Hepatitis B: Where Are We Today?

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Key Words

Hepatitis B · Geographic distribution · Life cycle ·
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Summary

Hepatitis B still is a major challenge of modern medicine. Here, we summarize the disease, the life cycle of the virus, the emergence of drug-resistant mutant strains and resulting consequences for new drug development. Hepatitis B has a worldwide prevalence of 0.1–20%. It occurs in an acute and a chronic phase. The acute phase is characterized by viral replication, onset of immune response leading to destruction of infected hepatocytes and, in most cases, viral clearance and lifelong immunity. Clinical symptoms are observed in this phase. The chronic phase is characterized by lifelong persistence of the virus, often without clinical symptoms but bearing the risk of hepatic cirrhosis or hepatocellular carcinoma. Both phases are distinguished by detection of viral antigens and hepatitis B antibodies in the blood. The hepatitis B virus (HBV) shows several features unique for hepadnaviridae: i) formation of covalently closed-circle DNA (cccDNA) as template for viral DNA replication and synthesis of viral RNAs and ii) a reverse transcription step in the synthesis of new viral DNAs. These features are the prime targets in antiviral drug development. Unfortunately, with the exception of interferon, resistant mutant strains of HBV were found after widespread use of every new drug so far. Therefore, combination therapies aimed at different targets are being discussed so that the selective pressure can no longer be compensated by HBV. This paper is the first part of a trilogy concerning HBV which will also discuss the situation regarding transplantation (second part) and blood transfusion (third part).

Schlüsselwörter

Hepatitis B · Geographische Verteilung · Lebenszyklus ·
Antigene · Mutanten · Resistenz

Zusammenfassung

Hepatitis B ist nach wie vor eine der größten Herausforderungen der modernen Medizin. Wir geben einen Überblick über die Erkrankung, den Lebenszyklus des Virus, das Auftreten von Resistenzmutationen und die daraus resultierenden Konsequenzen für die Entwicklung neuer Medikamente. Hepatitis B tritt weltweit mit einer Prävalenz von 0,1–20% auf. Die Krankheit manifestiert sich in einer akuten und einer chronischen Phase. Die akute Phase zeichnet sich aus durch virale Replikation, Einsetzen der zur Zerstörung befallener Hepatozyten führenden Immunantwort und in den meisten Fällen Eliminierung des Virus und lebenslange Immunität. Klinische Symptome treten gewöhnlich in dieser Phase auf. Die chronische Phase zeichnet sich durch lebenslange Persistenz des Virus aus, oft symptomlos, jedoch mit der Gefahr einer Leberzirrhose oder eines Leberzellkarzinoms. Beide Phasen können durch den Nachweis zirkulierender viraler Antigene sowie Hepatitis-B-Antikörper identifiziert werden. Die Biologie des Hepatitis-B-Virus (HBV) zeichnet sich durch bestimmte Eigenschaften aus, die für die Hepadnaviridae einzigartig sind: 1) Bildung eines kovalent geschlossenen, zirkulären DNA-Strangs (cccDNA) als Matritze für die Replikation viraler DNA und die Synthese viraler RNAs und 2) eine Reverse-Transkriptase-Funktion während der Synthese neuer viraler DNAs. Diese Eigenschaften sind bevorzugte Ziele bei der Entwicklung antiviraler Medikamente. Leider sind bisher bei jedem neuen Wirkstoff (mit Ausnahme von Interferon) nach weitreichendem Einsatz Resistenzmutationen beobachtet worden. Daher werden nun Kombinations-therapien mit unterschiedlichen Angriffspunkten diskutiert, um den Selektionsdruck soweit anzuheben, dass er durch das HBV nicht mehr kompensiert werden kann. Die vorliegende Arbeit ist Teil einer Trilogie, die im zweiten Teil das Gebiet der Organtransplantation und im dritten Teil das Thema Bluttransfusion betrachten will.

Introduction

Hepatitis B is still one of the most prevalent communicable diseases worldwide. It is estimated that over 2 billion people alive today have been infected with the hepatitis B virus (HBV) at some time in their lives. Of those, 350 million people are suffering from chronic hepatitis and are persistent HBV carriers and thus one of the main sources of infection. Annually, 1 million deaths are attributed to hepatic cirrhosis, hepatocellular carcinoma and acute liver failure as a consequence of HBV infection [1]. The geographic distribution shows a significant gradient between Western countries and developing nations. It is estimated that in Southeast Asia, China and Africa where prevalence is high, more than half the population are exposed to HBV at some point in their lives, resulting in 8–20% being chronic carriers. The predominant ways of infection in these areas are perinatal transmission (vertical) or transmission from child to child (horizontal). In areas with low levels of infection (low prevalence), such as North America, Western Europe and Australia, the predominant way of infection is horizontal transmission between young and sexually active adults [1, 2]. The currently recognized modes of transmission are: i) infected mother to child at birth (perinatal/neonatal), ii) close contact with an infected person, iii) sexual contact, iv) parenteral exposure to blood (blood to blood) or other body fluids or specimens, such as surgical instruments or laboratory equipment [1, 2]. As HBV serum levels in infected individuals can be very high (10^8 – 10^{10} infectious virions/ml) [1, 3, 4], even invisible traces of serum are a potential source of infection. HBV is also detectable in semen, saliva, breast milk, urine and cervical discharge [2–4, 162].

Since the discovery of HBV in 1965 by Baruch Blumberg [5], tremendous effort has been put into understanding the virus and the disease caused by it, leading to the successful development of vaccines and a good understanding of the biology and complexity of HBV. Vaccination has proven to be an effective way of protection against HBV infection, as demonstrated by several national vaccination programs [1]. However, in its chronic state, the virus can currently not be eradicated by medical treatment, and there is no specific treatment for acute hepatitis B [6]. In this article we will summarize the current understanding of HBV-induced liver disease and the biology of HBV. We will also focus on current prophylaxis protocols other than vaccination, with emphasis on current developments in antiviral drugs, especially anti-HBV immune globulin (HBIG).

Hepatitis B: Geographic Distribution

As mentioned above, there is a clear gradient between so-called developed countries and the Third World. Bearing in mind the large number of unreported cases, the prevalence of

hepatitis B in the various areas of the world is estimated as follows [1, 7, 8]: <2% in Northern, Western and Central Europe, North America and Australia, with a prevalence of carriers particularly among blood donors in Northern Europe, North America and Australia of 0.1% or less; 2–8% in Eastern Europe, the Mediterranean, Russia and the Russian Federation, Southwest Asia, Central and South America; >8% in parts of China, Southeast Asia, tropical Africa, with high endemic areas where as many as 20% of the apparently healthy population may be asymptomatic carriers and thus a huge reservoir of infection. In low-prevalence areas the predominant way of infection is horizontal transmission between young and sexually active adults and members of certain risk groups, such as intravenous drug users. This leads to a mostly acute course of disease with either acute hepatitis, hepatic failure or spontaneous clearance. In high-prevalence areas, infection is acquired neonatally or transmitted from child to child at a very early age. This results in predominantly chronic hepatitis with the consequence of liver failure or liver cancer (hepatocellular carcinoma, HCC) at a later age in a high number of HBV carriers (see below) [1, 4, 9]. Hence, the risk of infection is high in such countries, with consequences in relation to global travel and migration due to war or famine.

Several attempts have been made to eradicate or at least significantly reduce the prevalence of hepatitis by vaccination programs. Initial attempts, e.g. in the USA, were aimed at high-risk groups to prevent the virus from spreading amongst the adult population. The failure of such programs was largely attributed to the lack of compliance of the targeted group, combined with problems in assessing and identifying vaccination candidates [10]. Epidemiological calculations revealed that the only way to reduce the prevalence of hepatitis B in a population is the consequent vaccination of newborns to eliminate the number of chronic carriers and thus the main reservoir of infection [11]. This hypothesis was shown to be correct by the infant vaccination program in Taiwan, dramatically reducing infections in newborns and thus the overall numbers of carriers in the population [12]. The authors describe that in 1982, 15–20% of the general population were carriers of hepatitis B surface antigen (HBsAg). As a consequence, HCC accounted for 20% of all malignancy-related deaths, and hepatitis-related chronic diseases, such as cirrhosis, caused about 90% of deaths due to liver diseases, thus putting hepatitis B-related fatalities at rank 6 of the 10 main causes of deaths in Taiwan [12]. After the vaccination program was initiated in 1982, strictly all newborns were vaccinated. Babies born to high-titer HbsAg-positive mothers are at particularly high risk, with 90% of such infants becoming chronic carriers without intervention. These infants received a single dose of HBIG at birth in addition to the regular vaccination regimen, a combination which proved to be highly effective in preventing perinatal infection for these high-risk babies [13, 14], thus assuring the success of the vaccination program [12]. This strictly executed program was subsequently shown to have re-

duced the prevalence of HBsAg to 1.7% in children aged 6 between 1989 and 1993. If the program is maintained with the same strictness, it is estimated that by the year 2010 the carrier rate in children in Taiwan will have declined to <0.1% [12, 15]. Similar results were reported for the Italian hepatitis B vaccination program [16, 17] as well as by a survey of worldwide vaccination programs [18], emphasizing the importance of protecting newborns by vaccination against HBV to eliminate the prime source of chronic carriers.

Hepatitis B: The Disease

HBV hepatitis is initiated by infection of hepatocytes with HBV, followed by viral replication and spread of the infection to other hepatocytes (see below). It is important for the understanding of the disease that infection and virus propagation per se does not destroy the hepatocytes. On the contrary, the virus can be maintained and propagated inside hepatocytes for the entire life span of the infected cell. The initial phase of the infection is characterized by a feeling of sickness, fever, weakness, jaundice (rare) and flu-like symptoms, thus making diagnosis difficult. During this acute phase in which infective virions and viral antigens are released into the bloodstream, the patient's immune system initiates an immune response and starts to destroy infected hepatocytes, which in turn elicits the clinical picture of hepatitis with jaundice and elevation of serum transaminases. The liver disease caused by the HBV infection is therefore the result of the host's immune system trying to eliminate infected hepatocytes rather than of direct damage elicited by the virus [19]. This acute form of disease often resolves spontaneously after 4–8 weeks, with most patients recovering without significant consequences or recurrence. In a number of cases, the disease develops into a fulminant hepatitis with acute hepatic necrosis resulting in death [1, 4, 9, 19, 20].

Acute hepatitis B is characterized by the presence of anti-HBc IgM serum antibodies converting to IgG during convalescence and recovery, and the transient (<6 months) presence of HBsAg, hepatitis B e antigen (HBeAg) and viral DNA, with clearance of these markers followed by seroconversion to anti-HBs and anti-HBe. During this acute phase, in most adults, the immune system is able to eliminate infected cells and circulating virus, resulting in eradication of the infection and a lifelong immunity [6]. More than 90% of infections in adults fall into this category. The remaining 5–10% of adult-onset infections and >90% of neonatal infections become chronic and can continue for the life span of the patient, with the consequence of hepatic cirrhosis and subsequent liver failure or HCC in the majority of patients. Chronically infected individuals in the carrier state represent the predominant reservoir of infection. The chronic and thus carrier state is defined as persistence of HBsAg in the circulation for >6 months [1, 4, 6]. Chronic hepatitis B is a prolonged infection which

generally develops over many years. It is characterized by persistent serum levels of HbsAg and anti-HBc IgG and the absence of an anti-HBs antibody response. HBV DNA and HBeAg are often detectable at high concentrations, but may disappear if viral replication ceases during the course of the chronic disease. The associated inflammatory liver disease is characterized by varying severity. It is generally milder than acute hepatitis B, however, it usually lasts for decades, progresses to cirrhosis in approximately 20% of cases and is associated with an up to 100-fold increase in the risk of HCC [4, 21]. In patients with chronic hepatitis B, 3 phases of viral replication typically occur during the course of an HBV infection [9]: i) High-replicative phase: HBsAg, HBeAg and HBV DNA are present and detectable in the patient's serum with usually increased aminotransferase levels and moderate, histologically apparent inflammatory activity. ii) Low-replicative phase: this phase is associated with the loss of HBeAg, decrease in or loss of HBV DNA in the patient's serum and appearance of anti-HBe-IgG. iii) Non-replicative phase: low levels of the viral replication markers HBsAg and HBV DNA may be present. However, the majority of patients with chronic hepatitis B do not progress into the non-replicative phase. Due to the permanent presence of HBV, the regenerating liver parenchyma is constantly reinfected and subsequently destroyed by the host's immune system. Hence, there is a delicate balance between excessive liver destruction (autoaggression with the possibility of a fulminant and thus potentially lethal course of disease), complete clearance of the virus and immunity and failure to eliminate the virus (chronic infection), with the patient's chances of recovery poised between extremes. For patients with chronic hepatitis B, the difference between the chronic persistent and the chronic active course is all-decisive, as the latter may lead to cirrhosis and/or HCC [3, 4, 9, 22, 23].

The course of infection as described above varies according to superinfection with other pathogens, the patient's general health and, most important, age. As indicated above, the age of the individual at the point of infection largely determines the course of disease. The risk of chronic infection and thus of becoming an asymptomatic carrier is inversely proportional to age. It is highest for infants who become infected during the perinatal period and lowest for older children and adults. These findings can be summarized as follows [11, 22–24]: i) infection during the perinatal period: acute symptoms in <5% of cases, almost exclusively progression to chronic infection and carrier state (70–90%); ii) infection at the age of 1–5 years: acute infection in 50–80% of cases; iii) infection at the age of >5 years: predominantly acute hepatitis (>90%), with about 5% of patients developing long-term chronic infections. For the USA it is estimated that only 8% of acute infections occur between birth and the age of 10 years. However, infection at this age accounts for approximately one third of all chronic infections [25]. Besides becoming a disease carrier, infection at an early age also bears an elevated risk of developing cirrhosis

or HCC, which is correlated with the length of time a person is chronically infected. Infants who become chronically infected have a lifetime risk of developing cirrhosis or HCC of approximately 25%, whereas for adults acquiring a chronic infection the estimated lifetime risk is 15% [22, 23, 26].

The Extrahepatic Reservoir of the HBV

Despite the strong hepatocellular tropism of the virus, HBV was detected in a variety of extrahepatic sites and cell types, including peripheral blood mononuclear cells (PBMCs) [27–29]. Subsequently, it became evident that PBMCs represent a stable long-term extrahepatic reservoir for HBV and that these infected PBMCs may cause reinfection after orthotopic liver transplantation [30–34]. This notion was then further supported by demonstration of HBV replication, active transcription in PBMCs and in vitro infectibility of these cells [35–37], although Kock et al. [163] were not able to detect replication-competent HBV DNA in lymphocytes. Transcriptional activity and the rate of HBV replication may be low enough in these cells for the presence of HBV not to be detected by conventional methods, thus mimicking a HbsAg-negative patient. This was supported by the findings of Roche et al. [38] demonstrating HBV DNA persistence 10 years after liver transplantation despite successful HBIG treatment with or without concomitant antiviral therapy. As a consequence, this extrahepatic reservoir is a current threat for reinfection after liver transplantation and may thus argue for a long-term supply of protective immunoglobulins to prevent graft reinfection [30, 31, 38, 39].

In conclusion, HBV hepatitis is a complex disease, which is reflected in the complicated life cycle and genetic makeup with features that are unique for hepadnaviridae. As there is no available treatment for the elimination of the virus once the infection has established itself, the main goal in national and global anti-hepatitis programs is the prevention of new infections. As mentioned above, this may only be achieved by consequent immunization of newborns to eradicate future reservoirs of the virus, as infection in early childhood predominantly leads to chronic disease and thus to a lifelong source of infection. Therefore, the prevention of infection ‘under all circumstances’ has to also be a main concern in cases of infants born to HBV-positive mothers and in connection with liver transplantation due to HBV-related liver failure.

HBV Serotypes and Genotypes

The envelope or surface protein of HBV, the HBs protein or HBsAg is highly immunogenic and induces anti-HBs antibodies. It is antigenically heterogeneous with a common antigen designated a. The 4 variant antigenic determinants are designated d, r, w and y, with the 2 pairs d/y and w/r being mutually

exclusive, resulting in 4 major antigenic subtypes or phenotypes: adw, ayw, adr, ayr [40, 41]. Subsequently, 4 subdeterminants of w (w1–w4) were identified, resulting in the differentiation of 8 HBs subtypes: adw2, adw4, ayw1, ayw2, ayw3, ayw4, adr, ayr [42]. These subtypes differ in their geographic distribution [43] but not in the clinical features of the corresponding HBV infection and thus provide useful epidemiological markers, allowing subtle analysis of HBV molecular epidemiology [1, 6, 21, 44, 45].

After the first complete HBV DNA sequence was published in 1979 [46], sequencing data from numerous HBV isolates led to the definition of 8 recognized genotypes A to H which vary by approximately 8–15% at the nucleotide level [47–50]. These genotypes show a distinct geographic distribution. Genotype A is prevalent in North America, North-Western Europe and Central Africa. Genotypes B and C are predominantly found in Asia. Genotype D is found worldwide with the highest prevalence in the Mediterranean region. Genotype E is typically found in Africa, whereas F is typical for the aboriginal population in America and Polynesia. Genotype G has been detected in the USA, France, the UK and Germany, and genotype H seems to be limited to the native population of Central and South America [164–166]. Some authors have provided data showing that the differences on the nucleotide level might also influence the clinical characteristics of these strains. Analyses of data from several studies indicate that, for example, genotype A was more associated with chronic hepatitis B, whereas genotype D was predominant in patients with acute disease. The comparison of genotype B and C pointed to a more severe disease progression, worse outcome and a higher risk of severe liver dysfunction and HCC for genotype C than genotype B. The influence of the genotype on the response to antiviral therapy is less clear, but it appears that there are differences among HBV genotypes, e.g. to the response to IFN- α , where patients infected with genotypes A and B tend to respond better than patients infected with genotypes C and D. A similar picture seems to emerge for therapy with lamivudine, where patients infected with genotype B seem to have better response rates than patients infected with genotype C. Although the data provided thus far clearly point to differences in severity and progression of the disease depending on the HBV strain, further systematic analysis is needed to substantiate these indications [44, 45, 51, 52, 164–166].

Hepatitis B: The Virus

HBV belongs to the family of hepadnaviridae which infect birds (e.g. ducks), mammals (North American woodchuck, squirrels) and primates and consists of 2 subgroups: orthohepatnaviridae and avihepatnaviridae. They are characterized by a unique genetic makeup and particularities in their life cycles specific to for these viruses [1, 4, 21, 53]. The infec-

tive virion consists of an outer envelope composed of the S protein or HBsAg. This envelope encloses the virus DNA associated with the core protein or HBc antigen (HBcAg). A third protein, HBeAg, is a circulating peptide derived from the core gene which is modified and exported from hepatocytes. The P protein, the viral polymerase and the HBx-protein complete the set of 5 virus-encoded polypeptides [1, 4, 21, 53]. The S protein is synthesized in 3 different forms which differ in size and are termed large, middle and small S protein. They originate from different transcription initiation sites within the S gene. All 3 proteins share specific antigenic determinants and are responsible for eliciting protective cellular and humoral immunity to the HBsAg. These determinants are the basis for the development of currently available HBV vaccines. Antibodies to HBsAg which confer protective immunity are detectable in patients who have recovered from acute hepatitis B and in those immunized with HBV vaccines. They may, however, decrease to a non-detectable level in patients who have fully recovered from the infection [4, 9]. HBcAg which encloses the viral DNA induces a cellular immune response crucial for the killing of infected liver cells. Antibodies to HBcAg can be detected in all patients who have been exposed to HBV. Anti-HBc antibodies are protective since vaccination was achieved by using the HBc protein [167]. Their presence alone cannot be used to distinguish acute from chronic infection, and unlike anti-HBs, these antibodies are found in patients with persistent infections as well as in patients who have recovered from a HBV infection [4, 9]. HBeAg is a circulating protein. It is derived from the core gene, modified and exported from liver cells. It serves as a marker of active viral replication. It is thought that this protein might serve as a tolerogen, as some authors associate the presence of HBeAg with a diminished immune response [52, 54–56]. Generally, HBeAg is only detected in persons who also are positive for circulating serum HBV DNA, the most predictable indicator for active viral replication. A fourth protein, the P protein, is the viral polymerase which contains reverse transcriptase functions. The properties of this enzyme are discussed below. The fifth protein encoded by the HBV genome is the HBx protein, a polypeptide of 154 amino acids. Expression of full-length HBx protein is important for *in vivo* infectivity [57]. It acts as a transcriptional activator of several viral and cellular promoters through direct interaction with cellular transcription factors and is also involved in the activation of signal transduction cascades [51, 52]. Indications are provided by some authors that the HBx protein might play a role in the development of HBV-mediated carcinogenesis and thus HCC [58]. The mechanisms by which the HBx protein is involved in HCC development are unknown, but HBx-mediated transcriptional transactivation, its binding to the tumor suppressor p53 and deregulation of cell cycle checkpoints indicate an alteration in cellular gene expression and thus a contribution to hepatocyte transformation [52, 59–62].

The viral DNA, called RC DNA (relaxed circular DNA molecule), is partially double stranded, arranged in a circular structure but not covalently closed. The genome size is about 3,200 base pairs and contains 4 open reading frames (ORFs) which are overlapping (see below) and encode 4 protein families. Both strands differ in length thus creating gaps in the circular structure. At the 5' end of the short plus strand, a ribooligonucleotide is covalently attached which serves as primer in the initial replication steps. At the 5' end of the minus strand, the viral polymerase is linked covalently to the DNA strand, a feature unique to the hepadnaviridae [51, 63, 64].

The life cycle of the virus starts with the attachment of the virus particle to the surface of hepatocytes by binding to a yet unidentified receptor. The pre-S1 region (amino acids 21–47) of the large S protein is essential for hepatocyte entry [52, 65, 66]. Inside the cell, the virion is disassembled, and core particles are then transported to the nucleus where the viral DNA is repaired to form a covalently closed circle, the cccDNA, and viral DNA replication is initiated. Unlike the subsequent steps, this process of attachment, incorporation, disassembly and nuclear transport is not yet fully understood [51, 63, 64]. The cccDNA serves as a template for viral DNA replication as well as synthesis of viral RNAs [51, 52, 64]. The majority of the cccDNA molecules are then (after their synthesis) associated with histones to form a minichromosome in a quasi-chromatin (nucleosome) structure [67–69] which is assumed to favor stable propagation in the hepatocyte even through cellular divisions, thus, in addition to cccDNA replication, also accounting for viral persistence [70–73]. It is assumed that cccDNA formation is performed by the cellular machinery since the viral polymerase is not required for this process [72]. Since the process of cccDNA formation is unique for the hepadnaviridae, it may serve as a target for drug development [69, 73]. However, the use of this process as a drug target may have the disadvantage that cccDNA formation is performed by cellular proteins. After viral DNA replication, transcription of the various viral RNAs is initiated from the minichromosomes by using the host cell RNA polymerase II [74]. This transcription is a highly orchestrated process producing several RNA species with distinct functions. In the first step, genomic and subgenomic RNAs are transcribed from the minichromosomes. The subgenomic RNAs function as mRNA for the translation of the envelope (HBs) and X proteins only. This translation occurs at the rough endoplasmic reticulum (ER), and the 3 HBs species are translocated into the intermediate compartment where they assemble to form a pre-envelope structure. The 2 genomic RNAs are longer than genomic length. Due to different starting points of transcription, 2 distinct RNAs of about 3.5 kB are produced which encode the pre-C, core and pol ORF. The longer RNA (start of transcription at nucleotide 1,785) is responsible for translation of the pre-C/C ORF producing the soluble form of the HBc protein, the HBeAg. The second genomic RNA, the pregenomic RNA (start of transcription at nu-

cleotide 1,818) is responsible for translation of the core protein (HBcAg) and the viral polymerase. In addition, this pregenomic RNA species serves as template for reverse transcription of the viral minus DNA strand after encapsidation [75]. Since the rather small HBV genome of 3.2 kb has only limited coding capacity, the viral RNAs are synthesized from frame-shifted overlapping ORFs [51, 52, 64, 76, 77]. The HBc protein which is synthesized in the cytosol assembles independent of the HBs proteins into pre-core structures. These structures associate with mature pregenomic RNA species to form subviral core particles by an encapsidation-like process. Once the pregenomic RNA is encapsidated, it associates with the viral polymerase, a multifunctional enzyme. This enzyme then undergoes conformational changes and initiates reverse transcription within the pre-core particles. In a highly complex process which also requires accessory cellular proteins the negative DNA strand is synthesized, followed by synthesis of the complementary plus DNA strand which is also performed by the viral polymerase. At the end of this process, the mature, partially double stranded DNA genome of HBV (RC DNA) is formed within the nucleocapsid with the polymerase protein covalently attached to the HBV RC DNA. Details of this highly complex mechanism are reviewed in [4, 51, 52, 64, 72, 73] and references therein.

This process of viral DNA replication via an RNA intermediate and reverse transcription is unique for the hepadnaviridae which makes it a prime target for antiviral drug development as discussed below. The assembly of mature nucleocapsids containing full-length RC DNA occurs in the cytosol. They are then transported to the golgi apparatus, where they are incorporated into the preformed envelope structures made by the 3 HBs species. Once this multistep process is completed, the newly formed mature virions are then secreted to the cell surface via the constitutive pathway of secretion and subsequently released from the cell surface in a non-cytolytic manner [64, 78, 79]. The 3 HBs proteins have distinct and essential functions during morphogenesis and secretion, which was shown by analysis of mutant strains which were replication-competent but showed deficiencies in maturation and secretion [51, 80, 81].

HBV Mutants

Knowledge of HBV mutants arose from the development of vaccines and treatments against hepatitis B. For each treatment/vaccine developed, thus far resistant strains arose. The resistance could in large be attributed to specific mutations or genetic changes which in turn has led to further understanding of the molecular biology of the virus and further research towards treatment. Three major lines of prophylaxis and therapy can be summarized: i) vaccination; ii) treatment with HBIG or interferon; iii) drugs inhibiting specific steps in the life cycle of the virus, e.g. virostatic drugs.

One has to bear in mind that each therapeutic approach exerts a selective pressure on the corresponding pathogen to evade these constraints by e.g. mutations which will allow the pathogen to survive under the new conditions. Silent mutations which until then were undetected may now become the predominant variant since they allow survival under new therapeutic constraints. As with a lot of viruses and bacteria, each new approach for eradication has produced resistant genotypes, thus reflecting the genetic variability and plasticity of microorganisms, with the spread of resistance to antibiotics for the treatment of bacterial infections being a prime example for this dilemma. With the development of each new treatment or drug, one has to be aware of this problem, even in the case of viruses with genomes as small as that of the hepadnaviridae.

Vaccination Escape Mutants

Most currently available hepatitis B vaccines are based on cross-protecting epitopes of the S antigen, predominantly the a epitope. During a study on the immunogenicity and efficacy of hepatitis B vaccines in Italy, it was observed that some patients who had mounted a successful immune response following vaccination (anti-HBs-positive) subsequently became infected with HBV. Molecular analysis of these virus variants by sequencing their S gene revealed a distinct mutation within the a epitope resulting in an amino acid replacement from glycine to arginine at amino acid position 145. This point mutation was subsequently detected in isolates from resistant virus mutants worldwide [82–84]. The region in which this mutation occurred, the a epitope of HBs, is an important epitope for the binding of vaccine-induced neutralizing antibodies. It was shown that antibodies of this specificity were no longer able to bind to this region in the mutant virus, thus failing to efficiently neutralize the mutant virus. Since this mutant virus replicates at a normal rate, it was implied that the mutation in the S protein does not affect the overall efficiency of the binding of virions to the hepatocyte [8, 85]. Since these initial reports, several types of vaccination escape mutant strains were described. Initial fears that such mutants might become the predominant strains in a population and thus could pose a potential threat to vaccination have so far not proven true. In addition, Ogata et al. [168] showed that licensed recombinant hepatitis B vaccines could protect chimpanzees against infection with such an S gene HBV mutant (G145R) at amino acid position 145. However, close monitoring of the distribution and prevalence of these and especially of new strains should be maintained to minimize the risk of HBV outbreaks [85–89]. Further characterization of the immune response towards epitopes of the S protein showed that failure to respond to vaccination may also exist at the T-cell level [90]. In addition, such mutations in the S gene may also affect the polymerase protein and its function because of the overlapping reading frames for the surface and the polymerase protein (see below) [91].

HBIG Resistance Mutants

During treatment of liver transplant patients with HBIG, escape mutations were detected in a number of cases. Most isolates contained the same glycine to arginine shift as observed in the above mentioned vaccination escape mutants. This observation also confirms the finding that changes in the a epitope of the S protein allows the virus to evade antibody-mediated selective pressure [91–97]. The average overall reinfection rate under HBIG monotherapy ranges from 20 to 35%. Different rates were observed according to the treatment protocol (high vs. low dose), the clinical status of the patient, superinfection with hepatitis D virus and most importantly the virus load and replication status before transplantation [23]. HBV reinfection during the first 6 months after transplantation is usually related to inadequate HBIG levels in patients with a high viral load and/or active viral replication before transplantation, whereas late recurrence is mostly attributed to HBIG escape mutants [23, 94, 96].

Polymerase Mutations

The understanding of the unique features of viral DNA replication in hepadnaviridae via an RNA intermediate using reverse transcription has led to the development of several antiviral drugs aimed at inhibiting the HBV polymerase. Since retroviruses also use reverse transcriptase during their life cycle, most drugs which are now used and tested against HBV are derived from retroviral research and were initially developed as anti-HIV therapy. These substances are nucleoside analogues which block the active site for reverse transcription of the enzyme. Several molecules have been investigated, and among the first substances to be released as an antiviral drug was lamivudine, an enantiomer of 3'-thiacytidine. This drug showed significant reduction in viral replication, thus reducing the viral load to a non-detectable level [98]. However, shortly after this drug was widely used in hepatitis B treatment, including liver transplant patients, often fulminant cases of recurrence of viral replication were reported [99–107]. Analysis of the corresponding HBV strains revealed a mutation in the YMDD (Tyr-Met-Asp-Asp) motif of the polymerase protein located in domain C of the protein [52, 108, 109]. Numerous reports on lamivudine-resistant HBV strains have been published since [23, 110–116].

Although, compared to the wild type, strains with a single YMDD mutation have a markedly reduced replication rate, they become the clinically dominant viral species after only a few months of lamivudine treatment [52, 100, 109, 115–119], with the possible consequence of exacerbation of liver disease and graft failure in transplant patients, as described above. If the selective pressure of lamivudine is sustained, these YMDD mutants may regain their full replicating capacity by compensatory mutations, leading to fulminant progression of the infection [52, 115, 116, 119, 120–123]. Lamivudine resis-

tance is selected in approximately 14–32% of patients per year of therapy. More importantly, the prevalence of such mutations rises to approximately 70% after 4 years of treatment, with 100% of lamivudine resistance observed in some studies after several years of lamivudine monotherapy. Similar results were observed with famciclovir, another nucleoside analogue [23, 110, 112–114, 116, 124–128]. With regard to reinfection prophylaxis after liver transplantation, combination therapy with lamivudine and HBIG was shown to significantly reduce the reinfection rate to almost 0% in patients who were HBV-negative at the time of transplantation and 10% in patients showing viral replication at this time, and is currently the state of the art protocol for prevention of graft reinfection [23, 126, 129–138].

A resistant strain has also been identified for one of the most recently approved drugs of the nucleoside analogue family, adefovir dipivoxil [139], which is now widely used to replace lamivudine in patients with lamivudine-resistant HBV strains [112, 113, 116, 140–143]. Molecular and biochemical analysis of this strain showed that resistance was due to a mutation in the polymerase gene conferring resistance to adefovir dipivoxil. The mutation was identified by DNA sequencing and was shown to be not identical with the YMDD mutant strains, still susceptible to lamivudine and located in domain D of the HBV polymerase [139]. Another report of adefovir resistance which has been published since [144], describes a case of particular interest, since this strain first developed lamivudine resistance with a viral breakthrough under lamivudine therapy. The patient was then switched to adefovir, and lamivudine was discontinued. After the outgrowth of an adefovir-resistant strain, lamivudine was reintroduced while adefovir was maintained. Analysis of samples during the course of treatment revealed that after initiation of adefovir without lamivudine the lamivudine mutation reverted back to wild type followed by subsequent selection of adefovir resistance and regained susceptibility to lamivudine. It would therefore be important to elucidate whether these 2 distinct types of resistance are mutually exclusive or whether corresponding double mutations will be observed during a combination therapy with adefovir and lamivudine, with obvious consequences for such combination therapies [145].

Double Mutants

As described above, each antiviral agent or therapy has elicited specific resistance mutants, and combination therapy with e.g. HBIG and lamivudine was able to drastically reduce the incidences of reinfection. However, in the case of lamivudine resistance, compensatory mutants restoring replication fitness have been described which can be regarded as double mutants. Sequence information about the YMDD mutants revealed that due to the overlapping reading frames for the polymerase and the HBs protein, mutations in the YMDD

motif also affect the HBs coding region. It was speculated that due to such mutations, the antigenicity of the a determinant of the HBs protein might be altered, thus leading to double mutants with HBIG and lamivudine resistance. Furthermore, it was speculated that a sequential or a combination therapy with HBIG and lamivudine would produce the corresponding double mutations. Unfortunately, these predictions were confirmed by isolation of several double mutant strains [104, 106, 122, 123, 146–149]. Taking these findings into account, it can be stated that for every given antiviral agent or therapy resistance can be expected, which calls for close monitoring of patients under such treatment.

Conclusion

In this brief overview of the biology of HBV we showed that an effective therapy for hepatitis B resulting in complete elimination of the virus is still not available. The only way to prevent new infections and spreading of the disease is strict vaccination of newborns to achieve immunity across the entire population. Current prophylactic measures, such as HBIG, nucleoside analogues and vaccines, induce the outgrowth of escape mutants when used as monotherapy. Even with current combination therapies, double mutations are being detected, with the fatal potential of spreading within a population [150].

Despite the rather small genome size of 3.2 kb with overlapping reading frames, the HBV genome is capable of accommodating a variety of mutations to escape the selective pressure of current treatments. This may be made possible by 2 features of the HBV genome: the high mutation rate and the vast amount of virions produced in the phase of active replication. The mutation frequency for HBV is estimated to be approximately $1.4\text{--}3.2 \times 10^{-5}$ nucleotide substitutions per site and year, which is approximately 10-fold higher than for other DNA viruses [52, 151]. This can be explained by the re-

verse transcriptase function of the HBV polymerase which lacks a proofreading function [52]. In addition, the enormous viral replication rate leading to peak viral loads of 10^{10} virions/ml serum and an estimated half-life of the serum HBV pool of about 1–2 days lead to an estimated daily peak rate of de novo HBV production of up to 10^{11} [52, 152] and even 10^{13} virions [153]. These high viral loads and turnover rates coupled with the poor replication fidelity may account for a high prevalence of mutant HBV and thus an extended HBV quasi-species pool at any given time during active replication. The estimated daily peak rate of de novo HBV production led to the assumption that at the time of maximum viral replication every possible single and most double mutations would be produced each day [153]. The vast majority of these mutants will go unnoticed since they may not be as replication-competent as the wild-type virus. However, if a certain selective pressure is applied, as is the case with antiviral treatments, such resistant mutants may become the predominant species in numbers that allow for the selection of compensatory mutants which in turn may restore wild-type replication rates leading to fulminant outbreaks of the disease under therapies as described above. Most resistant HBV strains with either single or compensatory mutations were identified after or during monotherapy with one antiviral drug or treatment. Hence, in order to achieve full control of viral replication, a combination therapy aiming at different targets may be advisable and even necessary in order to raise the selective pressure to a level which can no longer be compensated by the HBV genome [20, 110, 116, 134, 154–161]. However, since even with current combination therapies double mutations are being detected with the potential of spreading in a population [150], close monitoring of such treatment programs is essential to avoid the emergence and spread of truly multiresistant HBV strains, similar to multiple resistance against antibiotics with its known set-backs in the treatment of bacterial infections.

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