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The diagnostic and clinical impact of the genetic variability of the S (surface) gene of hepatitis B virus

Diagnostische und klinische Relevanz der genetischen Variabilität des S- (surface) Gens des Hepatitis B Virus

Bernard Weber*

Laboratoires Réunis Kutter-Lieners-Hastert, Luxembourg Institut für Medizinische Virologie, Universitätskliniken Frankfurt, Germany

Abstract

The genetic variability of hepatitis B virus (HBV) represents a challenge for the sensitivity of immunodiagnosis, especially for the detection of surface antigen (HBsAg). There are two types of variants of HBV. Naturally occurring variants are the results of random changes selected over years of population pressure. These variants include HBV genotypes and unusual sequences, which may be poorly detected by immunoassays. The selected variants are mutants that arise in individuals under medically (vaccine, hepatitis B immune globulin and antiviral therapy) or naturally (chronic hepatitis B) induced immune pressure. HBV S-gene mutants have been identified in successfully immunized people worldwide. Based on the assumption that current vaccines containing S protein do not cross-protect against S gene mutants, a mathematical model predicts the disappearance of wild-type HBV in areas with HBsAg endemicity and the emergence of S gene mutants in approximately 100 years as a consequence of universal HBV vaccination. Mutant viruses may escape detection by commercial HBsAg kits. There are several reports on HBsAg negative carriers (HBV-DNA positive) of S gene mutants with immunosilent infection or "unusual" serologic constellations. Although S gene mutants have been found to be associated with a more severe clinical course of HBV infection and hepatocellular carcinoma, the clinical significance of the genetic variability of HBV genotypes and HBsAg mutants needs to be further investigated. Detection of HBsAg needs to be improved by the introduction of new HBsAg assays able to recognize S gene mutants described so far and with a lower detection threshold than current immunoassays in order to detect smallest amounts of HBsAg in low-level carriers. There is also a need for more complete epidemiological data on the prevalence of HBsAg mutants in Western Europe and assays for the (differential) screening of mutants need to be developed and evaluated.

Keywords: genotype; escape mutant; antiviral therapy; vaccination.

Zusammenfassung

Die genetische Variabilität des Hepatitis B Virus (HBV) stellt eine Herausforderung für die Sensitivität der immunologischen Diagnostik, insbesonders zum Nachweis des Oberflächenantigens (HBsAg) dar. Es gibt zwei Klassen von HBV-Varianten. Natürlich vorkommende Varianten sind das Ergebnis von zufälligen Änderungen, welche über Jahre in einer Population selektiert wurden. Zu diesen Varianten zählen die HBV-Genotypen und ungewöhnliche Sequenzen, welche unter Umständen mit Immunoassays schlecht nachweisbar sind. Selektierte Varianten sind Mutanten, welche in Personen, die einem iatrogenem oder natürlichem (chronische Hepatitis B) Selektionsdruck ausgesetzt sind, vorkommen. Diese Ausweichmutanten werden durch Impfung, Hepatitis B Immunglobulin- und antivirale Therapie selektiert. HBV S-Gen-Mutanten wurden weltweit bei erfolgreich immunisierten Probanden beobachtet. Basierend auf der Annahme, dass gängige HBV-Impfstoffe keine Kreuzimmunität gegenüber S-Gen-Mutanten induzieren, ist auf Basis einer mathematischen Modellrechnung, eine komplette Verdrängung des Wild-Typ-Virus durch eine S-Gen Mutante in ungefähr 100 Jahren in Regionen mit hoher HBsAg-Prävalenz zu erwarten. Virusmutanten werden z. T. nicht von diagnostischen Tests erkannt. Es gibt zahlreiche Berichte in der wissenschaftlichen Literatur über HBsAg-negative Virusträger (HBV-DNA-positiv) mit einer immunologisch-negativen Infektion oder einer ungewöhnlichen Serokonstellation. Obwohl S-Gen-Mutanten bei Patienten mit progredienter Hepatitis B oder hepatozellulärem Karzinom vorkommen, ist die klinische Bedeutung der genetischen Variabilität von HBsAg noch nicht eindeutig abgeklärt. Die Labordiagnose der HBV-

*Correspondence: Prof. Dr. Bernard Weber, Laboratoires Réunis Kutter-Lieners-Hastert, Centre Langwies, L-6131 Junglinster, Luxembourg
Fax: +35 2 78 88 94
E-mail: Web@labo.lu

Infektion sollte durch den Einsatz von neuen Tests, welche S-Gen-Mutanten erkennen und eine niedrigere Detektionsgrenze im Vergleich zu herkömmlichen Immunoassays besitzen um geringe HBsAg-Konzentrationen bei "low-level"-Träger zu erkennen, verbessert werden. Es besteht ein Bedarf für umfassende epidemiologische Daten zur Prävalenz von HBsAg-Mutanten in Westeuropa und für die Entwicklung und Evaluierung von Methoden zum (differenzierten) Screening von Mutanten.

Schlüsselwörter: Genotyp; Ausweichmutante; antivirale Therapie; Impfung.

Introduction

The surface antigen (HBsAg) is the most important marker for laboratory diagnosis of hepatitis B. HBsAg detection is used for the diagnosis of acute and chronic hepatitis B virus (HBV) infection, and it indicates potential infectiousness. It is also useful as a follow-up marker since declining concentrations are observed in resolving hepatitis B. Commercially available tests are based on the sandwich enzyme immunoassay (EIA) principle which uses monoclonal antibody (Mab) and/or polyclonal antibody against the "a" determinant of wild-type (and mutant) HBsAg. The specificity of HBsAg assays is over 99%, false positive results are observed with heparinized samples or are due to interferences with haemoglobin or bilirubin. Higher rates of false positives than in the general population are observed during pregnancy and in individuals with acute or chronic infections or suffering from autoimmune diseases or chronic liver diseases [1, 2]. The sensitivity of HBsAg assays is continuously improving, and the detection limit of licensed tests is under 0.5 ng ml^{-1}. "False" negative or atypical results are observed under the following circumstances [3]:

- Window period at the end of the incubation period prior to the onset of HBsAg synthesis in a high enough concentration to be detectable with immunoassays and in the post-acute or reconvalescence phase when HBs-antigenemia declines and the anti-HBs antibody response is under the detection limit of serological tests, the immune response is delayed or immune-complexes are present.
- Low-level carrier.
- Resolving infection "tail end" carrier, which corresponds to the progressive decay of HBsAg under the detection limit of the assay in individuals with chronic HBV infection who eliminate HBsAg over many years.
- S gene mutants and variants.
- HCV/HDV co-infection may interfere with HBV replication and/or HBsAg expression.

Since the late 1980s, there have been an increasing number of reports of HBV patients with atypical HBV serological markers, some of them even lack any HBV immunological markers. Analysis of HBV clones from these patients demonstrated variants and mutants. There is growing concern about the impact of HBV mutants on health care systems, since their prevalence is increasing in areas with high HBV prevalence, due to the selective pressure of national and international universal vaccination programs and the advent of antiviral therapy for chronic hepatitis B.

Molecular organization of the HBV genome and S gene

HBV, which is the prototype member of the family *Hepadnaviridae*, is a circular, partially double-stranded DNA virus of approximately 3200 nucleotides [4]. This highly compact genome contains the multiple overlapping open reading frames (ORF), including the preS/S gene (envelope), preC/C gene (preCore precursor protein, HBeAg and HBcAg), P gene (polymerase) and X gene (X protein); 67% of the genome is multiply coding. HBV encodes three closely related envelope proteins: small (S, HBsAg), middle (M, preS2) and large (L, preS1) [5, 6]. The envelope proteins of HBV span the lipid bilayer of the virus. They are involved in receptor binding, viral assembly and secretion. While the M protein is dispensable for virion secretion, both S and L proteins are required. Functional characterization via mutagenesis of the S and L genes has mapped two envelope regions essential for virion secretion [7, 8]. One region is localized in an internal domain of the S protein. The other region within the large envelope protein is localized in the C terminus of the preS1 domain (amino acids 107 to 119 of the adr subtype) and extends into the adjacent amino acids of the preS2 domain [7, 9, 10].

HBsAg is the major envelope protein, consisting of 226 amino acids. Computer analysis of topogenic element of HBsAg shows that there are at least three hydrophobic and two hydrophilic domains. The second hydrophilic region is exposed on the outer virion surface and is called major hydrophilic region (MHR) (amino acids 99 to 169) because it contains the major group and subtype-specific antigen determinants. The major B-cell epitope, called the "a" determinant, is located in the second hydrophilic region and consists of short chain of about 24 amino acids (amino acids 124 to 147, Figure 1). The "a" determinant is the major neutralizing epitope and is common to all the HBV genotypes and exposed on the surface of the virions. Antibodies directed to "a" confer protection against all the HBV genotypes. The "a" determinant is a conformational epitope made up of a hydrophilic two-loop structure kept by disulfide bonds between Cys124 and Cys137, Cys139 and Cys147, respectively, and projects out from the surface of the HBV particle (Figure 1). Based on the data of amino acid substitutions of the S gene and their effect on the antigenicity and secretion of surface antigen, the MHR may be separated in five functional areas related to the anti-

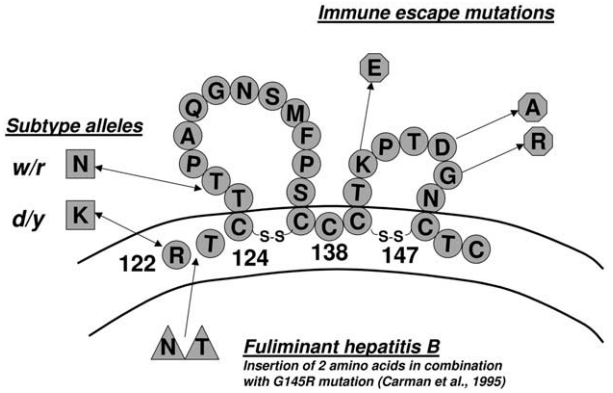

Figure 1 Amino acid sequence between aa 122 and 147 ("a" determinant) and its relationship to selected observed mutational changes.

genic effect of variants and mutants and their selection pressure, indicated as HBs1 to –5 [11].

S gene variants of hepatitis B virus

The HBV DNA template is transcribed by cellular RNA polymerase to pre-genomic RNA, which in turn is reverse transcribed to DNA by viral polymerase. A consequence of the unique way of HBV replication is a significant tendency to mutation. Due to the specific selection pressures within a host, a population of viral quasi-species emerges [12]. HBV mutants with survival advantages over the wild-type virus appear within the selective in vivo environment.

The mutation rate and the origin of HBV are uncertain. As the virus contains a polymerase enzyme without proofreading activity, error frequencies on RNA or DNA copying are likely to be of the order measured for retroviruses and other RNA viruses. Measurement of the rate of sequence change of HBV is complicated by the existence of overlapping ORFs and the lack of synonymous sites in most of the coding sequence, and amino acid changes in the pre-core and S gene region have a positive selective value and may occur as an immune evasion strategy [13].

HBV seems to exhibit a mutation rate more than 10-fold higher than other DNA viruses. The rates for nucleotide substitutions vary depending on the stage of disease. The natural evolutionary rate for the HBV genome in chronic hepatitis B is approximately 1.4 to 3.2×10^{-5} substitutions/site/year [14], whereas in the liver transplantation setting, it is almost 100-fold higher [15]. Mutations tend to cluster into mutational patterns: in particular, the basal core promoter (BCP), the pre-core region and the "a" determinant of the viral envelope [16].

There are two types of variants of HBV. Naturally occurring variants are the results of random changes selected over years of population pressure. These variants include HBV genotypes and unusual sequences, which may be poorly detected by immunoassays.

The selected variants are mutants that arise in individuals under medically or naturally (chronic hepatitis B) induced immune pressure. They include vaccine and hepatitis B immune globulin therapy (HBIg) escape mutants. In liver transplant recipients, high titer antibody in a situation with low titer virus and a large number of susceptible cells (liver transplant) is an ideal breading ground for mutants. In chronic HBV infection, naturally occurring escape mutants may be selected by the anti-HBs response of the HBV carrier [17, 18]. As a consequence, some chronic carriers have both HBsAg and anti-HBs, the antibody being directed against HBsAg epitopes not shared by the circulating antigen and thus not protective [17, 19].

Naturally occurring variants

By using subtype-specific antibodies against HBsAg, nine different serological subtypes were defined [20]. One determinant is common to all subtypes ("a"). There are also two pairs of mutually exclusive subdeterminants ("d" or "y", and "w" or "r"). Initially, HBsAg subtypes were used for studies of geographic distribution of HBV [20, 21]. However the HBsAg subtype does not reflect true genotypic variation [22]. The HBV subtype classification was based on a limited number of amino acid substitutions; sometimes the HBsAg subtype can be changed by a nucleotide point mutation of the S gene. Two amino acid residues encoded by S gene at codon positions 122 (d/y) and 160 (w/r) have been postulated to determine the different antigenic subtypes [23–25]. Since the HBV genotype is due to the entire nucleotide sequence, genotyping is more appropriate for investigation of geographic distribution and epidemiology.

Genotypically, based on sequence divergence in the entire genome of $>8\%$, HBV genomes have been classified into seven groups, designated A to G [4, 26]. The genotypes of HBV have distinct geographical distributions [27] (Figure 2). Genotypes A and D have global distributions, genotypes B and C are predominantly in East and South East Asia, genotype E is predominant in West Africa, and the most divergent genotype F is found exclusively amongst indigenous peoples in Central and South America [28–30].

HBV genotypes are associated with different clinical disease [11, 31–33]. Patients' responses to interferon therapy seem also to be associated with specific HBV genotypes [31, 32]. It was suggested that HBV genotype C is associated with a more severe liver disease and with a lower rate of response to interferon therapy [31, 32]. The emergence of resistance to antiviral therapy with lamivudine seems to be independent of the genotype but may be related to the presence of HBeAg. HBV carriers infected with genotype C are more frequently positive for HBeAg [34]. HBV genotype distribution is also closely

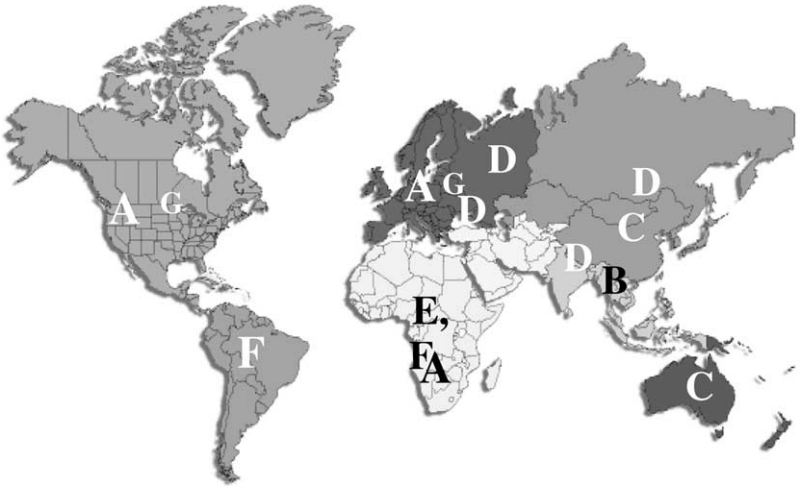

Figure 2 Geographic distribution of HBV genotypes.

related to the ethnic backgrounds of HBV-infected persons. Genotype A is predominant in whites, while genotypes B and C are predominant in Asians [27, 28, 35]. Since vertical infection is a major mode of transmission of infection in Asia, there is the question, which factor, viral genotype, duration of infection or ethnicity of the host, plays the most important role in the determination of the clinical course of HBV carriers.

The seventh genotype was proposed for an HBV isolate (AF160501) recovered in France which has a sequence divergence of $>11.8\%$ from HBV isolates of the other six genotypes [26, 36]. This atypical HBV strain was already detected and partially sequenced in 1991 but never recognized as a new genotype [37]. The virus structure is essentially identical to that of the other genotypes. Two remarkable traits characterize genotype G that are not shared by any HBV isolates of the other six genotypes. HBV/G has a slightly longer genomic length of 3248 bp in comparison to the other genotypes. The longer length of the HBV/G genome is attributed to an insertion of 36 bp at codon 2 of the core gene [26]. HBV/G has also two stop codons at positions 2 and 28 of the pre-Core region, either of which prohibits the translation of the HBeAg precursor [38, 39]. It seems as if HBV/G prevails in restricted areas in the world, possibly via particular routes of transmission. Its prevalence is particularly high in the US, since it exceeded 11% of all infections [26]. Despite the two stop codons in the precore region characteristic of HBV/G, HBeAg is detected in sera from individuals from whom HBV/G isolates are recovered [40]. Coinfection of HBV/G with HBV/A is frequent [26, 36, 41]. HBeAg in the sera of individuals infected with HBV/G would be attributed to the HBV/A with which they are coinfected. There is a close association between HBV/G and HBV/A genomes, since the homology within the S gene sequence of 94.6 to 97.5% is closer than the divergence of $>4\%$ in the S gene separating the six major genotypes A-F [42]. The similarity may be due to coinfection and the recombination between HBV/A and HBV/G [43]. HBV/G may be selected with seroconversion to anti-HBe, since the number of clones of HBV/G recovered from patients at different time points increases with the duration of follow-up [43].

Viruses selected by external pressure: mutants

Amino acid substitution within the "a" determinant can lead to conformational changes, which can affect the binding of neutralizing antibodies with several possible consequences:

1. Mutant viruses may escape detection by certain commercial HBsAg kits.
2. Clones of these viruses may have a selective advantage in carriers treated with passive/active immunization (Hepatitis B immune globulin (HBIg) or vaccine) and become the dominant clone. This process of selection and escape has been described during passive therapy with HBIg in HbsAg-positive liver transplant recipients and in newborns of HBsAg and HBeAg positive mothers who have become HBV carriers despite treatment with HBIg and vaccine.
3. Double escape mutants to immunologic detection and selective immune pressure.

The emergence of a HBV mutant able to escape vaccine-induced response was first suggested in Italy some 15 years ago [44]. Thirty-two (2.0%) of 1590 vaccinated people, including babies born to HBsAg carrier mothers, became HBV infected despite successful immunization [45]. All cases showed co-existence of HBsAg and anti-HBs. In one infant, serious disease occurred. The virus from this patient was an escape mutant with a different sequence from that of the isolate from the mother. Meanwhile, vaccine-induced escape mutants have been reported from many countries throughout the world [45–51]. The far most important and best-documented mutation is the replacement of the glycine residue at position

145 by arginine (G145R) due to a point mutation (G to A) at nucleotide position 587.

This mutant is stable over time and may retain its ability to replicate at high titer for several years [45]. It can be transmitted horizontally to other humans. Intra-familial horizontal transmission was reported among 3 of 10 infants who carried the G145R mutant despite the presence of high levels of anti-HBs [52]. G145R mutant can also persist in the peripheral blood leukocytes (PBL) of HBsAg negative individuals from the non-vaccinated random population and can be transmitted horizontally among the family members although none of them has received immunoprophylaxis against HBV or has clinically apparent disease or any other known risk factors of HBV infection [53].

Meanwhile, there have been several reports on HBV S-gene mutants affecting amino acid positions 120, 123, 124, 126, 129, 131, 133, 141 and 144 of HBsAg [54–61]. The most relevant mutations seem to be amino acid substitutions of G145R, K141E and T131I and insertion of 3 amino acids between residues 123 and 124, since they markedly affect the antigenic structure of HBsAg [54]. The epidemiology of HBV mutants has been studied in African and Asian countries. The situation in Europe remains unclear. It is expected that the prevalence in the general population, with the exception of Mediterranean regions, is relatively low. In Western Europe and the US, the occurrence of HBV mutants is mainly restricted to risk groups, including chronic carriers, liver transplant recipients and breakthrough infections in newborns despite successful immunization [55, 58, 60]. While S gene mutants are responsible only in 0.2 to 4.6% of the cases for breakthrough infections despite passive/active immunization of the newborn [55, 62], up to 40% of HBIg treated liver transplant recipients may develop S-gene escape mutants [58, 60].

The prevalence of S gene mutants in the general population is modulated by the interaction of three factors:

1. Prevalence of HBV infection and chronic carriers: In regions (Sub Saharan Africa, South East Asia, Alaska and certain South American countries) with high HBsAg endemicity (HBsAg prevalence $>8\%$), the prevalence of mutants is high since during chronic infection, mutants are selected out of the quasi-species circulating in the peripheral blood by the immune pressure due to the natural cellular and humoral immune response. In Papua New Guinea, 2% of HBV infected pregnant women are carriers of HBV escape mutants [11].
2. Vertical transmission of HBV: It is estimated that at least 23% of the carriers in Asia and 8% of the carriers in Africa occur as a result of perinatal infection [63]. The most important feature of HBV infection in the newborn is its chronicity. Due to the immature immune response of the new born, HBV infection persists lifelong in a high percentage of vertically infected individuals and constitutes an excellent breeding ground for escape mutants.
3. Immunoprophylaxis of vertical HBV infection and universal vaccination campaigns in high endemicity areas: Due to the high prevalence of virus circulating in the general population and the selective immune pressure, the incidence of emerging vaccine escape mutants is increasing (see also chapter on vaccination).

Clinical significance of HBV S-gene mutants

The clinical significance of S gene mutants needs, in analogy to that of HBV genotypes, to be further investigated. There have been reports of mutant strains recovered from patients with fulminant HBV infection, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [60, 62, 64]. However, HBV S-gene mutants may be also present in HBsAg-negative asymptomatic carriers without any sign of hepatic injury. Grethe et al. [65] reported the presence of S gene mutants in sequential serum samples in an asymptomatic HBV carrier over a period of three years. The HBV strains were both diagnostic and immune escape mutants, the structure of the major antigenic determinants was severely affected by multiple amino acid exchanges which did not prevent oligomerization and secretion of envelope proteins. Mutations in the HBV genome associated with fulminant hepatitis B (FHB) include mutations affecting HBeAg production [66], HBV variants encoding changes in the core protein [67] the preS2 gene [68], and S gene including the hepatitis B immune globulin (HBIG), vaccine and antiviral therapy escape mutants (for review see [64]). The G145R mutation in combination with an insertion in the “a” determinant between amino acids 122 and 123 of the viral envelope was observed in a HBsAg negative case of fulminant reactivation of hepatitis B [46]. Ghany et al. [60] and Kalinina et al. [69] described G145R mutations in conjunction with clusters around codons 40–45, 114–122 and 198–208. The region around 44–49 is within the major histocompatibility class I-restricted T-cell epitope of HBsAg. Liver transplant patients infected with escape mutants 144 or 145 showed a worse clinical outcome compared to other patients on high-dose, long-term HBIg prophylaxis with “wild-type” virus (44% vs. 23% graft failure caused by HBV infection) [58]. A combination of viral factors and/or host factors directing the vigor and character of the antiviral inflammatory responses are probably involved in the pathogenesis of FHB [70].

Generally, there seem to be no amino acid substitutions in the “a” determinant in acute hepatitis. Genetic alterations of the genome and especially of the S gene are increasing with the progression of the disease, and there is an accumulation of S gene mutations in HBV-related end-stage liver disease [71, 72]. Insertions between positions 122 and 123 that have been reported

in combination with the G145R mutation in fulminant hepatitis [46] are also found in HBV isolates of HBsAg negative patients with chronic liver injury [17, 73].

Abnormal expression of certain HBV gene products can result in cytoxicity with the appearance of ground-glass cells and the eventual development of heopatocellular carcinoma [74]. Furthermore, the association of intracellular accumulation of viral proteins with cirrhosis and fibrosing cholestatic hepatitis has been described. A preS mutation that accounted for virus retention and misassembly was isolated from a patient with chronic HBV infection [75]. Mutations that account for viral retention may contribute to a more progressive form of liver disease [75]. Once a chronic infection is established, several mechanisms are potentially operative to perpetuate viral persistence. High mutational rates associated with hepadnaviral replication can lead to the selection of immune escape mutants at both the antibody and cell-mediated levels [70]. The question whether HBV S-gene escape mutants are rather the consequence of chronic infection, as they are selected as a minority self-variant by the immune pressure over time, or if they are themselves implicated in the generation of a chronic carriage or more severe clinical course than wild-type virus remains unanswered.

HBV S-gene mutants and antiviral therapy

The S gene is completely overlapped by the polymerase gene. Mutations in the S gene may produce functionally significant changes in the overlapping polymerase gene, conversely mutations in the polymerase gene may produce changes in the S gene [76]. With the advent of antiviral treatment and prophylaxis protocols using both HBIg and nucleoside analogues in the transplantation setting, new HBV mutants have been selected with changes in the polymerase as well in the envelope protein [64, 76, 77]. The selection of vaccine escape mutants by HBV vaccination or HBIg is associated with changes in the S gene that are accompanied by mutations in the fingers sub-domain of the polymerase protein (rt) of rtM204I and rtL180M/M204V [76]. The HBV polymerase mutants appear to be replication impaired, both in vitro and in vivo, however compensatory mutations in the polymerase protein may restore the replication phenotype of these HBV mutants [77]. The G145R mutation also results in a concomitant change in the HBV reverse-transcriptase region of the polymerase at codon rtR/W143Q [78]. Although drug resistance develops, the clinical course after selection of antiviral-resistant HBV mutants seems to be benign. However, mutants with amino acid changes in the "a" determinant at P120T or G145R, which also contained lamivudine-resistant mutations (YMDD (tyrosine, methionine, aspartate, aspartate) motif of the polymerase protein) were reported by Bock et al. [79] from cases of severe clinical course of hepatitis B infection in liver transplant recipients after the emergence of lamivudine resistance. These "double" mutants replicated to a greater extent in vitro in the presence of lamivudine than in control cultures. The lamivudine-enhanced replication and the severe and fatal course associated with these mutants suggest that continuation of therapy could be deleterious in some patients. The ability to change a viral protein by mutations in overlapping but unrelated viral gene may produce HBV mutants with altered antigenicity and/or replication and a natural history that may be distinctly different to wild-type HBV.

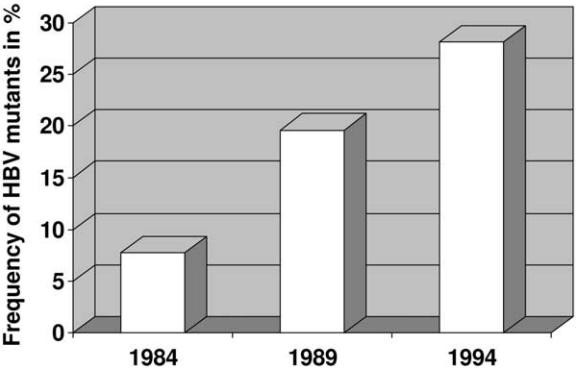

Figure 3 Increase in the prevalence of HBV mutants (mostly G*145*R) over 10 years after the introduction of universal vaccination in Taiwan. The rate of mutation was higher among fully immunised children than among those unvaccinated (data from Hsu et al. [80]).

S gene variants/mutants and vaccination

In Taiwan, the frequency of escape mutants (mostly G145R) in the population has increased from 7.8% to more than 25% over ten years after the introduction of universal vaccination [80] (Figure 3). The rate of mutations was higher among fully immunized children than among those unvaccinated. Based on the assumption that current vaccines containing S protein do not cross-protect against S gene mutants, a mathematical model predicts the disappearance of wild-type HBV and the emergence of G145R in approximately 100 years [81]. Immune escape of mutant strains of HBV in vaccinated individuals exists as well at the B- and at the T-cell level. Anti-HBs binding to synthetic peptides from the "a" determinant is significantly reduced by the G145R substitution and by changing the amino acid sequence from adw2 to adr in vaccinated individuals [82]. By comparing the class-II restricted cell responses to the so far known 4 T-cell epitopes of wild-type virus and naturally occurring variants, a significant loss of T cell reactivity was observed with certain variants in a high percentage of vaccinated individuals [83]. Stimulation of T cells also induces the secretion of antibody to HBsAg by specific B cells. However, those peptides that fail to activate T

cells are also unable to induce any significant anti-HBs production [83].

With universal vaccination, it is of concern that S gene mutants may become dominant strains, infecting even people with protective levels of anti-HBs. The addition of appropriate HBsAg to current vaccine may be a possible solution for the prevention of infections by escape mutants.

Not only escape mutants may account for vaccine failure. There are antigenic differences between the vaccine strains of HBsAg/ adw subtype and the predominant HBsAg subtype circulating in West Africa [84]. The consequence is that, for example, children in Gambia vaccinated against hepatitis B show serological evidence of breakthrough infections, particularly if antibodies induced by the vaccine are low in titer. These findings raise the questions whether the international admitted cut-off of 10 mIU ml^{-1} for immunity is sufficient to warrant protection against all HBV genotypes and whether a higher value, for example 100 mIU/ml^{-1}, should be considered, especially in geographic areas where variant HBV infections emerging in the face of increasing herd immunity as a result of herd vaccination are frequent.

Mechanisms of the loss of antigenicity of HBsAg

Different mechanisms intervening at the translational or post-translational level, including conformational changes, hydrophobic changes, insertion of basic residues and reduced synthesis or secretion of HBsAg may account solely or in conjunction for escape mutations, immunosilent HBV infection or unusual serological patterns. Mutation in other parts of the gene (preS1) or genome (precore and core) of HBV may also affect the secretion of HBsAg and assembly of mature virus particles. When virus multiplication is strongly decreased through mutation in other parts of the genome and HBsAg expression is inhibited, very low HBsAg titers (low-level carrier) might be responsible for the failure of HBsAg detection, independently of substitutions concerning the surface antigen [85–87].

Substitution at position 129 introduces a new asparagines-linked (N-linked) glycosylation site [88]. The created putative glycosylation site in the mutant HBV may change the antigenicity of HBsAg. Mutation in simian immunodeficiency virus, which newly introduced glycosylation, allowed the virus to escape neutralization by antibodies that could neutralize the parental virus [89].

The "a" determinant is a highly conformational array of overlapping epitopes whose structure has not yet been solved. So, hydrophobic changes may cause structural alterations of the antigenic epitope in the MHR, leading to difficulty in detecting HBsAg. A reduction in hydrophilicity is often accompanied by the loss of antigenicity [88]. Transfection experiments with escape mutants to HBIg and lamivudine therapy after liver transplantation revealed that the D144E mutation reduces HBsAg affinity to anti-HBs [90].

Mutations close to the "a" determinant can change the immunodominant region structure and therefore alter the group-specific determinant antigenicity even though mutations are not present within this region, for example mutations of amino acid positions 118 and 120 adjacent to the "a" determinant change its structural configuration [91, 92]. The lack of detection of HBsAg in the presence of low levels of viral replication may be caused by the existence of viral genomes harboring deletion in the pre-S1 region that affects the S promoter, thereby producing a reduction of the HBsAg synthesis [93]. Cabrerizo et al. [93] observed that HBsAg-negative hemodialysis patients and dialysis-unit staff members who had suffered acute hepatitis B that resolved previously were infected by a mixture of the wild-type virus and a deletion mutant in the pre-S1 region. This deletion (amino acids 58–118) affects the S gene promoter with a reduction of the synthesis of HBsAg. Substitutions at amino acid positions 112 to 114 dramatically reduce the virion secretion and give raise to a low secretion phenotype [94]. A change of threonin to alanin in position 118 in a case of chronic liver disease with no serological markers of HBV infection but HBV-DNA positive had a destabilizing effect on the structural integrity of the "a" determinant and altered the antigenicity profile of the mutant HBsAg [92]. Thus, a considerable decrease or complete absence of properly folded surface antigen may explain the absence of reactive HBsAg in the serum of the chronic HBV carrier. Furthermore, a RNA hairpin loop was predicted for the transcript generated by the small surface protein of this mutant, which could have an inhibitory effect at the translational level. Another case of a preS mutation isolated from a patient with chronic hepatitis B infection was shown to lead to virus retention and misassembly [75]. Sequence analysis of the HBV genome revealed two deletions and a point mutation at the regulatory CCAAT element of the S promoter. Analysis of the mutant strain revealed an inverse ratio of S gene products in comparison to wild-type HBV that leads to intracellular viral retention, atypical intracellular distribution of HBV proteins, enhanced nuclear localization of HBV DNA and malformation of the extracellular viral particles.

The mutation Pro 120 Ser is another example for an escape mutation outside the "a" determinant in immunized individuals, which affects the binding of antibodies to the second loop of the "a" determinant [95]

A core promoter mutant dinucleotide substitution may be frequently found in patients with occult HBV infection. This mutant can be associated with the absence of circulating HBsAg in these patients [96].

The interaction of mutations may be very complex, an example is given by the case of a cirrhotic HBsAg negative patient who harbored two different HBV populations, one of genotype A and the other of genotype D [97]. The genotype D virus was a precore mutant, both

genomes had a mutation at the basal core promoter and the genotype D virus was also mutated in the "TATA box" of the large surface antigen promoter. HBsAg of genotype D showed multiple mutations of the "a" determinant. HBsAg of the genotype A virus was very weakly recognized by commercial tests, whereas HBsAg from HBV/D was recognized but was mainly retained within transfected cells, probably because of an excess of large surface antigen.

HBV variants/mutants and diagnostic assays

Mutant viruses may escape detection by commercial HBsAg kits. There are several reports on HBsAg-negative virus carriers (HBV-DNA positive) with immunosilent infection or "unusual serologic constellation, these include [11, 45, 65, 85, 88, 98]:

- Individuals with isolated anti-HBc reactivity
- Patients with discordant results between HBsAg assays.
- Patients seronegative for HBsAg but HBeAg positive
- Patients seronegative for HBsAg but anti-HBc and anti-HBs positive (serologic constellation of resolved HBV infection)
- Presence of both HBsAg (HBeAg) and anti-HBs (mostly at low titer <100 mIU ml^{-1})

The great danger represented by HBV S-gene variants/mutants is that they can be transmitted horizontally by the peripheral blood of asymptomatic carriers with no known risk factors of HBV infection. The presence of G145R mutants in the PBL may be associated with immunosilent carriage (HBsAg and anti-HBc negative) [53]. Alternatively, immunosilent HBV infection may be observed in HBV carriers of double mutants with amino acid substitutions in the "a" determinant of the S antigen as well as in the C gene leading to truncated HBeAg and core proteins [99]. There is great concern that escape mutants may be spread by blood transfusion, sine HBV DNA detection by nucleic acid amplification technology (NAT) is not mandatory for blood donor screening in many countries, especially in those areas where the prevalence of escape mutants is expected to be high.

Isolated reactivity to anti-HBc (HBsAg and anti-HBs negative) is a relatively frequent seroconstellation (up to 1% of volunteer blood donors) that is generally observed after resolved HBV infection (loss of anti-HBs or low-level anti-HBs). This serological pattern is also very frequently associated with HBsAg negative chronic low-level HBV DNA carriage [3]. The reasons for the absence of HBsAg detection are the presence of HBsAg mutants, inhibition of expression by HCV or HDV co-infection and/or low-level HBsAg synthesis under detection limit of screening assays and the presence of immune complexes (for review see [100]). Weinberger et al. [101] observed that the exchange rate per amino acid in the "a" determinant in isolates from isolated anti-HBc positive individuals was significantly higher than in the residual parts of the molecule and even higher than in the same region of the HBsAg positive controls. On the other hand, sequences were found from solely anti-HBc positive individuals, who were previously published to stem from "normal" HBsAg-positive HBV carriers.

HBsAg (HBeAg) and anti-HBs (mostly at low titer <100 mIU ml^{-1}) may be present simultaneously if the antibody being directed against HBsAg epitopes is not shared by the circulating antigen and thus not protective [17, 19]:

- Deletion in the S gene may be associated with persisting viremia in the presence of anti-HBs. Direct sequencing of the HBV genome revealed a major population of DNA molecules with a deletion of nucleotide 31 of the HBs gene in a patient with anti-HBs seroconversion but who still remained HBV carrier with a virus load of 10^4 DNA molecules ml^{-1} of serum [102]. This deletion led to a frame-shift and introduced a stop-codon after 21 amino acids of HBs. The resulting HBsAg lacking the major epitopes recognized by specific antibodies could favour ongoing viral replication despite the presence of anti-HBs. However, because the reading frame of the polymerase was also severely damaged by this deletion, it is possible that a minor population of intact genomes was present to help in the formation of virus particles.
- Amino acid replacement from Thr or Ile to Ser at codon 126 in the "a" determinant was observed in the majority of the clones from two HBV carriers who had antibodies to HBs and were also HBeAg positive [103]
- Mutations close to the "a" determinant may induce changes in the structural configuration so that it is not efficiently neutralized by anti-HBs antibody but still recognized by the antibody of the anti-HBs antibody of the diagnostic enzyme immunoassay [91].

The analytical sensitivity of HBsAg assays is also dependent on HBV genotype or subtype. By testing dilution series of HBV subtypes from the French Centre National de la Transfusion Sanguine up to 10-fold differences the sensitivity of three commercial assays were observed (Figure 4). HBsAg detection with monoclonal antibody-based diagnostic assays may be unreliable in populations where the circulating subtypes/genotypes or variants are distinct from the virus strain used for the production of monoclonal antibody.

Future perspectives

Laboratory diagnosis of HBV mutants

Laboratory diagnosis of HBsAg mutants needs to be improved by the introduction of new HBsAg assays able to recognize so far described S gene mutants and with lower detection thresholds than current immunoassays in

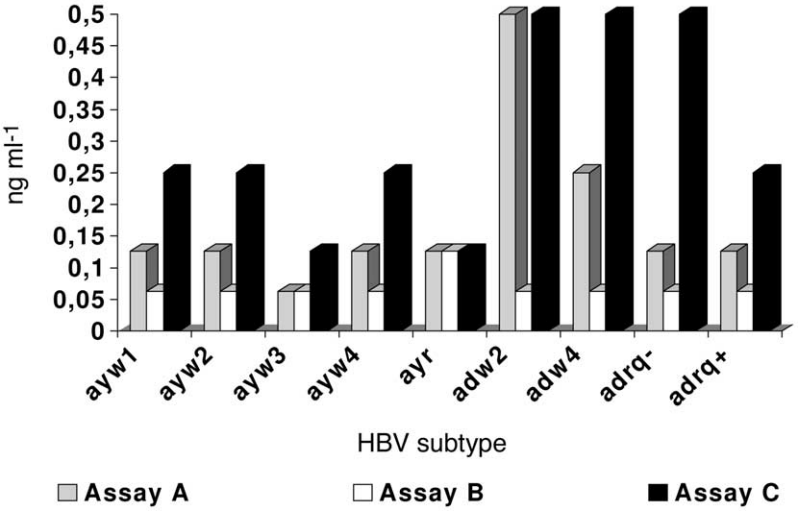

Figure 4 Sensitivities of HBsAg immunoassays for HBV subtypes with the "Centre National de Transfusion Sanguine" (CNTS) panel. Assay A shows a comparable analytical sensitivity for all HBV subtypes, the two alternative HBsAg immunoassays show a highly variable detection threshold dependent of the HBV subtype.

order to detect smallest amounts of HBsAg in low-level carriers.

Modification of commercial assays is necessary to avoid false "negative" results due to the presence of diagnostic escape mutants [46, 57]. Polyclonal-antibody-based assays do not guarantee full sensitivity [88, 104]. Mutations of cysteine 149 to serine or of glycine 145 to arginine (G145R), lysine or glutamic acid lead to a loss of cross-reactivity [105]. Preliminary data indicate that immunoassays using a mixtures of monoclonal antibodies which detect wild-type as well as mutant HBV or assays that are monoclonal antibody cross-reactive based with wild-type and mutant strains achieve a higher sensitivity in dilution series of recombinant expressed HBsAg variants than conventional assays (Figure 5). However, evaluation of the immunodetection of various HBsAg mutants remains difficult, as the occurrence of these mutants is not very common and sufficient volume of serum samples is difficult to obtain. As a substitute, recombinant antigens are constructed to reflect mutations described in the literature occurring throughout the S gene [100]. Dilution series of crude extracts or purified antigens in HBV-negative pooled sera are used for the optimization and evaluation of the performance of diagnostic immunoassays. Several studies comparing the ability of licensed immunoassays EIAs to detect S gene mutants have been performed [72, 100]. A new HBsAg assay showed a significantly better performance for the detection of the most relevant HBV S-gene mutants [100]. However this assay was less sensitive for the detection of wild-type virus.

More sensitive HBsAg assays may also detect low-level HBsAg in HBV mutant carriers. Of 2000 antenatal clinic attendees in Papua New Guinea, 5% of HBsAg positive

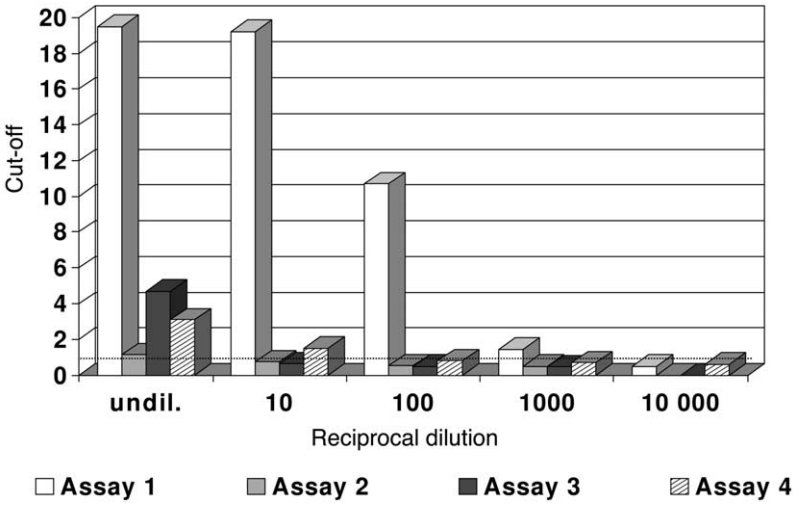

Figure 5 Sensitivities of HBsAg immunoassays for a recombinant HBV S-gene mutant. Assay 1, based on a mixture of monoclonal antibodies against S gene mutants, shows a significantly higher sensitivity in dilution series of a recombinant mutant than conventional assays.

subjects were negative in a widely used monoclonal assay but PCR positive. The monoclonal assay had a sensitivity of 0.5–1 ng ml^{-1}. These samples were reactive in another assay with a sensitivity of 0.1 ng ml^{-1}. Over 50% of these discordant samples had rare or unique variants of the major hydrophilic region of HBsAg [81]. These data demonstrate that the sensitivity of current screening HBsAg assays should be increased, arguing for implementation of detection of HBV DNA in blood donors. With a sensitive PCR protocol (detection limit of less than 50 genome equivalents ml^{-1}), a prevalence of HBV DNA up to 40% in isolated anti-HBc reactive individuals was observed [106]. These individuals are mostly chronic HBV carriers and show very low virus concentrations in their sera but can nevertheless transmit the infection [107–109]. Alternatively, anti-HBc testing should be used in combination with HBsAg for the screening of blood donors, especially in those countries where HBV DNA detection by nucleic acid amplification technology (NAT) is not mandatory or in lower-income regions of the world. A further argument for anti-HBc screening is that the viral load in chronic isolated anti-HBc positive carriers is low and there is a potential risk for failure of HBV DNA detection with pool-PCR in blood donors. Anti-HBc screening would reduce the residual risk (for review see [3]). The probably relatively poor cost/benefit relation and the crucial issue of increased loss of donors by poor specificity or incorrect result interpretation are major arguments against the routine testing of blood donors, especially in countries with low incidence of HBV infection and where donor screening by pool HBV DNA testing is already performed. In order to permit a more cost-effective screening of blood donations, combined assays for anti-HBc/HBsAg need to be developed. In the field of HIV screening, combined antigen/antibody assays have now achieved, after five years of experience and technical improvements, a high sensitivity and specificity [110]. Combined anti-HBc/HBsAg should be from a technical point of view more easily achievable than p24Ag/HIV antibody detection.

Epidemiology and vaccine development

There is a need for more complete epidemiological data on the prevalence of HBsAg mutants in Western Europe and assays for the differential screening of mutants need to be developed and evaluated. Current methods for the detection of HBV mutations are time consuming, labor intensive and not suitable for screening large numbers of samples.

Recently, an immunoassay based on a monoclonal antibody able to recognize both wild-type and mutant HBsAg was evaluated for its ability to detect HBV escape mutants in patients transplanted for HBsAg-positive cirrhosis and receiving HBIg therapy. By running the new assay in parallel with an immunoassay that does not recognize HBsAg mutants, 4 of 12 patients who reinfected the graft despite receiving HBIg were identified as having a recurrence due to surface antibody escape mutants. In the remaining 8 patients, reinfection was due to wild-type HBV [111]. This serological approach allows rapid and cost-effective screening for HBsAg escape mutants in the liver transplant setting. Furthermore, an assay able to reliably recognize mutants could be used for the screening of blood donors and for epidemiological studies.

Real-time PCR with fluorescent hybridization probes represents a specific, sensitive, quantitative and rapid method for the detection of HBV mutants [112]. With a detection limit of 100 copies HBV DNA ml^{-1}, as few as 5% of mutants among wild-type virus can be detected.

There is also a need for the development of a true neutralization assay in order to evaluate the potential of mutants to escape passive or active immunization or natural immunity. A surveillance network should be created to detect HBV mutants worldwide in order to determine the risk of HBV infection from S gene mutants following prophylactic or post-exposure immunization. In those countries where there is a rapidly growing increase in the prevalence of G145R mutants due the selective pressure of universal vaccination, the inclusion of mutant HBsAg in HBV vaccine should be emphasized.

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