

To date five drugs belonging to the class of NA have been approved for treatment of CHB. These are lamivudine (LMV), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TDF). These drugs are divided into three groups, the L-nucleosides (LMV and LdT), acyclic phosphonates (ADV and TDF), and D-cyclopentane (ETV) groups. The effectiveness of many of these drugs is limited by the selection of resistant strains of HBV during treatment [19]. This is because the "error prone" nature of the HBV reverse-transcriptase results in a high mutation rate (1 in every 10^5 nucleotide substitutions for each cycle of replication [20]) leading to a large population of variant HBV sequences, some of which encode preexisting mutations in the HBV polymerase gene which confer resistance to one or more DAAs. These mutant strains of HBV are typically selected on therapy, giving rise to a dominant population of drug resistant viruses. The different DAAs have different propensities for selection of resistance, with the most widely used drug LMV driving selection of resistant HBV in up to 23 % of patients following 12 months of therapy, rising to 80 % by 5 years of treatment [21]. In contrast it is encouraging that there is no evidence to date for selection of HBV strains that encode resistance to TDF, even following 5 years of treatment [22, 23] (Table 14.1). Although rates of resistance are low for adefovir and entecavir monotherapy, in the setting of prior resistance to LMV, high rates of resistance to these drugs is observed because of cross-resistance (Table 14.1, adapted from Zoulim and Locarnini, 2009 [19]).

Resistance Profiles of Current DAAs

The molecular mechanisms of resistance to drugs for treatment of CHB have been eloquently described previously [19, 30] but are briefly summarized herein.

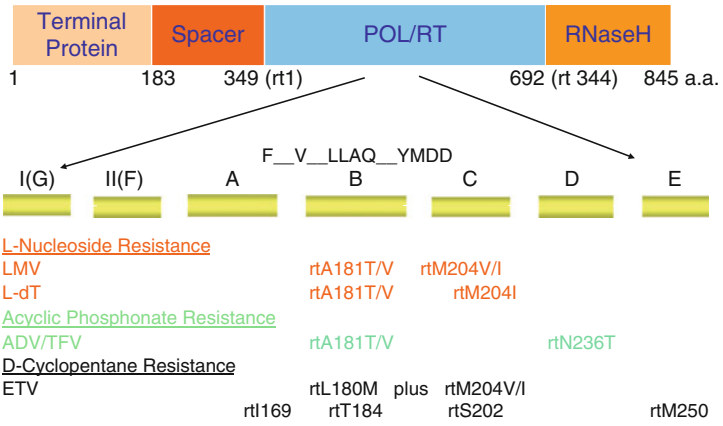

Fig. 14.2 Primary resistance mutations to NA identified within the HBV polymerase. (Reproduced from Zoulim and Locarnini 2012 [31])

To date, eight codons within the HBV polymerase are associated with primary drug resistance to NAs. These are codons 169, 180, 181, 184, 202, 204, 236, and 250 [19] (Fig. 14.2).

The reason for the strong barrier to selection of resistance to the acyclic phosphonate TDF is unclear. It remains to be determined whether continued use of this drug as monotherapy leads to selection of TDF resistant HBV. Nonetheless, the high degree of potency exhibited by TDF (and related compounds such as TAF—see below), and the high genetic barrier to selection of HBV resistance shows there is a role for these compounds in first line therapy against CHB. These compounds also have an important role when first line therapy with alternative NA treatments (such as LMV) has led to the selection of HBV encoding resistance mutations, or in the setting of failed immunomodulatory therapy.

New DAAs in the Pipeline: Tenofovir Alafenamide (TAF)

Although TDF is a very effective antiviral compound with no evidence to date of selecting antiviral resistance, there are reports of small numbers of HBV infected patients who do not respond to TDF therapy [130]. The reasons for this are unclear—it is likely that poor compliance is a contributor in these studies, but where compliance was not an issue it suggests reduced TDF efficacy might be related to quasispecies diversity or coinfection with multiple genotypes, such as genotypes A and G. Tenofovir alafenamide (TAF) is a TDF prodrug that provides efficient delivery of active drug to hepatocytes at reduced dosage, with improved plasma stability [32]. A recent study of 51 CHB subjects with HBeAg negative HBV infection showed that doses as low as 8 mg per day for 4 weeks resulted in similar levels of viral decline as the standard 300 mg daily dose of TDF [33]. Further clinical studies in larger cohorts are currently underway at a dosage of 25 mg per day.

New Compounds Undergoing Clinical Trial

DAAs are limited in their effectiveness in that they only inhibit active viral replication. They have no effect on viral entry, nor does it on the preexisting pool of viral cccDNA. These mRNAs express a continual source of HBV proteins such as the HBsAg and HBx, both associated with persistence and disease progression. There is an unmet need for new HBV treatments which complement existing antiviral therapies. In the following section new strategies currently under phase II clinical development targeting HBV entry, or expression of HBV mRNAs and small molecules targeted to the core protein will be discussed.

Myrcludex B

Although the discovery of the NTCP receptor for HBV entry has been relatively recent [3, 34, 35], it has been known for some time that synthetic peptides derived from the large envelope protein block HBV entry, as well as entry of the related hepatitis delta virus (HDV), which utilizes the HBV envelope protein for viral entry [36–39]. This discovery has led to the production of an HBV "entry inhibitor" Myrcludex B, which is a myristlyated PreS1 peptide currently under clinical trial. It has recently been shown that Myrcludex B not only prevents HBV spreading from infected human hepatocytes in vivo, but also hinders amplification of the cccDNA pool in initially infected hepatocytes [40]. This important finding suggests Myrcludex B could be a useful tool in the treatment of CHB, as well as more obvious applications such as the prevention of reinfection following liver transplantation.

RNA Interference

RNA interference (RNAi) is gaining increasing credence as a treatment strategy for chronic HBV. RNAi is a process by which small interfering RNA molecules of 21–25 nucleotides (short interfering or siRNAs) induce gene silencing at the post-transcriptional level, to effectively knock down gene expression [41]. The overlapping nature of the HBV genome means that multiple HBV RNAs can be targeted by a single siRNA molecule [42]. Cell culture and murine studies have shown that RNAi, delivered as a HBV plasmid, inhibits HBV replication in these models [43]. In transgenic mice, RNAi expression reduced HBsAg secretion in serum, as well as HBV mRNAs and genomic DNA in the liver, and also reduced the number of hepatocytes staining positive for core antigen (HBcAg) to undetectable levels [44].

Although effective at reducing HBV replication in cell culture and murine models, progress towards RNAi as an effective therapy has been limited by difficulties in delivering siRNA molecules to the liver. A recent major advance in the field has overcome the problem of liver-specificity, using cholesterol-conjugated siRNAs [45] which localize the siRNAs to the hepatocyte. Using this approach, researchers

at Arrowhead have demonstrated liver-specific knockdown of HBV replication and protein expression in murine models [45]. These studies have now been extended to a chronically infected chimpanzee [46] and Arrowhead has successfully treated patients in a phase 2 multicenter randomized, double-blind placebo controlled dose-escalation study in patients with HBeAg-negative CHB whose viremia was controlled by entecavir [47]. The RNAi ARC-520 was shown to be safe and well-tolerated, with a 50 % drop in HBsAg observed in treated subjects compared to placebo controls. In addition to the Arrowhead molecules, RNAi approaches are being developed by other biotech companies including Alnylam [ALN-HBV: ESC-GalNAc-SiRNA multicomponent lipid nanoparticles] and Tekmira [TKM-HBV: Lipid Nanoparticle (LNP)]. These too show promise, with preclinical evaluation of the Alnylam RNAi demonstrating a 2.3 log reduction in HBsAg in chronically HBV-infected chimpanzees. Since prolonged expression of the HBsAg is associated with increased risk of progression to HCC [48], it remains to be determined whether siRNA-mediated reductions in HBsAg levels would positively impact on the long-term risk of progression to liver cancer.

Emerging Viral Targets

HBV Core Protein

The HBV core protein (nucleocapsid) is critical for viral RNA packaging, reverse transcription and intracellular trafficking. It is also an important for cccDNA generation and stability, binding directly to the cccDNA [49, 50]. These properties suggest the core protein is a suitable therapeutic target, enabling regulation of multiple facets of the HBV life cycle. Elucidation of the core crystal structure in 1999 [10] has provided new insights in core protein assembly, including defining the core dimer–dimer interface [10] leading to development of a range of compounds with therapeutic potential.

Packaging and Capsid Assembly Inhibitors

Several non-nucleoside analog (NNA) inhibitors of pgRNA packaging and HBV capsid assembly have been identified that dysregulate or selectively inhibit either pgRNA encapsidation, nucleocapsid assembly, or both. These include the phenylpropenamide derivatives AT-61 and AT-130 developed by Avid Therapeutics (later managed by Gilead Sciences) [51]. These compounds selectively inhibit viral pgRNA packaging [52] and are active against both wild-type and lamivudine-resistant HBVs [53, 54], inducing structural changes in HBV capsids. AT-130 has been shown to bind to a promiscuous pocket at the core dimer–dimer interface that favors a unique binding site in the capsid [55, 56]. This binding decreases viral production by initiating virion assembly prematurely in the replication cycle, resulting in morphologically normal but empty capsids that are noninfectious [52].

Hetero-aryl-dihydropyrimidines (HAPS) are a class of antiviral agent which inhibit HBV replication by preferentially stabilizing non-capsid polymers of the core protein [57–62]. In addition to reducing HBV replication and pgRNA levels, they markedly reduce cccDNA [59] and prevent interaction of the core protein with the minichromosome, thereby inhibiting cccDNA transcription and stability [57]. Like the phenylpropenamides, the HAPs are also active against nucleos(t)ide analogs (NA)-resistant strains of HBV [54].

Other compounds targeting the viral nucleocapsid include core inhibitors being developed by Novira Therapeutics (NVR-1221) [63] and 2-amino-*N*-(2,6-dichloropyridin-3-yl) acetamide which binds in the groove structure within the HBV capsid [64].

HBV cccDNA

The continual presence of cccDNA in the hepatocyte nucleus is a major impediment to HBV treatment and cure, as it is not directly targeted by current treatments and is a continual source of the pgRNA transcriptional template and additional viral mRNAs. Although a proportion of cccDNA molecules are removed during resolution of acute (transient) HBV infection, due to immune-mediated clearance of HBV infected cells during hepatocyte turnover [65–71], reactivation of HBV infection in immunosuppressed patients that may have previously resolved their acute infection [72–76] shows that cccDNA is not completely cleared during disease resolution in all patients. Hence the removal of cccDNA, or the suppression of its transcriptional activity, is a desired aim for treatment and cure regimens.

Epigenetic Regulation of cccDNA

Studies of the related hepadnavirus duck hepatitis B virus (DHBV) show that cccDNA extracted from infected duck livers exists as a heterogeneous pool of viral minichromosome of 20–21 topoisomers, present as either a half-chromatized (transcriptionally active) or fully chromatized (transcriptionally silent) molecules [7]. Studies of cccDNA extracted from HepG2.2.15 cells which are stably transformed with an overlength copy of the HBV genome showed that HBV cccDNA is also present as a minichromosome [77].

HBV replication and gene expression is controlled by epigenetic regulation of cccDNA, by acetylation and methylation of histone proteins which surround the cccDNA minichromosome [49, 61]. Histone deacetylases (HDACs) are a class of enzymes that remove acetyl groups from histones, allowing them to wrap the DNA more tightly, thereby regulating transcription. A second class of enzymes, the histone acetyl transferases (HATs) facilitate binding of transcription factors to DNA through the acetylation of histones bound to cccDNA. Together these molecules play a critical role in transcription of viral mRNAs from the HBV minichromosome, including pgRNA, the major replicative intermediate.

Recent advances in the HIV arena have shown that molecules which inhibit HDAC activity (HDAC inhibitors or HDACi) reactivate latent HIV proviral DNA incorporated within resting CD4 cells. This has led to the development of the "Shock and Kill" approach for HIV cure [78], whereby latent provirus in resting CD4 cells is reactivated by HDACi's, with subsequent replicating virus then destroyed using DAAs [79, 80]. It is over 20 years since Newbold and colleagues [7] first suggested that HBV chromatin could represent a unique target for novel antiviral therapies. However it is not clear that the aforementioned HIV approach is directly applicable to HBV treatment, since HBV is never truly "latent." Indeed, acknowledged reactivation of HBV cccDNA would be an outcome, leading to increased viral replication, with elimination or deactivation of cccDNA likely to be the best means of achieving HBV cure. However, such reactivation could be managed clinically with potent NAs such as TDF or ETV. Transcriptionally active cccDNA is associated with histone acetylases (PCAF, p300, CBP), and HBx regulatory protein [58, 81] and it has recently been shown that interferon alpha (IFN-α) represses cccDNA transcription by recruting a range of transcriptional co-repressors to the cccDNA, providing a molecular mechanism for IFN-α mediated repression of HBV transcription.

Stimulation of HDAC activity decreases both pgRNA transcription and HBV replication [82] suggesting that treatment with HDAC inhibitors would increase transcription and replication. However for reasons that are unclear, treating HBV infected cells with the HDAC SIRT1 inhibitor Sirtinol *reduced* HBV replication and pgRNA transcription [83]. It has also been shown that inhibition of cccDNA bound HAT activity results in detachment of PCAF and p300, decrease HBV replication and pgRNA transcription from cccDNA [82]. Together these findings show that epigenetic regulation of cccDNA is possible, although a complete understanding of factors regulating cccDNA biogenesis and expression will be required before these molecules are further progressed with confidence.

DNA methyltransferases. HBV gene expression is regulated in part by DNA methylation, with transfection of methylated HBV DNA in HepG2 cells leading to reduced HBV mRNA levels, decreased surface and core protein expression and decreased secretion of HBV viral proteins [84]. Methylated cccDNA was also identified in tumor and nonneoplastic human liver tissues [84]. Proof of principal utilizing HBV DNA methylation as a method for reducing gene expression has been provided by Xirong et al. [85], who showed that DNA methyltransferase 3a (Dnmt3a) targeting the HBV X promoter (XP) suppressed HBV replication and HBsAg expression in HepG2 cells and transgenic mice. These studies are yet to be performed using infection models which would demonstrate specificity for HBV cccDNA, but they show promise as an additional mechanism for regulating HBV replication and gene expression.

Selective Removal of cccDNA

The ability to eliminate cccDNA from the infected hepatocyte would be a major advance towards HBV cure. The development of designer targeted endonucleases that specifically recognize and cleave selected DNA sequences [86], resulting in

gene disruption due to imprecise DNA repair, suggests this approach is indeed feasible. These methodologies include transcription activator-like effector nucleases (TALENs) [87] as well as the CRISPR (clustered regularly interspaced short palindromic repeats) CAS9 system [88, 89].

Talens. DNA targeting transcription activator-like effect ors (TALEs) derived from the plant pathogen *Xanthomonas* [90] been coupled with nuclease domains to form transcription activator-like effector nucleases (TALENs), capable of directed cleavage of specific DNA sequences. Recently, Bloom and colleagues reported that TALENS targeting HBV core and envelope gene sequences led to targeted disruption of up to 31 % of cccDNA molecules in HepG2.2.15 cells, with concomitant reductions in other HBV markers such as the secreted HBsAg [87]. This is a promising finding; however, it must be noted that this cell line, similar to other stably transduced cell lines commonly used for analysis of cccDNA (i.e., AD38 cells) contain integrated forms of the HBV genome, and true cccDNA is not the transcriptional driver in these systems. It remains to be determined whether the observed changes in DNA levels were mediated against newly synthesized cccDNA molecules, or had resulted from disruption of HBV DNA integrated into the HepG2.2.15 genomes. The effectiveness of Talens against cccDNA will become clearer when they are tested in the NTCP expressing cells that mimic more closely natural infection. Importantly though, TALENs demonstrate high specificity for HBV sequences [87], with "off-target effects" and toxicity not evident in cell culture or murine studies.

CRISPR. The CRISPR (Clustered Regularly Interspersed Palindromic Repeats) Cas9 (**C**RISPR **as**sociated protein **9**) gene editing technology has recently been utilized to target HBV DNA in cell culture [88, 89] and murine models [89]. This approach uses RNA "guide" sequences which target the CAS9 endonuclease to the desired sequence for specific cleavage, which is then disrupted following DNA repair via a nonhomologous end joining (NHEJ) process. Importantly, Seeger and Sohn [88] have recently demonstrated the utility of this approach to specifically target HBV cccDNA, using the NTCP-HepG2 infection model. They showed that CRISPR/CAS9 targeting of the HBV ENII/CP region and the PC regions resulted in deletions in HBV cccDNA up to 2 kb in size, providing proof of principal that this approach can be used for the targeted disruption of cccDNA.

Limitations to Epigenetic Regulation of cccDNA as Therapy

Epigenetic regulation of HBV cccDNA as anti-HBV therapy needs to be carefully considered due to possible off-target effects on acetylation of host DNA and their ability to reactivate HBV infection. Indeed, reactivation of HBV has been observed following treatment of patients with the anticancer HDAC inhibitory drug Romidepsin [91] and in patients receiving immunosuppressive therapy [72–76, 92]. This suggests that not all cccDNA is eliminated in some patients who have previously cleared an acute infection, raising questions about the true definition of HBV cure. With the development of increasingly sensitive diagnostic tools it may

become evident that some level of cccDNA persists in all patients, even with the clearance of the majority of infected hepatocytes, meaning that natural eradication of HBV is unachievable. Through specifically targeting cccDNA, it may be possible to induce cure, although this approach is currently not being considered based on current knowledge and clinical experience. A further problem with this approach for controlling HBV infection are "off-target" effects, since they may also affect the epigenome of host chromosomes. Approaches need to be developed that are specific to HBV cccDNA epigenome and not host DNA. Additional important questions include whether such alteration of cccDNA promotes integration of the modified DNA and whether this in turn may lead to expression of novel protein(s) and or a greater propensity for progression to HCC.

Future Targets for HBV Therapies?

HBeAg

The HBeAg is required for the establishment of persistent infection, with CHB rarely establishing in babies borne to HBeAg-negative mothers, in contrast to 90 % of newborns from HBeAg-positive mothers developing CHB [93]. In turn, low levels of HBeAg in patient serum are associated with HBeAg seroconversion in the setting of IFN-α treatment [94], this being an accepted marker of treatment response that usually precedes HBsAg clearance. Taken together these findings suggest that the HBeAg itself may be a suitable therapeutic target, with reductions in the levels of HBeAg perhaps driving the immune response to clear infection, or reducing mother to baby transmission in newborns. Unlike the related HBcAg, the HBeAg crosses the placenta [95], and is thought to establish tolerance to the HBcAg and HBeAg in the developing fetus [96, 97]. The HBeAg protein sequence differs from the HBV core protein by the addition of ten highly conserved [98] amino acids at the N-terminus and removal of the C-terminal arginine rich core domain [99]. Recent delineation of the HBeAg crystal structure [9] shows that the cysteine molecule at position −7 in the N-terminal 10-mer sequence (SKL**C**LGWLWG) bonds with cysteine 61 to form a dimer whose structure differs markedly from the HBcAg dimer [10]. It has been suggested that these differences may explain some of the biophysical, biochemical and functional properties of the HBeAg and HBcAg molecules [9, 10]. For example, the HBeAgs inability (usually) to form capsids, instead forming high molecular weight soluble forms in the blood [100], its propensity to initiate tolerogenic rather than immunogenic T-cell responses [101–104], inability to bind and activate B cells without T cell support [102–104], and suppression of innate immune signaling pathways [105–107]. It is hoped that the characterization of the HBeAg crystal may lead to development of molecules such as humanized mAbs which specifically target the HBeAg that may in turn drive HBeAg and HBsAg seroconversion. This approach should also be considered for other HBV proteins as it is becoming increasingly important for treatment of chronic conditions

such as rheumatoid arthritis and Crohn's disease [108]. Our group has recently identified a novel single variable domain (VNAR) of the shark immunoglobulin new antigen receptor (IgNAR) antibody which displayed biologically useful affinity for recombinant and native HBeAg, and recognized a unique conformational epitope [109]. This molecule was subsequently engineered for ER-targeted in vitro delivery to function as an intracellular antibody (intrabody), which effectively regulated precore/HBeAg expression, showing potential as a HBeAg therapeutic that is worthy of further investigation, exploiting the possible importance of FcRn expression on hepatocytes and their role in intracellular antibody "neutralization" [110].

HBx

The hepatitis B virus X protein is a non-structural protein that is essential to initiate and maintain virus replication after infection [111]. Although HBx does not bind directly to cccDNA, its recruitment is mediated through its interaction with a wide range of cellular transcription factors and cofactors [112]. Modulation of cccDNA transcription and HBV replication by "HBx-knockout" strains of HBV which transcribe less pgRNA that wild-type HBV strongly implicates the HBx protein in regulation of cccDNA transcription [58, 111]. These findings suggest that the HBx protein may be a suitable therapeutic target. Indeed numerous studies have shown that siRNA mediated knockdown of the HBx protein reduces HBV replication [113, 114]. The overlapping nature of the HBV genome means that siRNAs targeting the HBX gene also target the major pgRNA transcriptional template, as well as the 3′ end of the HBV polymerase. Consequently it is unclear if HBV replication is reduced in these models due to reduced HBx-mediated transcription of cccDNA, or decreased levels of the pgRNA itself, or both. Studies using the NTCP infection model as well as in non-transformed hepatocytes are required. It should also be noted that the overlapping nature of the HBV genome means that mutations at positions A176T2/G1764A which frequently occur in the HBV basal core promoter also alter the HBx coding sequence at positions 130 and 131. These changes are associated with increase virulence and disease progression, with Tseng and colleagues showing that the A1762T mutation in particular was associated with increased risk of liver cirrhosis [115]. The role of HBx in this association is unclear. Identifying the crystal structure of the HBx protein may enable identification of the exact location of binding sites with host transcription factors, facilitating the HBx protein as a novel therapeutic target.

HBsAg

HBsAg clearance and seroconversion to anti-HBs is the closest outcome to natural HBV "cure." However, this rarely occurs during therapy, thought mainly due to the persistence of the cccDNA and associated minichromosome transcriptional tem-

plate which is not affected by the most potent antiviral currently available, TDF. A recent clinical study of HBeAg-positive patients treated with TDF showed that HBsAg loss was observed in 10 % of patients following 5 years of therapy [22, 27]. Independent predictors of HBsAg loss in these patients included high levels of HBsAg at baseline and the log 10 IU/ml decline in HBsAg levels by week 24. This contrasts with studies on patients treated with IFN, where treatment response (HBsAg loss) was associated with low levels of HBsAg at baseline [116]. Since the continued expression of HBsAg in the absence of viral replication is associated with adverse disease outcomes, strategies which target the HBsAg itself may be a useful addition to current NA therapies and reduce the likelihood of progression to liver cancer in CHB patients. One such approach may be therapeutic mAbs. This approach was initially tested by van Nunen and colleagues [117], who evaluated the efficacy of the anti-HBs mAb Tuvirumab in chronic HBV patients, either as monotherapy or in combination with IFN. Although long-term efficacy (neutralization of HBsAg in serum) was not observed, temporary reductions of HBsAg levels by at least 50 % were observed in all patients. In three patients receiving combination therapy, serum HBsAg was reduced to background levels. In addition, loss of serum HBV-DNA was observed in three patients in the combination group, with subsequent HBeAg seroconversion in two patients. Subsequently Lever and colleagues showed that although treatment of two patients with an anti-HBs mouse monoclonal (RFHBs1) had no impact on serum HBsAg levels, serum HBeAg levels were reduced to below the level of detection in both patients, and HBV DNA was reduced to below the level of detection in one patient [118]. Taken together, these findings suggest that therapeutic mAbs targeting the HBsAg are worth revisiting.

Host Targets

Toll-Like Receptors

New therapeutic approaches for treatment of CHB are not limited to targeting HBV itself, with promising studies showing upregulation of cell defense mechanisms by TLR agonists activating antiviral signaling pathways that reduce HBV replication [119]. For example, TLR7 and TLR8 agonists limit HBV replication by upregulating IFN-α expression, with these compounds currently in phase II clinical trials [120–122].

APOBEC

APOBEC proteins are host cytidine deaminases that hypermutate single stranded DNA. Lucifora and colleagues recently demonstrated that IFN-α mediates its antiviral effect at least in part by upregulating APOBEC3A enzymes that hypermutate the HBV genome [123]. The antiviral effect was also mediated following stimulation of the lymphotoxin beta receptor, which activated the related APOBEC3B [124]. Interestingly, the HIV Vif protein binds to and downregulates Apobec3, and

is thought to be a potential therapeutic target for HIV, although a HBV protein which downregulates Apobec analogous to the HIV Vif protein is yet to be identified. Although the HBV core protein binds Apobec3, it does not downregulate Apobec expression, instead facilitating the interaction of Apobec with viral cccDNA, thought to be critical in mediating the antiviral effect of IFN-α [123]. Since HBV encodes proteins such as the HBeAg that downregulate antiviral signaling pathways [105–107, 125, 126] it would not surprise if it also encodes a suppressor of Apobec3A. Studies determining whether HBV encodes a mediator of Apobec3 expression similar to the HIV Vif protein are worthy of consideration.

Cyclophilin

Cyclophilins are host cofactors essential for replication of a number of viruses including hepatitis C virus (HCV) and HIV [127, 128], although their role in the HBV life cycle is yet to be elucidated. Cyclophilin inhibitors have antiviral activity against HCV, and it has recently been shown in cell culture studies that the cyclophilin inhibitors alisporivir and NIM811 have antiviral activity against HBV, reducing HBV DNA and HBsAg production [129]. The effect was enhanced following co-treatment with the NA telbivudine. The potential of cylophilin inhibitors as an HBV therapy has been recognized by biotechnology companies such as OnCore, with the cyclophilin inhibitor OCB-030 currently under development for this purpose (http://www.oncorebiopharma.com).

Conclusion

There is an urgent need for improved HBV therapies and curative regimens for chronic HBV. Although there is a highly effective preventative HBV vaccine, it has no impact on existing infections affecting over 300 million people globally. The development of new treatments, including more potent DAAs, entry inhibitors, epigenetic or enzymatic manipulation of HBV cccDNA, viral protein targets and host targets, suggests that we are on the dawn of a new era for chronic hepatitis B treatment. The recent identification of the HBV NTCP entry receptor and the establishment of appropriate cell culture and animal models enabling studies of the complete HBV life cycle including cccDNA should speed development of new therapeutic approaches that may lead to HBV cure in the foreseeable future.

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Chapter 15
IFN-Based Therapy and Management of Patients

Victor C.K. Lo and Harry L.A. Janssen

Introduction

Interferon alfa (IFN) is an endogenously produced 128-amino acid cytokine that acts as an immunomodulator, enhancing cell-mediated immunity against viruses. Specifically, they are named because of their ability to interfere with viral replication. They also activate natural killer cells and macrophages, and upregulate antigen-presenting cells. Recombinant IFN alfa was approved in the 90s as the first agent for the treatment of chronic HBV. It is administered parenterally.

A study in 2014 investigated the mechanism by which IFN alfa induces a direct antiviral effect. It was found that interferon alfa and lymphotoxin-β receptor activation may induce non-cytolytic degradation of covalently closed circular DNA (cccDNA) via upregulation of proteins of the APOBEC3 family [1]. The degradation of intrahepatic cccDNA would prevent HBV reactivation. Since the genomic DNA was not affected, the authors suggest that the induction of nuclear deaminases, such as those induced by lymphotoxin-β receptor activation, may have potential as a new therapeutic for hepatitis B.

The pegylation of interferon (pegIFN), in which a polyethylene glycol is attached to the interferon protein, extends the half-life of IFN, stabilizes serum concentrations,

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and reduces the frequency of dosing and side effects, thereby improving patient compliance with treatment. Additionally, it was demonstrated that the use of pegIFN alfa leads to comparable or higher rates of HBeAg loss, HBV DNA suppression, and ALT normalization as conventional IFN [2]. Thus conventional (standard) IFN has largely been replaced with the use of pegIFN alfa. The treatment of chronic hepatitis B (CHB) uses recombinant pegIFN alfa-2a or 2b. PegIFN alfa-2a has a 40 kDa branched peg that is attached to IFN, whereas pegIFN-alfa-2b has a 12 kDa straight chain peg.

Interferon Monotherapy

The advantage of using IFN over oral nucleos(t)ide analogue (NUC) therapies is that treatment duration is finite, usually 48 weeks as recommended by current guidelines for treatment-naive patients, and there is no evidence for drug resistance. However, the frequency of adverse events is higher and is dose-dependent, and patient compliance may be lower due to the need for injection in contrast to the convenience of an oral agent. With the introduction of pegIFN alfa, injection schedules can be reduced from thrice weekly with conventional IFN to only once weekly.

While pegIFN alfa does not result in HBV suppression rates as great as NUCs, it has been shown that there exists post-treatment antiviral benefits which are more durable—a "delayed" effect. In patients with HBeAg-positive CHB, pegIFN alfa induces an early reduction of HBV replication, sometimes with a subsequent hepatitis flare. Using pegIFN alfa for a finite 1-year in HBeAg-positive patients resulted in HBeAg seroconversion rates of 32 % and 29 % respectively, found at 6 months post-treatment follow-up [3, 4]. These HBeAg seroconversion rates are significantly higher and more durable than what has been found with 1 year of NUCs such as LAM, ETV, or TDF. PegIFN alfa has also been shown to induce HBV DNA suppression, HBsAg clearance, normalization of ALT, and histological improvement [5]. Patients who achieve an IFN-induced HBeAg seroconversion also have a reduced risk of developing cirrhosis and HCC, leading to better clinical outcomes for patients.

PegIFN alfa has been used in the treatment of HBeAg-negative patients in whom HBV DNA continues to replicate despite the presence of anti-HBe immunity [6]. Treatment in HBeAg-negative patients with pegIFN alfa has shown a response (defined as HBV DNA below 400 copies/mL for up to 24 weeks after cessation of therapy according to the Peginterferon Alfa-2a Negative Chronic Hepatitis B Study Group) rate of approximately 20 % [5, 7], which is also durable and leads to better clinical outcomes [8]. Thus, a 48 week course of treatment with pegIFN alfa is currently recommended for HBeAg-negative CHB patients who have no contraindications to pegIFN alfa, as treatment is finite and virological response is durable.

A study in 2012 compared the treatment response between HBeAg-negative patients who received pegIFN alfa for 48 weeks with those who received pegIFN alfa for 96 weeks (with a dose reduction from 180 to 135 μg in the latter 48 weeks) [9].

This study examined a group of 128 patients with mostly HBV genotype D. It was observed that more than twice the number of patients who received pegIFN alfa for 96 weeks had a combined response of HBV DNA <3400 IU/mL and ALT normalization at 48 weeks post-treatment. Thus they concluded that extending treatment duration to 96 weeks improves sustained virological responses. However, it is important to consider side effects, as 12 % of patients withdrew from the extension group due to adverse events (AEs). The extended use of pegIFN alfa incurs a higher cost and risk of patient non-adherence, as well, so careful consideration is required before extending pegIFN alfa therapy. Nonetheless, pegIFN alfa is currently only therapeutic option that offers a reasonable chance of sustained off-treatment response.

Several studies examined the long-term effects of interferon use in CHB patients, particularly on survival and hepatocellular carcinoma (HCC). A placebo-controlled study analyzed 101 patients with a follow-up period of 1.1–11.5 years [10]. It was found that the cumulative incidence of HCC development was significantly higher, and the cumulative survival rate was lower in the placebo group compared to the treatment group. These findings were replicated in another study of 165 HBeAg-positive patients treated with IFN with a median follow-up period of 8.8 years [11]. Thus, the authors concluded that IFN results in long-term beneficial effects in terms of reduction of HCC and prolongation of survival.

Interferon Safety and Adverse Event Management

The most frequently reported side-effects associated with the use of conventional IFN were flu-like symptoms including: fever, fatigue, irritability, chills, headache, muscle aches, and local reaction at the injection site [12, 13]. Less common are: anorexia, nausea, insomnia, neutropenia, thrombocytopenia, alopecia, weight loss, and depression. A study in 2005 investigated the safety of pegIFN alfa for the treatment of CHB in 300 patients [13]. They found that all patients reported one or more of the adverse effects known to conventional IFN treatment with no reports of new adverse effects. The rate of dose reduction in the study was 22 % and therapy discontinuation was 9 %. The higher frequency of dose reduction using pegIFN alfa-2a compared to conventional IFN (rate of 10 %) was attributed to increased occurrence of neutropenia. However, while pegIFN alfa induced thrombocytopenia and neutropenia, it was found that the number of infections was low and relatively mild and that bleeding complications were also mild (epistaxis). Patients with liver cirrhosis should have more frequent monitoring, as they are at an increased risk of thrombocytopenia and bleeding complications.

Informing patients about adverse events and adequate treatment of symptoms, such as specific serotonin reuptake inhibitors (SSRIs) for depression, may lead to an increased proportion of patients capable of completing treatment without dose reduction. The use of anti-pyretics and analgesia may also help to relieve other side effects associated with the use of pegIFN alfa, such as flu-like symptoms.

Predicting Response to pegIFN Alfa

Since pegIFN alfa treatment is expensive and associated with considerable side effects, it is of clinical interest and significance to be able to predict which patients will have a high probability of response. Much investigation has been done on serum HBV DNA, ALT and HBsAg levels, HBV genotype and IL28B polymorphisms [14].

Pretreatment Response Prediction

Pretreatment virological, serological, and biochemical parameters such as viral load, HBsAg, HBeAg, and ALT levels as well as host and virus genetic factors have been investigated for their role in predicting response to pegIFN alfa. Being able to reliably predict and identify which patients will likely and unlikely benefit from pegIFN alfa or continuation of pegIFN will serve to increase cost-effectiveness and reduce patient side effects. Pretreatment ALT and HBV DNA levels have been demonstrated to be a reliable factor in helping clinicians form treatment plans [15]. Specifically, high pretreatment ALT levels and low HBV DNA were associated with a higher rate of sustained response to pegIFN alfa.

However, as viral load and ALT levels fluctuate throughout the natural course of disease, they are somewhat unreliable as predictive variables to treatment response. Thus, both host and viral genetic factors, like HBV genotype, have been investigated as a predictor of response to pegIFN alfa treatment. It has been found that CHB patients with HBV genotype A have the best response and that HBV genotype D is associated with poorer responses to treatment compared to other genotypes. Genotype A and D are more common among the Caucasian population, whereas genotypes B and C are more common among Asian populations [3, 16].

A 2012 study demonstrated that the presence of precore (PC) and basal core promoter (BCP) mutations in the viral genome affect the serological and virological response to pegIFN alfa [17] in HBeAg positive disease. Specifically, those with detectable mutant PC/BCP have a lower probability of response (HBeAg loss and suppressed HBV DNA) compared to those with wild-type, irrespective of HBV genotype. Thus, the authors conclude that the presence of wild-type virus is a strong predictor of response and HBsAg clearance. However, another failed to confirm these findings [18], suggesting that further investigation is needed to fully understand the effect of PC and BCP mutations and their predictive value to pegIFN alfa response.

Another study investigated the effects of host genetic polymorphisms on the interleukin 28B gene (IL28B, also known as IFN-λ-3) [19] on pegIFN alfa treatment response in 208 HBeAg-positive CHB patients. While the exact mechanism by which these genetic polymorphisms affect treatment response remain unclear, it was shown that there exists favorable IL28B genotypes (AA for rs12980275 and CC for rs12979860) which increase the probability of achieving a sustained HBeAg

seroconversion with pegIFN alfa. However, some patients maintain detectable HBV DNA and elevated ALT level despite HBeAg seroconversion [20]. While favorable IL28B polymorphisms were a strong predictor for serological response to pegIFN alfa in terms of HBeAg seroconversion, it was found that they are poor predictors for combined responses of HBeAg seroconversion and HBV DNA suppression (HBV DNA <2000 IU/mL). Thus, the authors recommend that genotypic variations in IL28B can be used in combination with other predictors of response such as HBV genotype and pretreatment HBV DNA and ALT levels, but not as a replacement. Another study in 2011 looked at other host genetic polymorphisms such as HLA-DPA1 and HLA-DPB1 as predictors to response to pegIFN therapy in HBeAg-positive patients [21]. Their findings suggest that genetic variations in HLA-DP regions may influence spontaneous and/or treatment-induced HBV clearance, but that further research is required to fully characterize the effects. Altogether, these studies provide evidence that host genetic factors are also important in the response to pegIFN alfa therapy, in addition to viral genotypic factors.

With regard to HBeAg-negative disease, a 2013 retrospective study also examined the effects of IL28B on pegIFN alfa response in CHB patients with mostly HBV genotype D [22]. They similarly found that particular IL28B genotypes are more favorable towards a positive response. Specifically, HBeAg-negative patients with HBV genotype D who carry the CC genotype of rs12979860 IL28B had an increased rate of sustained virological response and HBsAg clearance (3.9-fold higher) than those with the CT or TT genotype.

In contrast to the above two studies, other studies on CHB patients treated with pegIFN alfa demonstrated evidence that polymorphisms near IL28B gene were not associated with on- and post-treatment kinetics of HBV DNA and HBsAg levels [23, 24]. In light of conflicting results, further investigation is needed to fully characterize the effect of IL28B polymorphisms on pegIFN alfa response, particularly for HBeAg-negative patients, and its clinical relevance as a treatment predictor.

In a 2013 study, it was found that baseline HBsAg was the only independent predictor of loss of HBsAg at week 144, after combination pegIFN + ADV treatment for 48 weeks [25]. Specifically, the authors noted that a low baseline HBsAg was a strong predictor for HBsAg loss for HBeAg-negative CHB patients. However, two large multinational studies of patients treated with pegIFN alfa-2a found that baseline serum HBsAg levels did not correlate with antiviral response, regardless of HBeAg status [26, 27]. These conflicting results suggest that further studies are required to validate the predictive value of baseline HBsAg levels for pegIFN alfa response.

Response-Guided Therapy

Strategies to evaluate the effectiveness of pegIFN alfa during the course of therapy is important as well, as stopping treatment early for patients whom it will be ineffective serves to improve cost-efficiency and reduce side-effects. A number of

studies have examined on-treatment parameters for their predictive value in determining treatment-response.

A study in 2013 analyzed the HBsAg levels of 803 HBeAg-positive patients treated with pegIFN [28]. The authors found that on-treatment levels of HBsAg could predict off-treatment response. Specifically, for patients who had serum levels of HBsAg <1500 IU/mL by week 12 of therapy, 45 % achieved response (defined as HBV DNA <2000 IU/mL and HBeAg loss). In contrast, only 14 % achieved a response in those who did not experience HBsAg decline, and only 6 % of patients responded if they had serum HBsAg levels >20,000 IU/mL by week 12. This effect was found to be HBV genotype-dependent, as response rates were low in patients with genotype A or D if there was no HBsAg decline, and B and C if HBsAg levels were >20,000 IU/mL by week 12. By week 24, nearly all patients with serum HBsAg >20,000 IU/mL failed to respond regardless of HBV genotype. This study demonstrates that on-treatment serum HBsAg can be used to guide treatment decisions, particularly with regard to discontinuing pegIFN when HBsAg levels remain >20,000 IU/mL after 24 weeks of therapy.

For HBeAg-negative patients, a study in 2009 observed that an on-treatment HBsAg decline of greater than 1 $\log_{10}$ IU/mL and <10 IU/mL at week 48 was significantly associated with sustained HBsAg clearance 3 years after pegIFN alfa treatment [29].

A 2010 study investigated early on-treatment kinetics of HBV DNA and HBsAg and their predictive power on pegIFN alfa treatment response in HBeAg negative patients [26]. It was found that patients with HBV genotype D who do not experience a decline in HBsAg levels and achieve <2 $\log_{10}$ copies/mL change in HBV DNA by week 12 of treatment do not achieve HBV DNA suppression or ALT normalization 6 months post-treatment. Follow-up studies were conducted to validate this finding, and thus the stopping rule for this study was incorporated into current treatment recommendations for patients with HBV genotype D [30].

Combination of IFN with NUCs for the Treatment of Chronic Hepatitis B

Combination therapy of IFN with NUCs has been investigated as an approach to treating chronic hepatitis B. Theoretically, the antiviral effects of NUCs would strongly suppress HBV DNA replication, and the immunomodulating effects of pegIFN alfa would enhance the host response to eliminate infected hepatocytes. However, the following sections on pegIFN alfa combination with specific NUCs demonstrate that the superiority of combination therapy to monotherapy is not well established. Thus current international guidelines do not support the use of pegIFN alfa in combination with NUCs for the treatment of CHB. However, studies investigating the use of more recently approved NUCs and their combination with pegIFN alfa are currently underway in both HBeAg-positive and HBeAg-negative CHB patients. Additionally, other strategies such as add-on or switch-over to pegIFN alfa from NUC have been explored as alternatives to concurrent pegIFN + NUC therapy.

Combination IFN and Nucleoside Analogues

Lamivudine (LAM)

Many studies have been conducted on the use of pegIFN alfa combined with LAM for the treatment of chronic hepatitis B, as LAM is the first approved NUC for the treatment of chronic hepatitis B. A number of pivotal studies on both HBeAg-positive and HBeAg-negative disease have been described in this section in chronological order respectively.

In a study published in 2005, 307 HBeAg-positive patients were randomized to either pegIFN alfa-2b with LAM or with placebo for 52 weeks [3]. At the end of the follow-up period of 26 weeks, it was found that combination pegIFN + LAM therapy was not superior to pegIFN alfa monotherapy in terms of achieving a sustained response. Specifically, while combination therapy initially had higher response rates on-treatment (lower HBV DNA, higher rates of HBeAg seroconversion), the rates of HBeAg seroconversion, ALT normalization, HBV DNA suppression, and HBsAg clearance at the end of follow-up were similar between the two treatment groups. It was also observed that patients with HBV genotype A and B had a higher response rate than those with HBV genotype C and D. The authors concluded that combined pegIFN alfa-2b + LAM therapy is not superior to pegIFN alfa-2b monotherapy.

Another study in 2005 examined pegIFN alfa-2a monotherapy, LAM monotherapy and combination pegIFN alfa-2a + LAM, for 48 weeks of treatment with a 24 week follow-up [4]. They examined 814 HBeAg-positive patients, mostly infected with HBV genotype B or C. Those who received pegIFN alfa-2a + LAM or pegIFN alfa-2a alone had higher rates of HBeAg seroconversion (32 and 27 % compared to 19 % on LAM monotherapy) and also HBV DNA suppression (32 and 34 % compared to 22 % on LAM monotherapy). Furthermore, 16 patients who received pegIFN alfa-2a had HBsAg seroconversion, whereas none of the patients on LAM monotherapy did. The authors concluded that pegIFN alfa-2a offered superior efficacy over lamivudine in the treatment of HBeAg-positive CHB, on the basis of HBeAg seroconversion, HBV DNA suppression, and HBsAg seroconversion.

A large registration trial for HBeAg negative disease investigated the efficacy and safety of pegIFN alfa-2a alone, LAM alone, or a combination of the two for 48 weeks with a 24 week follow-up [5]. Their trial was placebo-controlled and had approximately 180 patients in each of the three treatment groups. It was observed that pegIFN alfa-2a monotherapy alone or in combination with LAM yielded higher rates of ALT normalization (59 and 60 %) compared to LAM alone (44 %) after follow-up. A similar finding was observed with respect to HBV DNA suppression (<20,000 copies/mL), where rates were higher in pegIFN alfa-2a monotherapy and combination (43 and 44 %) compared to LAM alone (29 %). Using a threshold of HBV DNA <400 copies/mL, the rates were 19 and 20 % compared to 7 % with LAM alone. Thus, while adverse event rates were higher in patients taking pegIFN alfa-2a, such as pyrexia, fatigue, myalgia, and headache, these patients also achieved a higher combined response, which was sustained at least up to 24 weeks post-treatment. The authors also concluded that the addition of LAM to pegIFN alfa-2a

did not improve response rates. This study provides support for the use of pegIFN alfa-2a monotherapy for HBeAg-negative CHB patients over LAM.

The study investigating the treatment responses of pegIFN alfa-treated patients for 48 versus 96 weeks of treatment also found that the concurrent administration of LAM during the first 48 weeks did not improve outcome in HBeAg negative patients [9].

These studies taken together suggest that the concurrent administration of LAM or monotherapy of LAM does not have superior rates of response compared to pegIFN alfa in both HBeAg-positive and HBeAg-negative CHB patients. Other treatment strategies such as add-on or switch-over could be investigated for benefit as well, but as LAM resistance has been well-established, future research may utilize more recent and potent NA like ETV and TDF.

Telbivudine (LdT)

A large randomize-controlled trial performed in HBeAg-positive CHB patients investigating the combination of pegIFN alfa-2a with LdT was published recently [31]. A total of 159 patients were randomized to LdT monotherapy, pegIFN alfa monotherapy, or pegIFN alfa + LdT combination therapy. Although HBV DNA reduction was more pronounced and rapid in the combination therapy group, the rate of occurrence of serious peripheral neuropathy was also significantly higher in this treatment group (7 cases in 50 patients, compared to 1 in 109 in the monotherapy groups). Thus, the trial was terminated prematurely due to these severe side effects, and the authors concluded that the combination of pegIFN + LdT should not be used.

Entecavir (ETV)

There have been few studies on the combinatory use of pegIFN alfa and ETV, a third generation NUC with more potent antiviral activity and lower incidence of resistance compared to older NUCs. ETV is currently recommended by AASLD, EASL, and APASL guidelines for treatment-naive chronic hepatitis B patients, so it is certain that new studies will be published on its use in combination with pegIFN alfa.

A study published in 2014 investigated the serological response rates of add-on pegIFN to ETV therapy in 175 HBeAg-positive CHB patients [32]. Add-on pegIFN treatment (180 μg/week) during weeks 24–48 was associated with a higher rate (19 %) of HBV DNA reduction to <200 IU/mL, compared to patients who continued ETV monotherapy (10 %). Therapy was also discontinued in patients who achieved HBV DNA <200 IU/mL, and it was found that 13 % of patients receiving add-on pegIFN achieved remission compared to 2 % of patients on ETV monotherapy. At 96 weeks post-treatment follow-up, those in the combinatory treatment group also experienced a higher rate of HBeAg seroconversion than those on ETV

monotherapy (26 % compared to 13 %). Thus the authors conclude that the addition of pegIFN to patients already on ETV monotherapy could be a useful strategy to further reduce viral load, prevent relapse, and facilitate the discontinuation of ETV therapy. Unfortunately, this study lacked a pegIFN monotherapy arm.

Another recent study from Asia investigated the efficacy and safety of switching long-term ETV therapy to pegIFN alfa-2a therapy [33] in highly selected patients with low HBeAg levels. A total of 192 HBeAg-positive patients on ETV for 9–36 months were randomized to either switch-over to pegIFN for or continued ETV monotherapy for 48 weeks. It was found that patients who switched to pegIFN achieved higher rates of HBeAg-seroconversion than those who continued on ETV monotherapy (14.9 % vs. 6.1 %), and that the only occurrences of HBsAg loss were confined to the pegIFN treatment group, at a rate of 8.5 %. Thus the authors concluded that switch-over to pegIFN after long-term viral suppression with ETV could be a viable strategy for inducing HBeAg seroconversion and potentially HBsAg loss.

In another 2014 study, 218 treatment-naive, HBeAg-positive Chinese patients were randomized to either pegIFN alfa-2a monotherapy for 48 weeks, concurrent ETV and pegIFN alfa-2a treatment during weeks 13–36, or lead in treatment with ETV for 24 weeks followed by pegIFN alfa-2a [34]. Response rates were evaluated at the end of pegIFN alfa-2a treatment and also at the end of 6-months follow-up. While the addition of ETV suppressed HBV DNA during treatment, the response was not sustained off-treatment at the end of 6-months follow-up. Although therapy was effective, as all three treatment groups achieved significant reduction rates in HBeAg, there was no evidence that combination treatment in either of the treatment sequences yielded superior benefit compared to pegIFN alfa-2a monotherapy in terms of immunological response. Rates of HBeAg seroconversion, HBsAg clearance or seroconversion were also similar between the three groups. Thus, in contrast to the above two studies, the authors concluded in that ETV add-on or pretreatment with ETV was not superior compared to pegIFN alfa-2a monotherapy, and that further investigation on the optimal combination of NUC with pegIFN alfa was required. However, it should be noted that ETV pretreatment in this study was 24 weeks, a shorter duration than the aforementioned studies.

These large studies taken together indicate that there is potential benefit in either switching over or adding-on pegIFN to prior long-term ETV monotherapy, but that the optimal combination or timing has yet to be determined.

Combination IFN and Nucleotide Analogues

Adefovir (ADV)

Relatively fewer clinical trials have been conducted on the use of pegIFN alfa in combination with ADV for the treatment of chronic hepatitis B. As with LAM, ADV is an older generation of NUC, and is likely to see less frequent use for the

treatment of CHB. Studies on both HBeAg-positive and HBeAg-negative CHB patients have been conducted and discussed here.

A study published in 2006 investigated cccDNA change after 48 weeks of combined pegIFN alfa-2b + ADV and its correlation to serological, virological, and histological markers [35]. Twenty-six HBeAg-positive patients were involved in this single arm study, and had biopsies done at baseline and end of treatment. They observed a 2.4 $\log_{10}$ decrease in cccDNA and 2.2 $\log_{10}$ decrease in intrahepatic HBV DNA after 48 weeks of combination treatment, along with a reduction in the number of HBsAg- and HBcAg-positive hepatocytes by 2.5- and 2.3-fold, respectively. Additionally, it was found that serum HBV DNA became undetectable in 13 (54 %) of the patients, 8 patients lost HBeAg with 5 of those patients experiencing HBeAg seroconversion, and 4 patients developed anti-HBsAg. Those who had lost HBeAg had significantly less intrahepatic cccDNA than those who did not. There was also a strong correlation between intrahepatic HBV DNA and serum HBsAg titre. Thus the authors concluded that the combination of pegIFN alfa-2b + ADV can effectively diminish intrahepatic cccDNA, HBV DNA, reflected by a reduction in serum HBsAg levels, and induce a positive serological, virological and histological on-treatment response.

A 2013 study investigated the sustained curative efficacy of ADV add-on therapy to HBeAg-positive patients already on pegIFN alfa-2a monotherapy with poor virological response [36]. They examined a total of 85 patients, with 34 receiving add-on ADV therapy and the remainder continuing on pegIFN alfa-2a monotherapy, both for 6 months. At the end of treatment, it was found that the addition of ADV significantly improved sustained virological and biochemical responses, and higher rates of HBeAg loss (55.9 % vs. 19.6 %) and seroconversion (41.2 % vs. 13.7 %). Thus, they concluded that add-on ADV was beneficial for patients experiencing poor virological response to pegIFN alfa-2a monotherapy. However, this study does not include a follow-up period after treatment, so it is uncertain what the sustained response and relapse rate is.

A study in 2014 investigated a small group of 61 HBeAg-positive CHB patients, randomized to receive either pegIFN alfa-2b alone or pegIFN alfa-2b + ADV for 52 weeks [37]. Analysis at the end of treatment revealed no significant differences in the HBeAg seroconversion rate, but that the rates of undetectable HBV DNA was significantly higher in the combination group. It was also found that thyroid dysfunction was significantly higher in patients receiving combination therapy. However, this study also does not include a follow-up period after treatment and had a relatively small sample size, so it remains to be determined if these findings are sustained in the long-term.

A prospective, randomized trial published in 2009 investigated the safety and efficacy of pegIFN alfa-2a + ADV compared to pegIFN alfa-2a monotherapy in 60 HBeAg-negative CHB patients [38]. Patients received therapy for 48 weeks and follow-up was conducted at 24 weeks post-treatment. It was found that combination therapy resulted in greater on-treatment viral suppression and ALT normalization rates, but that they were not sustained upon treatment cessation at 24 weeks post-treatment. Thus they concluded that while combination pegIFN alfa-2a + ADV

is safe, it was not superior to pegIFN alfa-2a monotherapy in terms of sustained virological and biochemical response.

A study published in 2011 assessed the virological and serological impact of sequential ADV therapy followed by pegIFN alfa-2a in 20 HBeAg-negative patients [39]. Patients received 20 weeks of ADV, followed by 48 weeks of pegIFN alfa-2a with an overlap of 4 weeks, and were then followed up at 24 and 48 weeks post-treatment. It was found that ten (50 %) of the patients experienced a sustained combined response (ALT normalization and suppressed HBV DNA). However, the authors acknowledge their limited sample size and lack of a control group. Thus, they suggest further investigation is needed to fully evaluate the strategy of sequential therapy.

In conclusion, these studies suggest that the use of ADV with pegIFN alfa has potential beneficial on-treatment serological and virological effects, but there is evidence that such benefits are not sustained in the long-term.

Tenofovir (TDF) Therapy

There have not been many clinical trials published on the combinatory use of TDF with pegIFN alfa yet as TDF is the most recent NUC approved for the treatment of CHB. However, as with ETV, TDF is recommended by AASLD, EASL, and APASL guidelines for treatment-naive CHB patients, more studies will inevitably be published on its use in combination with pegIFN alfa.

Preliminary data from a prospective, global randomized controlled trial investigating HBsAg loss using the combination of pegIFN alfa-2a + TDF have been recently published [40]. A total of 740 HBeAg-positive and HBeAg-negative patients of varying HBV genotypes were randomized to four treatment groups: concurrent pegIFN + TDF for 48 weeks, concurrent pegIFN + TDF for 16 weeks followed by TDF monotherapy for 32 weeks, TDF monotherapy for 48 weeks, or pegIFN monotherapy for 48 weeks. At the end of 48 weeks of treatment, it was found that combined pegIFN + TDF for 48 weeks resulted in a higher rate of HBsAg loss (7.5 %) compared to either TDF or pegIFN monotherapy (0 % and 2.4 % respectively), and also a higher rate of HBsAg seroconversion (5.9 %) compared to all other treatment arms (0.6 %, 0 % and 1.8 % respectively). Of the patients who experienced HBsAg loss, 73 % were HBeAg-positive, and most had HBV genotype A or B (A: 31.8 %, B: 36.4 %, C: 18.2 %, D: 13.6 %). Rates of HBeAg loss were also higher in combination treatment arms (24.3 % and 20.2 % respectively) compared to monotherapy arms (8.3 % and 12.5 % respectively). HBV DNA suppression (<15 IU/mL) was significantly higher in the TDF-treated patients (69.2 %, 71.2 %, 60.5 % compared to 20.8 % respectively). Thus, the authors concluded that the combination of pegIFN + TDF for 48 weeks is superior to either treatment given alone at the end of 48 weeks of treatment. It remains to be seen if these effects are sustained at a longer 72 weeks timepoint.

Conclusion

New antiviral agents against chronic HBV infection have resulted in significant advances but disease control and clearance is still difficult to attain due to persistent HBV replication from cccDNA in infected hepatocytes. While NUC therapy is safe, effective in suppressing HBV DNA, and convenient for patients, immune-based antiviral strategies are likely needed for the immune-mediated clearance of infected hepatocytes. Thus at the current time, the antiviral and immunomodulating effects of pegIFN alfa make it an important tool for clinicians to consider in treating their chronic hepatitis B patients, as it is practically the only licensed therapeutic option that can offer off-treatment, sustained response. Sustained off-treatment response can be achieved in about 20–30 % of HBeAg positive or negative patients. Pretreatment prediction rules using in particular HBV genotype, ALT and HBVDNA levels as well as response-guided therapy using quantitative HBsAg can optimize treatment response and help to individualize therapy. Further research into both viral and host genetic factors will also lead to better identification of patients likely to benefit from pegIFN alfa and minimize the number of patients receiving pegIFN alfa with little clinical benefit. Future studies on the use of pegIFN alfa in combination with newer antiviral agents, such as ETV and TDF, are required to determine which treatment regimens lead to the best clinical outcomes.

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Chapter 16
Nucleos(t)ide Analogue Based Therapy and Management of Patients

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Introduction

Antiviral therapy of chronic hepatitis B (CHB) is aimed to improve quality of life and survival by halting progression of liver damage to cirrhosis, end-stage liver disease, liver cancer (HCC), thus preventing anticipated liver-related death [1–3]. These goals are achieved by suppression of hepatitis B virus (HBV) replication either by short-term treatment with pegylated interferon (Peg-IFN) or by long-term therapy with potent nucleos(t)ide analogues (NUCs). According to the most recent international guidelines, Peg-IFN and third generation NUCs such as entecavir (ETV) and tenofovir disoproxil fumarate (TDF) are the first-line drugs recommended for CHB naïve patients [1–3]. One year of Peg-IFN treatment induces a durable suppression of viral replication in nearly 30 % of patients. However, Peg-IFN requires parenteral administration, has a limited efficacy, causes side effects which are generally mild in nature, and is contraindicated in patients with advanced liver disease due to the risk of decompensation associated with interferon-related hepatitis flares and/or infections. Conversely, management of patients receiving NUCs is very easy and these drugs are the treatment of choice in patients with compensated or decompensated cirrhosis, in patients of advanced age, in pregnant women, and in those not responder, contraindicated or unwilling to Peg-IFN.

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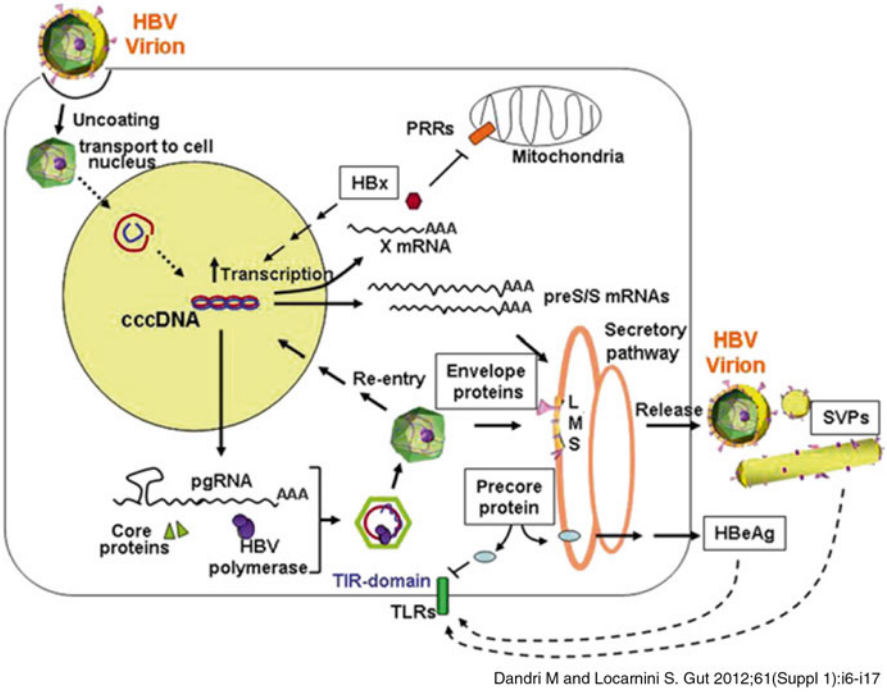

Fig. 16.1 The life cycle of hepatitis B virus (HBV)

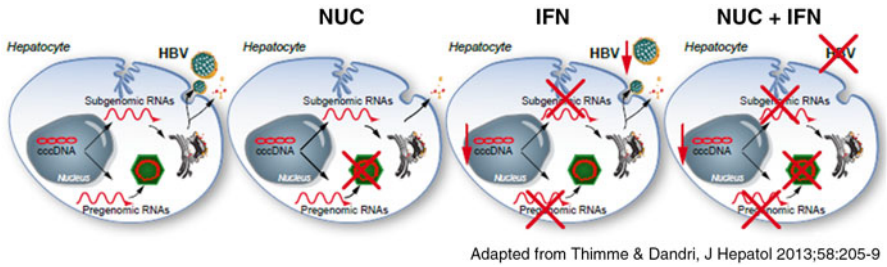

Fig. 16.2 Mechanisms of action of NUC and IFN

All NUCs belong to the same class, i.e., HBV polymerase inhibitors affecting the reverse transcription step of HBV replication (Figs. 16.1 and 16.2). They inhibit the reverse transcription of the pregenomic RNA into HBV DNA but have no direct effect on covalently closed circular DNA (cccDNA), explaining why they have only modest effects on the production of circulating viral antigens, i.e., HBsAg and HBeAg, and why, at variance from interferon-based treatment, immunological control of HBV infection is rarely achieved. However, long-term administration of NUCs is hampered by the selection of drug resistant mutants, leading to loss of efficacy, that differ according to the drug. NUCs can be subdivided into nucleoside

analogues, which include lamivudine (LMV), ETV, telbivudine (LdT) and nucleotide analogues including adefovir dipivoxil (ADV) and TDF. LMV, ADV, and LdT are not any more recommended due to the limited efficacy and moderate to high resistant rates whereas due to the long-term efficacy, the excellent tolerability and the negligible risk of drug-resistance ETV or TDF should be considered as the first-line drugs for CHB patients [3]. Worldwide, these latter drugs have become the preferred option for most patients with CHB, independently on the hepatitis B antigen (HBeAg) status, having the indefinite duration of treatment as the only potential disadvantage. In this chapter, we review the NUCs-based therapy in CHB patients, including HIV-coinfection and pregnant women, mainly focusing on the efficacy and safety of ETV and TDF therapy.

First and Second Generation NUC in Naïve Patients

Lamivudine

LMV was the first nucleoside analogue for the treatment of both HBeAg-positive and -negative patients. One year of LMV treatment achieved virological suppression in 36–44 % of HBeAg-positive patients and in 60–73 % of HBeAg-negative patients while HBeAg seroconversion rate in HBeAg-positive patients was approximately 20 % [4–6]. Notwithstanding, long-term LMV therapy inexorably ends with the selection of specific mutations in the HBV polymerase gene at rates that increase from 20 % after 1 year to peak 70 % after 5 years of therapy [7]. In HBeAg-positive patients, non-Asian ethnicity, high pretreatment serum HBV DNA level, male sex, longer treatment duration, and high body mass index are likely predictors of LMV-resistance (R) [8], whereas factors associated with the development of resistance in HBeAg-negative patients are poorly defined [9, 10]. As a rule, patients with incomplete suppression of HBV replication at week 24 have higher risk of generating mutated strains [11, 12]. The emergence of LMV-R leads to virologic rebound, alanine aminotransferase (ALT) flares, histological worsening, clinical decompensation, and HCC [7, 9, 13]. For those patients developing LMV-R, early add-on ADV or switch to TDF monotherapy is the recommended rescue treatment, whereas switching to another nucleoside analogue such as LdT or ETV is contraindicated as these drugs share a similar resistance profile [7].

Adefovir Dipivoxil

ADV was the first nucleotide analogue approved for use in patients with CHB showing significant HBV DNA reductions and liver histology improvement compared with placebo [14, 15]. In the 48-week registration trials, ADV achieved undetectable serum HBV DNA in 13–21 % of HBeAg-positive, with 12 % of HBeAg seroconversion and in 50–65 % of HBeAg-negative patients [14–16]. In HBeAg-negative

patients, 5-years of ADV treatment achieved a virologic and biochemical response in nearly 70 % of subjects with 5 % of patients achieving hepatitis B surface antigen (HBsAg) seroclearance [17]. Because of the significant rates (29 % after 5-year of treatment) of genotypic resistance (rtN236T and/or rtA181V/T mutations) over long-term administration and the suboptimal rates of virological response, ADV monotherapy is no longer considered in HBV patients [3, 17, 18]. Moreover, treatment with ADV may be limited by renal toxicity. Although none of the patients treated with ADV 10 mg/daily for 48 weeks showed a ≥0.5 mg/dL increase of the serum creatinine [14], this occurrence was reported in up to 9 % of patients after 5 years of ADV treatment [19]. Moreover, several cases of ADV-related Fanconi syndrome have also been reported [19].

Telbivudine

In the phase III GLOBE study, LdT demonstrated superior efficacy in achieving undetectable serum HBV DNA levels compared to LMV [20] and similar results were reported in the second year of the trial, both in HBeAg-positive (56 % vs. 38 %) and HBeAg-negative patients (82 % vs. 57 %) [21]. Among the 596 patients without genotypic resistance to LdT at the end of the 2-year GLOBE trial, two additional years of treatment increased the rates of virological and biochemical response to 76 % and 86 % and to 86 % and 90 % in HBeAg-positive and HBeAg-negative patients, respectively, while the cumulative rate of HBeAg seroconversion increased to 53 % [22]. However, at the second year of treatment the frequency of LdT-R increased to 25 % [21]. LdT was well tolerated even though asymptomatic grade 3/4 increases in creatine kinase levels were more common in LdT than in LMV-treated patients (13 % vs. 4 %, $p<0.001$) [21]. Interestingly enough, long-term LdT therapy was associated with an improvement of renal function particularly among patients with increased risk of renal impairment. Estimated glomerular filtration rate (eGFR) significantly increased by 15 mL/min/1.73 m^2 from baseline to year 4 of treatment [22, 23]. However, because of the significant rates of resistance, current international guidelines do not recommend LdT as a first line therapy for CHB patients.

First and second generation NUCs have been now replaced by third generation NUCs, like ETV and TDF, characterized by high potency and genetic barrier, and low rates of resistance.

Efficacy and Safety of Entecavir in Naïve Patients

ETV in Registration Trials

One year of ETV led to undetectable HBV DNA in 67 % of HBeAg-positive patients with normalization of ALT and HBeAg loss in 68 % and 22 % of patients, respectively [24]. Although ETV showed a continuous viral decline beyond week 48, rates of

HBeAg loss and seroconversion remain relatively low [25, 26]. ETV discontinuation after a 48-week treatment period causes virological and biochemical breakthrough in the majority of patients [27] whereas continuous ETV use for up to the year 5 (0.5 mg/day the first year and then 1 mg/day) resulted in a virological and biochemical response in 94 % and in 80 % of patients, respectively, with HBeAg seroconversion and HBsAg seroclearance of 23 % and 1.4 %, respectively [28]. ETV-R in NUC-naïve CHB patients appears at rates of 1.2 % after 5 years of therapy [29].

In HBeAg-negative patients, 1-year of ETV treatment led to undetectable serum HBV DNA and ALT normalization in 90 % and in 78 % of subjects, respectively. Virological rebound occurred in 2 % of the patients without however emergence of genotypic resistance [30]. ETV discontinuation after the first year of treatment resulted in a virological rebound in the vast majority of patients while patients who continued treatment for up to the third years maintained a virological response [31].

ETV in Cirrhotic Patients

ETV treatment was reported to have good efficacy profile in patients with advanced fibrosis or compensated cirrhosis resulting in undetectable HBV DNA in >90 % and ALT normalization in over 60 % of the patients after 1 year of treatment [32]. Rates of virological response and HBeAg clearance after 1-year of ETV treatment were 89 and 48 % in decompensated patients compared to 78 and 41 % in those with compensated liver disease. Moreover, among patients with decompensated cirrhosis, 65 % achieved a Child-Pugh A score (CPS) and 49 % showed improvement of at least 2 points in the CPS, with a cumulative transplantation-free survival of 87 % [33]. In a randomized, open-label study in 195 CHB patients with decompensated cirrhosis (mean pretreatment MELD score = 16), 1-year treatment with ETV 1 mg daily showed significant greater viral suppression compared to ADV 10 mg daily (57 % vs. 20 %) however with similar HBeAg seroconversion, CPS improvement and survival rates [34].

ETV in Field Practice Studies

In two European field practice studies including 1162 CHB patients (mean age 51 years, 76 % HBeAg-negative, 36 % with cirrhosis) treated with ETV, the 5-year cumulative probability of a virological response was 97 % and 99 %, respectively [35, 36]. One patient only developed ETV-R (L180M, M204V, S202G) at year 3, and was successfully rescued by TDF [36]. The same efficacy results were also reported in Asian studies [37–40] including 1126 treatment-naïve patients. At year 5, 98 % and 95 % of patients achieved undetectable serum HBV DNA and normal ALT, while two patients developed ETV-R within the fourth year of treatment [37]. Rates of long-term virological and serological response in NUC-naïve CHB patients treated with ETV in clinical practice are reported in Fig. 16.3.

HBeAg seroconversion*	15%	38%	58%	55%	67%	33%
HBsAg seroclearance	0.4%	0.2%	4.1%*	3.5%	0.4%	1.5%

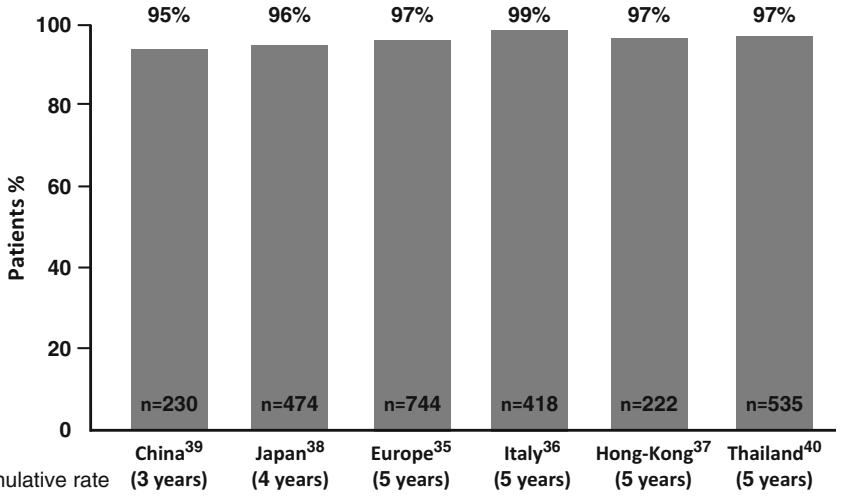

Fig. 16.3 Rates of long-term virological and serological response in NUC-naïve CHB patients treated with ETV in clinical practice

Safety and Tolerability of ETV

Long-term administration of ETV was associated with low rates of severe adverse events (AEs) and drug discontinuation. Analysis from phase III clinical trials showed that after a median of 184 weeks of treatment, 5 % of patients had drug-related grade 3/4 AEs, ultimately leading to treatment discontinuations in 1 % of cases, while 1 % of patients reported a >0.5 mg/dL serum creatinine increase from baseline [41]. Although in 2009 five cases of lactic acidosis were reported in decompensated cirrhotic patients (all with a baseline MELD score >22 points) under ETV treatment [42], this risk was not confirmed in other studies including patients with severe liver disease treated with ETV for 2 years, as only one out of 113 patients developed this AE [34, 43]. Notwithstanding, particular caution should be exercised when administering ETV to patients with severe liver disease and high baseline MELD scores, with ETV treatment to be withdrew in any patient who develops clinical or laboratory findings suggestive of lactic acidosis [44]. The overall favorable safety profile of ETV was also confirmed in a field practice studies. Among 3823 patients exposed to ETV for 12–66 months no major safety issues have been reported [36, 45–48].

Efficacy and Safety of Tenofovir in Naïve Patients

TDF Efficacy in Registration Trials

In two double-blind studies, 1-year treatment with TDF was compared to ADV in HBeAg-positive and HBeAg-negative patients [16]. A significantly higher proportion of patients receiving TDF reached viral suppression compared to those treated with ADV: 76 % vs. 13 % and 93 % vs. 63 % in HBeAg-positive and HBeAg-negative patients, respectively. Significantly more HBeAg-positive patients treated with TDF normalized ALT levels and lost HBsAg compared to those treated with ADV (68 % vs. 54 %; 3 % vs. 0 %). At week 48, no amino acid substitutions within HBV DNA polymerase associated with phenotypic resistance to TDF have developed. The long-term follow-up of the registration trial reported that 98 % of the 146 HBeAg-positive patients and 99 % of the 264 HBeAg-negative patients achieved undetectable HBV DNA after 8 years, without evidence for TDF-R. HBeAg seroclearance was achieved in approximately 30 % of the patients treated for 8 years, while HBsAg loss occurred in 12 % and 1 % of the HBeAg positive and negative patients, respectively [49].

TDF in Patients with Cirrhosis

A phase 2, double-blind study randomized 112 patients with CHB and decompensated liver disease to receive either TDF ($n=45$), combination therapy with Emtricitabine (FTC) plus TDF ($n=45$), or ETV ($n=22$) [50]. After 48 weeks of treatment, virological and biochemical responses were similar among the three treatment arms (71 % vs. 88 % vs. 73 %; 57 % vs. 76 % vs. 55 %). A 2 point median MELD score reduction and a 1 point median CPS reduction were observed in all the three treatment arms.

TDF in Field Practice Studies

Four European field practice studies including 1597 CHB patients (mean age 47 years, 75 % HBeAg-negative, 26 % with cirrhosis) reported that a 3–4 year course of TDF treatment achieved virological response ranging from 92 to 100 % without emergence of TDF-R [51–54]. Rates of long-term virological and serological response in NUC-naïve CHB patients treated with TDF in the registration trial and in clinical practice are reported in Fig. 16.4.

HBeAg seroconversion*	23%	NA	27%	37%	31%
HBsAg seroclearance	5.7%	2.5%	1.6%	17%*	7.5%

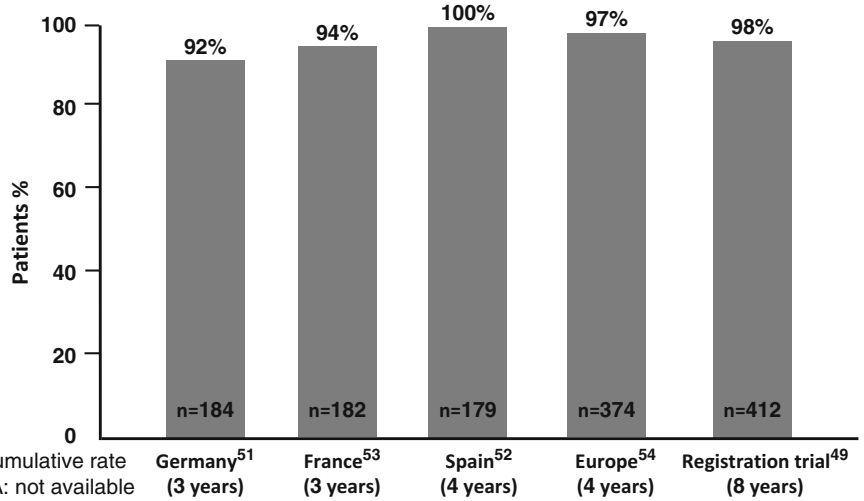

Fig. 16.4 Rates of long-term virological and serological response in NUC-naïve CHB patients treated with TDF in the registration trial and in clinical practice

Safety and Tolerability of TDF

TDF was well tolerated over the 8 years of the long-term follow-up study as only 20 (3.4 %) patients had dose reduction ($n = 18$), temporary treatment interruption ($n = 1$) or drug discontinuation ($n = 1$) for a renal event that consisted of ≥0.5 mg/dL increase in serum creatinine from baseline (2.2 %), phosphorus <2 mg/dL (1.7 %), or eGFR <50 mL/min (1 %) [49]. No significant renal safety difference was observed among decompensated cirrhotics treated with TDF ± FTC or with ETV as the proportion of subjects with a confirmed increase in serum creatinine ≥0.5 mg/dL from baseline or confirmed serum phosphorus <2.0 mg/dL were 9, 7, and 5 % among the three arms of treatment [50]. No major changes of renal function were observed during the 3–4 years of TDF in three European cohort studies [51, 52, 54]. However, in the latter study, enrolling 374 NUC-naıve patients treated with TDF for 4 years, the proportion of patients with eGFR <50 and <60 mL/min increased from 2 to 3 % and from 7 to 11 %, respectively; the rates of patients with serum phosphate <2.3 mg/dL increased from 2 to 5 %, and 1 % of the patients had phosphate <2.0 mg/dL throughout the study period. Overall, the 4-year probability of TDF dose reductions or discontinuations for renal-related AEs was 11 % [54]. An Italian field practice study in 156 NUC-naıve patients treated with TDF for 2 years reported de novo hypophosphatemia (≤2.5 mg/dL) in 6 % of the patients [55]. In a study investigating the

safety of a 2-year course of TDF among patients with mild baseline renal impairment, i.e., eGFR 50–80 mL/min, none of the patients had a ≥0.5 mg/dL increase of serum creatinine, whereas nine patients, all with baseline eGFR <61 mL/min, had eGFR declining <50 mL/min that, however, stabilized after dose adjustment [56]. To date, five cases of TDF-induced Fanconi syndrome have been reported in HBV monoinfected patients [57–59]. To prevent this severe AE, and more in general, chronic tubular damage and phosphate wasting syndrome, TDF dose should be proactively reduced as recently suggested [60].

How Should Patients Be Monitored During NUCs Therapy

Once a NUC is started, viremia should be tested with sensitive PCR assay every 3 months until undetectability (<10–15 IU/mL) is confirmed on two separate occasions, and every 6 months for the following years. Monitoring of HBV DNA is important also to differentiate between treatment failures. Primary non-response, defined as less than 1 $\log_{10}$ IU/mL decrease in HBV DNA levels from baseline to month 3 of therapy, occurs in 2–3 % of the patients only; partial virological response (PVR), i.e., detectable serum HBV DNA at week 48 of treatment in a compliant patient, ranges from 5 to 50 % according to baseline levels of viremia; virological breakthrough, defined as a confirmed increase in HBV DNA level of more than 1 $\log_{10}$ IU/mL compared to the lowest HBV DNA level, is a rare event during long-term ETV or TDF therapy. In HBeAg-positive patients, HBeAg/anti-HBe should be assessed every 6 months whereas HBsAg should be tested every 6–12 months in patients who are HBeAg-negative with persistently undetectable serum HBV DNA to detect HBsAg seroclearance.

As all NUCs are excreted through the kidneys, appropriate dosing adjustments are recommended. All patients should be tested at baseline and during treatment for serum creatinine to calculate the eGFR by MDRD formula to adjust NUC dose if eGFR falls below 50 mL/min, or <60 mL/min for some TDF treated patients [60], or had a rapid decrease during treatment. In addition, the baseline renal risk should be assessed for all patients. High renal risk includes one or more of the following factors: decompensated cirrhosis, creatinine clearance <60 mL/min, poorly controlled hypertension, proteinuria, uncontrolled diabetes, active glomerulonephritis, concomitant nephrotoxic drugs, solid organ transplantation. All CHB patients receiving TDF should be monitored every 3 months with serum creatinine, eGFR and serum phosphate whereas CHB patients on ETV should be monitored with serum creatinine levels and eGFR only if at high renal risk [3]. Closer renal monitoring is required in those patients with mild, or at risk for, renal impairment. While there is no enough evidence to recommend monitoring of bone density by DEXA scan in all patients receiving TDF-based antiviral regimens, bone mineral density should be assessed in selected patients, i.e., those who have a history of pathologic bone fractures or other risk factors for osteoporosis or bone loss, such as cirrhosis, independently of NUC therapy.

Management of Partial Virological Response to ETV or TDF

Antiviral therapy with ETV or TDF has negligible rates of resistance, though the few cases of ETV-R in NUC-naïve patients occurred in patients with PVR [61]. The optimal management of such patients is currently debatable, it seems reasonable that the HBV DNA levels at week 48 and their kinetics must be taken into account. Patients with residual viremia ≤1000 IU/mL or with continuous decline of serum HBV DNA levels could be maintained on the same drug given the progressive increase of virological responses over time and the negligible risk of resistance. For those with a flat pattern of HBV DNA or with a residual viremia >1000 IU/mL a rescue strategy with a non cross-resistant analogue, i.e., TDF for partial response to ETV and contrariwise, can be recommended [62].

Long-Term Benefits of NUCs Treatment

CHB patients with advanced fibrosis or cirrhosis demonstrated histological improvement and reversal of fibrosis and cirrhosis after long-term treatment with both ETV and TDF. In 57 patients under long-term ETV treatment, a second liver biopsy evaluation after a median of 6 years showed a significant histological improvement (a ≥1 point improvement in the Ishak fibrosis score) in 88 % of patients, including all 10 patients with advanced fibrosis or cirrhosis at baseline [63]. A reduction in Ishak fibrosis score to 4 or less was observed for all four patients who had cirrhosis at baseline [64]. More strong evidence of beneficial effect on fibrosis and cirrhosis regression was reported during 5-year TDF treatment [65]. Of the 348 patients who completed 240 weeks treatment and had biopsy results at baseline and at week 240, 304 (87 %) had histological improvement (≥2 point reduction in Knodell necroinflammatory score with no worsening of fibrosis) and 176 (51 %) had regression of fibrosis (≥1 unit decrease in the Ishak staging score). Of the 96 patients with cirrhosis (Ishak score 5 or 6) at baseline, 71 (74 %) had cirrhosis histologically reversed, whereas 3 (1.2 %) of 252 patients without cirrhosis at baseline progressed to cirrhosis during treatment. Low BMI, absence of diabetes mellitus, normal ALT levels, and mild or absent necroinflammation at year 5 of treatment were associated with a higher likelihood of cirrhosis regression [65]. Clinical decompensation is fully prevented in compensated cirrhotic patients through the 3–5 years of effective ETV and TDF treatment [36, 54, 66–68], whereas among patients with decompensated liver disease survival is significantly improved by antiviral therapy as persistent HBV DNA suppression led to reversal of clinical decompensation in most patients [69]. Recently, several studies evaluated the role of NUC on HCC risk reduction. Annual incidence of HCC among NUC-naïve CHB patients without cirrhosis ranged from 0.6 % to 1.4 % and 0.8 % to 1.4 % in Asian and European patients treated with ETV, respectively [36, 68, 70–73] whereas among TDF-treated

non cirrhotic patients the annual HCC risk ranged from 0.4 to 1 % [54, 73]. In ETV-treated cirrhotic patients, annual incidence of HCC ranged from 2 to 4.1 % in Asian studies [68, 71, 72, 74] and was 2.6 % in European studies [36, 73] while data from European studies in TDF-treated cirrhotics revealed that the risk ranged from 3.7 to 4 % [54, 73]. These HCC rates are very similar to what has been estimated from natural history studies in untreated patients [75].

When Can NUC Treatment Be Stopped?

The best stopping rule for NUC-treated patients is HBsAg loss and anti-HBs seroconversion, the latter is the sole safe stopping rule for cirrhotic patients. This endpoint is however rarely achieved (~1 %) in HBeAg-negative patients and in HBeAg-positive patients infected at birth. By converse, in NUC-treated HBeAg-positive patients with good predictors of response, such as short duration of infection, genotype A, elevated ALT levels and moderate levels of HBV DNA, this stopping rule can be achieved in up to 20 % of the patients after 5 years of treatment. In HBeAg-positive patients without cirrhosis, NUC treatment could be stopped after a confirmed and maintained (≥12 months) anti-HBe seroconversion combined with undetectable HBV DNA, an event that is observed in approximately 40–50 % of the HBeAg-positive treated patients after 5 years of therapy. However, viremia and hepatitis will relapse in up to 50 % of these patients after NUC discontinuation, thus suggesting a very strict monitoring strategy in the post-treatment follow-up to early detect virological rebound and restart therapy. For HBeAg-negative CHB patients there is no consensus between international guidelines about timing of treatment discontinuation. European (EASL) and American (AASLD) guidelines recommend HBsAg seroclearance as NUC stopping rule while Asian-Pacific (APASL) guidelines suggests that NUC cessation could be tempted after at least 2 years of treatment if HBV DNA is undetectable on three separate occasions 6 months apart [1–3]. Two Asian studies evaluated the off-treatment durability of response in HBeAg-negative CHB following ETV discontinuation according to APASL guidelines. Both studies reported high relapse rates (45 % and 91 %, respectively) in the year after treatment discontinuation, suggesting that NUC therapy should be continued indefinitely until the recognized treatment end-point of HBsAg seroclearance [76, 77]. However, this remains a major discussion point as strategies may be country specific [78, 79]. In countries where drug cost is an issue, full reimbursement for therapy and or monitoring is not in place and compliance tends to fade over time, NUC withdrawal might be worth to be carefully explored in selected HBeAg-negative patients. By converse, for patients leaving in countries where oral therapy, and HBV management in general, is fully reimbursed, and/or for those with cirrhosis or poor compliance to off-treatment monitoring, long-term administration till HBsAg clearance might still be the best strategy.

HBV and Pregnancy

Chronic HBV infection in pregnancy is an important global health problem as mother-to-child transmission is the most common mode of acquiring chronic HBV infection in endemic areas [80]. Data on the natural history of CHB during pregnancy are conflicting: some data suggest no worsening of liver disease in the majority of HBV-infected pregnant women while case reports show hepatic exacerbations and fulminant hepatic failures during pregnancy [81–85]. Some additional studies suggest that HBV infection is associated with adverse pregnancy outcomes, including higher rates of preterm birth, gestational diabetes, and antepartum hemorrhage [81–85]. All women should be routinely tested for HBsAg during their first trimester of pregnancy and those resulting positive should be referred for additional assessment and medical management [2, 3]. Without immunoprophylaxis with hepatitis B immunoglobulin (HBIG) and HBV vaccination within 12 h of birth, up to 90 % of infants born to HBeAg-positive mothers become HBV chronically infected [86, 87]. However, up to 28 % risk of perinatal transmission still persist in HBeAg-positive mothers with high HBV DNA levels despite immunoprophylaxis and vaccination [88–91], whereas antiviral prophylaxis in the third trimester of pregnancy has been shown to decrease the risk of HBV transmission [92–98]. Maternal viremia plays a significant role in vertical transmission, with increased risk which starts from HBV DNA levels greater than 6 $\log_{10}$ IU/mL [87, 88, 99]. For this reason, all pregnant women with serum HBV DNA >6 $\log_{10}$ IU/mL in the third trimester, or with HBV perinatal transmission in a prior pregnancy, need to be treated with NUC to initiate between weeks 28–32, with careful discussion of the risks and benefits. In fact, no anti-HBV agent has been approved for use in pregnancy and all NUCs are classified as Food and Drug Administration (FDA) pregnancy risk category C, except for TDF and LdT, which are category B. However, LdT has limited efficacy and moderate to high resistant rates therefore the drug of choice is TDF, due to its potency, leading to a rapid reduction of serum HBV DNA, the null risk of resistance and the excellent safety profile without significant increase in birth defects or adverse outcomes, so far [100–102]. Despite infant plasma TDF concentrations are lower than maternal plasma or breast milk, the label recommends against its use during breastfeeding [103]. However, recent study identified that the exposure to the drug is lower from breastfeeding than from in utero exposure concluding that there is no contraindication to TDF use during breastfeeding [104]. If administered only for prevention of mother-to-child transmission TDF may be discontinued within the first 3 months after delivery whereas in pregnant women who require anti-HBV treatment for their own health, therapy should be maintained. Moreover, pregnant women who need antiviral therapy due to the advanced liver disease may be safely treated with TDF starting from the first trimester while women with advanced liver disease who becomes pregnant under category C NUC need to be immediately switched to TDF, due to the risk of withdrawal flare that could result in reactivation and even decompensation of liver disease.

HIV and HBV Coinfection

Current estimates place the prevalence of CHB among human immunodeficiency virus (HIV)-infected patients between 5 and 20 %. Thus, 2–4 million out of 35 million people living with HIV worldwide have CHB [105, 106]. In some regions in sub-Saharan Africa and Southeast Asia, HBsAg can be found in up to 15–20 % of the HIV population. In Europe, nearly 10 % of HIV-infected individuals have CHB, more than 100-fold the rate in the general population. It is estimated that half of HIV-positive persons have been exposed to HBV and, therefore, exhibit markers of spontaneously self limited HBV infection, i.e., hepatitis B core antibodies (anti-HBc) with or without hepatitis B surface antibodies (anti-HBs) or have current HBsAg [107]. In the case of HIV patients with CHB living in Europe, HBV genotype A is the most prevalent; it is found in approximately three-quarters of HIV–HBV coinfected individuals whereas in Southern Europe, HBV genotype D is equally prevalent to genotype A in this population [107].

Natural History of CHB in Persons Living with HIV

Compared with HBV-monoinfected individuals, HIV–HBV coinfected patients have lower chances for spontaneous HBeAg and HBsAg seroclearance. Serum HBV DNA levels are more elevated, which may in part explain the faster progression to end stage liver disease and HCC characteristically seen in coinfected patients [108]. Following the advent and broader use of highly active antiretroviral therapy (HAART), opportunistic complications have declined dramatically. However, liver-related complications are on the rise in patients coinfected with hepatitis C and B viruses. Current knowledge suggests that treatment of both HIV and HBV may prevent or slow down the development of hepatic complications in such patients [109]. The enhanced risk of liver toxicity of antiviral agents, particularly among cirrhotic HIV–HBV coinfected patients should not preclude prescription of HIV plus HBV therapy, although antiviral with the safest liver profile should be preferred [108]. Patients should be warned against stopping HAART with anti-HBV drugs for any reason because abrupt resumption of HBV replication may lead to a flare in liver enzymes and even fulminant hepatic failure [110].

Diagnosis

All HIV-infected persons must be tested for HBV markers: HBsAg, anti-HBc, and anti-HBs. HBsAg testing must be refreshed yearly in all patients or in case of unexplained ALT elevations, visits or living in endemic areas, new diagnosis of sexually

transmitted diseases. Persons who are anti-HBc-positive and HBsAg-negative, in particular those with elevated ALT, should be screened for HBV DNA in addition to HBsAg, to rule out occult HBV infection. Hepatitis delta antibodies should be screened for in all HBsAg-positive persons [110].

Treatment of Patients with HIV–HBV Coinfection

In patients with HIV–HBV coinfection, HBV therapy is indicated in all individuals with cirrhosis, CD4 counts less than 500 cells/mL, serum HBV DNA >2000 IU/mL, and/or elevated ALT. For most patients, the best option is triple combination of antiretrovirals, including two reverse transcriptase inhibitors with anti-HBV activity, that is, TDF plus LMV or FTC [110]. Some experts strongly believe that any person with HBV infection requiring antiretroviral therapy (ART) should receive TDF plus LMV or FTC unless history of TDF intolerance, particularly with advanced liver fibrosis (METAVIR score: F3/F4). TDF administration should be adapted to eGFR. In persons with no history of treatment with LMV and strict contraindication of TDF, ETV can be used in addition to fully suppressive combination ART without FTC or LMV. In fact ETV displays weak activity against HIV and may select for resistance mutations, thus it should always be administered only in the context of a fully suppressive HIV treatment. ART-naïve Asian, HBeAg-positive, HIV-coinfected persons initiating ART with TDF or TDF + FTC reached unexpectedly high rates of HBe and even anti-HBs seroconversion, strengthening the rationale for early ART. One-year course of Peg-IFN could be considered as therapy for CHB in coinfected patients unwilling to start HAART who have normal CD4 counts, HBeAg-positive, with low HBV DNA, elevated ALT, genotype A, and without advanced liver disease. The addition of anti-HBV NUCs has not been proved to increase Peg-IFN efficacy. In ART treated patients where the nucleoside backbone needs to be changed, anti-HBV therapy may be stopped cautiously in HBeAg-positive persons who have achieved HBeAg-seroconversion for at least 6 months or after confirmed HBs-seroconversion in those who are HBeAg-negative. In persons with liver cirrhosis, stopping of effective anti-HBV treatment is not recommended in order to avoid liver decompensation due to ALT flares. In some cases of TDF intolerance, i.e., renal disease, TDF in doses adjusted to renal clearance in combination with effective ART may be advisable. In persons with no prior LMV exposure, ETV may be used alone. NUCs substitution should only be performed if feasible and appropriate from the perspective of maintaining HIV suppression. Caution is warranted to switch from a TDF-based regimen to drugs with a lower genetic barrier, i.e., FTC or LMV, in particular in LMV-pretreated cirrhotic patients as viral breakthrough due to archived mutated variants is likely to happen. This has also been described in individuals with previous LMV-R who have been switched from TDF to ETV. The addition of ETV to TDF in persons with low persistent HBV replication has not statistically proved to be efficient and should therefore be avoided [110].

Vaccination

The response to the HBV vaccine is influenced by the CD4 cell count and level of HIV loads. In persons with low CD4 cell count (<200 cells/μL) and ongoing HIV replication, ART should be initiated first prior to respective vaccination. Because of the lack of data on the impact of immunization in isolated anti-HBc IgG positive persons (HBsAg negative, anti-HBc positive, and anti-HBs negative profile), vaccination is not presently recommended in this population. In HIV-positive persons vaccinated for HBV with insufficient response (anti-HBs <10 IU/L), re-vaccination should be considered. Double-dose (40 μg) at three to four time points (months 0, 1, 6, and 12) may help to improve response rates to the HBV vaccine. Persons who fail to seroconvert after HBV vaccination and remain at risk for HBV should have annual serological tests for evidence of HBV infection. TDF based cART has been associated with prevention of HBV infection in these persons [110].

Conclusion

The possibility of treatment of CHB patients have evolved fast, several therapeutic options are now available and nowadays hepatitis B is a treatable disease. The most popular and effective anti-HBV therapeutic strategy in CHB patients is the administration of third generation NUC such as ETV and TDF with the aim to maintain HBV DNA to as low a level as possible. Advantages of this strategy include excellent tolerability, long-term viral suppression without emergence of drug-resistance in the majority of patients resulting in biochemical remission, histological improvement, with also cirrhosis regression, and prevention of clinical decompensation while in patients with decompensated liver disease survival is significantly improved though early mortality and HCC do still represent a major clinical challenge. In fact, effective antiviral treatment reduces but does not eliminate the risk of HCC development both in cirrhotics but also in patients with less advanced liver disease. However, long-term administration of ETV or TDF cannot eradicate HBV infection making long-term therapy necessary in most patients with increasing cost and the potential issues of compliance and of unproven safety profiles in lifetime. NUC are the treatment of choice in patients with severe liver disease, in old patients, in those contraindicated or unwilling to Peg-IFN and in patients with concomitant diseases. Moreover, TDF is the first line NUC therapy for pregnant women with serum HBV DNA >6 $\log_{10}$ IU/mL in the third trimester of pregnancy and in pregnant women who need antiviral therapy due to the advanced liver disease. For patients with HIV–HBV coinfection requiring ART and who need anti-HBV treatment the best option is triple combination of antiretrovirals that includes two reverse transcriptase inhibitors with anti-HBV activity such as TDF plus FTC, whereas 48 weeks of Peg-IFN could be considered for HBeAg-positive CHB coinfected patients unwilling to start HAART, having low HBV DNA, elevated ALT, genotype A and without advanced liver disease.

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Chapter 17
Organ Transplantation in HBV-Infected Patients

Tsung-Hui Hu and Chao-Long Chen

Introduction

Hepatitis B virus (HBV) infection is associated with liver-related complications that can lead to end stage liver disease (ESLD) and liver failure [1]. Liver transplantation (LT) offers the ultimate cure for patients with chronic hepatitis B (CHB) and is the only treatments available for patients with ESLD [2]. However, HBV recurrence in LT recipients (LTR) can lead to rapid liver disease progression, graft failure, and death [3]. By the 1990s, HBV was considered as a contraindication for LT due to poor outcomes, with a survival rate of only ~50 % at 5 years [4]. The landmark study by Samuel et al. in 1991 [5] showed that passive immunization with Hepatitis B immunoglobulin (HBIG) reduced the HBV recurrence rate to around 30–40 %. Since the approval and use of the first nucleos(t)ide analogue (Nuc) lamivudine (LAM), the combination of HBIG plus LAM has further reduced HBV recurrence and improved survival of HBV-related LT [6–8], and become the standard of care for prophylaxis against HBV recurrence after LT [9]. However, HBIG is expensive, inconvenient, and there is no clear consensus on the optimal dose and schedule for

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the HBIG regimen [6, 8, 10, 11]. The advent of more potent Nuc with high genetic barrier to resistance, i.e., entecavir (ETV) and tenofovir (TDF), has further reduced long-term recurrence rates [12–16]. Recent strategy has suggested the use of HBIG for only a period of time after LT, followed by long-term Nuc alone [17–19]. Till now, the consensus has not been documented.

HBV infection after non-liver organ transplantation is also a problem and was studied more in the setting of renal transplantation (RT). HBV infection is an established cause of morbidity and mortality in RT recipients (RTRs) [20–23]. Immunosuppression post-RT may affect the host's immune responses against HBV [24, 25]. Rates of HBV DNA reactivation of 50–94 % have been reported in the absence of prophylactic antiviral therapy, thereby leading to fatal liver complications [21, 22, 26, 27]. Due to poor patient and graft survivals, RT was not preferred to hemodialysis for HBsAg-positive patients with end-stage renal failure [21]. However, there is a lack of alternative therapy [like hemodialysis for end stage renal disease (ESRD)] in patients with other organ failure [28–33]. With the availability of Nuc since 1998, HBV infection is no longer a risk factor for death or graft failure in organ transplant recipients [34–36].

The advance and the current status of organ transplantation in HBV-infected patients are reviewed in this chapter.

Liver Transplantation

Clinical Course After LT

Definition of HBV Recurrence

Most studies have defined HBV recurrence as the reappearance of hepatitis B surface antigen (HBsAg) and/or HBV DNA post-transplant. Although the reappearance of HBsAg has been considered the marker of recurrent HBV infection, the reappearance of HBV DNA in serum is the most important determinant of prophylaxis failure. With newer and more potent antiviral therapies with high barriers to resistance, patients with the reappearance of HBsAg used to have undetectable HBV DNA in serum and were not associated with graft dysfunction [37–39].

Risk of HBV Recurrence

Many related factors may be responsible for HBV recurrence, including recipient host factors, donor factors and perioperative treatment (use of antiviral agents and immunosuppressants, drug resistance, viral mutations) [40]. Natural history studies from the era before the use of prophylactic therapies showed that the level of HBV DNA at the time of transplantation was the principal factor for HBV recurrence [10, 37, 41, 42]. Of the 372 European HBsAg-positive patients who underwent LT

from 1977 to 1990, the 3-year HBV recurrence rate was highest (83 %) in HBV-related cirrhosis with HBV DNA greater than 10^5 copies/ml at time of LT, intermediate (58 %) in those without detectable HBV DNA or HBeAg, lower (32 %) in those with hepatitis D virus (HDV) co-infection and lowest (17 %) fulminant HBV infection [41, 43]. Even in the current era of routine prophylactic therapies (HBIG + Nuc), HBV recurrence is most consistently associated with levels of HBV DNA before LT [10, 37, 41, 42, 44].

Among other potential factors, HBV variants with antiviral drug-resistant mutation and/or HBIG resistant mutation are the main causes of HBV reinfection [10, 39, 45, 46]. HBsAg escape mutants that harbor single or double point mutations may significantly alter the immunological characteristics of HBsAg, in which most mutations are located within the second "a" determinant loop, with an arginine replacement for glycine at amino acid 145 [47, 48]. It was shown that mutations in the HBsAg (D144E) and the polymerase (L426I/L526M/M550I) of the HBV genome may be responsible for viral breakthrough under combination antiviral prophylaxis with HBIG and LAM [49]. There are also a few studies that investigated the potential influences of precore or BCP mutants on the outcomes of LT [50, 51]. A study showed that infection with precore mutant strains predisposes a patient to early graft loss following transplantation [50]. However, this association has disappeared in the modern era of antiviral prophylaxis of ETV or TDF with or without HBIG.

Other factors identified as being of potential importance are the presence of drug-resistant HBV strains [10, 41, 52] and the recurrence of HCC, possibly due to HBV replication in HCC cells as a source for the recurrence of HBV infection [37, 53]. A recent study in 354 HBV patients with HCC who underwent LT found that patients who had HBV recurrence were 3.6 times more likely to develop HCC recurrence [54]. A study of 154 patients under HBIG + ETV therapy showed an overall HBV recurrence rate of 0.6 %, 1.6 %, and 6.2 % at 1, 2, and 4 years, respectively in which recurrent HCC was an independent risk factor (hazard ratio = 13.5, 95 % confidence interval, 2.4–74.4; $P = 0.006$) [55]. HCC at the time of LT was also a risk factor for post-LT virological rebound. The study of Fung et al. [37] showed a more than sevenfold higher risk of HBV recurrence in patients who had HCC at transplant. In a recent study using pooled data from two cohorts (HBIG + LAM in 171, and HBIG + ETV in 145 patients), predictors of HBV recurrence were Nuc used (LAM), pre-LT HCC, post-LT low anti-HBs (<100 mIU/ml), male gender, and HBsAg (+) in the explanted liver tissue [39].

Evolution of HBV Prophylaxis in LT

HBIG Monotherapy

In 1991 and 1993, Samuel et al. demonstrated that the recurrence rate of HBV after LT is significantly reduced by the intravenous administration of high-dose HBIG [5, 43]. Other studies also demonstrated significantly reduced HBV recurrence after LT

from 90 to 20–40 % by administering high doses of intravenous HBIG 10,000 IU in the anhepatic phase and in the first postoperative week, then monthly [5, 44, 56, 57]. However, HBIG administration is costly, inconvenient and a high dosage of intravenous HBIG after LT may lead to side effects [57], HCV transmission, and allergic reactions [58]. Long-term use of HBIG may also result in the development of genetic HBV mutants, which may cause the virus to become resistant to neutralization [59–62]. Titration of HBIG dose based on anti-HBs titer is an alternative to reduce the need for HBIG. Anti-HBs titer greater than 500 IU/l for the first 3 months, 100–250 IU/l between 3 and 6 months, and 100 IU/l after 6 months post LT are considered to be safe targets of HBV prophylaxis [63].

Subsequently, intramuscular (IM) HBIG has been shown to be as effective as IV HBIG [64, 65]. It can achieve adequate anti-HBs titer to a dose of about 400–2000 IU/month due to slow release. Franciosini et al. [66] noted that patients receiving low-dose IM HBIG reported significantly better health-related quality of life scores, but worse scores on side effects scales compared to patients using IV HBIG. It was also shown in some studies that subcutaneous (SC) HBIG could effectively maintain anti-HBs levels above 100 IU/l, in addition to the advantages of convenience for patients, stable anti-HBs plasma levels, lower dosages of HBIG, and fewer adverse effects [10, 67–69]. But notably, due to it's late introduction, to use intramuscular (IM) or subcutaneous (SC) HBIG for monoprophylaxis post LT is not suggested.

LAM/ADV Monotherapy

At earlier times, LAM has been shown to be safe and effective in patients awaiting LT [70–72]. A multicenter trial conducted at ten centers evaluated the use of LAM as a monotherapy in the pre- and post-liver transplant settings and found that after >12 weeks of post-transplant LAM therapy, 60 % remained HBsAg-negative, a rate comparable to that seen with long-term HBIG monotherapy [73]. Subsequent studies demonstrated that LAM monotherapy in the post-LT setting was associated with 8–32 % HBV recurrence rate at 16–24 months [74–77]. However, high drug resistance rates of 25 %, 30–40 %, and 50 % are found at 1, 4, and 6 years post-LT [73, 78–80].

Adefovir (ADV) appears to be an effective antiviral agent for LT recipients with recurrent HBV infection and LAM-resistance. However, nephrotoxicity was reported and dose adjustment is needed in patients with impaired renal function [8, 81]. In a study of 42 LTRs who developed recurrent HBV or de novo HBV infection with LAM-resistant HBV, switch to ADV achieved complete virological suppression in 27 (64.3 %) during 31 months follow-up without renal dysfunction [82]. Another study showed that ADV monotherapy prior to transplant reduced post-transplant HBV recurrence to only 9 % during a median of 35 months follow-up [83]. Furthermore, HBIG was not required in 18 patients whose pre-LT serum HBV DNA level was suppressed to <3 $\log_{10}$ IU/ml and no HBV recurrence was observed during combined LAM + ADV therapy for a median period of 22 months after LT [18].

Combination of HBIG + Nucs

High-Dose IV HBIG with LAM

The first trial of long-term HBIG combined with LAM was reported in 1998. With monthly HBIG administration plus LAM 150 mg/day, all patients survived without serum HBV DNA positivity 1 year after LT [8]. Thereafter, combination of HBIG and LAM has proved to be more effective in minimizing graft reinfection (≤10 %) and has thus become the standard of care for HBV-infected LTRs [8, 10, 84–86]. Three recent meta-analyses have clearly demonstrated that combination of HBIG and LAM is superior to LAM or HBIG alone [6, 87, 88]. In addition, there was a significant reduction in the development of YMDD (rtM204V) mutants with HBIG + LAM as compared with LAM monotherapy [88].

High-Dose HBIG with ETV vs. LAM

After availability of ETV, a case control study compared the combination of either ETV or LAM with IV HBIG at a dose of 200 IU/Kg intraoperatively and daily for 5 days post-LT followed by interval administration of 1000 IU to maintain anti-HBs titers >500 IU/l during the first 6 months and 200 IU/l thereafter. The results showed no HBV reinfection after 2 years in 26 patients using ETV, but HBV recurred in 4 % after 3 years and 6 % after 5 years in the 63 patients using LAM [13].

Low-Dose IM HBIG with Nuc

Low-dose IM HBIG (300–800 IU) has been suggested as being as effective as intravenous HBIG. A large prospective study of 233 patients receiving IM HBIG 2000 IU intraoperatively, 800 IU IM/day for the first post-LT week and 800 IU IM/month thereafter in combination with LAM reported a 6 % HBV recurrence rate during a mean follow-up of 30 months [89]. A study of 120 patients with prophylaxis using IM HBIG combined with LAM or ETV reported a HBV recurrence rate of 11.1 % in 90 patients in the LAM group but none in the ETV group [90]. Subsequent reports of ETV plus low-dose HBIG revealed that the recurrence rate of HBV was 0–3.2 % [12–16], which was lower than that reported with HBIG + LAM combination [89, 90]. A recent large cohort study of 145 patients using ETV plus low-dose, on-demand (when anti-HBs <100 IU/l) IM HBIG prophylaxis showed a HBV recurrence rate of 1.37 % during a median follow-up of 36 months, in contrast to a rate of 6.4 % (P=0.055) during a median follow-up of 77 months in 171 patients using LAM plus on-demand IM HBIG prophylaxis [39]. The experience of TDF/FTC plus low-dose HBIG therapy was relatively limited, but was associated with good safety and efficacy [12, 19, 91, 92]. A systematic review reported that antiviral prophylaxis with TDF/FTC plus HBIG combination is associated with negligible HBV recurrence post LT [93].

HIBG Discontinuation Followed by Nuc Maintained Therapy

Among the parameters of HBIG evaluated in a systematic review, a high-dosage HBIG during the first week after LT was found to be the only significant factor associated with HBV recurrence [94]. Therefore, the efficacy of HBIG discontinuation has been challenging. Table 17.1 illustrates 4 randomized trials with both study group (HBIG discontinued with Nuc maintained) and control group (HBIG continued with/without Nucs) [17, 19, 95–97]. In an earlier study in 2001, 24 patients (all HBV DNA negative pre-LT) who had received HBIG monotherapy for at least 6 months after LT were randomized into two groups; 12 were switched to LAM, 12 were maintained on HBIG. At 1.5 years post-LT, recurrence of HBV occurred in 2 of 12 in the LAM group compared to 1 of 12 in the HBIG group [95]. In the second randomized study, HBV recurrence was not observed in 29 patients who had HBV DNA levels <2.5 pg/ml spontaneously or with LAM therapy at the time of LT. They received LAM + HBIG combination therapy for the first month after LT then were randomized into either LAM alone or LAM + HBIG therapy. HBV recurrence was not observed during a follow-up of 18 months [97], but developed in 15 % of LAM + HBIG group and 11 % of LAM monotherapy group when follow-up was extended to 83 months. It seems that maintained HBIG has no benefit for the prevention of HBV recurrence [96]. In the third randomized study, LTRs after 1-year therapy with LAM + HBIG were randomized to continue LAM + HBIG or LAM + ADV. HBV recurrence rate in 2-years was 6 % (1/18) in LAM + ADV group and 0 % (0/18) in the LAM + HBIG group [17]. In a recent study, 37 patients maintained on FTC/TDF + HBIG after LT were randomized to either stop the HBIG or continue. No patient experienced HBV recurrence through a median follow-up of 72 weeks [19]. Based on these four studies, we performed a subsequent meta-analysis by using the software package RevMan 5 [98] according to the PRISMA guidelines [99], in which heterogeneity was assessed by formal statistical testing with χ^2 and I^2 [100, 101]. We found that there was no difference in HBV recurrence between the two regimens among four trials ($P = 0.37$; RD = 0.04; 95 % CI = −0.05 to 0.14) (Fig. 17.1). Nuc with continued HBIG did not achieve a favorable outcome compared to Nuc with HBIG discontinued though the HBV recurrence rate was relatively higher in the HBIG discontinued group (6/66, 9.09 %) than that in the HBIG continued group (2/58, 3.44 %).

In addition to randomized control studies, there are also 19 prospective or retrospective studies without control group [16, 18, 41, 102–117] dealing with issues on the discontinuation of HBIG with Nuc maintained (Table 17.2). Maintained Nuc after HBIG withdrawal includes LAM monotherapy in five, ETV in one, LAM + ADV combination in four, TDF + FTC combination in three, and mixed regimens in six studies, all used post LT HBIG + Nuc for a period of time (at least 4 days, mostly 6–12 months) before HBIG withdrawal (Table 17.2). Follow-up periods ranged from 9 to 57 months, with median 24 months. If we combine data from Tables 17.1 and 17.2, in patients with HBIG discontinuation and Nuc maintained, the highest HBV recurrence 8.49 % was observed in the LAM group followed by 4.42 % in the TDF + FTC group, 3.87 % in the LAM + ADV group, and 3.85 % in the ETV group

Table 17.1 Published studies of randomized trials with study (HBIG discontinued with Nucs maintained) and control groups (HBIG continued with/without Nucs) for HBV prophylaxis after liver transplantation

Authors (year) [ref.]	Study year	Patients	Follow-up (median; mo)	Antiviral agent after HBIG withdraw (no. of patients)	Antiviral agent (HBIG continued; no. of patients)	HBV recurrence (%)
Buit, 2007 [97]	1998–2007	*N*=29 (HBIG 1 month)	83	LAM (*n*=20)	LAM+HBIG (*n*=9)	3/20 (15) (LAM) 1/9 (11.1) (LAM+HBIG)
Teperman, 2013 [19]	2007–2011	*N*=37 (HBIG 6 months)	18	FTC+TDF (*n*=18)	FTC+TDF+HBIG (*n*=19)	0 (0) for both group
Naoumov, 2001 [96]	–	*N*=24 (HBIG>6 months)	13	LAM (*n*=12)	HBIG (*n*=12)	1/12 (8.3) (HBIG) 2/12 (16.6) (LAM)
Angus, 2008 [17]	2004–2006	*N*=34 (HBIG 12 months)	21.2	LAM+ADV (*n*=16)	LAM+ HBIG (*n*=18)	1/16 (6.2) (LAM+ADV) 0/18 (0) (LAM+HBIG)

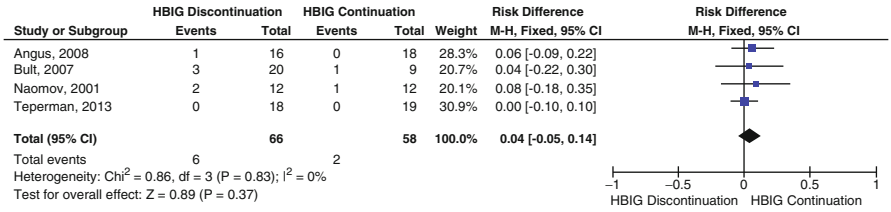


Fig. 17.1 Meta-analysis of four randomized trials with both study group (HBIG discontinued with Nucs maintained) and control group (HBIG continued with/without Nucs) of HBV prophylaxis after liver transplantation

[16–19, 41, 95, 96, 102–117]. There is no significant difference between the four groups (Fig. 17.2). Only the LAM group exhibits a borderline significance of higher rates of HBV recurrence than that of other groups.

Potent Nuc Monotherapy

ETV and TDF are the most recently introduced Nucs with both high antiviral potency and high barriers to resistance. TDF/FTC, TDF, and ETV are all safe and effective antiviral treatment in patients with decompensated liver disease and achieved undetectable HBV DNA (<400 copies/ml) at 48 weeks of treatment in 70.5, 87.8 and 72.7 % of the patients respectively [118]. In a recent study of ETV monoprophylaxis pre and post-LT, HBsAg reappeared in 18/80 patients (22.5 %) by 2 years post-LT, However, all of the patients with HBV DNA <5 $\log_{10}$ IU/ml and HBsAg <3 $\log_{10}$ IU/ml at the time of LT achieved HBsAg seroclearance ad none had genotypic antiviral resistance [38]. In a subsequent report including 362 patients, 176 (49 %), 142 (39 %), and 44 (12 %) were treated with LAM, ETV, and combination therapy (predominantly LAM + ADV) respectively at the time of transplant. The rate of HBsAg seroclearance and HBV DNA suppression to undetectable levels at 8 years was 88 and 98 %, respectively. Overall 8-year survival was not different among the three treatment groups [37]. Wadhawan et al. [119] conducted a prospective trial to evaluate Nuc with HBIG regimen in 89 patients between 2005 and 2012, in which only patients with HBV DNA levels >2000 IU/ml were given HBIG ($n = 14$). Of the remaining 75 patients not receiving HBIG, 19 patients received LAM + ADV, 42 received ETV, 12 received TDF, and 2 received ETV + TDF. At the last follow-up (median = 21 months), 66 patients cleared HBsAg with a HBV recurrence rate of 12 %, and without mortality due to HBV recurrence. Based on these, current data did not recommend LAM monotherapy for post LT prophylaxis due to inadequate potency and high resistance rates. There are now increasing number of reports of HBIG-free antiviral prophylaxis in using ETV or TDF alone or in combination. A completely HBIG-free protocol seems to be better adopted for patients who are HBV DNA negative at the time of LT [37, 38, 93].

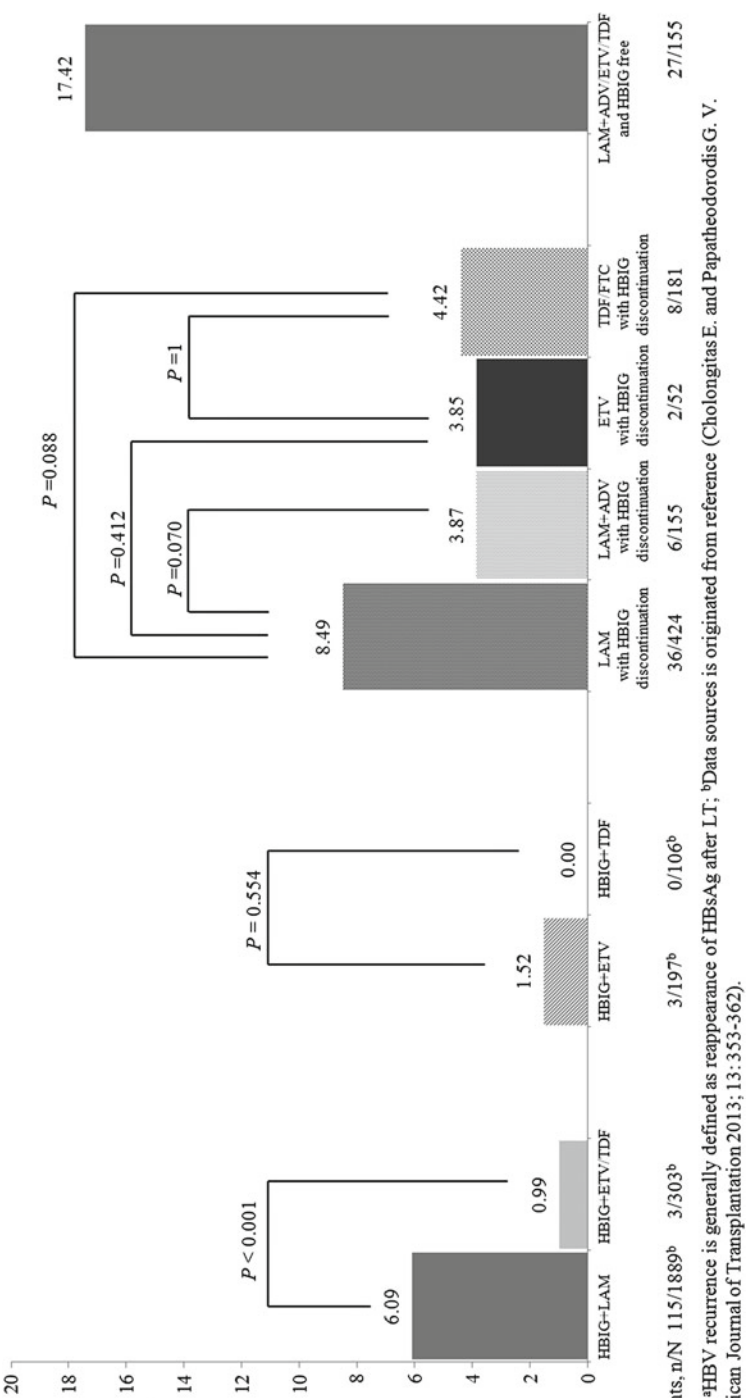
Table 17.2 Published studies on the discontinuation of HBIG with Nucs maintained for HBV prophylaxis after liver transplantation (without control group). They are listed according to the period of HBIG used

Authors, year [ref.]	Study year	Patients	Follow-up (median; months)	Antiviral agent after HBIG withdraw (no. of patients)	HBV recurrence (%)
Park, 2002 [104]	1996–2000	*N*=30 (HBIG 7 days)	9	LAM	3/30 (10.0)
Nath, 2006 [109]	2002–2005	*N*=14 (HBIG 7 days)	14.1	LAM+ADV	0/14 (0)
Gane. 2013 [18]	2003–2007	*N*=20 (HBIG 7 days)	57	LAM+ADV	0/20 (0)
Wong, 2007 [105]	1994–2005	*N*=21 (HBIG>3 months)	40	LAM	1/21 (4.8)
Ahn, 2011 [116]	2002–2007	*N*=24 (HBIG 4 days-6 months)	15.5	LAM (*n*=9) LAM+ADV (*n*=14) TDF+FTC (*n*=1)	0/9 (0) (LAM) 2/14 (14.3) (LAM+ADV) 1/1 [100] (TDF+FTC)
Neff, 2007 [110]	2004–2005	*N*=10 (HBIG 6 months)	31	LAM+ADV	0/10 (0)
Cholongatis, 2014 [118]	2010–2013	*N*=28 (HBIG 6 months)	21	ETV [11] TDF [17]	0 (0)
Shiffman, 2009 [112]	–	*N*=21 (HBIG>6 months)	10.8	TDF+FTC	1/21 (4.7)
Stravitz, 2012 [113]	1958–2009	*N*=21 (HBIG>6 months)	31.1	TDF+FTC	3/21 [14]
Wesdrop, 2013 [111]	1997–2010	*N*=17 (HBIG>6 months)	26.5	TDF+FTC	1/15 (6.7)
Cholongitas,2012 [115]	2007–2011	*N*=47 (HBIG 12 months)	24	LAM+ADV (*n*=23) LAM+TDF (*n*=5) ETV (*n*=9) TDF (*n*=10)	2/23 (8.7) (LAM+ADV) 0/5 (0) (LAM+TDF) 1/9 (11.1) (ETV) 0/10 (0) (TDF)
Yi, 2013 [16]	2007–2009	*N*=26 (HBIG 12 months)	24	ETV	1/26 (3.8)
Tanaka, 2014 [117]	2005–2011	*N*=24 (HBIG 12 months)	29.1	LAM+TDF (*n*=9) TDF (*n*=15)	0 (0)
Lu, 2008 [106]	2002–2006	*N*=122 (HBIG >12 months)	12	LAM	11/122 (9.0)
Sevmis, 2011 [107]	2001–2009	*N*=53 (HBIG>12 months)	46.5	LAM	4/53 (7.5)

(continued)

Table 17.2 (continued)

Authors, year [ref.]	Study year	Patients	Follow-up (median; months)	Antiviral agent after HBIG withdraw (no. of patients)	HBV recurrence (%)
Saab, 2011 [114]	2008–2010	*N*=61 (HBIG >12 months)	15	LAM+ADV (*n*=19) LAM+TDF (*n*=41) ETV+ADV (*n*=1)	0/19 (0) (LAM+ADV) 2/41 (4.9) (LAM+TDF) 0/1 (0) (ETV+ADV)
Dodson, 2000 [103]	1993–1997	*N*=16 (HBIG 24 months)	16.1	LAM	0/16 (0)
Lo, 2005 [108]	1999–2004	*N*=8 (HBIG >24 months)	21.1	LAM+ADV	0/8 (0)
Degertekin, 2010 [41]	2001–2007	*N*=185 (HBIG discontinued after a varying period)	42	LAM (*n*=141) ADV (*n*=16) TDF (*n*=3) ETV (*N*=5) LAM+ADV (*n*=15) LAM+TDF (*n*=3) ADV+TDF (*n*=2)	12/141 (8.5) (LAM) 0/16 (0) (ADV) 0/3 (0) (TDF) 0/5 (0) (ETV) 1/15 (6.7) (LAM+ADV) 0/3 (0) (LAM+TDF) 0/2 (0) (ADV+TDF)



Note. [a]HBV recurrence is generally defined as reappearance of HBsAg after LT; [b]Data sources is originated from reference (Cholongitas E. and Papatheodoridis G. V. American Journal of Transplantation 2013; 13: 353-362).

Fig. 17.2 Risk of HBV recurrence after liver transplantation according to various types of prophylaxis in published studies

Overall Comparison

HBIG Plus Potent Nuc Promise Lowest HBV Recurrence Rates

A systematic review [93] has shown that HBV recurrence was observed to be significantly higher in patients who received Nuc monotherapy or HBIG monotherapy than that of HBIG plus Nuc combination therapies, if the definition of HBV recurrence was based on HBsAg positivity (26 % vs. 5.9 %, $P<0.0001$). In our analysis, HBV recurrence occurred in 27 (17.42 %) of 155 patients with either LAM + ADV, ETV or TDF HBIG-free monotherapy, which was significantly higher than that of HBIG contained regimens [38, 119] (Fig. 17.2). However, if the definition of HBV recurrence was based on HBV DNA detectability, the HBV recurrence rate was similar between HBIG + Nuc combination and potent Nuc monotherapy (0.9 % vs. 3.8 %, $P=0.11$), especially for monotherapy with ETV or TDF [93]. In addition, unlike patients receiving HBIG or Nuc monotherapy, high preoperative viral load seems to be no longer associated with an increased post-LT HBV reinfection in patients given HBIG plus Nuc [39, 120, 121]. Furthermore, LAM + HBIG developed HBV recurrence significantly more frequently when compared to patients under ETV/TDF + HBIG combination (6.1 % vs. 1.0 %, $P<0.001$) [93]. ETV and TDF had similar antiviral efficacy when they combined with HBIG (1.5 % vs. 0 %, respectively, $P>0.05$) [93] (Fig. 17.2). Therefore, the strategy of ETV/TDF + HBIG may still be recommended for patients who are HBV DNA positive at the time of LT.

HIBG Discontinuation Leads to a Higher Rate of HBsAg Reappearance

In considering the fact that waiting list patients are more likely to undergo LT with undetectable HBV DNA, a recent strategy has been to use HBIG for only a finite period of time after LT, followed by long-term Nuc monotherapy. With the encouraging results of previous ETV/TDF + HBIG studies, the experience is increasing. Although the preliminary results of LAM maintained after HBIG withdrawal were good [97], longer follow-up showed that 14 % of patients eventually experienced the recurrence of HBV [96]. Theoretically, ETV and TDF may allow a safer discontinuation of HBIG than LAM due to high potency and very low resistance. In the analysis from Tables 17.1 and 17.2 and Fig. 17.2, LAM maintained group exhibits the highest HBV recurrence (8.49 %) following HBIG discontinuation, but LAM + ADV exhibited a similar HBV recurrence to that of ETV/TDF + FTC following HBIG discontinuation (3.87 % in LAM + ADV; 3.85 % in ETV; 4.42 % in TDF/FTC) (Fig. 17.2). In addition, ETV/TDF + FTC after HBIG discontinuation seems to be slightly inferior to ETV/TDF + FTC with maintained HBIG (4.42 % vs. 0 % in TDF/FTC regimen). But ETV/TDF + FTC after HBIG discontinuation is still superior to ETV/TDF + FTC monoprophylaxis in totally HBIG free regimen ($P<0.05$) (Fig. 17.2). However, there should be some bias in the interpretation of HBV recurrence, because the dose and duration of these studies were highly variable and the data numbers were relatively limited. Nevertheless, HBIG discontinuation under

LAM + ADV, ETV or TDF/FTC therapy may lead to a higher rate of HBsAg reappearance, although with low HBV DNA detectability, than when HBIG is continued long term. Larger studies with longer follow-up are needed for definitive conclusions.

Total Withdrawal of Prophylaxis

Withdrawal of all antiviral prophylaxis with no maintenance HBIG or Nuc therapy can be considered in patients whose intrahepatic HBV DNA, and cccDNA are controlled below the positive titers. A study [122] included 30 patients who were transplanted 64–195 months earlier and were HBsAg-positive, HBeAg and HBV-DNA negative at LT. After verification of no detectable intrahepatic total HBV DNA and ccc-DNA by liver biopsy, all patients underwent HBIG withdrawal and continued LAM with monthly HBsAg and HBV-DNA monitoring and sequential liver biopsies. Thereafter, those with confirmed intrahepatic total and ccc-DNA undetectability 24 weeks after stopping HBIG also underwent LAM withdrawal and were followed-up without prophylaxis. Five of these 30 became HBsAg-positive during a median follow-up of 28.7 months (range 22–42) after LAM withdrawal, but none of these patients experienced clinically relevant events. Of the patients with HBsAg reappearance, one remained HBsAg-positive with detectable HBV-DNA and was successfully treated with TDF. HBsAg-positivity in the remaining patients was transient and followed by anti-HBs seroconversion. They conclude that patients with undetectable HBV viremia at LT and no evidence of intrahepatic total and cccDNA may safely undergo the cautious weaning of prophylaxis. In such a strategy, LAM is cheap and the cost effectiveness on the management of reactivated HBV may be high.

Patient and Graft Survival

It was reported that a high reinfection rate of HBV may accelerate the progression of disease, which resulted in a 5-year survival rate of less than 50 % [3, 123, 124]. The availability and advances in the prophylactic therapies have changed such outcomes of LTRs. In a retrospective study of HBV-infected adults undergoing primary LT in the USA between 1987 and 2002, the 1-year survival probability significantly improved from 71 % in year 1987–1991 to 87 % in year 1997–2002, and the corresponding 5-year survival rate increased from 53 to 76 % ($P < 0.01$) [4]. A large study in 5912 HBV-related LT in Europe over 20 years (1988–2010) showed that the patient and graft survival at 1 and 3 years before 1995 was significantly lower (73%, 65 % and 69 %, 60 %, respectively) when compared with year 1996–2000 (86 %, 81 % and 83 %, 75 %, respectively; each $P < 0.001$), year 2001–2005 (88 %, 83 % and 84 %, 79 %, respectively; each $P < 0.001$), and year 2006–2010 (86 %, 81 % and 83 %, 77 %, respectively; each $P < 0.001$) [125]. This incremental improvement in survival over time reflects the influence of the newer Nuc of ETV and TDF.

After prophylaxis with post-LT HBIG + Nuc, patients' survival continued to improve as 90 % 1-year patient survival was reported in 2007 [126], and 1, 3, 5, and 10 years survival of 93.9, 90.0, 86.9, and 84.1 %, respectively, in 2012 [127]. Even with a totally HBIG-free regimen, patient survival in LTRs could reach 95, 88, and 83 % at 1, 5, and 8 years under potent Nuc prophylaxis [37]. The impact of HBV recurrence on the survival after LT is no longer a significant problem.

Liver Graft from HBsAg-Positive or Anti-HBc-Positive Donor

Regarding donor factors, HBsAg-positive liver grafts can be transplanted to patients with HBV-related diseases [128–130]. Given the shortage of donors, the use of HBV positive grafts in patients with HBV-unrelated diseases could expand the donor pool. A recent study in 42 HBsAg-negative patients using HBsAg-positive liver grafts showed no differences in complications and the patient and graft survivals were comparable to those receiving HBsAg-negative grafts. However, HBsAg persisted after transplant in all patients that received HBsAg-positive grafts though no HBV flare-ups were observed under Nuc therapy with/without HBIG combination [131]. Another study [130] reviewed the outcome of 92 LT using allografts from HBsAg-positive donors in the USA (1990–2009). Allograft and patient survival were comparable between the HBsAg-positive and HBsAg-negative ($n = 82108$) allografts. Utilization of HBsAg-positive liver grafts seems not to increase postoperative morbidity and mortality in the LTR. However, there remains concern of the use of HBsAg-positive live donors, because of the risk of postoperative reactivation and possible liver failure in the donors.

The use of anti-HBc-positive liver grafts is another solution to the current deceased donor shortage. However, the major concern of transplanting such grafts is the transmission of de novo HBV infection to non-HBV recipients. A systematic review [132] including 13 studies showed a 2.7 % incidence of de novo HBV infection during a median period of 25.4 months in patients receiving LAM monotherapy and 3.6 % in patients receiving HBIG + LAM combination therapy during a median period of 31.1 months. Another systematic review [133] including 39 studies showed recurrent HBV infection in 11 % of HBsAg-positive LTRs who received anti-HBc-positive grafts, while survival was similar to HBsAg-positive recipients of anti-HBc-negative grafts. Furthermore, if LTRs did not receive any anti-HBV prophylaxis, de novo HBV infection developed in 47.8 % of 186 HBV naïve recipients, significantly higher than 15.2 % of 138 recipients with serological markers of past HBV infection ($P < 0.001$) or 9.7 % (3/31) of recipients with successful pre-LT vaccination ($P < 0.001$) [134–138]. A study showed that LTRs maintained on ADV therapy had a numerically higher rate (15 %, 5 of 33) of de novo HBV infection than patients maintained on LAM (8 %, 5 of 62) [139]. LAM may be the most cost-effective option for prophylaxis of de novo HBV infection from anti-HBc-positive liver grafts, when compared with newer antivirals (ETV or TDF) [140]. HBIG seems to be unnecessary either as monotherapy or in combination with LAM.

Vaccination Before and After Liver Transplantation

The active immunization of post-LT recipients with HBV vaccine has been tried. Earlier studies reported a successful response to HBV vaccination after LT [141, 142]. However, most studies of post-LT HBV vaccination were of low response rates [143–145]. Patients who were not chronic HBV carriers used to respond well to vaccination. In contrast, the effect of vaccination was disappointing in patients with liver cirrhosis due to immune tolerance [146, 147]. In addition, donors from their spouses with high anti-HBs titers before donation may respond well to vaccine. They undergo adoptive immune transfer from the donor [148, 149]. A study has shown that a high anti-HBs titer (>1000 IU/l) in donors is essential for protective adoptive transfer [150]. Pre-LT HBV vaccination for candidate living donors may facilitate improved post-LT vaccine responses in recipients with liver cirrhosis. LAM or HBIG prophylaxis after LT may be also associated with recurrence due to escape mutants in which second generation recombinant HBV vaccine is not effective [151]. Third-generation recombinant pre-S containing vaccine Sci-B-Vac™ is effective in about 50 % in prevention HBV recurrence due to escape mutants [152].

Notably, considering the extremely high rates of de novo HBV infection after LT in HBV naïve recipients [133] and the successful prevention of de novo HBV infection by pre-LT vaccination [134–138], HBV vaccination should be offered to all naïve HBV patients pre-LT to minimize the need for post-transplant Nuc prophylaxis. Vaccination post-LT may be also tried to enable withdrawal of Nuc prophylaxis if mounting a protective anti-HBs response. However, HBV vaccination alone (without any Nuc) post-LT has been reported to be ineffective in preventing de novo HBV infection [133].

Renal Transplantation

Prevalence of HBV Infection in Renal Transplant Recipients

The prevalence of HBV infection in renal transplant recipients (RTRs) varies between countries, as shown in Table 17.3. With the availability of HBV vaccine in 1980s, the prevalence has been decreasing over time [22, 58, 153, 154]. It decreased from 24.2 % before 1982 to 9.1 % after 1982 ($P<0.001$) in a study [22], and from 6.2 % in 1994 to 2.3 % in 2006 in another study [153]. In countries where hepatitis B is endemic, the prevalence rates are much higher [23, 35, 155–157]. In a 2009 Taiwan study [156], the prevalence of HBV infection in RTRs was 9.2 % (51/554), which is lower than what was reported previously from Taiwan in 2001 (12.9 %, 62/477) and 1994 (20.9 %, 14/67) [23, 157]. The decreasing prevalence of HBV infection may also be attributed to the use of EPO for anemia that consequently decreased the need for blood transfusions during the pre-transplantation period.

Table 17.3 Prevalence rates of HBsAg positivity in renal transplant recipients

Authors, year [ref.]	Study year	Country of origin	HbsAg rate % (no. of patients)
Mathurin, 1999 [22]	1972–1996	France	15.3 (128/834)
Aroldi, 2005 [135]	1972–1989	Italy	14.2 (77/541)
Hu, 1994 [138]	1988–1992	Taiwan	20.9 (14/67)
Lee, 2001 [23]	1984–1999	Taiwan	12.9 (62/477)
Tsai, 2009 [137]	1988–2006	Taiwan	9.2 (51/554)
Santos, 2009 [133]	1992–2006	Portugal	3 (37/1224)
Morales, 2004 [134]	1990–1998	Spain	2.2 (76/3365)
Chan, 2002 [35]	1983–2000	Hong Kong	13.2 (67/509)
Wong, 2001 [136]		Hong Kong	15 (39/265)

Natural History and Outcome of RTRs with HBV Infection

Factors Affecting Progression in HBV-Related Disease After RT

In chronic HBV-infected patients, viral (viral load, genotype, and genomic mutations) host (gender, age, and immune status) and external factors (coinfection with hepatotropic viruses, immunosuppressive therapies, and heavy alcohol consumption) may contribute to the progression of liver disease [1]. Immunosuppression post-RT may affect the host's immune responses against HBV in RTRs [24, 25]. Persistent viral replication and reappearance of HBeAg was observed in 50 % and 30 %, respectively, after RT in 151 HBsAg-positive RTRs [158]. A longitudinal study in 51 HBsAg-positive RTRs showed that 13 (25.5 %) developed cirrhosis (LC) during 57 months follow-up after RT. The study further showed that HBV DNA levels at baseline could not predict LC development while persistent elevation of serum HBV DNA $\geqq 10^5$ copies/ml after RT was a significant risk factor for the development of LC [156]. In contrast, a study in 944 RTRs with HBV infection showed that high pre-RT HBV DNA level $>5 \times 10^4$ IU/ml was a significant predictor ($P=0.007$) for HBV reactivation post-RT [159].

Precore and core promoter mutations are significantly associated with advanced liver disease during the natural course of chronic HBV infection [160]. Similarly, a study with serial HBV DNA sequencing in nine RTRs showed that seven with persistent or increasing amounts of the HBV core gene deletion mutants developed LC, and five died of ESLD [161]. The other study showed that development of T1762/A7164 mutants predicted an increase in HBV DNA, which was associated with eventual development of LC after RT [156]. Another study indicated that in HBV RTRs infected with core promoter mutants, the additional appearance of deletions in the C gene and/or the pre-S region was accompanied by development of LC and ESLD [162].

Histological Progression

The impact of RT on the natural history of HBV has been controversial. A study in 26 HBsAg-positive and 42 HBsAg-negative RTRs showed that HBsAg-positive patients had more severe histological findings, namely chronic persistent hepatitis (CPH) in 38 %, chronic active hepatitis (CAH) in another 38 % and LC in 42 %, in contrast to 17 % ($P=0.08$), 14 % ($P=0.04$) and 19 % ($P=0.07$), respectively, in HBsAg-negative RTRs. During a mean follow-up of 82 ± 58 months, 54 % of HBsAg-positive patients died from liver failure, compared with 12 % of the HBsAg-negative group ($P=0.002$) [163]. This study confirms that HBsAg-positive RTRs had more liver-related complications than HBsAg-negative RTRs.

A prospective study in 20 HBsAg-positive RTRs with serial biopsies during a mean follow-up of 83 months showed that 82 % of RTRs developed CAH or LC. The outcome was much worse than that of ten HBsAg-positive patients who were treated by hemodialysis. They therefore concluded that RT might be inadvisable for HBsAg-positive patients with end stage renal failure [164]. Another large single center study with 310 follow-up liver biopsies in 131 HBsAg-positive RTRs showed that histological deterioration was observed in 85.3 %, with LC development in 28 % and CAH in 42 %, and only 6 % showed a normal liver biopsy during a mean interval of 66 months [158].

Development of Hepatocellular Carcinoma (HCC)

As liver disease may progress in HBV-infected RTRs, HCC may also develop. A nationwide large scale study in 3826 RTRs in Taiwan from 1997 to 2006 showed a higher incidence of HCC in HBV-RTRs than that of non-HBV RTRs, during a mean follow-up period of 7.4 years, despite the availability of anti-HBV drug therapy [165]. The incidence of HCC was significantly greater in the HBV group at years 1 (7.84 vs. 0.70 per 100 person-years), 3 (2.82 vs. 0.26 per 100 person-years), and 5 (1.86 vs. 0.17 per 100 person-years) [165]. Another study reported a 10-year HCC incidence of 4.2 % in HBV-infected RTRs with post-transplant LAM therapy in contrast to 34 % ($P=0.008$) in HBsAg-positive RTRs who did not receive any antiviral therapy [166]. Notably, the histological progression was all reported before the era of antiviral therapies.

Anti-HBV Therapy for RTRs

The efficacy of currently available antiviral therapy options in RTRs with HBV infection is presented in Table 17.4. In general, interferon (IFN) based therapy is not recommended for RTRs. Previous studies reported an increase in acute allograft rejection, immne-mediated renal allograft injury, and graft loss following IFN therapy [167–170].

Table 17.4 Characteristics of antiviral agents for HBV therapy in patients of renal transplant recipients

Antiviral agent	Approved therapy (year)	Consideration in RTRs of HBV [Ref.]
LAM	1998	• Approved worldwide for the treatment of chronic hepatitis B both in organ transplant patients, with evidence of meta-analysis (high rate of drug resistance) [35, 74, 171–183]
ADV	2002	• Good evidence of treatment in LAM resistant RVRs • Potential renal toxicity for RTRs [184–186]
ETV	2005	• Good effect but relatively limited data in RTRs • Preferred choice for first line treatment HBV reactivation of RTRs • No nephrotoxicity [183, 187, 188, 192, 196–198]
LdT	2006	• Lack of evidence for RTRs • May be considered combination therapy in patients of renal function impairment who need ADV or TDF treatment [196–201]
TDF	2008	• Rare evidence for RTRs • Reported renal toxicity in HIV patients [189, 190, 194, 195]

Lamivudine

It has been approved worldwide for the treatment of chronic hepatitis B in organ transplant patients [35, 74, 171–183]. A meta-analysis including 181 RTRs in 14 clinical prospective cohort studies showed that LAM therapy resulted in a mean overall HBV DNA clearance in 91 % and HBeAg loss in 27 % but LAM resistance was reported in 18 %. The increased duration of LAM therapy was directly correlated with the frequency of HBeAg loss ($r=0.51$, $P=0.039$) and LAM resistance ($r=0.620$, $P=0.019$).

Adefovir Dipivoxil

A retrospective study showed that ADV add on LAM therapy was superior to ADV monotherapy in achieving undetectable HBV DNA at month 24 (44.4 vs. 20 %) in RTRs with LAM resistance, but 4 (29 %) of the 14 RTRs developed moderate to severe impaired renal function [184]. Another study showed that both serum creatinine and 24-h proteinuria increased significantly during 2-year ADV therapy in 11 HBV-infected patients with LAM resistance [185]. In contrast, no significant renal function impairment has been observed during long-term ADV plus LAM combination therapy in RTRs with LAM resistance [186]. However, with the availability of ETV and TDF, ADV may no longer be used to treat HBV in patients with renal impairment or post RT

Entecavir and Tenofovir

More recent study on ETV monotherapy in 27 Nuc-naïve or LAM experienced HBV-infected RTRs showed undetectable HBV DNA in 96 % at month 12 and 100 % at months 24 of therapy without viral resistance [187]. Studies also show that ETV is more effective than LAM in reducing HBV DNA levels in RTRs [183, 187, 188]. The experience of TDF for RTRs was very limited, only described in sporadic case reports [189, 190].

Selection of Antiviral Therapy

Given the drug potency, safety, and resistance issues during long-term therapy, LAM, ADV, and telbivudine (LdT) are no longer recommended for patients with organ transplantation [58, 183, 191–194]. Instead, potent Nuc with low resistance should be used for RTRs. Since long-term use of TDF in HIV patients has been associated with possible renal toxicity, as well as metabolic bone disease and osteomalacia [194, 195], it has been suggested that ETV may be preferred over TDF in RT population because no nephrotoxicity has been reported in chronic hepatitis or cirrhotic populations [187, 192, 196–198]. TDF adapted to creatinine clearance could be a safe alternative in RTRs with drug resistance [189]. If renal allograft dysfunction is in progress, the inception of LdT, in theory, could potentially lead to renal function improvement. This is attributed to LdT having exhibited a better eGFR evolution among HBV patients during long-term antiviral therapy [196–199]. LdT is also associated with improvement of renal function in liver transplant setting [200, 201] who are considered at high risk for renal dysfunction due to the concomitant use of the nephrotoxic calcineurin inhibitors (CNIs) [202].

Timing and Duration for Antiviral Therapy

At present, the general consensus is that Nuc therapy should be commenced pre RT in those with active CHB and start at time of transplant in those without CHB as the majority of patients will have increase in HBV DNA under immunosuppression [193]. Actually, there are two principal approaches to preventing HBV reactivation after RT: prophylactic and preemptive. A study showed that preemptive LAM therapy improved the survival of HBV-infected RTRs [35], while others showed that prophylactic LAM treatment might provide benefits in RTRs [177, 182], but salvage treatment after hepatic dysfunction during HBV recurrence was less effective [180].

The duration of anti-HBV therapy in RTR should also be considered. In the era of LAM, prolonged therapy is associated with drug resistance [183, 203], while withdrawal of LAM may be adversely associated with a high risk of relapse and liver failure. A recent small study showed a high rate (75 %, 9/12) of virological relapse (defined as HBV DNA >2000 IU/ml) during a median follow-up of 65 weeks (range 8–194 weeks) in patients who had completed 2-year LAM treatment and

discontinued therapy after demonstration of undetectable HBV DNA at two occasions 6-month apart [183]. However, another study in 12 low risk RTRs (more than 9 months therapy, HBV DNA and HBeAg-negative, stable immunosuppression) showed that five (41.7 %) of them achieved successful Nuc withdrawal, with two (16.7 %) patients maintaining undetectable serum HBV DNA for more than 18 months after cessation of LAM therapy [35]. It was also reported that no liver related mortality was recorded in 20 HBsAg-positive kidney or heart transplant recipients after LAM treatment was discontinued [204]. Recent study also reported the successful withdrawal of antiviral agents in six of 14 HBV-RTRs who met the following criteria: no cirrhosis; normal liver biochemistry; negative HBeAg; no viral resistance; antiviral therapy >9 months; maintenance dosage of immunosuppressant for >3 months; and no acute rejection during recent 6 months. Four (66.7 %) of these six patients successfully withdrew Nuc and remained HBV DNA negative for a median period of 60.5 months [205]. Taken together, the therapeutic strategy is complex and the results inconsistent, making it difficult to reach a conclusive recommendation. In high risk patients with high levels of HBV DNA at baseline, or those who are maintained with a high dose of immunosuppressant, long-term therapy may be needed [192, 193].

Patient and Graft Survival After Renal Transplantation

The impact of HBV infection in the survival of RTRs has also been debated and remains controversial. Some studies showed no significant difference in 5-year survival between HBsAg-positive and negative RTRs [206, 207]. Other larger and longer studies showed negative impact of HBV infection on patient and graft survival [21–23, 36, 208]. Lee and colleagues [23] reported that the 10 year patient and graft survival was significantly higher in the HBV-negative RTRs (82.8 and 74.2 % respectively) than in the HBV-infected RTRs (51.4 and 44 % respectively). Mathurin and colleagues [22] further showed that the 10-year survivals of HBV-infected patients (55 ± 6 %) and HCV-infected patients (65 ± 5 %) were significantly lower than that of patients without HBV or HCV infection (80 ± 3 %, $P < .001$). The most important predictor of outcome following RT in HBsAg-positive RTR is the presence of cirrhosis prior to transplant. A meta-analysis including 6050 RTRs indicated clearly that serum HBsAg was an independent risk factor for death (relative risk: 2.49, $P < 0.0001$) and allograft loss (relative risk of 1.44, 95 % CI of 1.02–2.04) after RT [21]. However, most of these studies were conducted in the era before oral anti-HBV therapy was available. A guideline has suggested that the best predictor for liver mortality following renal transplantation in an HBsAg-positive recipient is with cirrhosis at the time of transplant, and liver biopsy should be considered in all potential HBsAg-positive renal transplant candidates. Established cirrhosis with active viral infection should be considered a relative contraindication to RT [209].

The availability of LAM in 1998 marked the new era of oral therapy. A study from Hong Kong showed that the survival of HBsAg-positive RTRs who received preemptive LAM treatment (transplanted after 1996) was similar to that of

HBsAg-negative controls, whereas HBsAg-positive RTRs who did not receive LAM treatment (transplanted before 1996) had significantly increased liver related mortality (relative risk 68, 95 % CI, 8.7–533.2) and lower survival (relative risk, 9.4, $P<0.001$) [35]. A large study in RTRs in the USA from 2001 to 2007 also reported that HBV infection was no longer a risk factor for death or kidney failure, although 5-year cumulative incidence of hepatic failure was higher in 1346 HBV-RTRs (1.3 % vs. 0.2 %; $P<0.001$), compared with 74,355 HBV-negative RTRs [34]. Notably, a large retrospective study showed that the 10 year patient and graft survival rates in 66 HBsAg-positive RTRs were significantly lower than those in 2054 non-HBV RTRs (64.4/36.6 % vs. 88.2/70.5 %, respectively, $P<0.0001$). In contrast, patients with LAM therapy had significant improvement in both 10 year patient and graft survivals, as compared to HBV RTRs who did not take LAM (85.3/59.2 % vs. 49.9/22.7 %, respectively, $P<0.0001$) [36]. A nationwide large-scale study of 3826 RTRs in Taiwan from 1997 to 2006 also reported that there were no differences between the HBV and non-HBV groups in patient or graft survival rates during a mean period of 7.4 years follow-up [165]. A more recent study indicated that patient and graft survival rates of LAM prophylactic HBV-RTRs were significantly higher than those of historical control (never LAM treated HBV-RTRs) ($P=0.001$ and 0.017, respectively) from 2000–2009 [166].

HBsAg-Positive Renal Transplant Donors

Kidneys from HBsAg-positive donors were previously not acceptable for RT, because of the potential risk of HBV transmission to recipients. Obviously, the extremely high prevalence of HBsAg in Asian populations would limit the donor pool. In some situations, it is acceptable for renal grafts from HBsAg-positive donors to HBsAg-positive or HBsAg-negative recipients with long-term Nuc administration with or without HBIG [210–213]. One study compared 14 anti-HBs-positive patients who received kidneys from HBsAg-positive donors and 27 HBsAg-positive patients who received kidneys from HBsAg-negative donors, and found that the ten year patient survival (92.8 % vs. 62.5 %, $P=0.14$) was higher but not significantly different [214]. There are also reports on LAM combined with HBIG in anti-HBs-positive recipients who received grafts from HBsAg-positive donors [213, 215]. A prospective non-randomized controlled study in 373 HBsAg-positive RTRs who received a kidney from either HBsAg-positive donor ($n=65$) or HBsAg-negative donor ($n=308$) using a standardized immunosuppressive and antiviral regimen (400 U HBIG once for HBsAg-negative graft recipients and twice for HBsAg-positive graft recipient, 400 U HBIG weekly for 3 months and LAM 100 mg daily for 6 months for recipients with HBV DNA-positive grafts) showed no significant differences in liver injury and patient survival among these 2 groups of RTRs [213]. A latest study from Thailand used the propensity score matching technique to compare outcomes of 43 HBsAg-negative recipients with anti-HBs titer above 100 mIU/ml (by natural or vaccination) who received RT from HBsAg-positive donors versus 86 HBsAg-negative donors, and found no significant difference in

graft and patient survival during a median follow-up duration of 58.2 months and no HBV-infective markers were detected in the HBsAg-positive donor group [216]. Notably, most of these reports regarding the safety of HBsAg-positive renal donors to HBsAg-negative recipients were all from Asia where HBV infection is highly endemic. Therefore, considering the remarkable impact of renal transplantation on patients' survival and life quality as well as recent progress in anti-HBV therapy, the benefit of renal graft absolutely overweighs the risk of HBV transmission, which was also shown in liver transplant recipients [129, 130].

Anti-HBc-Positive Renal Transplant Donors

The exclusion of anti-HBc-positive renal donors would limit the donor pool because of the extremely high prevalence of natural immunity from childhood HBV exposure in Asian populations. However, it was shown that the de novo HBV infection rate from anti-HBc-positive kidney and heart allografts was significantly lower than that from liver allografts [217]. In a systematic review of 1385 anti-HBc-seropositive renal donors, seroconversion of anti-HBc, anti-HBs or both occurs in 3 % of RTRs, and only 0.28 % of the recipients develop HBsAg seroconversion. Furthermore, there was no symptomatic hepatitis, higher mortality, or shorter renal graft survival among these patients [218]. Since there was a very low risk of seroconversion, renal grafts from anti-HBc-positive donors is not contraindicated [219, 220]. However, monitoring of serum HBV markers is still required after RT. Nuc therapy initiation is indicated only when there is seroconversion of HBsAg or an increase in viral load, and may be interrupted after immunosuppression is reduced and complete viral clearance has been achieved [221]. Pre-transplant immunization may be helpful to further reduce the risk of HBV transmission [210, 222].

Comments on HBV-Positive Renal Transplant Donors

Finally, it is important to emphasize that use of either HBsAg-positive or anti-HBc-positive donors in RT is a completely different scenario and risk profile than the risks in LT. In RT, anti-HBc-positive kidneys have never been an issue whilst HBsAg-positive kidneys can be safely used provided the recipient has protective immunity (natural or post-vaccination) or receives antiviral prophylaxis following transplantation [129].

Renal Recipients with Markers of Past HBV Infection

Reactivation of HBV infection can also occur at a rate of 0.9–5 %, during a period ranging from 8 weeks to 15 years in HBsAg-negative but anti-HBs- and anti-HBc-positive RTRs [25, 223–228]. It may sometimes be difficult to distinguish these

from patients with de novo infection by receiving anti-HBc-positive renal graft. It is indicated that the odds ratio for HBV reactivation in patients without anti-HBs antibodies at transplantation compared to those with anti-HBs antibodies was 26 (95 % CI [2.8–240.5], $P=0.0012$) [227]. Notably, the 1-, 3-, 5-, and 10-year patient survival was 86.7, 79.4, 72.2, and 65.0 % respectively in the de novo HBV group, and was 96.1, 93.8, 91.5, and 84.5 % respectively in the non-HBV reactivation group (log-rank 4.12, $P=0.042$) [228]. However, since there are low rates of de novo HBV infection, routine antiviral prophylaxis in this group cannot be recommended. Suggestions have advocated monitoring of HBsAg or HBV DNA and institution of preemptive antiviral therapy if HBV DNA progressively rises [192].

Organ Transplantation Other Than LT and RT

Besides RT, there is less data available for other non-liver organ transplantation [229, 230]. HBV reactivation after heart transplantations was common but usually well controlled with LAM treatment. HBsAg-positive donor hearts were safely transplanted into anti-HBs-positive recipients; Therefore, HBV carrier status should not contraindicate heart transplantation [230]. It is also reasonable to consider recommendations similar to that for the RT setting [28–33]. Among these, bone marrow transplantation (BMT) is the most serious one that should be briefly addressed. Immunosuppression in BMT can result in reactivation not only among HBV patients, but also in those immune to HBV. Among patients with resolved hepatitis B before BMT, the anti-HBs titer may decline and serum HBV DNA may become detectable [231]. Chemotherapy which was used before BMT may further reactivate HBV infection. An earlier study reported 100 Hong Kong patients undergoing chemotherapy for lymphoma and found that the development of HBV-related hepatitis in 13 (48 %) of 27 HBsAg-positive patients; 2 (3.9 %) of 51 HBsAg-negative, anti-HBc-positive patients; and none (0 %) of 22 HBsAg-negative, anti-HBc-negative patients [232]. A study of 137 consecutive patients (23 HBsAg-positive, 37 anti-HBs-positive, and 77 negative for HBV) who underwent hematopoietic cell transplantation (HCT) showed that hepatitis due to HBV reactivation was more common in HBsAg-positive patients than in HBsAg-negative patients (hazard ratio, 33.3; $P<0.0001$). Furthermore, HBsAg-positive patients with detectable HBV DNA before HCT had a significantly higher risk of hepatitis flare than HBsAg-positive patients without detectable HBV DNA (adjusted hazard ratio, 9.35; $P=0.012$) [233]. It has also been reported that adoptive transfer of immunity against HBV leading to clearance of HBV infection was found in patients undergoing BMT in which the donors had recovered from prior HBV infection or had been actively immunized against hepatitis B [233, 234]. Overall, prophylactic antiviral therapy is recommended for all HBsAg-positive patients undergoing BMT regardless of HBV DNA status, and should be continued for at least 6 months or longer according to baseline serum HBV DNA levels [235–238]. Finally, transplanting avascular organs such as the cornea carries very low risk of HBV transmission, even from HBsAg-positive donors [239–241]. Antiviral prophylaxis is not recommended for this transplant setting.

Summary and Conclusion

Organ transplantation in the HBsAg-positive patient is effective and life saving, but the prevention or management of HBV recurrence and/or reactivation has been a challenge. For LTRs, high genetic barrier Nuc plus HBIG is still the standard of care to prevent HBV recurrence post LT. HBIG discontinuation after a period of time after LT seems to be safe, but might lead to a higher HBsAg reappearance rate, although most are with undetectable HBV DNA after HBsAg reappearance. Even with higher rates of HBsAg reappearance than HBIG contained regimens, HBIG free with potent Nuc therapy could also achieve similar clinical outcomes. However, the clinical significance and long-term outcomes of HBsAg reappearance in LTRs are unknown. Larger studies with longer follow-up are needed for a definitive conclusion.

The reported prevalence of chronic HBV carriers receiving RT is decreasing, but it is still not negligible, especially in endemic areas of HBV infection. HBV has conferred a high risk of morbidity and mortality in RTRs before the advent of Nuc. At present, HBsAg-positive or anti-HBc-positive donors can be safely used in RTRs. Flow diagram of management algorithm for RTRs with HBV infection is illustrated in Fig. 17.3. Considering long-term treatment, antiviral agents with a high genetic barrier to resistance (ETV or TDF) and lack of nephrotoxicity (e.g., ETV) are recommended.

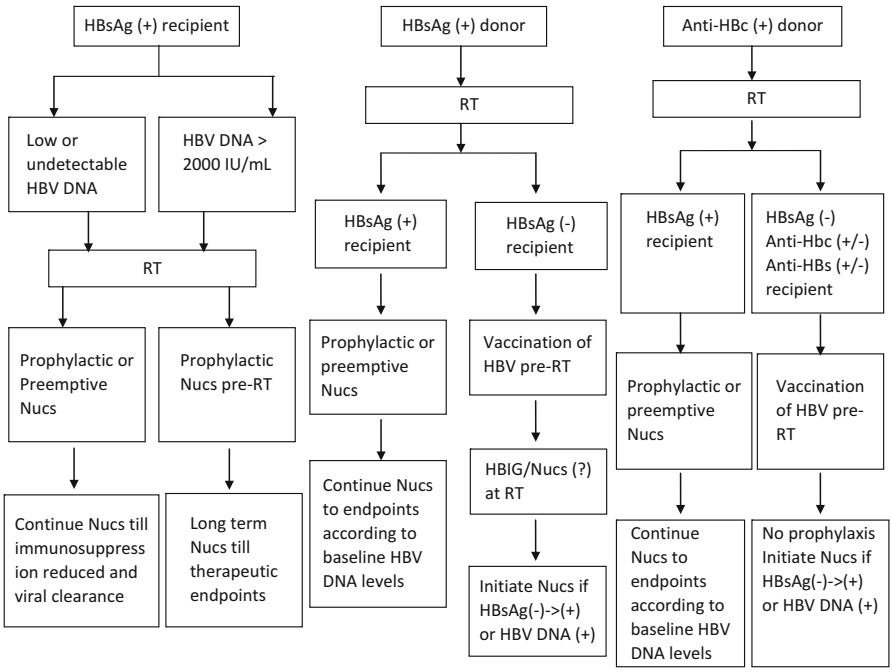


Fig. 17.3 Flow diagram of management algorithm for renal transplant recipients with HBV infection

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Chapter 18
Reactivation of Hepatitis B Virus Due to Cancer Chemotherapy and Other Immunosuppressive Drug Therapy

Stevan A. Gonzalez and Robert P. Perrillo

Introduction: The Many Guises of Hepatitis B Reactivation

Hepatitis B virus reactivation (hereafter HBVr) was first described 40 years ago in the setting of cancer chemotherapy and kidney transplantation. Initial reports were only small case series, but they nonetheless provided landmark observations [1, 2]. These seminal reports not only demonstrated that immune suppressive drug therapy (ISDT) was associated with marked flares of hepatitis, some of which proved fatal, but also that hepatitis B surface antigen (HBsAg)-negative patients with detectable antibody to hepatitis B core antigen (anti-HBc) were also at risk [1, 2].

Since these early studies, HBVr has been shown to occur as a complication of coinfection with human immunodeficiency virus and with the use of ISDT for varied chronic inflammatory disorders [3, 4]. These clinical associations have led to the central hypothesis that the initiating event in reactivation during ISDT is a change in innate and adaptive cell mediated responses directed against the virus.

Reactivated hepatitis B also occurs spontaneously in which case the initiating events that lead to spontaneous HBVr are less apparent than in the above settings. Early description of spontaneous HBVr indicated that it occurred in 5–10 % of persons with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B annually, and it has been shown to occur even more frequently in those with antibody to hepatitis B e antigen (anti-HBe)-positive chronic hepatitis B [5–7]. Even here changes in the immunologic control of viral replication may be a contributing factor.

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For example, the core gene is highly conserved in HBeAg-positive chronic hepatitis B but marked sequence variability has been observed after HBeAg seroconversion, and changes in amino acid structure within immunodominant core gene epitopes have been shown to be associated with diminished efficiency of cytoxic T cells to viral antigens [8, 9].

The current chapter will focus on HBVr during ISDT. It is disquieting to consider that this association will expand in the future as an expanding array of newer biologics agents find clinical use across a number of medical specialties. It is also sadly ironic that forty years have elapsed since the initial description of HBVr due to ISDT and yet it remains an important clinical association even though it is preventable with safe and effective antiviral therapy.

Defining the Syndrome

Characteristic features of HBVr include an acute rise in serum hepatitis B virus deoxyribonucleic acid (HBV DNA) that is often associated with a hepatitis flare and rising serum aminotransferase levels [4, 10]. There is substantial variability as to how the components of the definition are defined in the literature.

Virologic criteria: This is defined in clinical studies in one of three ways: (1) either as *de novo* detection of HBV DNA in a patient who was previously found to not have detectable HBV DNA, (2) an increase of at least one log (tenfold) IU/mL in a patient previously found to have detectable serum HBV DNA, or (3) a value of serum HBV DNA that is above an arbitrary cutoff in a patient with biochemical worsening [11]. An example of this would be to use a cutoff of 20,000 IU/mL or greater. Accurate estimation of HBVr demands that the assay be as sensitive as possible. The first two criteria above also require that a similarly sensitive method of HBV detection be applied to both time points. For example, use of a relatively insensitive assay with a cutoff of 2–4 log IU/mL at baseline can lead to overestimation of the frequency of HBVr if a more sensitive method is used on a second sample and underestimation of its occurrence if the order is reversed. The third virologic criterion can initially be potentially misleading in active cases of chronic hepatitis B because high levels of HBV DNA are typically seen in this situation and one needs to take into account other causes of biochemical exacerbation such as superinfection with other hepatotrophic viruses and hepatotoxicity due to medications. Reactivated hepatitis B may be accompanied by HBsAg reverse seroconversion (sero-reversion) in patients who were initially HBsAg-negative but positive for anti-HBc. HBsAg sero-reversion may occur with or without concomitant HBeAg sero-reversion.

Biochemical criteria: Clinical studies also exhibit variability in the biochemical criteria needed for the definition of HBVr. Most studies have used alanine aminotransferase (ALT) as the reported variable rather than aspartate aminotransferase level (AST). Criteria for hepatitis flares typically involve a two- or threefold rise in

ALT above baseline levels. Some studies have further required that values exceed 100 U/L at the time virologic criteria are reached. The presence of five- to tenfold elevations of ALT has been used to define severe hepatitis flares and this is often expressed as a multiple of the upper reference range rather than a comparison to baseline levels.

It is important to recognize that a standardized formal definition of HBVr has not been established [11–14]. The availability of a standardized measure of severity would allow for a more uniform characterization of cases across studies, assist in risk stratification, and allow better discrimination of prognosis [13]. Several systems have recently been proposed, some of which incorporate changes in clinical outcomes other than severity of liver injury (Table 18.1) [15, 16].

Clinical Spectrum and Outcomes

HBVr can be associated with a significant risk of morbidity and mortality or it can be very mild and clinically inapparent. Practical experience has shown that many mild cases are not detected because changes in serum aminotransferase levels are attributable to the chemotherapy and the patient is not known to have HBV infection. Many observational studies include a number of cases in which no alteration of liver chemistries are appreciated. The overall frequency with which biochemical abnormalities are seen is often in excess of 50 % in observational studies that report data on all patients who meet the virologic criterion [11].

Mortality from HBV reactivation is reported in most clinical series. It is difficult to determine the absolute risk of death from HBVr. Some studies have reported a high mortality risk (greater than 50 %) [17, 18]. However, in most studies liver-related mortality from HBVr has been considerably lower varying from 0 to 10 % [11]. Mortality has generally been observed to be a less frequent outcome in patients who reactivate during treatment of nonmalignant conditions with tumor necrosis factor (TNF) alpha inhibitors [11, 19]. The relatively low mortality rate is unlikely to be due to treatment with antiviral therapy because studies that predated antiviral therapy have also revealed relatively low mortality. Even though fulminant liver failure is uncommon when HBVr is precipitated by cancer chemotherapy, it is important to emphasize that these patients lack the usual option of liver transplantation due to the underlying malignancy [20].

Observational studies tend to give less emphasis and inconsistent reporting on outcomes other than severity of hepatitis and case fatalities. One of the important clinical concerns, however, is the relatively high frequency with which cancer chemotherapy needs to be interrupted, either temporarily halted or prematurely discontinued. This has been shown to occur in as many as 20–40 % of patients with breast cancer [21]. There has also been little concerted effort to report late complications of chemotherapy interruption such as cancer-related fatalities due to the progression of underlying malignancy. Another underreported outcome is disease morbidity. An international survey of the American Association for the Study of Liver Disease (AASLD) membership

Table 18.1 Proposed grading criteria for HBV reactivation

NIH system [15]		University of Toronto System [16]				Current authors	
Grade	Features	Grade	Hepatic features	Grade	Immuno-suppression	Grade	Features
1, Silent	Without change in ALT	H_0	No hepatitis, ALT <2×ULN	I_0	No consequence	1, Silent	Without change in ALT or AST
2, Mild	Change in ALT greater than twofold, no jaundice	H_1	Hepatitis, ALT 2–10×ULN without change in bilirubin or INR	I_1	No interruption but increased need for HBV DNA and ALT monitoring	2, Very mild	ALT or AST increased <2×baseline
3, Moderate	Change in ALT and jaundice	H_2	Severe hepatitis, ALT >10×ULN and/or bilirubin 2×ULN or INR (>1.3)	I_2	Interruption of ISDT with reinitiation after hepatitis flare resolves	3, Mild	ALT or AST increased 2–5×baseline, no jaundice or change in INR
4, Severe	Change in ALT, jaundice, and signs of liver failure	H_3	Fulminant hepatitis as with H_2 and ascites or encephalopathy	I_3	Interruption of ISDT with reinitiation of second line therapy	4, Moderate	ALT or AST increased >5×baseline; no jaundice or change in INR
5, Fatal	Liver failure leading to death or transplant	H_4	Death due to liver failure	I_4	Discontinuation of ISDT	5, Severe	As above with jaundice and/or change in INR (>1.5)
						6, Life threatening	Fulminant[a] requiring transplant consideration or fatal

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *INR* international normalized ratio of prothrombin time

[a]Assumes presence of ascites, encephalopathy, or renal dysfunction

** V = virological, H = hepatic, I = immunosuppression

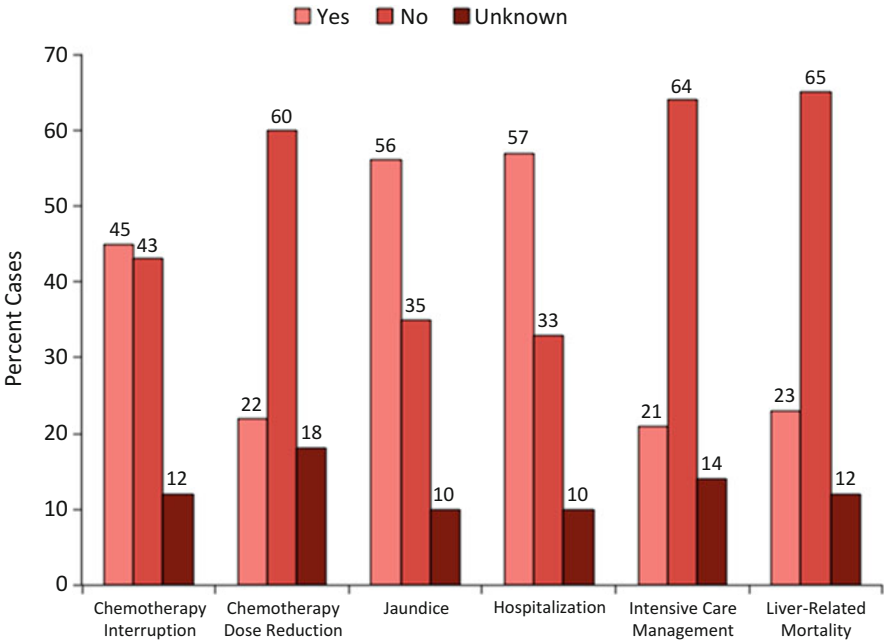

Fig. 18.1 Clinical outcomes in hepatitis B reactivation. Distribution of clinical outcomes in 188 cases of hepatitis B reactivation associated with cancer chemotherapy. Data were derived from an international survey of hepatologists [12]

described 188 cases of HBVr induced by cancer chemotherapy in which nearly half of the patients had chemotherapy interruption and more than 50 % required hospitalization with a large number requiring intensive care management. In addition, liver related mortality was reported in 23 % of patients (Fig. 18.1) [12].

Global Reach and Changing Epidemiology

HBVr occurs worldwide and there is reason to suspect that the incidence may be increasing due to the convergence of a number of factors: (1) the rapid development of immunologically potent biologic agents that are used across medical specialties; (2) relatively poor provider awareness of the benefits of HBV screening and antiviral prophylaxis of at risk patients; and (3) ambiguous and weak specialty practice guidelines [11, 13, 22]. In a recent international survey distributed to liver disease specialists, 40 % of whom practiced outside of the United States, a disproportionate number of cases (131 of 188, or 70 %) were patients born in areas of intermediate to high HBV endemicity (Fig. 18.2) [12]. A major concern in the findings from this survey was that only 40 % of the 188 HBVr cases were screened for HBsAg and anti-HBc before

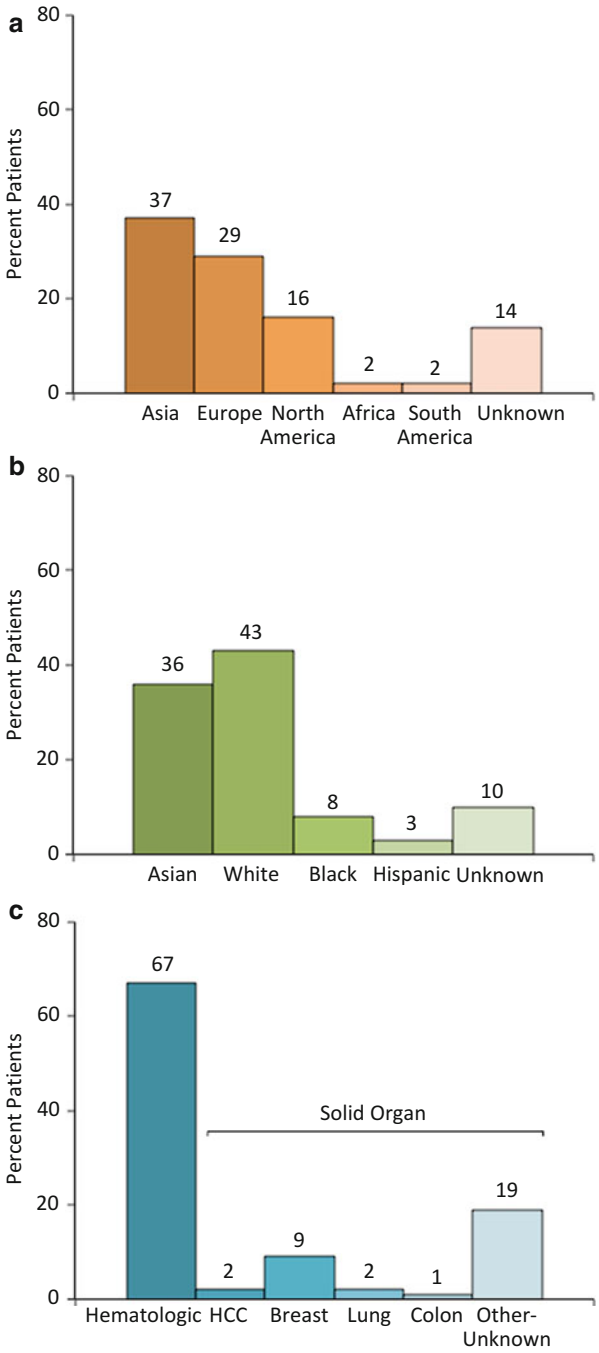

Fig. 18.2 Geographic, ethnic, and clinical distribution of hepatitis B reactivation during cancer chemotherapy. Results of international survey of 99 North American, Asian, and European hepatologists and gastroenterologists 40 % of whom practiced outside of North America [12]. The geographic distribution (**a**), ethnicity (**b**), and type of malignancy (**c**) are reported in 188 cases. Other types of cancer included bone, brain, gastrointestinal, retinoblastoma, round cell, sarcoma, and teratoma. *HCC* hepatocellular carcinoma

initiation of chemotherapy and an additional 13 % had HBsAg screening alone. Further disappointing, only 10 % received prophylactic antiviral therapy.

The widespread use of immunosuppressive therapy for nonmalignant conditions is a major contributing factor to a rising incidence of HBVr from ISDT. There is broad availability of TNF alpha inhibitors and broad licensing of potent biologic agents with inhibitory effects on T cell signaling, cell adhesion molecules, tyrosine kinase activity, and cytokines including interleukin (IL)-12 and IL-23. Within this landscape of therapeutic options have been increasing reports of HBVr induced by anti-CD20 B cell depleting agents used in chemotherapeutic regimens for lymphoma as well as in the management of rheumatologic diseases [11, 14, 23]. The routine use of some of these agents in the treatment of various chronic inflammatory disorders has greatly widened the spectrum of patients potentially at risk of HBVr beyond those receiving cancer chemotherapy or traditional immunosuppressive therapy such as glucocorticoids, methotrexate, or azathioprine. A diverse range of medical specialties such as gastroenterology, dermatology, and rheumatology now utilize these biologic agents on a regular basis.

Immunopathogenesis and Timeline of Virologic and Biochemical Events

It has been difficult to directly study the immunopathogenetic events linked to HBVr due to limited biologic samples, which by necessity are harvested after the disorder is clinically apparent [24]. A sequence of events has been depicted for HBV infected persons during and after cancer chemotherapy administration, which is supported by a limited number of in vitro and numerous observational studies (Fig. 18.3). Longitudinal cohort studies evaluating the kinetics of viral replication in patients with resolved HBV infection undergoing cytotoxic chemotherapy have provided insight into the timeline of evolving HBVr in individuals at risk. During the course of immunosuppressive drug therapy, an initial phase of enhanced viral replication appears to take place, in which up to a 100-fold or greater increase in serum HBV DNA levels can be observed as early as 3–6 months prior to the onset of overt clinical HBVr [25]. During this period of increased viral replication, serum aminotransferases remain normal (Fig. 18.3b) A second phase ushered in by immunologic restitution often follows after discontinuation of the immunosuppressive agent. During this phase, onset of clinical hepatitis may occur with biochemical evidence of hepatocellular injury and risk of hepatic failure (Fig. 18.3c). Preliminary evidence using biologic samples from patients with HBVr due to cancer chemotherapy suggests that an acute resurgence of HBV-specific cell mediated immunity plays a primary role in liver cell injury during the second phase of reactivation. In these patients increased HBV-specific CD8+ T cells have been detected along with diminished regulatory T cells; similar findings were observed in patients with active chronic hepatitis B but not in inactive HBsAg carriers [26]. A similar pattern may

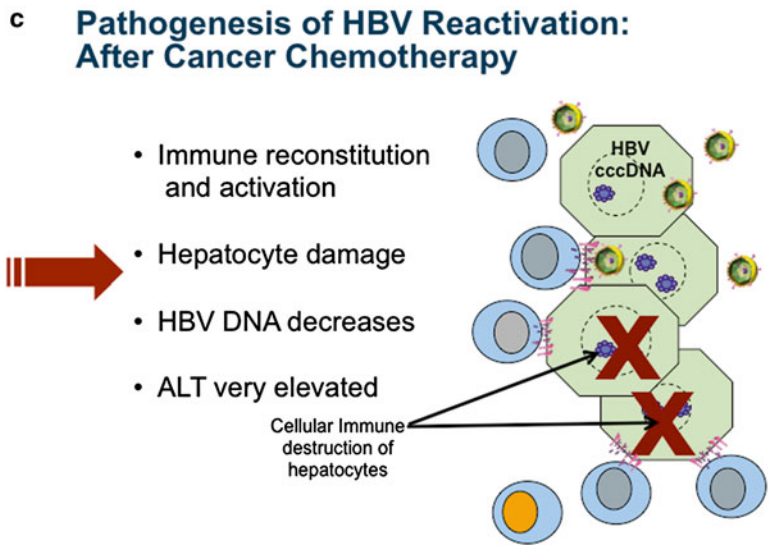

Fig. 18.3 Proposed immunopathogenesis of hepatitis B reactivation in the setting of cancer chemotherapy in an inactive HBsAg carrier. (**a**) Low-level viral replication occurs in an inactive HBsAg carrier who is about to undergo cancer chemotherapy. Small amounts of covalently closed circular HBV DNA reside in the nucleus of the cell (see in *purple*). It is this form of HBV DNA that serves as the genomic template for future viral transcription. *ALT* alanine aminotransferase, *HBsAg* hepatitis B surface antigen, *HBV* hepatitis B virus, *WNL* within normal limits. (**b**) Administration of cancer chemotherapy blunts T cell immune responses, which in turn permits expanded viral replication. Immunogenic core and other viral antigens are avidly displayed on the surface of infected hepatocytes in conjunction with HLA class I molecules during the administration of chemotherapy (*magenta* colored structures). *HLA* human leukocyte antigen. (**c**) Following discontinuation of a chemotherapy cycle, there is an immunologic rebound against the more greatly expressed viral antigens, resulting in hepatocytolysis and biochemical exacerbation. Serum HBV DNA levels often decline but at the expense of liver cell injury

occur when HBVr occurs in association with other immunosuppressive agents; however, immunologic changes occurring in other forms of immunosuppression have yet to be described.

Reconstitution of the host immunity appears to be a critical factor leading to liver injury after discontinuation of immunosuppressive agents, in the setting of cancer chemotherapy, or abrupt glucocorticoid withdrawal [4]. As the immunologic events leading up to HBVr are thought to occur several weeks before the development of overt clinical signs or elevated aminotransferases, a strategy of HBV DNA monitoring and deferred antiviral therapy is unlikely to avoid liver injury or potential liver failure in many patients [27–29]. This is well illustrated in the case depicted in Fig. 18.4.

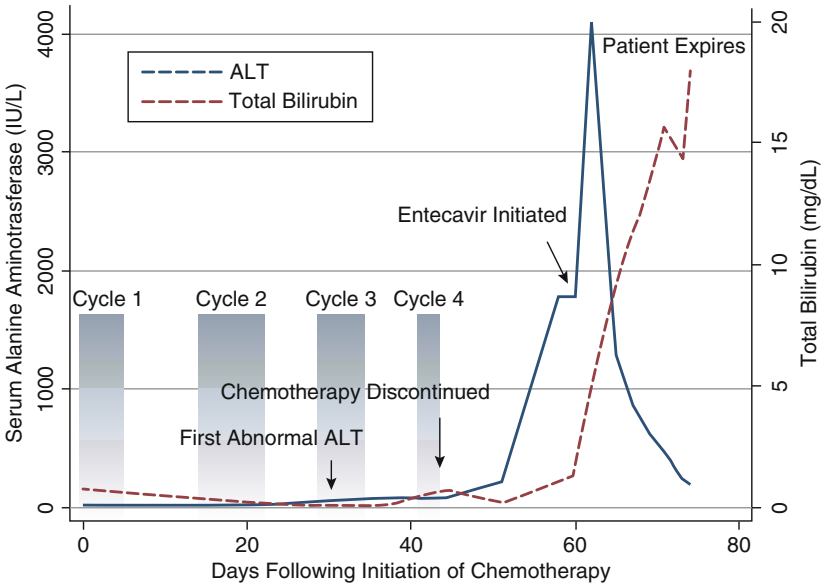

Fig. 18.4 Clinical case of hepatitis B reactivation. Graphic representation of a fatal case of HBVr in a 55 year-old Chinese woman treated with doxorubicin, paclitaxel, dexamethasone, and cyclophosphamide for stage II breast cancer. Elevated ALT was observed during the third cycle of therapy approximately 5 weeks after beginning treatment. She had not been screened for HBV prior to chemotherapy and the gradual increase in ALT did not prompt testing for HBV. Testing with HBsAg and anti-HBc was first done on day 60 after initiation of chemotherapy, at which point, the patient had already demonstrated fulminant liver failure. At that time, serum HBV DNA was detectable at 2 million IU/mL. Entecavir was initiated but she expired 2 weeks after starting antiviral therapy. *ALT* alanine aminotransferase, *anti-HBc* antibody to hepatitis B core antigen, *HBsAg* hepatitis B surface antigen, *HBV* hepatitis B virus, *HBVr* hepatitis B reactivation

Covalently Closed Circular HBV DNA and Viral Reactivation

As mentioned above, HBVr induced by ISDT occurs in patients with resolved as well as active infection and reactivation is precipitated by disruption of the balance between the host's immune control over viral replication and the inherent fitness of HBV to replicate. However, there is a key difference in these two clinical states in that very stringent immunosuppression is usually needed when patients with resolved infection undergo HBVr whereas this is not the case with HBsAg-positive individuals. This discrepancy is better understood by an awareness of the biological gradient of covalently closed circular DNA (cccDNA) that exists in hepatic tissue during the various phases of infection. Covalently closed HBV DNA is a highly stable molecule that acts as the genomic template for viral transcription and a concentration gradient that encompasses three orders of magnitude has been detected in hepatic tissue [30, 31]. The highest concentrations of cccDNA have

been demonstrated in patients with active chronic hepatitis B and high serum levels of HBV DNA, followed in descending order by the inactive HBsAg carrier state, and those with resolved infection [30, 31]. As patients with resolved infection have only minute amounts of cccDNA such individuals would be anticipated to undergo HBVr only under conditions in which highly aggressive ISDT is given. By contrast, inactive HBsAg carriers and those with chronic hepatitis B with high levels of serum HBV DNA have progressively higher amounts of cccDNA and are in a more ready state for uncontrolled viral replication with moderate and minimal immunosuppression, respectively. Much of this is inferential, but the hypothetical importance of different levels of cccDNA in determining the risk for HBV under varying conditions of immunosuppression is consistent with many of the clinical observations that have been made in this area.

HBV Reactivation During Cancer Chemotherapy

HBV reactivation occurs most commonly during chemotherapy for leukemia or lymphoma. Rates of 50 % or greater have been routinely reported in patients not given antiviral prophylaxis [11]. However, HBVr has been observed in a wide array of solid organ malignancies including breast, colon, lung, stromal tumors of the gastrointestinal tract, head and neck cancer, retinoblastoma, sarcoma, and teratoma. Among solid organ malignancies, HBVr occurs most commonly with breast cancer where rates of 20–40 % have been reported. The frequent occurrence of HBVr has been attributed to the use of anthracycline (doxorubicin or epirubicin)-based regimens [32]. Anthracyclines are also used for ovarian, uterine, and lung cancer, and are frequently incorporated into the treatment of hepatocellular carcinoma with transarterial chemoembolization (TACE). Reactivated hepatitis B may occur at any time during chemotherapy and may even occur several months after discontinuation of treatment. While the timing of HBVr has not been linked to any particular set of features, this most likely is determined by a number of interactive variables including the intensity of immunosuppression and the baseline virologic and serologic status of the host.

The inordinate frequency of HBVr during chemotherapy for hematologic and solid organ malignancies tends to parallel the degree of potency of the immunosuppressive regimen and in particular the incorporation of drugs like rituximab and high-dose glucocorticoids [33, 34]. It is unclear whether it also reflects a greater degree of baseline immunodeficiency in these populations due to the lymphotrophic properties of HBV. The extensive immunologic conditioning in conventional bone marrow or hematopoietic stem cell transplant recipients has been associated with reactivation in as many as 50 % or more of HBsAg-positive persons and in 10–20 % of HBsAg negative, anti-HBc positive patients further affirming that an important determining relationship exists between HBVr and the degree of immune suppression provided by drug therapy [35, 36].

Clinically Useful Predictors of Reactivation

A number of factors including age, gender, level of viral replication, and type of malignancy have been found to be associated with HBVr in patients undergoing cancer chemotherapy. Experiences in patients with lymphoma have described male gender to be a risk factor. The reason for this is unclear but it is not thought to be explained by the fact that males are more commonly infected. In a comprehensive analysis of risk factors for HBVr in 138 HBsAg positive cancer patients, multivariate analysis revealed detection of pre-chemotherapy HBV DNA, the use of anthracyclines or glucocorticoids, and a diagnosis of breast cancer or lymphoma as predictors of HBVr [37]. Of these, perhaps the most useful is the pre-therapy level of serum HBV DNA using a sensitive PCR assay such as TaqMan. It has been reported that HBVr occurs more frequently in patients with HBV DNA levels above 2,000 IU/mL when sensitive PCR assays have been used [37–39], whereas other studies using less sensitive methods of detection such as branched DNA hybridization have found an association of HBVr with detectable versus non detectable HBV DNA [37].

The HBeAg status of the patient has been found to be predictive of a higher risk of HBVr in some but not all studies. In general, the presence of HBeAg can be considered to be a surrogate for high serum HBV DNA because HBeAg reactivity is almost always associated with high-level serum HBV DNA (>20,000 IU/mL) during active chronic hepatitis B as well as in the immune tolerant stage of infection where levels typically exceed 1 x 10^6 IU/mL [40].

There has been considerable debate about the possible protective role of concomitant antibody to HBsAg (anti-HBs) in patients who are HBsAg negative and anti-HBc positive. Several reports suggest that the presence or absence of anti-HBs in patients with resolved infection may play an important role in determining risk of HBVr. In cohorts of bone marrow hematopoietic stem cell transplant recipients and patients with non-Hodgkin's lymphoma, the presence of anti-HBs in the setting of resolved infection has been associated with a decreased risk of HBVr [33, 35, 41–43]. In one study of 29 patients with B cell lymphoma and resolved HBV with positive anti-HBs at baseline, anti-HBs concentration was measured before and after rituximab therapy [43]. A significant decline in anti-HBs concentration was noted throughout the cohort, including a subgroup of 19 patients with low concentration (<100 IU/mL) at the onset of rituximab therapy in which eight patients lost anti-HBs entirely. However, in a recent systematic review the detection of anti-HBs showed only a weak trend when cancer chemotherapy was given for a broad range of malignancies. These seemingly discordant observations may be potentially explained by the absence of information about anti-HBs concentration in observational studies. Due to the poor quality of the data, the American Gastroenterological Association (AGA) institute management guidelines recently suggested against using anti-HBs status to guide antiviral prophylaxis in HBsAg negative patients until more is known [11]. It is possible that high level humoral immunity is more likely to be protective when ISDT is given for a relatively short period of time and

Table 18.2 Gradient of risk of hepatitis B reactivation based on serological status and degree of immunosuppression

Risk Level	Serological Risk Status	Immunosuppressive Agent Risk Status	*If* Serological Risk →	Anticipated HBVr Frequency
High	HBsAg positive Detectable serum HBV DNA or HBeAg positive	Systemic chemotherapy Anthracycline derivatives B cell T cell depleting agents* High dose glucocorticoids**	High	>20%
			Intermediate	>10%
			Low risk	>5%
Intermediate	HBsAg negative, anti-HBc positive, anti-HBs negative	TNF alpha inhibitors*** Calcineurin inhibitors Tyrosine kinase inhibitors and other cytokine inhibitors**** Moderate dose glucocorticoids (>4 weeks) Transarterial chemoembolization	High	>10%
			Intermediate	1-5%
			Low	0-2%
Low	HBsAg negative, anti-HBc positive, anti-HBs positive	Low dose glucocorticoids (<4 weeks) Methotrexate Azathioprine 6-MP	High	1-5%
			Intermediate	<1%
			Low risk	<<1%

* B cell depleting agents include rituximab, ofatumumab, and ibritumomab tiuxetan; T cell depleting agents include alemtuzumab, which depletes both B and T cells.
** High dose considered >20mg daily.. Moderate dose is 10-20 mg. Low dose is < 10 mg.
*** More data are needed (particularly individuals treated with TNF alpha inhibitor monotherapy) because of a low degree of confidence in the risk of HBVr derived from observational studies.
**** Although data are limited in tyrosine kinase inhibitors such as imatinib and nilotinib as well as other cytokine inhibitors such as abatacept, ustekinumab, and integrin receptor inhibitors, these likely represent an intermediate or possibly high risk of HBVr.
Abbreviations: 6-MP, 6-mercaptopurine; anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

is not primarily operative solely through its effects on B cell function. This is an area needing more study(Table 18.2, Fig. 18.5).

It is difficult to provide an absolute risk calculation for HBVr in an individual who is about to undergo cancer chemotherapy even when major predictive factors such as HBV DNA level and potency of the immunosuppressive drug or regimen are known because a number of covariates with the potential to effect incidence rates may not be recognized when ISDT is begun. Examples of this include the possible need for maintenance therapy, the use of novel anticancer drugs with limited post-marketing experience, and the potential effects of comorbid illnesses (for example, serious infection or poorly controlled diabetes) which may further decrease the overall integrity of the immune system.

B Cell Depleting Agents

Rituximab and a similar drug ofatumumab are B cell depleting agents which are associated with a high risk of HBVr, particularly when used in conjunction with other chemotherapeutic agents for B cell lymphoma and chronic lymphocytic leukemia [23]. Both drugs target the B cell surface antigen CD20 and inhibit B cell

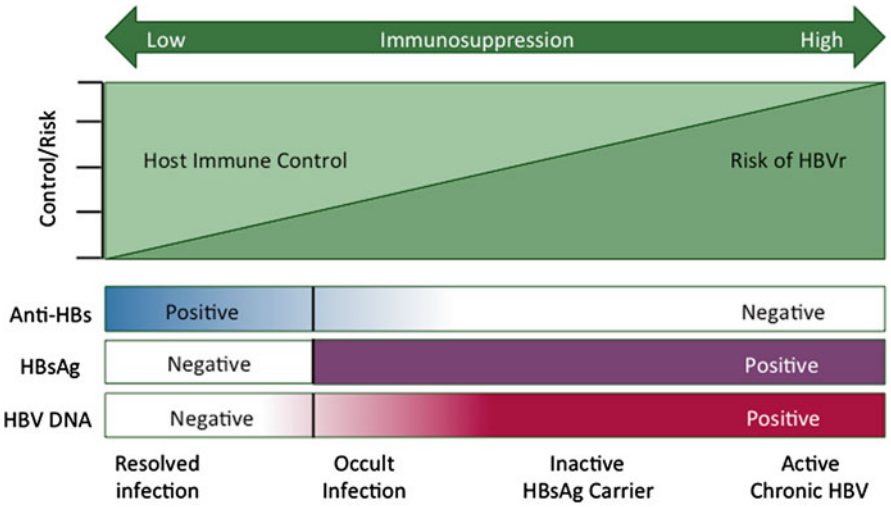

Fig. 18.5 Spectrum of hepatitis B reactivation risk based on potency of immunosuppressive drug therapy and degree of host immune control. The risk of HBVr occurs in a broad continuum based on the background of the host immune system's ability to control viral replication. This is reflected by the individual's serological status and/or the presence and level of HBV DNA. The HBVr risk increases in relation to the immunologic potency of the administered immunosuppressive drugs as indicated in the *top horizontal bar*. In this graph, it is assumed that all anti-HBs patients are anti-HBc positive. *Anti-HBs* antibody to hepatitis B surface antigen, *HBsAg* hepatitis B surface antigen, *HBV DNA* hepatitis B virus deoxyribonucleic acid, *HBVr* hepatitis B reactivation

activation and these effects remain ongoing for several months after their discontinuation. Similarly purposed agents include ibritumomab tiuxetan, which constitutes radioimmunotherapy targeted against CD-20, and alemtuzumab, which targets CD52 present on B and T cells. The latter has been associated with cases of HBVr.

Rituximab was licensed for use in the USA in 1997 and was approved in Europe as maintenance therapy for follicular lymphoma in 2012. It is currently widely used in these regions and Asia. Ofatumumab has been more recently licensed for use in the USA (2014) and has been approved in Europe and Canada (2010 and 2012, respectively). Due to lead-time differences, a recent review of the US Food and Drug Administration (FDA) Adverse Event Reporting System identified 109 cases of HBVr caused by rituximab and three cases caused by ofatumumab. More information on HBVr risk with ofatumumab is likely to be forthcoming but it can be anticipated to have the same risk as rituximab due to the similarity in mechanism of action.

Rituximab has been associated with a particularly high risk of HBVr in patients treated for non-Hodgkin's lymphoma in combination with a traditional CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone). The reported frequency of HBVr associated with regimens involving rituximab plus CHOP (R-CHOP) has been shown to vary considerably between studies in HBsAg positive and HBsAg negative cases [33, 41, 44–52]. Rates of HBVr in HBsAg-positive patients have varied between 16 and 70 % [53].

Variability in HBVr frequency seems to be particularly true in HBsAg negative, anti-HBc positive patients. In a recent large multicenter retrospective study from Korea, China, Taiwan, Singapore, and Malaysia, investigators identified HBVr in 28 % of HBsAg-positive non-Hodgkin's lymphoma patients treated with rituximab [44]. In the same study, reactivated hepatitis B occurred in 10 % of similarly treated patients who were HBsAg negative and anti-HBc positive. The range with which reactivation has been reported in anti-HBc positive patients has varied from 2 to 41 % in other studies [25, 29, 33, 42, 44, 54, 55]. In a recent meta-analysis, a relative risk of 5.5 was found in patients who were HBsAg negative and anti-HBc positive when rituximab-containing therapy was compared with traditional CHOP [53]. Even given the wide range of reported frequencies of HBVr, the high rate of reactivation in HBsAg negative anti-HBc positive patients remains a particular concern in highly endemic regions for HBV such as Asia because a large percentage of the general population (30–50 %) have resolved infection and this has important implications for HBV DNA monitoring and consideration for antiviral prophylaxis.

Predictive factors for HBVr in anti-HBc positive patients receiving rituximab-containing chemotherapy have not been studied extensively. In a large retrospective study involving 190 patients with resolved hepatitis B and diffuse large B-cell lymphoma, HBVr was observed in 14 %. Risk factors associated with HBVr in this cohort included an increased number of rituximab cycles (>8) and decreased pre-treatment lymphocyte–monocyte ratio as a marker of lower baseline host immunity [52]. The potential impact of occult viremia was not studied but has generally been considered to be important.

One of the hallmarks of HBVr occurring with B cell depletion is HBsAg sero-reversion during treatment. HBsAg sero-reversion is a clinically important event because it has been associated with frequent hepatitis and severe liver injury. With the exception of extensive immunologic conditioning associated with bone marrow hematopoietic stem cell transplantation, sero-reversion to HBsAg-positive rarely occurs with immunosuppressive therapy other than B cell depleting agents. In one study, sero-reversion of HBsAg occurred in 40 % of patients who developed HBVr [50]. In a prospective study including 150 patients with resolved infection who underwent R-CHOP treatment of non-Hodgkin's lymphoma, 27 developed reactivation of which 16 (59 %) developed hepatitis, 9 (33 %) underwent HBsAg sero-reversion and 5 (19 %) HBeAg sero-reversion [54].

Another interesting feature of rituximab induced HBVr is that it may occur several months after discontinuation of the agent. This most likely can be explained by its long lasting effects on B cell activation. In a review of 183 cases reported in the literature it was shown that HBVr appeared on average 3 months after treatment discontinuation with a range of 0–12 months [56]. Rarely have longer post-withdrawal intervals been linked to HBVr. This signifies a need for long-term antiviral prophylaxis as discussed in the section on management of HBVr.

In addition to hematologic malignancies, B cell depletion therapy has recently found extensive use in patients with severe rheumatoid arthritis, idiopathic thrombocytopenic purpura, and cryoglobulinemia. Use for nonmalignant conditions will be discussed further below.

Transarterial Chemoembolization for Hepatocellular Carcinoma

A number of studies over the past 10 years have attempted to characterize the risk of HBVr from local ablation, surgical resection, or systemic chemotherapy of hepatocellular carcinoma in HBsAg positive and HBsAg negative, anti-HBc positive patients. Reactivated hepatitis B has been well documented in patients treated with TACE. There are data to suggest that arteriovenous shunting can occur within malignant tissue or at the time of the procedure itself and this can lead to systemic exposure to chemotherapeutic agents [57].

A recent review described more than 540 HBsAg carriers to be enrolled in studies in which TACE was the only modality of treatment [11]. Rates of HBVr as high as 30–40 % were reported. Multivariate analysis in one study revealed that baseline HBV DNA level in excess of 2,000 IU/mL independently predicted HBVr [58]. In another study, 119 patients were treated with TACE using either adriamycin or the more immunologically suppressive combination of epirubicin and cisplatin. These patients were compared with those who received other forms of local ablation including TACE combined with radiotherapy. High-level viremia and increased treatment intensity were the major risk factors for HBVr. When compared with local ablation as the reference population, the adjusted hazard ratio for TACE with adriamycin was 2.5; for TACE with epirubicin and cisplatin, 4.2; and for TACE with the two drug regimen and radiotherapy, 10.2 [59].

Immunosuppressive Therapy for Nonmalignant Disorders

The risk of HBVr during cancer chemotherapy has been recognized for several decades but the past 10–15 years has witnessed increasing attention to the risk that exists when ISDT is used for nonmalignant disorders. Table 18.3 lists some of the nonmalignant disorders commonly treated with ISDT that have been associated with HBVr. The most common settings have been with the treatment of rheumatologic, dermatologic, and inflammatory bowel diseases. The range of agents used for these disorders includes antimetabolites, glucocorticoids, biologic agents, monoclonal antibodies, and calcineurin inhibitors. The most commonly used biologic agents are disease-modifying antirheumatic drugs (DMARDs), which as a broad category, includes azathioprine, methotrexate, cyclosporine, and a range of biologics such as TNF alpha inhibitors. Newer agents which block costimulation of lymphocytes, tyrosine kinase inhibitors, and integrin inhibitors have been developed for a variety of indications but have not had sufficient use to allow determination of the magnitude of risk for HBVr. B cell depleting agents such as rituximab or ofatumumab have potent and long lived effects on B cell function and have come under close scrutiny recently. The US Food and Drug Administration has recently mandated a box warning on these agents and strongly recommends HBV screening for HBsAg and anti-HBc and antiviral therapy when appropriate due to numerous reports of severe HBVr when used for rheumatic as well as malignant indications.

Table 18.3 Nonmalignant diseases commonly treated with immunosuppressive drug therapy

Rheumatoid arthritis
Plaque psoriasis
Psoriatic arthritis
Juvenile idiopathic arthritis
Ankylosing spondylitis
Crohn's disease
Ulcerative colitis
Granulomatosis with polyangiitis (Wegener's granulomatosis)
Microscopic polyangiitis
Eosinophilic granulomatosis with polyangiitis (Churg–Strauss syndrome)
Essential mixed cryoglobulinemia
Systemic mastocytosis
Myelodysplastic and myeloproliferative diseases
Multiple sclerosis
Solid organ transplantation
Severe asthma[a]
Nephrotic syndrome

HBVr hepatitis B reactivation

[a]The risk of HBVr has been shown to be largely restricted to persons treated with moderate to high doses (20 mg or more of prednisone or equivalent) given for more than 3 months [107]

The determination of the magnitude of risk for HBVr with any biologic agent is generally difficult until there is broad clinical experience. However, there are several circumstances other than limited clinical experience that may confound estimate of risk. Patients who are started on newer biologic therapies for conditions such as ankylosing spondylosis, plaque psoriasis, and ulcerative colitis have often failed previous therapy with other disease modifying agents, suggesting that there may be a lengthier period of immunologic suppression prior to the initiation of the new agent. This may explain why in one large case series of TNF alpha inhibitor therapy, HBVr occurred significantly more commonly in patients given prior immunosuppressive therapy when compared to individuals who lacked prior exposure (96 % vs. 70 %, respectively, $p < 0.03$) [19]. It is also clear from observational studies that many patients continue with more than one immunosuppressive agent for control of refractory inflammatory disorders, thus making it difficult to assign direct causality to any one particular agent. In one review of the literature, two thirds of patients who developed HBVr while taking TNF alpha inhibitors were reported to be taking other immunosuppressive agents such as glucocorticoids, methotrexate, or calcineurin inhibitors. These considerations may explain why there are often wide estimates of the magnitude of risk with TNF alpha inhibitors, a biologic drug class in which HBVr has been reported to occur in 0–40 % of HBsAg positive patients [11, 19, 60, 61].

Antimetabolites

The antimetabolites include azathioprine, 6-mercaptopurine (6-MP), and methotrexate. These agents are frequently used for chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease. Overall, these agents are considered to be at low risk for precipitating HBVr when used alone. Azathioprine monotherapy in doses used for autoimmune diseases has only rarely been associated with HBVr. There are several cases of methotrexate induced HBVr but most of the reported cases have been on other adjunctive immunosuppression, including glucocorticoids [11, 62]. Flares of hepatitis have been reported upon withdrawal of methotrexate similar to what has been reported with glucocorticoids. However, given the large number of patient exposures to methotrexate over the past 30–40 years, this drug can be considered to be low risk for inducing HBVr (Table 18.2).

Glucocorticoids

Glucocorticoids are far more commonly associated with HBVr than other traditional immunosuppressive agents. These agents have a direct suppressive effect on T cell-mediated immunity and in addition, they stimulate a glucocorticoid responsive element in the HBV genome which results in increased viral transcription [63]. It has been known for decades that short term exposure to moderate to high doses of glucocorticoids enhances viral replication and often lowers serum aminotransferase levels whereas abrupt withdrawal results in an immunologic rebound that is typified by elevation of serum aminotransferases and a decline in serum HBV DNA. Knowledge that this occurred in 30–40 % of HBeAg positive patients has been used in the past in conjunction with interferon or lamivudine rescue as a therapeutic strategy for chronic hepatitis B [64, 65]. Other investigations that predated the development of nucleoside analogues showed that patients treated with several months of glucocorticoid therapy often had long-lasting flares of disease [66].

Glucocorticoids have been used inconjunction with cancer chemotherapy, particularly in the treatment of lymphoma where they have been shown to increase the risk of HBVr. [37] In a prospective randomized trial involving 50 patients with non Hodgkin's lymphoma, those treated with prednisolone, epirubicin, cyclophosphamide, and etoposide had a significantly higher cumulative incidence of HBVr (78 % vs. 38 %) when compared to patients who received a corticosteroid sparing regimen and more frequently had severe hepatitis, including increased ALT levels and development of jaundice [34].

The dose and possibly the duration of glucocorticoid therapy have a significant effect on risk of HBVr. Glucocorticoid doses of 30–60 mg of prednisone or prednisolone daily with tapering doses over 1–3 months duration have been associated

with a high risk of precipitating episodes of HBVr whereas low-dose (<10 mg) maintenance glucocorticoids are not [11]. The doses used in the management of patients with non-Hodgkin's lymphoma are conventionally 60 mg/m^2 on day 1–7 of each cycle, thus accounting for the increased proportion of patients having reactivation when corticosteroids are used as part of the regimen.

Biologics

Biologic agents, particularly TNF alpha inhibitors, have been associated with an intermediate risk of HBVr. As TNF alpha is an important pro-inflammatory cytokine that reduces HBV replication, its inhibition may significantly disrupt immune control of HBV. The TNF alpha inhibitors are useful in the management of a number of autoimmune disorders including rheumatoid arthritis, plaque psoriasis, and inflammatory bowel disease. All of the available agents including infliximab, eternacept, and adalimumab have been linked with HBVr.

A comprehensive review of HBVr attributed to TNF alpha inhibitor therapy demonstrated an overall frequency of 39 % in HBsAg carriers and 5 % in HBsAg negative, anti-HBc positive patients [19]. The great majority of these patients were taking DMARDs such as methotrexate or additional immunosuppressive agents such as glucocorticoids that affect other immunologic effector systems against HBV. Recent data specific to rheumatic diseases noted a lower incidence of HBVr (12 %) in HBsAg carriers treated with TNF alpha inhibitors and DMARDs, while HBVr was reported to be approximately 2 % in HBsAg-negative individuals who were positive for anti-HBc [67, 68]. The frequency of HBVr with TNF inhibitor therapy has been reported to be even lower than 2 % in patients with past infection. For example, a recent study of 146 rheumatic patients with resolved hepatitis B from Italy found no cases of HBsAg sero-reversion, changes in anti-HBs status, or elevated HBV DNA during a mean period of 56 months [61]. In general, HBVr has been reported less frequently when TNF alpha inhibitors are used to treat patients with plaque psoriasis, and this may be due to the greater tendency to use them alone or in combination with low-dose methotrexate only [69].

Other biologics such as tyrosine kinase inhibitors and monoclonal antibodies directed against various immune targets have also been reported to precipitate HBVr, mostly in the form of case reports or small case series. These agents inhibit distinct immune pathways and are used in settings that include treatment of hematologic malignancies, solid tumors, as well as inflammatory diseases. Agents reported in cases of HBVr have included the tyrosine kinase inhibitors imatinib and nilotinib, which have an inhibitory effect on T cell activation and proliferation. Of note, hepatitis flares have been described to occur after the patients achieved a complete response to the use of tyrosine kinase inhibitor therapy for leukemia, suggesting that the flare was due to restoration of the immune response [70]. Reactivated hepatitis B has been reported with abatacept, a T cell costimulation modulator used in rheumatoid arthritis that inhibits CD80 and CD86 signaling; and ustekinumab, an IL-12/

IL-23 inhibitor used in the treatment of psoriasis. Rituximab is seldom used for dermatological disorders but has been used for treatment of severe, refractory rheumatoid arthritis and anklyosing spondylitis. There are only rare reports of HBVr occurring in patients treated with rituximab for non malignant indications but more reports are likely to follow [71]. Additional biologics which are not specifically reported to be associated with HBVr at the time of this writing include the integrin receptor inhibitors natalizumab and vedolizumab, which are used in the treatment of multiple sclerosis and inflammatory bowel disease.

Calcineurin Inhibitors

The calcineurin inhibitors cyclosporine and tacrolimus are commonly used in the transplant setting and have also found clinical use in the management of inflammatory bowel disease [72]. These agents inhibit T cell activation and transcription of IL-2. In the setting of organ transplantation from HBsAg negative, anti-HBc positive donors, there is a well recognized risk of HBV transmission from donor to recipient followed by HBVr within the recipient. The risk of HBV transmission appears to vary by organ type, with the greatest risk in liver transplant recipients [73]. Consequently, antiviral prophylaxis is recommended for recipients of anti-HB positive liver transplants, particularly if recipients are both anti-HBs and anti-HBc negative, in which case patients are usually treated with indefinite antiviral prophylaxis [74].

Prevention and Management

Screening for Hepatitis B Virus

Early recognition of the HBV status of persons about to undergo ISDT is essential to the prevention of HBVr because it provides the information needed to judge whether antiviral prophylaxis is needed. Accordingly, guidelines that advocate routine screening for all individuals exposed to ISDT have been published by the Centers for Disease Control and Prevention (CDC), the US Preventive Services Task Force, the AASLD, the Asian Pacific Association for the Study of the Liver (APASL), and the European Association for the Study of the Liver (EASL) (Table 18.4) [75–80]. More recently, the AGA Institute guidelines have been seen to differ slightly in not recommending routine screening for individuals who will receive low risk immunosuppressive drugs such as azathioprine and methotrexate (Table 18.4). Instead, a recommendation is made that all such individuals should be screened if they are likely to have a 2 % or greater prevalence of infection such as persons born in intermediate or highly endemic regions of the world or persons with a past history of injecting drug use [79].

Table 18.4 Current guidelines in the prevention of hepatitis B reactivation associated with chemotherapy or immunosuppression

	Recommended screening test			Antiviral prophylaxis during chemotherapy or immunosuppression		
	HBsAg	Anti-HBc	HBV DNA	Candidates	Timing of initiation	Duration after completion
U. S. Centers for Disease Control (2008) [75]	✓	✓	–	HBsAg +	–	–
American Association for the Study of Liver Diseases (2009) [76]	✓ if high risk	✓ if high risk	–	HBsAg+	At onset	6 months if HBV DNA <2,000 IU/mL vs. >6 months if HBV DNA >2,000 IU/mL
Asian Pacific Association for the Study of the Liver (2012) [77]	✓	✓ if biologic agent	–	HBsAg+	Prior to onset	6 months
				Anti-HBc+ and biologic agent	Defer, monitor HBV DNA	
European Association for the Study of the Liver (2012) [78]	✓	✓	✓ if HBsAg– or anti-HBc+	HBsAg+ or HBV DNA+	Prior to onset	12 months
				Anti-HBc+	If high risk agent; otherwise defer, monitor HBV DNA	
American Gastroenterological Association Institute (2015) [79]	✓ if moderate–high risk	✓ if moderate–high risk	✓ if HBsAg+ or anti-HBc+	anti-HBc+	Prior to onset if high/intermediate risk agent	6 months vs. 12 months if B cell depleting agent

Anti-HBc antibody to hepatitis B core antigen, *HBsAg* hepatitis B surface antigen, *HBV* hepatitis B virus

However, despite these relatively uniform guidelines advocating screening, this occurs relatively infrequently among oncologists, rheumatologists, dermatologists, and other prescribers of these drugs. The factors involved in the lack of routine screening among oncologists and other specialist prescribers of ISDT differ and are discussed separately.

Screening by Oncologists

Two large retrospective reviews of screening practices in US cancer centers involving over 8000 and 10,000 patients, respectively, determined that only up to 17 % of patients underwent HBV screening before cancer chemotherapy [81, 82]. Similarly, data reported from surveys of oncologists in the United States, Canada, and Australia noted universal screening practices in only 13–22 % of respondents [83–86].

However, screening practices may vary globally by geography and provider groups. For example, a recent Italian survey among oncologists who specifically treat hematologic malignancies reported universal screening in over 90 % of respondents [87]. Likewise, a survey conducted in Turkey reported that universal screening was routinely performed by 59 % of respondents [88]. Variations in screening rates may reflect regional differences in HBV prevalence, local practice patterns, a particular concern for patients with hematologic versus solid organ malignancies, and recommendations set forth by regional or national professional societies [89].

In the latest iteration of the provisional guidelines of the American Society of Clinical Oncology (ASCO), HBV screening (HBsAg and anti-HBc) was recommended for patients with risk factors for HBV infection or those about to undergo highly aggressively chemotherapy such as R-CHOP or in the setting of bone marrow hematopoietic stem cell transplantation [90]. ASCO has expressed concerns about cost-effectiveness and potential harms of HBV screening and thus have not endorsed a similar approach in patients receiving other forms of chemotherapy. In a recently published systematic analysis of HBVr following initiation of cancer chemotherapy, there was little uncertainty that anthracycline derivative regimens cause HBVr in at least 10 % of cases and should be considered as high risk [11].

Screening Practices in Other Medical Specialties

In general, there has been a higher rate of adoption of HBV screening among other medical specialties such as rheumatology, dermatology and gastroenterology; however, physician awareness of the risk for HBVr with biologic agents remains less than optimal. In a national survey of American rheumatologists, 69 % reported routine screening for HBV before treatment with biologic agents, including TNF alpha inhibitor therapy and monoclonal antibody therapy. Depending on the agent, 19–53 % admitted to being aware of the manufacturer's warning for HBV reactivation

within drug package inserts [22]. A national survey of dermatologists indicated that 52 % were aware that HBVr may result from TNF alpha inhibitor therapy, and 81 % of those who were aware routinely screened for HBV versus 3 % of those who were not aware [91]. Part of the educational gap may extend from past ambiguous or weakly worded practice guidelines of their specialty organizations. For example, the American College of Rheumatology practice guidelines for rheumatoid arthritis issued in 2012 warned against the use of biologic agents in patients with untreated hepatitis B but there is no recommendation for HBV screening [92]. Similarly, the American Academy of Dermatology guidelines issued in 2008 stated that HBVr can occur with TNF alpha inhibitor therapy and advised that patients should be screened in the appropriate setting [93]. Concern about HBVr has been growing, however, as shown by a 2014 publication by the National Psoriasis Foundation in which it is recommended that all patients who are candidates for TNF alpha inhibitors, ustekinumab, cyclosporine, or methotrexate be screened for HBV [94]. In none of the existing guidelines within these subspecialties are recommendations made about referral to specialists for consideration of antiviral prophylaxis.

Universal Versus Targeted Screening

Strategies for HBV screening in patients undergoing ISDT may include a universal approach, in which all patients are screened, or a targeted approach, in which only patients considered to be at increased risk undergo screening. Decisions to pursue either approach may be influenced by factors including the type of disease requiring treatment, the degree of anticipated immunosuppression, and the duration of therapy. Most groups have recommended a universal approach to screening individuals with a moderate to high risk of HBVr based on the potency of the immunosuppressive agent or regimen (Table 18.4) [75, 77–79]. Targeted screening may be an alternative for individuals at low risk. A disadvantage to a targeted approach is the need to define which patients should be selected for screening. Patients often do not recognize or report risk factors, and one study of pregnant women in the United States found that fewer than 60 % of those who tested positive for HBsAg provided adequate information about risk [95]. An additional general concern about the utility of knowing risk factors beforehand comes from a study in a large cancer center which demonstrated that even when known risk factors for HBV infection are identified, fewer than 20 % of patients may ultimately be screened [81].

Universal screening for HBV has been reported to be a cost-effective practice in patients with hematologic malignancies but has not been shown for solid organ malignancies [96, 97]. In one decision analysis study involving patients with diffuse large B-cell lymphoma undergoing treatment with R-CHOP, the investigators found that a strategy in which all patients are screened for HBsAg prior to chemotherapy was associated with the least overall cost compared with strategies of screening only those considered to be at high risk or no screening [96]. In addition, it was estimated that screening all patients could reduce the rate of HBVr by approximately tenfold when compared with no screening.

Management Strategies and Antiviral Therapy

Once a person is identified to be at risk of HBVr based on serological status and planned immunosuppression, different management strategies have been considered. Strategies include initiation of prophylaxis in which all patients receive antiviral therapy with a nucleoside analogue before starting ISDT; preemptive therapy, in which antiviral therapy is initiated at an early time point when HBVr is apparent in the form of HBsAg sero-reversion, de novo detectable HBV DNA, or a rise in HBV DNA levels prior to onset of an overt hepatitis flare; and deferred therapy, in which therapy is initiated at the time of an overt hepatitis flare.

Initiation of prophylactic therapy has been proposed for all HBsAg-positive individuals. However prophylactic therapy is also recommended in some patients who are HBsAg negative and anti-HBc positive depending on the immunosuppressive medication or regimen used (Tables 18.2 and 18.4, see comments on rituximab above). A key factor in the approach to management is ensuring that patients found to be at high risk are initiated on antiviral therapy (Fig. 18.6). Retrospective data

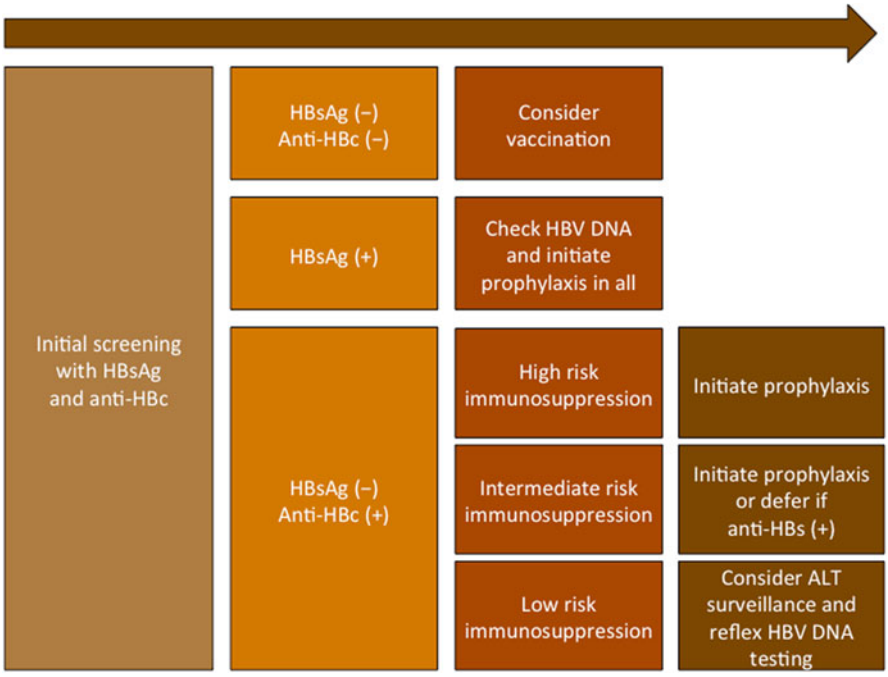

Fig. 18.6 Proposed algorithm of screening and management of hepatitis B reactivation. Following initial screening for HBV, subsequent management varies based on initial HBV serologies and degree of anticipated immunosuppression. The presence of anti-HBs in HBsAg negative and anti-HBc positive individuals may be associated with a decreased risk of HBVr, although this requires further study. All classes of ISDT are anticipated to result in diminution of anti-HBs titer with the most marked decline during B cell depleting therapy. *ALT* alanine aminotransferase, *anti-HBc* antibody to hepatitis B core antigen, *HBsAg* hepatitis B surface antigen, *anti-HBs* antibody to hepatitis B surface antigen, *HBV* hepatitis B virus

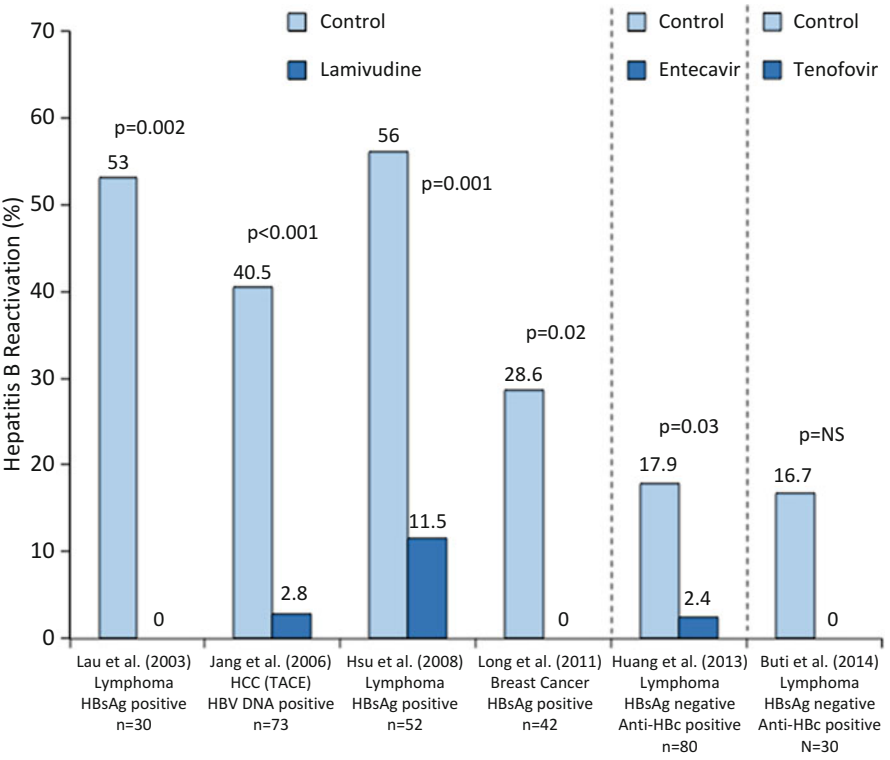

Fig. 18.7 Prospective randomized controlled trials evaluating antiviral prophylaxis vs. deferred therapy for hepatitis B reactivation. Prospective randomized controlled trials are shown involving a range of malignancies, all of which compared prophylaxis with lamivudine, entecavir, or tenofovir vs. a deferred approach (controls) [27, 29, 55, 58, 98, 99]. In all studies, the incidence of HBVr was significantly greater in controls with deferred therapy than in arms receiving prophylaxis. *HBsAg* hepatitis B surface antigen, *HBVr* hepatitis B reactivation, *HCC* hepatocellular carcinoma, *R-CHOP* rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, *TACE* transarterial chemoembolization

have revealed that in some cases, a large proportion of patients found to have HBV infection upon screening may ultimately not receive antiviral prophylaxis, leading to documented cases of HBVr [12, 82].

An alternative to prophylaxis is a strategy of preemptive or deferred therapy, in which decisions to initiate therapy are based on the appearance of increased viral activity or elevated aminotransferases, respectively, after initiation of immunosuppression or chemotherapy. However, these approaches can be associated with significant morbidity, as demonstrated in prospective studies in which delayed initiation of antiviral therapy did not entirely prevent severe hepatitis flares or liver failure [27, 54]. In contrast, several prospective randomized controlled trials and two systematic analyses, mostly involving the use of lamivudine and more recently entecavir or tenofovir, have demonstrated a significant decrease in HBVr associated with prophylactic therapy (Fig. 18.7). The randomized controlled trials encompass a range of malignan-

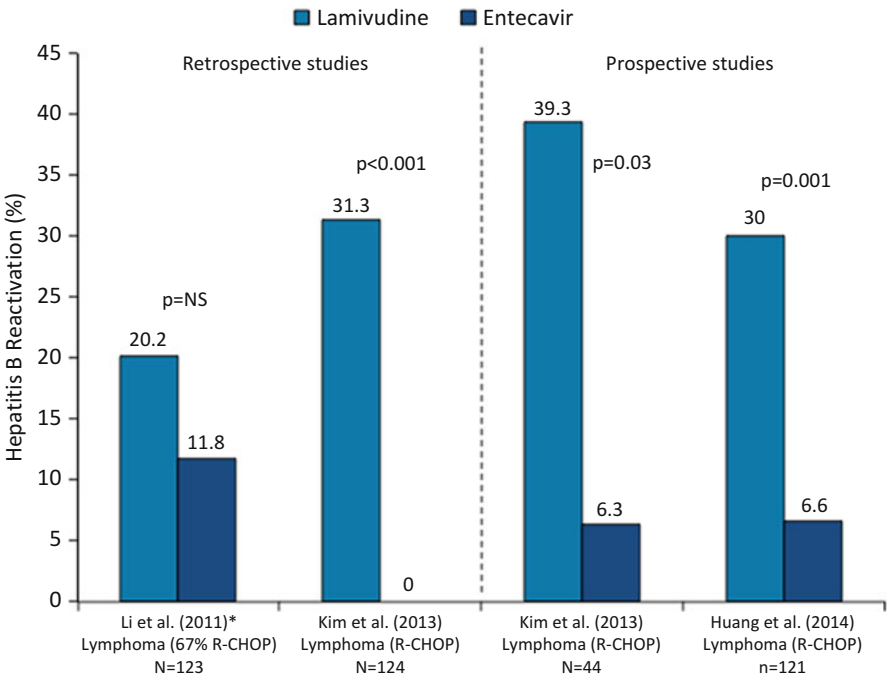

Fig. 18.8 Efficacy of lamivudine vs. entecavir as antiviral prophylaxis in hepatitis B carriers undergoing rituximab-based chemotherapy. Retrospective and prospective comparative studies are shown [44, 101, 102]. All patients were HBV carriers defined by positive HBsAg at baseline before initiating chemotherapy. (*Asterisk*) In retrospective study by Li et al., 67 % of patients received a rituximab-based chemotherapy regimen [101]. *HBsAg* hepatitis B surface antigen, *HBV* hepatitis B virus, *R-CHOP* rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone

cies, including breast cancer, HCC, and non-Hodgkin's lymphoma [27–29, 55, 58, 98, 99]. Altogether a strategy utilizing prophylaxis, in contrast with a deferred approach, has been associated with a greater than 80 % reduction in risk of HBVr, severity of hepatitis flares, and mortality [100].

Lamivudine has been the most widely studied antiviral used for prophylaxis; however, it may be considered inferior to newer agents in the prevention of HBVr due to their greater potency and higher barrier to resistance [101]. As a result, entecavir and tenofovir are often used as preferred antiviral agents. Several retrospective and prospective studies have now demonstrated a significant increase in efficacy of entecavir compared with lamivudine as prophylaxis in the setting of non-Hodgkin's lymphoma and R-CHOP therapy (Fig. 18.8) [44, 101, 102]. In these studies, entecavir was associated with a significantly decreased frequency of HBVr and HBV-related hepatitis. The low barrier to resistance associated with lamivudine may likely contribute to the observed decline in efficacy, as one study noted a high frequency of lamivudine resistance in lymphoma patients treated with rituximab containing chemotherapy [44]. In this prospective cross-sectional cohort, over one-half of HBVr

cases occurring in HBsAg carriers who received lamivudine prophylaxis were found to have lamivudine resistance, which occurred more frequently if treatment was given for greater than one year. In other studies, disruption of chemotherapy occurred much less frequently in entecavir arms (2–6 %) compared with lamivudine (18–20 %) [101, 102]. Tenofovir has now also been described as effective prophylaxis as well as in the treatment of established HBVr [55, 103].

Recommendations on duration of antiviral prophylaxis have ranged from 6 to 12 months after the completion of immunosuppressive therapy. Importantly, late onset HBVr has been a particular problem in the setting of rituximab-based therapy where this can occur up to a year following discontinuation of rituximab. A comprehensive review and meta-analysis of 183 cases of rituximab-associated HBVr noted that the majority of events appeared within the first 3 months after discontinuation of rituximab but 29 % occurred more than 6 months after discontinuation [56]. A recent publication describes an episode of HBVr accompanied by HBsAg sero-reversion and disappearance of anti-HBs 12 days after discontinuation of 23 months of rituximab [104]. Taken together, these studies indicate that antiviral therapy should be maintained for as long as the patient is taking rituximab, and it should not be stopped immediately after discontinuation even if HBVr has not complicated very prolonged therapy. Furthermore, antiviral therapy should be continued for at least 12 months after discontinuation of rituximab.

Other Considerations Related to Benefit of Antiviral Therapy

The eventual prognosis of patients who initially survive on demand antiviral therapy for HBVr has not been reported. However, in a prospective randomized controlled trial involving patients with spontaneous HBV reactivation unrelated to immunosuppression or chemotherapy, patients who received tenofovir benefited from a significant reduction in mortality at 3 months when compared to placebo [105]. As most of the patients with life threatening reactivation are likely to be continued on antiviral therapy for an indefinite period, it can be anticipated that long-term survival in regard to hepatitis B would be improved. In those with development of progressive liver failure, aggressive intensive care management may be required. Liver transplantation may be considered in selected patients whose malignancy has been controlled [106]. However, those who develop HBVr as a consequence of cancer chemotherapy are often limited by their cancer diagnosis in terms of transplant candidacy.

Summary and Future Perspective

As HBVr from ISDT is preventable, identifying patients at risk is of paramount importance. This allows identification of those will benefit from antiviral prophylaxis. Impediments to the adoption of HBV screening include poor physician

awareness of the risks for HBVr that extends from failure to screen for HBV. This knowledge gap occurs primarily among oncologists, rheumatologists, dermatologists, and even gastroenterologists but can affect any provider who prescribes these agents. Improved attempts at educating these specialists are urgently needed. Particular emphasis needs to be given to the fact that not only is HBVr a cause of significant morbidity and some mortality, but it also frequently leads to interruption of immunosuppressive drug treatments, which in the case of cancer chemotherapy, can impact patient survival. An expanded array of biologic agents that work quite effectively for chronic autoimmune disorders are in various phases of development and thus the problem of HBVr due to ISDT is likely to increase in the future unless there are systematic attempts at broader screening and more routine use of prophylactic antiviral therapy when appropriate. Many of these patients should be optimally referred to specialists who can make decisions about the appropriateness of antiviral therapy. Ideally, the language of any new management recommendations proposed by the practice guideline committees of the relevant medical organizations should state this last point clearly.

The choice of agent used for antiviral prophylaxis can be important when the patient has detectable HBV DNA in serum or when prolonged immunosuppressive therapy is planned. In such cases, nucleoside or nucleotide analogues with a high barrier to resistance such as entecavir or tenofovir are preferred. In many instances, however, lamivudine can offer successful prophylaxis without undue fear of drug resistance. An example of this would be when a patient is likely to be taking ISDT for a short interval (less than six months) and HBV DNA is not detectable prior to commencement of drug therapy.

There are many areas in which further research is needed. Some of the key areas with implications for a more universal system of diagnosis, evaluation, and management of HBVr are included below.

- *A standardized definition of reactivated hepatitis B during ISDT*. This would allow for valid cross-study comparisons and overcome inconsistencies in the way this condition is diagnosed. As reactivation is a virologic event, any new system should capture cases where serum aminotransferase elevations do not occur. These should be considered silent episodes rather than not included and will provide more reliable estimates of incidence.
- *A standardized grading systemfor the severity of reactivation*. Current grading systems rely heavily on ALT, bilirubin and INR to assess severity. Comorbidities contribute to the human toll that HBVr exerts and are not captured. In order to better assess the medical significance of HBVr, a newly derived system should optimally also link the degree of severity to outcomes such as need for hospitalization and frequency of interrupted therapy. These outcomes should be readily available to providers who care for these patients.
- *Consensus on screening*. There is need to more clearly define which populations should undergo universal screening and which should receive targeted screening. Factors which are important for policy decisions on screening need fully understood and consistent with a practical means of implementation, but two important ones are the risk associated with drug therapy (high, intermediate, low) and

the probability that the patient has chronic hepatitis B versus resolved infection. This has particular importance when resources are limited.

- *Better understanding of the risk for HBVr duringTNF alpha inhibitor therapy*. The current data are weak in quality yet the population at risk is potentially very large due to the common use of these agents. Risk calculations need to be made for cases in which monotherapy with a TNF alpha inhibitor is used and need to be distinguished from those in which TNF inhibition is utilized in combination with other immunosuppressive agents. This will lead to more accurate understanding of the level of risk for HBVr when these agents are used alone.
- *Consensus on how to evaluate patients with resolved infection*. There is a great need to know how to follow patients who are judged not to require antiviral prophylaxis.
  - Should involve HBsAg only or both HBsAg and HBV DNA to detect milder cases of reactivation?
  - What interval should be used for HBV DNA surveillance?
  - At what time during maintenance immune suppression therapy can monitoring be more relaxed to longer intervals? How long should HBV DNA surveillance continue during long-term or indefinite therapy?

  The authors recognize that measures enacted in clinical trials may not be easily applicable in clinical practice. Answers to the above questions would have important economic and resource management implications.
- *Further study of the intermediate and long-term outcomes of HBVr*. Clinical publications often focus on the acute manifestations of liver injury only. In the future, more attention needs to be given to capturing data on delayed outcomes such as the need for prolonged and costly hospitalization and the frequency of interruption of ISDT. The latter in turn has important implications for cancer progression when chemotherapy is withdrawn and lower quality of life when biologic agents are withdrawn from patients with nonmalignant disorders because of HBVr. This would provide important outcome data for specialists and provide further impetus for a change in specialty practice guidelines as well as increase the awareness of HBVr among prescribing practitioners.

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Chapter 19
Immunoprophylaxis of Hepatitis B Virus Infection and Its Sequelae

Mei-Hwei Chang

Introduction

Hepatitis B virus (HBV) infection is a major health problem in human. It can cause acute, fulminant, or chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). More than 780,000 people die every year due to the acute or chronic consequences of hepatitis B (http://wwww.who.int/mediacentre/factsheets/fs204/en/). It is estimated that 2 billion people had been infected by HBV, and *approximately 240 million people have chronic HBV infection worldwide* [1]. Liver cancer represents 6 % and 9 % of the global cancer incidence and mortality burden, respectively. With an estimated 746,000 deaths in 2012, liver cancer is the second most common cause of death from cancer worldwide [2]. Chronic HBV infection is a major risk factor for the development of HCC. The risk of HCC associated with seropositivity for HBsAg ranges from 5-fold to 98-fold with a population attributable risk of 8–94 % [3]. HCC is one of the five most common sites of cancer diagnosed. Unfortunately, the response to therapy for HCC is generally poor and the recurrence rate is high.

In spite of the progress of antiviral therapy in patients with chronic hepatitis B to suppress viral replication and to reduce liver inflammation and complication, the current result of viral and disease elimination is still very limited. To control hepatitis B virus infection and its sequelae, prevention is better than therapy. Immunoprophylaxis is the best method to prevent HBV infection. The development of HBV vaccine using HBsAg protein as the immunogen to induce the protective antibody (anti-HBs) against HBV infection shed light on the elimination of HBV infection and its sequelae. Through three decades' experience and cumulated data, we are confident that immunoprophylaxis is safe and successful to protect people from HBV infection and its related diseases.

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Transmission Routes of HBV: The Importance of Mother-to-Infant Transmission and Early Prevention

HBV infection is transmitted through either mother-to-infant route or horizontal route. An incubation period of 6 weeks to 6 months, mostly around 75 days, usually precedes the presentation of hepatitis B. In endemic areas such as Asia where mother-to-infant transmission is the major route of transmission, HBV infection develops mainly during infancy and early childhood. Even in endemic areas like Africa where horizontal transmission of HBV in early childhood was considered as the route of transmission previously, infants' HBsAg seropositive rates were reported to be 8.1 % among 0–6-months-olds, and 8.9 % among 6–12 months olds in one study [4], and 53.8 % among the live births of HBeAg positive mothers in another study [5]. The importance of early prevention against both mother-to-infant transmission and horizontal transmission of HBV is thus evident and stressed in Africa [6].

The age at which HBV infection occurs is an important factor affecting the outcome of HBV infection (Table 19.1). The earlier the infection occurs, the higher is the risk for chronicity. With no immunoprophylaxis, more than 90 % of infants who were infected by their HBeAg/HBsAg positive mothers will develop chronic HBV infection during follow-up [7] This may be explained by the very high virus amount transmitted to the neonate with physiologic immature immune system. The chronicity rate after HBV infection is decreased to approximately one quarter (23 %) in children infected at preschool age and in young adults to 2.7 % [8, 9]. Perinatal transmission decreased to <5 % if the mother was HBsAg positive but negative for HBeAg negative.

Intrauterine infection occurs rarely in <5 % of the infants to HBeAg and HBsAg positive mothers. In our study in Taiwan during 10 years, 2.4 % of the 665 infants of HBeAg and HBsAg positive mothers were seropositive for HBsAg at birth, suggesting intrauterine infection [10]. They remained HBsAg positive at 12 months of

Table 19.1 Maternal HBV sero-status and age at infection are important factors affecting the outcome of HBV infection in children born before versus after the HBV immunization program

	Without immunoprophylaxis	With immunoprophylaxis at infancy
Infant of HBsAg(+), HBeAg(+) mother	>95 % infected, ≥90 % chronicity rate[a] among infected infants [6]	Vaccine + HBIG → 10 % chronic infection [54]
Infant of HBsAg(+), HBeAg(−) mother	<5 % chronicity rate, with risk of FH or AH [11, 12]	0.29 % chronic infection if no HBIG at birth, 0.14 % if with HBIG, risk of FH or AH reduced [54]
Toddler	5.0 % infected, chronicity rate among infected 23 % [7]	–
Young adult	1.5 % infected, chronicity rate among infected 2.7 % [8]	–

FH fulminant hepatitis, *AH* acute hepatitis, *HBIG* hepatitis B immunoglobulin

[a]Chronicity Rate = rate of HBsAg(+) >6 months

age. Transplacental leakage of HBeAg-positive maternal blood, which is induced by uterine contractions during pregnancy and the disruption of placental barriers, is the most likely route to cause HBV intrauterine infection [11].

Acute or fulminant hepatitis B can occur in infancy. The incidence of fulminant hepatitis B is higher in infancy than in other age periods. Mother-to-infant transmission, mainly from HBeAg negative, HBsAg positive mothers, is the most important route of transmission for acute or fulminant hepatitis in infancy [12, 13].

Active and/or Passive HBV Immunization

HBV immunization can be classified into passive immunization and active immunization. Passive immunization using hepatitis B immunoglobulin (HBIG) provides temporary immunity, while active immunization by vaccine yields long-term immunity. Perinatal transmission is the most important transmission route of HBV, particularly in endemic areas, and therefore, prevention by active and passive immunization against HBV should be initiated at birth. Additional doses of HBV vaccine should be given during infancy.

Other prevention modalities, such as screening the blood products, proper sterilization of injection needles and syringes, and avoidance of risky behaviors, such as parenteral drug abuse, tattoo, or skin piercing to prevent horizontal transmission are also important. Many countries with low prevalence of HBV infection also have HBV vaccination program for adolescents to prevent the exposure to HBV by sexual contacts or other risk behaviors. But the program is not as successful as the infantile HBV immunization strategy.

Passive Immunization Against HBV Infection Using HBIG

HBIG is prepared from the pooled plasma of donors who have high levels of anti-HBs. During the process of extraction for anti-HBs, viruses are inactivated, and solvents used in the preparation are removed. It excluded the products tested positive for HBsAg, anti-HCV, and HIV. It is used for post-exposure prophylaxis (passive immunoprophylaxis) of HBV infection.

HBIG was given immediately after birth to infants of HBeAg-positive HBsAg carrier mothers. In comparison to the 91 % of HBsAg carrier rate among infants without immunoprophylaxis, the HBsAg carrier rate was 26 % among infants who received three doses of HBIG at birth, 3 and 6 months old, and was 54 % in those who received a single 1.0 ml dose of HBIG at birth. The prevention efficacy was 45 % by one dose of 1.0 ml HBIG and 75 % by three doses ofHBIG, respectively [14].

Active Immunization Against HBV Infection Using Hepatitis B Vaccine

Currently, there are two kinds of HBV vaccine on the market, the plasma-derived vaccine and the recombinant vaccine. The first HBsAg-based highly purified and inactivated vaccine was made by Dr. Maurice Hilleman from chronic HBV infected subject' serum [15]. In order to produce a safe vaccine, stringent treatments with pepsin, urea, and formaldehyde and rigorous filtration to destroy all viruses, and chimpanzees test was conducted [16]. The plasma vaccine was approved by FDA of the USA in 1981 [17]. By inserting the gene coding for HBsAg, HBsAg was expressed in yeast to develop the recombinant HBV vaccine [18] and was licensed in 1986 [19]. Gradually, recombinant vaccine replaced plasma vaccine, and becomes the main vaccine used worldwide.

Active immunization with three or four doses of HBV vaccine without HBIG was proved to be immunogenic in more than 90 % of infants of non-carrier mothers or HBeAg-negative carrier mothers. Pilot clinical trial revealed that for infants of HBsAg negative mothers, the first dose of vaccine at 1 week stimulated anti-HBs within 1 month in 48 % of the neonates, and in 91 % at 2 months after the second dose. By the age of 6 months and 7 months, 96 % and 100 % vaccinees developed anti-HBs after a third dose, respectively [20].

For infants of HBeAg and HBsAg seropositive mothers, 23 % was HBsAg(+) after three doses of HBV plasma vaccine given at 1 week, 1 month, and 6 months of age, while the HBsAg positive rate was 88 % in the unvaccinated infants. The prevention efficacy of using HBV vaccines was around 75 % (Beasley RP, et al. Unpublished data).

Active Plus Passive Immunization Against HBV Infection

Clinical trial combining HBIG immediately after birth followed by HBV vaccination for infants of HBeAg positive, HBsAg carrier mothers was conducted in Taiwan. The prevention efficacy was 94 %, which is superior to HBIG alone (71 %) or vaccination alone (74 %) [21, 22]. This best result of HBV prevention against perinatal transmission of HBV infection by highly infectious mothers established the ground of the later universal HBV immunization strategies and program used currently. A subsequent study using HBIG at birth and three 5-μg doses of recombinant HBV vaccine, only 4.8 % of the high risk infants became chronic carriers, with a >90 % level of protection and a rate comparable with that seen with HBIG and plasma derived hepatitis B vaccine [23]

The Timing, Strategies, and Global Status of the HBV Immunization Programs

Since the most common and important transmission route is mother-to-infant transmission during perinatal period, the most appropriate timing for HBV immunization should be started at birth, and additional doses of vaccine should be given in infancy to elicit early and long term protection. In the world first universal hepatitis B immunization program in Taiwan, immunization was given at birth with passive HBIG, and then three or four doses of hepatitis B vaccine. The strategy of universal HBV vaccination in infancy is more effective than selective immunization for high-risk groups.

There are three major strategies of HBV Immunization and screening of maternal HBV markers during pregnancy in different countries, depending on their epidemiologic features of HBV infection and available resources (Fig. 19.1):

Strategy 1. Combination of active and passive HBV immunization with maternal screening of HBeAg and HBsAg; this is conducted in highly endemic areas such as Taiwan.

Strategy 2. Combination of active and passive HBV immunization with maternal screening of HBsAg; it is conducted in areas with adequate resources, such as in the USA and Italy [24, 25].

Strategy 3. Active HBV immunization without maternal screening and HBIG. It is conducted in areas with limited resources.

The cost-effectiveness per case prevented by Strategy 2 was estimated to be highest; for Strategy 3 was lowest [26]. However, the protection for high risk mothers' infants is higher in Strategy 1 or 2 than strategy 3.

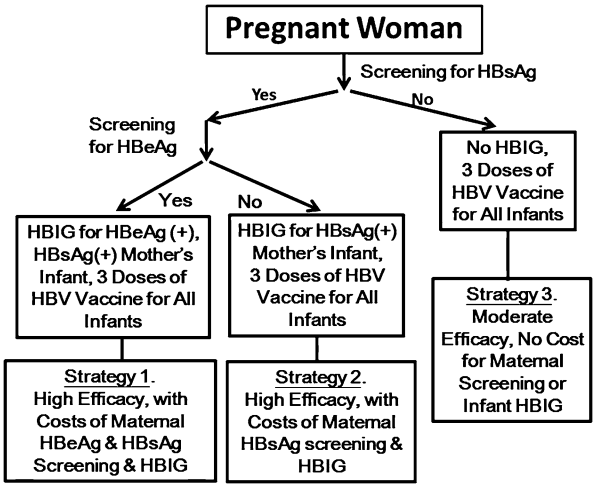

Fig. 19.1 Three major strategies of universal HBV immunization in the world countries

Universal Maternal Screening of HBeAg and HBsAg and Combining Active and Passive HBV Immunization for Infants (Strategy 1)

The world's first universal hepatitis B vaccination program was implemented in Taiwan using strategy 1 since July 1984. Screening for maternal serum HBsAg and HBeAg during pregnancy is conducted. Infants of highly infectious mothers with positive serum HBeAg and HBsAg received HBIG within 24 h after birth in addition to three or four doses of HBV vaccine during infancy. Infants of mothers with negative HBsAg, or positive HBsAg but negative HBeAg, or unknown HBV status received three or four doses of HBV vaccine only. The coverage rate of hepatitis B vaccine for neonates is around 94–99 % [27].

Universal Maternal Screening of HBsAg and Combining Active and Passive Immunization for Infants (Strategy 2)

In countries with low prevalence of HBV infection and better resources, HBIG is given to newborns of all HBsAg-positive mothers regardless of their HBeAg status, and three doses of HBV vaccine are given to all infants.

Since 1988, the Advisory Committee on Immunization Practices (ACIP), USA has recommended universal screening of pregnant women for HBsAg during the early prenatal period in each pregnancy. *All infants should receive HBV vaccination.* Infants of HBsAg seropositive mothers should receive appropriate immunization with HBIG and HBV vaccine to prevent perinatal transmission [24]. Although this strategy can save the cost of maternal screening for HBeAg, the wider use of HBIG in infants of HBsAg mothers regardless of maternal HBeAg status increases the cost.

Active HBV Immunization in Infancy Without Maternal Screening and HBIG at Birth (Strategy 3)

Using three or four doses of HBV vaccine to all infants without screening maternal HBV markers is a common practice of universal immunization program in the world. It can save the cost not only for maternal screening of HBV markers, but also the cost of HBIG. This policy is practically applicable in countries with limited resources. The results of prevention is good according the report of studies in Thailand and other countries [28].

In many endemic countries with limited resources, three doses of hepatitis B vaccine are given to all infants, regardless of the HBeAg status in HBsAg carrier mothers. This strategy offers an efficacy of around 75–80 % for infants of HBeAg-positive highly infectious mothers. Nevertheless, the cost of maternal screening and subsequent use of HBIG in the newborns can be avoided.

Global Status of the HBV Immunization Program

In 1992, the World Health Assembly passed a resolution to recommend global vaccination against hepatitis B. In 2009 WHO recommended that all infants receive the hepatitis B vaccine as soon as possible after birth, preferably within 24 h. The birth dose should be followed by two or three doses to complete the primary series.

Hepatitis B vaccine for infants was introduced nationwide in 183 countries by the end of 2013. Global coverage of infants with three doses of HBV vaccine in 1990 was only 1 %. It is gradually increased and was estimated to be as high as 81 % in 2013. A birth dose for hepatitis B vaccine was advocated by WHO, and was introduced in 93 countries by 2013, with a global coverage rate estimated as 38 %, reaching 79 % in the Western Pacific, but only 11 % in the African Region (WHO, Global immunization data) (http://www.who.immunization/monitoring_surveillance/data/en/).

The Preventive Effect of HBV Infection and Related Diseases by Immunization

Evidences support that hepatitis B vaccine provides effective protection against HBV infection and its complications, including fulminant hepatitis B, chronic hepatitis B, and its related HCC. It is the first successful cancer preventive vaccine in human [29]. It is also the first vaccine against a chronic disease [30].

Prevention of Acute Hepatitis B

Universal HBV immunization program has reduced the incidences of acute hepatitis B [31, 32]. After 25 years of universal HBV immunization in Taiwan, acute hepatitis B among adolescents and young adults ≤25 years old was reduced, making infants and the unvaccinated 25–39-year-old cohort additional targets for preventing acute hepatitis B (Fig. 19.2a). Vaccinated infants (0.78/100,000) had higher rates than those aged 1–14 years (0.04/100,000), due to breakthrough HBV infection from mother-to-infant transmission [32].

Prevention of Fulminant Hepatitis B

The mortality rateof fulminant hepatitis per 10^5 infants was reduced significantly from 5.1 in those who were born before the HBV vaccination program (1975–1984) to 1.71 in those born after the vaccination program in Taiwan (1985–1998) [33] *(Fig.19.2b). The mortality in vaccinated birth cohorts decreased further by more than 90 % from 1977–1980 to 2009–2011* [34].

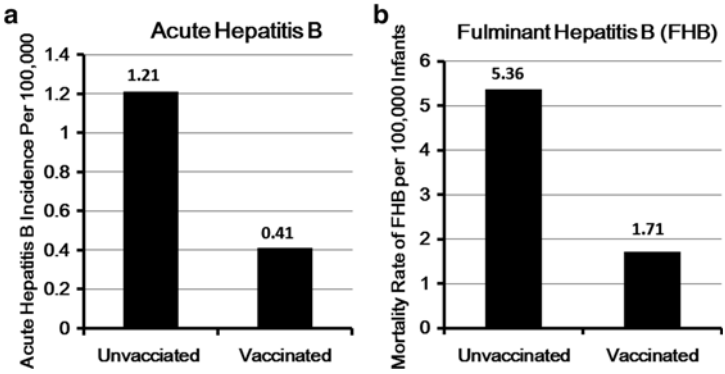

Fig. 19.2 (**a**) After universal hepatitis B immunization, the incidence rate of acute hepatitis B is also reduced in the vaccinated birth cohorts of 15–19 years old [32]; (**b**) the mortality rate of fulminant hepatitis (FH) B in infants is also reduced [33]

After universal HBV vaccination in Taiwan, HBV was found to very rarely cause fulminant hepatitis in children age ≥1 year, but remained a significant cause of fulminant hepatitis in infants [35]. The incidence rate ratio of patients age <1 year to those ages 1–15 years was 54.2 for HBV-positive fulminant hepatitis. HBV-positive fulminant hepatitis was prone to develop in infants born to HBeAg-negative, HBsAg-carrier mothers; these infants had not received HBIG according to the vaccination program in place. Maternal HBsAg was found to be positive in 97 % of the infants with fulminant hepatitis B, and maternal HBeAg was found to be negative in 84 % of these infants.

Reduction of Chronic HBV Infection Rate

Universal hepatitis B vaccination programs have effectively reduced the chronic HBV infection rate in many endemic countries. The protective efficacy of the hepatitis B vaccination program in infants born to highly infectious mothers and received HBIG and vaccine on schedule was approximately 85–90 %. The early mass survey data after the universal HBV vaccination program in Taiwan revealed that the protective efficacy was 86 % in the HBIG plus HBV vaccine group and 78 % in those with only three doses of HBV vaccine alone [36].

The HBsAg seropositive rates declined to below 1 % in most countries where universal vaccination programs have been successfully conducted [37]. Serial seroepidemiologic studies started just before the universal vaccination program and every 5 years in the post-vaccination era in Taiwan in the past three decades [38–42]. The results revealed that HBsAg seroprevalence rate among children declined from 9.8 % before the HBV immunization program to 0.5–1.2 % after the program. It implicates that Taiwan has been changed from an HBV endemic country to a low endemic coun-

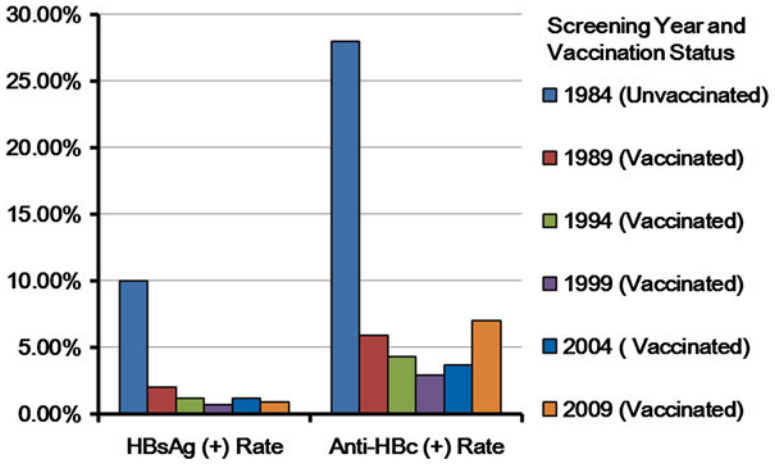

Fig. 19.3 Comparison of the HBsAg seropositive rates and anti-HBc seropositive rates among children born before versus after the launch of universal hepatitis B immunization program in Taiwan

try by the HBV immunization program (Fig. 19.3). The HBV infection rate (anti-HBc seropositive rate) declined from 38 to 16 % and further to 4.6 % in children 15–20 years after the HBV immunization program in Taiwan [42] (Fig. 19.3). Twelve years after integration of universal hepatitis B vaccine into the national expanded program on immunization (1992) in Thailand, the HBsAg and anti-HBc seropositive rate was reduced from 4.3 % and 15.8 % among those born before the program, to 0.7 % and 2.9 % among 6 months to 18 years old born after, respectively [28].

In Gambia, villages with good HBV immunization program, vaccine efficacies in 1993 against HBV infection and chronic HBsAg carriage were 94.7 % and 95.3 %, respectively [43]. During 1986–1990, the Gambia Hepatitis Intervention Study (GHIS) comparing fully vaccinated vs. unvaccinated GHIS participants among the allocated 125,000 infants. HBV infection was 0.8 % (2/255) vs. 12.4 % (59/475), suggesting a vaccine efficacy of 94 % [44]. Study in Gambia also showed a lower anti-HBc positive rate of 27.4 % (70/255) among vaccinated participants in comparison to 56.0 % (267/475) among the unvaccinated subjects.

Prevention of Liver Cancer by HBV Immunization

Prevention of chronic HBV infection by immunization can effectively reduce the incidence of liver cancer. HBV vaccine is the first human cancer preventive vaccine successfully preventing the development of liver cancer [29]. Taiwan has high incidence rates of HBV infection and HCC in both children and adults. Children with HCC in Taiwan are nearly 100 % HBsAg seropositive, 86 % of them are HBeAg negative, and their mothers are mostly (94 %) HBsAg seropositive [45]. The

Table 19.2 Impact of universal HBV immunization on HCC incidences among <20 Years old

	Diagnosed age of HCC	HCC incidence/100,000 person-year		% Reduction of HCC (after immunization)
		Before HBV immunization	After HBV immunization	
Taiwan [47][a]	6–9 Years 10–14 Years 15–19 Years	0.51 0.60 0.80	0.15 0.19 0.16	70 % 68 % 70 %
Khon Kaen, Thailand[b]	5–18 Years	0.097	0.024	75 %
Alaska Natives, USA[b]	<20 Years	0.5–3.0	0 after 1999	[c]Nearly 100 %

[a]Analysis for HCC incidences according to the birth year before versus after the vaccination program
[b]Analysis for HCC incidences according to the year of diagnosis before versus after the vaccination program
[c]Due to small population

histologic features of the HCC are similar to that in adult HCC. Most (80 %) of the non-tumor liver tissues have liver cirrhosis. Integration of HBV genome into host genome was demonstrated in the childhood HCC tissues [46].

The world first universal hepatitis B vaccination program in Taiwan has demonstrated significant reduction of the average annual incidence rate of HCC in children aged 6–14 years. It decreased from 0.52–0.54 cases per 100,000 children of the birth cohort born before the HBV vaccination program, to 0.13–0.20 cases in those born after the HBV vaccination program [47] (Table 19.2). According to a 20-year follow-up study of national cancer surveillance in Taiwan, the effect of HCC prevention by universal HBV vaccination was observed not only in children but also extended to adolescents, with an age- and sex-adjusted relative risk of 0.31 for persons vaccinated at birth [48]. Studies in Khon Kaen of Thailand also showed declines in the incidence of childhood HCC as a result of at-birth HBV immunization program [49]. Another study in Alaska, USA revealed effective reduction of HCC incidence, from 3 per 100,000 in 1984–1988 to undetectable after 1999 among Alaska Native children and adolescents under 20 years old, after 25 years of universal neonatal HBV immunization [50].

Remaining Problems for a Better Control of Hepatitis B and Its Sequelae

Low Vaccine Coverage Rate Due to Inadequate Resources or Ignorance

According to WHO global immunization data, global coverage rate of infants with three doses of HBV vaccine was estimated to be 81 % in 2013, as high as 92 % in Western Pacific Region, 89 % in Americas, 83 % in Eastern Mediterranean Region,

and 81 % in European Region, but lower rate as 74 % in South-East Asia Region, and 76 % in Africa. The coverage rate of a birth dose for hepatitis B vaccine reached 79 % in the Western Pacific, but only 11 % in the African Region (http://www.who.immunization/monitoring_surveillance/data/en/)

In some countries although universal HBV vaccination have been launched, the cost of vaccine is not covered by the government which may hamper the increase of immunization coverage rate. Further increase of the global coverage rates of neonatal dose and infantile HBV vaccination is important toward a better control of hepatitis B and related diseases. To provide free charged HBV vaccines for infants in developing countries may enhance effectively the vaccine coverage rate. It is particularly urgent in areas where HBV infection and HCC are endemic.

Poor compliance of the HBV vaccination due to ignorance or anxiety induced anti-vaccine act is still a problem in areas with adequate resources. *Incomplete vaccination had an independent effect on the mortality of FHF, showing an HR of 4.97 (3.05–8.11; $P \leq 0.0001$) after adjustment for maternal HBsAg serostatus* [51].

Education to enhance the understanding of the benefit and the extremely low vaccine-related adverse reactions of HBV vaccine is needed to improve the coverage rate. Previously an association between central nervous system demyelinating diseases and hepatitis B vaccine was implied [52]. Later evidence indicated that HBV vaccine does not increase the risk of onset or relapse of central nervous system demyelinating diseases [53].

HBV vaccination has not captured sufficient attention from the government in developed countries with relatively low prevalence of HBV infection, particularly under the competition of other new vaccines [54]. Competition of how to persuade the government of those countries to pay more attention to the low cost and very high efficacy of disease prevention is another important task to be done.

Breakthrough HBV Infection In Spite of Complete Immunization

In spite of complete immunization with combination of passive (HBIG) and active (vaccine) immunization, breakthrough infection may still occur. The most important risk factor is highly infectious mother with positive HBeAg and high viral load [10, 11]. The predictive breakthrough HBV infection rates of vaccinated infants at maternal viral load levels of 7, 8, and 9 log10 copies/ml were 6.6 %, 14.6 %, and 27.7 %, respectively [55].

In children born to HBeAg seropositive HBsAg carrier mothers, despite HBIG and three doses of HBV vaccine, 9.26 % still became HBsAg seropositive. In contrast, children born to HBeAg negative, HBsAg seropositive mothers, only 0.29 % became HBsAg positive if no HBIG was given at birth, and 0.14 % became HBsAg positive if HBIG was given at birth [56].

Another cause of breakthrough HBV infection is the emergence of hepatitis B surface gene mutants [57]. The prevalence rate of the hepatitis B surface gene *a* mutant increased from 7.8 % in the unvaccinated to 22–28 % among vaccinated HBsAg positive school children. The prevalence rate of the mutants among the total population has remained stationary for 20 years after the launch of the HBV immunization program because HBV vaccination reduced the HBsAg seropositive rates in the vaccinated population.

The natural course of surface gene mutant infected subject remains unclear. A recent study revealed that HBsAg-mutant HBV was detected in three of eight (38 %) HBV DNA-positive children with HCC. Higher frequency of HBV genotype C and a higher ALT level during surface mutant viremia were observed in codon 110–129 surface gene mutants than in codon 144–145 mutants. Immunized children carrying HBsAg-mutant HBV may develop hepatitis activity, HBeAg seroconversion, and a low viremic state earlier than those carrying wild-type HBV [57, 58].

Genetic hypo-responsiveness to vaccine, and immune compromised hosts are other causes of breakthrough HBV infection [59]. Immunosuppressive conditions, such as advanced HIV infection, chronic liver disease, chronic renal failure and diabetes have been demonstrated to be associated with reduced immunogenicity of hepatitisB vaccine.

The Possibilty of Blocking Mother-to-Infant Transmission of HBV Using Antiviral Agent in Addition to Immunoprophylaxis

Continuing efforts are ongoing to seek for other method to prevent breakthrough HBV infection by highly infectious mothers. Preliminary clinical trials using nucleoside analogue during the last trimester of pregnancy to prevent mother-to-infant transmission have been reported [60–62]. Lamivudine or telbivudine during late pregnancy in mothers with high viral load may reduce, but cannot prevent all the mother-to-infant transmission of HBV. It appeared safe in short term follow-up for mothers and infants [63]. *A study in pregnant cohorts with HBV DNA ≥7 log IU/ml showed significant reduction of perinatal transmission to 2 and 0 % in tenofovir disoproxil fumarate or lamivudine treated, compared with 20 % in untreated cohort* [64]. Tenofovir disoproxil fumarate for highly viremic mothers at 30 to 32 weeks of pregancy was also studied. The results indicated a significant reduction of HBV DNA sero-positive rate at birth and HBsAg sero-positive rate at 6 month old in their children, in comparison to non-treated control group [65].

In addition to the cost for screening viral load before enrolment and the cost for antiviral agent, the problems of discontinuation of oral antiviral agent in postpartum mothers need to be addressed. Further studies to clarify the long term safety, benefit, and efficacy of nucleoside analogue in the prevention of intrauterine infection are needed.

Future Prospects

Existing data already demonstrated the remarkable effectiveness of HBV immunization in preventing approximately 90 % of chronic HBV infection and 65–70 % of acute/fulminant hepatitis B. To eliminate HBV infection and its sequelae in the world, further increase of the global coverage rates of HBV vaccine particularly in areas with limited resource and countries with no universal HBV immunization program, and better strategies against breakthrough HBV infection mainly from mother-to-infant transmission are of vital importance.

Hepatitis B vaccine is the first cancer preventive vaccine in human. With the success of HBV vaccination to prevent liver cancer, the concept of a cancer preventive vaccine can be extended further to prevent the infection of other microorganisms and their related cancers.

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Chapter 20
Towards HBV Eradication: Future Perspective

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Since the discovery, substantial improvement in the understanding of hepatitis B virus (HBV) virology, host immune response, and natural course, combined with the advent of effective vaccine and antiviral drugs, has led to better control for chronic HBV infection, as well elaborated and demonstrated in the chapters in this book. In particular, 183 countries have implemented vaccination program as of 2013 [1]. The estimated global population with chronic HBV infection has decreased from 350 million to 240 million in the most recent estimate in 2012 [2]. In addition, potent nucleos(t)ide analogs (NUC) with high genetic barrier to drug resistance are able to maintain long-term HBV suppression, improve liver histology, reverse hepatic fibrosis, and reduce hepatocellular carcinoma (HCC). However, there are challenges ahead to achieve eradication of HBV.

First, the most important and critical challenge is the economic or financial problem. According to 2013 data of gross national income (GNI) per capita [3], the majority of high intermediate and high HBsAg prevalence countries have low-income economies. Conceivably, the infrastructure of the healthcare system is not satisfactory in many of these countries. For example, only 81 % of the infants received 3 doses of vaccine and only 93 of the 183 countries with nationwide HBV vaccination program for infants introduced the birth dose with a coverage rate estimated to be 38 % globally and only 11 % in the African region [1]. Even in a country like Taiwan

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where complete vaccine coverage was 97 %, the HBsAg carrier rate still remains at 0.5 % in the vaccinated population. Thus, nonsatisfactory outcome of incomplete vaccination is predictable. Reduction of vaccine price and international technical or financial support had greatly extended the HBV vaccination program in early years. Since universal HBV vaccination is the most important and effective step towards HBV eradication, further support to extend and enhance (birth dose) vaccination program is needed to reduce HBV infection and its adverse sequelae including HCC. Another important issue in the prevention of vertical transmission is the need for hepatitis B immune globulins (HBIG) administration in the newborns to maximize the prophylactic effect of vaccination, and the need for NUC administration during the third trimester of pregnancy in women with high viral load (see Chap. 19). These strategies have to face practical issues in terms of cost for both HBIG and NUCs and continuous supply for HBIG.

Second, there are large number of existing HBsAg carriers worldwide. Most patients with chronic hepatitis B or compensated cirrhosis have few or no symptoms; therefore persons with chronic HBV infection largely remain undiagnosed. It was estimated that only as low as 35 % of the chronically infected Asian-American adults were aware that they were infected. In Taiwan, a country with high HBsAg prevalence and HCC incidence, a 2005 estimate showed that only 46 % of the adults living with chronic HBV infection were aware that they were infected in spite of active campaign activity through media and public education since 1980s [4]. A recent survey in nearly 10,000 Greek adults showed that 47 % had never been tested for serum HBsAg and only 32.4 % of the diagnosed hepatitis B patients had ever been treated [5]. Since chronic HBV infection is now amenable to treatment, the importance of active screening programs to identify unrecognized victims of chronic HBV infection for appropriate monitoring and timely interventions is obvious. Lack of disease awareness or understanding of the disease and fear of stigmatization in society among patients are associated with inappropriate health-seeking behavior or poor patient adherence to therapy. Lack of disease awareness among governments and healthcare practitioners are also obstacles to the proper management of HBV disease. Lack of awareness among government officials results in lack of screening programs and inadequate reimbursement. Lack of adequate education and awareness among heath-care providers is obviously an even more serious problem because adequate explanation, counseling and individualized assessment are essential for successful anti-HBV therapy. In addition, lack of specialists and state-of-the-art laboratory assays are also problems in some countries. Even if the government and society are well aware of the problems, costs of screening, monitoring and therapy may be well beyond their threshold of willingness to pay. These factors not only are responsible for the low diagnostic rate of HBV, but also for the low treatment rate among diagnosed patients (4 % in Asia versus 20 % in the USA, 17–28 % in Europe, and 8 % in Japan) [4]. These contrasting figures reflect the difference in the level of development or income of the countries and also clearly indicate that lack of economic resources is the main obstacle to proper management of HBV. Obviously, to enhance awareness campaigns, active screening programs and other effective public policy responses such as national action plans need to be developed [6].

Third, currently available anti-HBV agents with their short-term/long-term efficacy are far from satisfactory and treatment strategies are still evolving. Problems in drug therapy per se include low therapy-induced HBeAg seroconversion rate, frequent unpleasant side effects that require close monitoring during IFN-based therapy, several decades of NUC therapy that is required to achieve HBsAg seroclearance which will otherwise make HBV suppression not durable after cessation of NUC therapy, drug resistance increase upon prolonged NUC therapy with cheap NUCs with low barrier to resistance (lamivudine, adefovir, telbivudine) though entecavir and tenofovir have very low or no drug resistance up to 7 years, and unknown adverse side effect beyond 10 years' drug therapy. These inherent problems have made treatment of chronic HBV infection a complex task that requires individualized assessment and decision, therefore representing a great challenge to general physicians. A more important and most critical challenge is the high cost of medical care and antiviral drugs. Cost analyses have shown that progression of liver disease is associated with increasing healthcare costs. The major cost of chronic hepatitis B and compensated cirrhosis is that of drug therapy while that of decompensated cirrhosis and HCC is that incurred with hospitalization. Lack of full or adequate reimbursement for treatment and diagnostic testing is so common in less privileged countries that adequate drug therapy is restricted only to those who can afford it and drug with high resistance rate such as lamivudine is still widely used especially in Asia and regions of poor economy [4]. In addition, these limitations have made adherence to treatment guideline impossible. Financial or technical support from international agencies and reduction of price by companies producing anti-HBV drugs or HBV assays would be the most direct and effective measures to improve the situation. This would allow to treat more patients with chronic hepatitis B who meet the current indications for treatment, but also consider earlier treatment intervention in patients with minimal inflammatory and/or fibrotic liver damage to prevent as much as possible the occurrence of oncogenic molecular events and the subsequent development of HCC [7].

Fourth, the currently available anti-HBV drugs are able to improve and halt the progression of liver disease but unable to achieve ideal therapeutic end point (HBsAg seroclearance and seroconversion) in the vast majority of the patients. New drug, new strategies, or new approaches are needed to achieve a better therapeutic outcome that would allow more patients to achieve HBsAg clearance. This endpoint is associated with a decreased risk of HCC development and is considered as sufficient to stop therapy. A functional cure (equivalent to resolved acute infection) would be defined by HBsAg loss with or without anti-HBs seroconversion, with undetectable HBV DNA, but persistence of cccDNA which is not transcriptionally active, allowing treatment cessation [8]. This seems to be the most pragmatic endpoint to achieve in a near future. The achievement of higher rate of functional cure would allow treatments with a finite duration with an expected lower cost than lifelong therapy. This would also allow to treat all infected patients in a global strategy to prevent HCC.

To achieve these goals it will be necessary to investigate a number of steps in the HBV replication cycle and specific virus-host cell interactions as potential targets for new antivirals [8]. These includes a direct inhibition of viral replication;

several approaches will have to be evaluated thoroughly and among them: entry inhibitors, targeting cccDNA formation or its degradation, silencing cccDNA, targeting viral transcripts with siRNA, capsid assembly modulators, targeting viral envelope proteins, and virus egress. Restoration of immune responses is a complementary approach which includes the restoration of innate immunity against HBV for instance with TLR agonists or delivery of specific antiviral cytokines, and restoration of adaptive immunity with inhibitors of negative check point regulators, therapeutic vaccine, or engineering of specific T cells. Novel targets and compounds could readily be evaluated using both relevant in vitro and newly developed in vivo models of HBV infection. Clinical evaluation of these new treatment concepts should cover all the real life situations including patients in the different phases of the disease, patients infected with different viral genotypes, infected by different routes of transmission, and patients with different treatment history [9]. The addition of one or several new drugs to current regimens should offer the prospect of markedly improving the response to therapy, thus reducing the burden of drug resistance, as well as the incidence of cirrhosis and hepatocellular carcinoma.

In this context, there is a need for the establishment of an International Coalition to Eradicate Hepatitis B (ICE-HBV) [9]. This coalition would consist of leaders in the HBV field across continents, committed to curing and eradicating HBV from the globe, similar to groups established by our HIV colleagues, who have established the HIV Cure Advisory Board and a Multidisciplinary International Working Group of researchers dedicated to HIV cure. This working group consists of subgroups for Virology, Immunology, Innovative Tools, and Clinical trials and aims at facilitating scientific discussion, exchange and collaboration to promote and accelerate research towards a cure for HIV; provide leadership in advocating for increased investment and resource optimization in HIV cure research; and provide clear and accurate information and disseminate knowledge to the broader community. This would be facilitated through consultation with industry (Pharma/Biotech), patients and advocacy groups, research funders, and regulatory agencies. The establishment of International Coalition to Eradicate Hepatitis B could drive changes in governmental policy and ensure funds are channelled to HBV cure research and drug development is urgently required. With this in place it may be possible to arrive at novel concepts enabling HBV cure within the next 5 years.

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