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Occult Hepatitis B Virus Infection: Detection and Significance

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Hepatitis B virus DNA · Nucleic acid amplification technique · Anti-HBc · Anti-HBs · HBsAg · Escape mutant

Abstract
The Taormina Consensus Conference defined 'occult hepatitis B virus (HBV) infection' (OBI) as the 'presence of HBV DNA in the liver of individuals testing HBsAg-negative with currently available assays'. Most occult is the so-called 'window period' after exposure before HBV DNA appears in the blood. We identified two blood donors whose donations tested HBsAg- and HBV DNA-negative, but transmitted HBV. Both subsequently developed HBsAg and acute hepatitis. However, such cases are not considered as true OBI. A true transient OBI remains HBsAg-negative during the entire course. One case of acute OBI showed a peak viremia of 15,000 IU/ml HBV DNA and sub-borderline HBsAg, suggesting a ratio of virions to subviral particles of 1:10, whereas 'normal' cases show at peak viremia a ratio of 1:3,000. Blood donors with OBI may transmit HBV. We studied 5 blood donors with OBI and 55 of their recipients. In 22 recipients, transmission was probable, but they remained healthy. However, in 3 recipients, who were immunosuppressed at the time of transfusion, fatal fulminant hepatitis B developed. The majority of anti-HBc-positive healthy individuals have HBV DNA in the liver which may start replication under severe immunosuppression. Nine such cases are described here. OBI or reactivated HBV infections often lead to selection of HBsAg escape mutations as we could show in 11 of 14 cases. Infection of vaccinated individuals favors development of OBI as we observed in 6 blood donors. HB vaccination may solve the problem of overt HBV infection but may favor OBI.

Introduction

Together, hepatitis B and C viruses (HBV, HCV) rank second after HIV in mortality caused by viruses. The HBV-associated diseases are mainly caused by cytotoxic immune reactions. Thus, the course of HBV infections is highly variable, ranging from transient silent infection to chronic hepatitis. About 40% of the world's population have been infected with HBV, and 370 million people are chronic carriers of hepatitis B surface antigen (HBsAg). Every year, one million people die from complications such as liver cirrhosis, liver failure or hepatocellular carcinoma. Usually, HBV-associated disease is accompanied by HBs antigenemia. The clinical significance of occult (i.e. HBsAg-negative) HBV infections is still under debate. In this brief article, we discuss (i) the significance of

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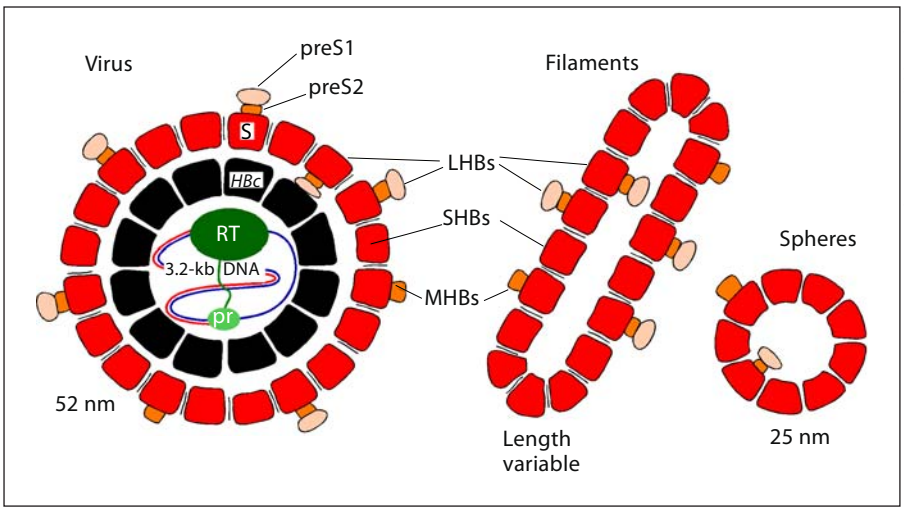

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Fig. 1. Model of HBV and subviral HBsAg particles. The diameter of 52 nm refers to the outer limit of the hydrated virus. The 3 co-terminal surface proteins with the 3 domains S, preS2 and preS1 are shown in grey or red. RT = Reverse transcriptase; pr = primer domain of the polymerase; HBc = core antigen.

occult HBV infections (OBI) for the safety of blood donations, (ii) the problem of OBI reactivation under immunosuppression, (iii) the role of escape mutants in OBI and (iv) the effect of vaccination on the incidence of OBI.

Brief Update on the Viral Life Cycle

HBV has several discoverers, but in 1970 David Dane first described virus-like particles in blood from hepatitis B patients. He and others immediately noted a large number of other, smaller particles in such samples. These subviral particles consist of the viral surface proteins, known as HBsAg. There are three co-terminal HBs proteins, small, middle and large, with the S, preS2 and preS1 domains (fig. 1). The viral core, HBc, encloses the small DNA and its polymerase. It is important to note that the round subviral particles are produced in large excess [1].

The intracellular life cycle of the virus is shown in figure 2. The virus comes from the blood, somehow passes the endothelium and reaches the hepatocytes. There, the preS1 domain interacts first with cell-bound heparan sulfate and, subsequently, with an unknown high-affinity receptor on the hepatocyte membrane [2]. Entry in the hepatocyte follows a novel, but not yet understood, endocytotic pathway [Kott and Glebe, unpubl.]. The core particles are released into the cytosol and transported along microtubules to the nuclear pore complex [3]. In the basket of the nuclear pore complex, the genome is released and enters the nucleoplasm [4] where it is transformed by cellular DNA repair factors to the covalently closed circular (ccc) DNA. This form of the viral genome can stay in the nucleus and replicate via reverse transcription for the entire life span of the infected hepatocyte. cccDNA expresses the viral pregenomic RNA which also functions as bicistronic mRNA for the core protein and the viral polymerase. The core protein, the polymerase and their mRNA/pregenome assemble in the cytosol into immature core particles. The RNA is reverse transcribed into DNA within the core particle [5].

The small circular DNA genome encodes only four open reading frames which are largely overlapping. In spite of this crowded genome, the virus can be extremely variable if it is under selective pressure. Following DNA synthesis, the mature core particles expose signal sequences which lead to nuclear transport and consequently to intracellular amplification of the viral genome [4]. Alternatively, the mature cores expose structures which interact with the so-called matrix domain of the large viral surface protein. This leads to envelopment of the cores by membranes containing the three HBs proteins [6], and finally to exocytosis via the multivesicular bodies [7]. Thus, entry and exit of the virus can function without damage to the cell. Since HBV is not well recognized by the innate immune system [8], it is not directly cytopathic.

The normal resolving acute hepatitis B is characterized by several months of asymptomatic replication whereby the virus can reach very high titers in the blood (fig. 3). Thereafter, the viral antigens are recognized by T cells which suppress viral expression and replication by secretion of cytokines and cytotoxic mechanisms. The decrease of viremia starts shortly before the onset of clinical disease. HBsAg levels initially seem to lag behind vi-

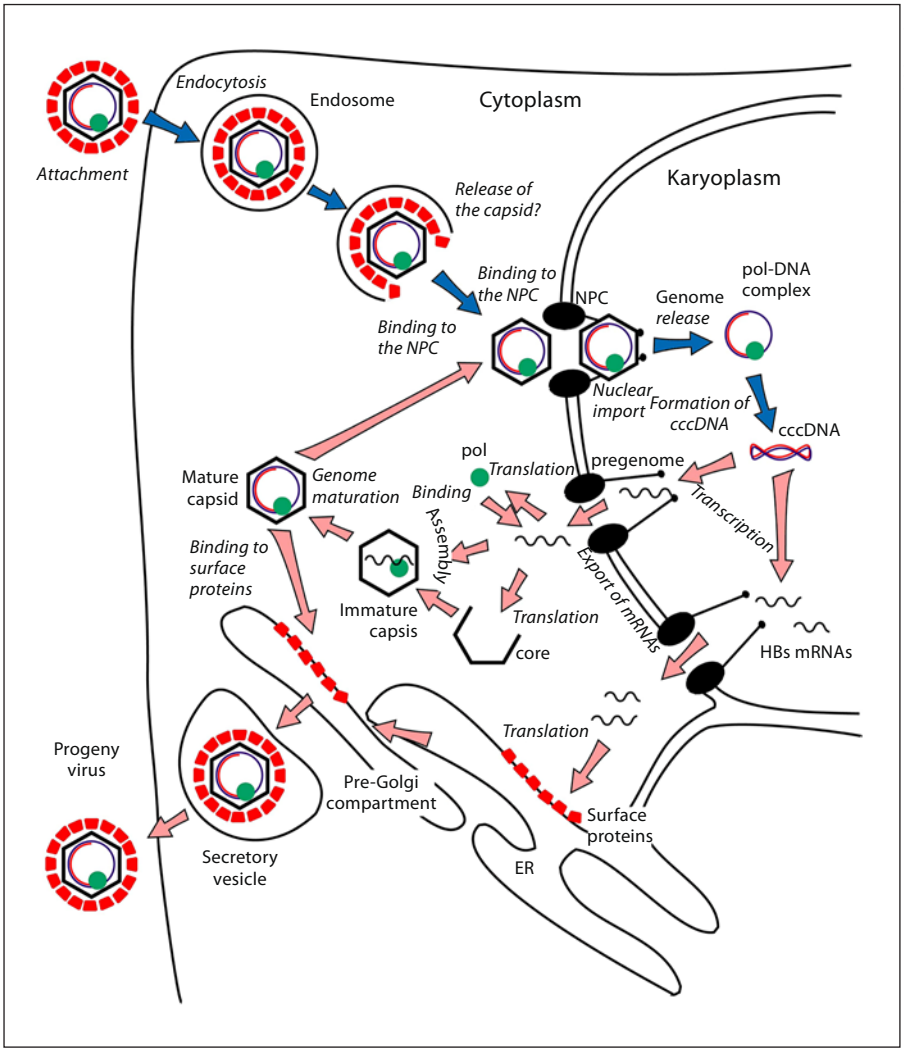

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Fig. 2. Intracellular life cycle of HBV. NPC = Nuclear pore complex; pol = polymerase; ER = endoplasmic reticulum.

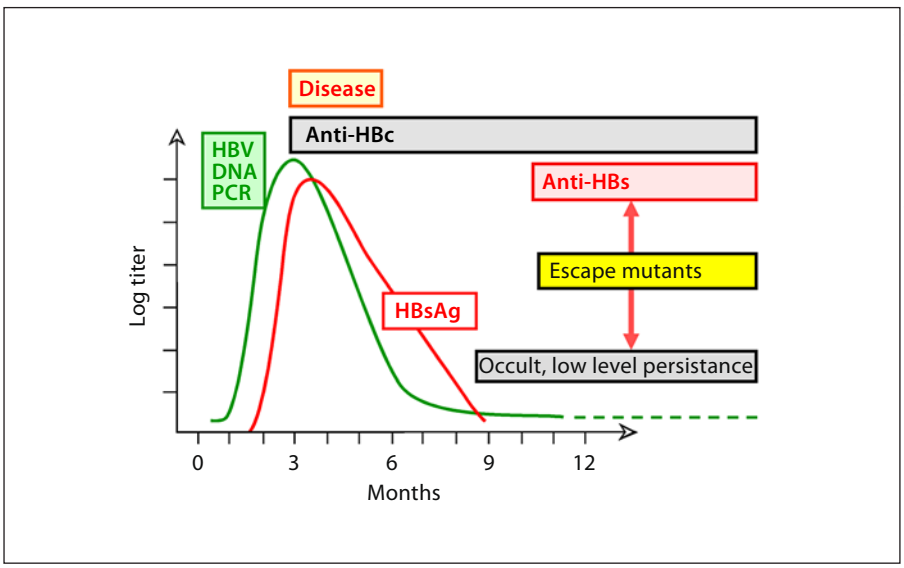

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Fig. 3. Typical course of acute resolving hepatitis B leading to occult persistent infection and selection of escape mutants.

remia levels because the immune assays for HBsAg are less sensitive than PCR for HBV DNA. During the acute phase of the disease, the decrease of HBV DNA is initially much faster than that of HBsAg because the subviral particles have a much longer half life (8 days on average) than the virions (1.6 days on average) [9]. Antibody against the viral core (anti-HBc) appears at the onset of disease, while antibody against the HBsAg (anti-HBs) appears much later. Recovery is characterized by the disappearance of detectable HBsAg from the blood.

Definition of OBI

Given the huge excess of subviral HBsAg particles over complete virus, it is surprising that something like OBI exists. It is defined as HBsAg non-reactivity of the serum in spite of ongoing HBV replication in the patient or carrier [10]. It deserves its name because a reliable detection of HBV genomes is only possible in liver tissue using a highly sensitive nucleic acid amplification technique (NAT), while serum is more often negative than positive.

One mechanism favoring development of OBI is an early and effective immune response. It can cause the level of HBs antigenemia to be too low or too short-lived for detection. In normal acute hepatitis B, the immune response is delayed and much HBsAg is produced. However, phases which may look like OBI occur even in the course of normal acute hepatitis B. These early and late window periods without detectable HBsAg are not considered as OBI, although the diagnostic pattern formally fulfils the definition. Viremic infections with >200 IU/ml HBV DNA and false-negative HBsAg results due to escape mutations are considered as 'false' OBI [10].

NAT-Negative Window Period Infections in Blood Donors

The most occult form of HBV infection is the period between the acquisition of the virus and the first appearance of detectable amounts of HBV DNA in the blood. It has been calculated that even a 50% detection limit of 10 copies/ml of HBV DNA would leave an infectious window period of 19.5 days in a newly infected blood donor [11]. In one blood donor, the day of infection became retrospectively known as it happened during an invasive medical procedure. The donation was collected 35 days later and tested negative for HBsAg and HBV DNA. However, the red cell unit was infectious and the recipient developed persistent HBV infection with a virus strain identical to that of the donor. Retesting of the donation with a 95% detection limit of 50 copies/ml still gave a negative result, which suggested that the window period may be even longer than calculated. The donor had been infected with a wild-type strain. He developed typical acute hepatitis B five months later [12]. A second similar case has also been described [13]. The long early window period leads to the relatively large residual risk of 1:360,000 for undetected HBV, even in a low-incidence country like Germany [14].

True Transient OBI

Due to an asymptomatic course, transient OBIs are very difficult to detect. Furthermore, this type of OBI is obviously quite rare. One possibility for detection is to screen blood donors not only for HBsAg, but also for HBV DNA by NAT. Recently, one such case was found in Spain [15]. This donor had a rather high viremia of 15,500 IU or 84,000 copies/ml HBV DNA but no other HBV markers. Five weeks later she had elevated transaminases, HBV antibodies and (still) HBV DNA. Four weeks later she was completely negative, except for the HBV antibodies.

Why was HBsAg undetectable in this case? Immune complexes of HBV and/or HBsAg with anti-HBs could be one explanation. However, the HBV DNA in the first sample could be immune precipitated with added exogenous anti-HBs or anti-preS1, but not with anti-human IgG.

Defective nonenveloped virions or free HBV DNA could be another explanation, but the HBV DNA-containing particles had the same density as normal Dane particles, the DNA was protected against DNase and the viral DNA could be extracted from immune precipitates generated with anti-HBs or anti-preS1. The HBsAg gene sequence was not mutated in the antigen loop, suggesting that a failure of the HBsAg assay due to escape mutations was not the reason for the negativity of HBsAg. Importantly, the sub-borderline, but elevated result of the HBsAg assay suggested that the ratio of HBV to HBsAg subviral particles was only 1:10 and not 1:3,000 as usual. A frame shift in the S-ORF on one of three clones suggested that there was a counter-selection against production of HBsAg.

Furthermore, the precore region contained the well-known mutation precW28stop leading to a loss of HBeAg expression. In addition, the core promoter contained the

nucleotide mutations G1764A and T1765C, which also decrease the expression of HBeAg. A decreased ability of the virus to produce the viral immunomodulator HBeAg may have favored a more rapid and vigorous immune response against HBsAg and HBcAg than in wild-type infection. While the major HBsAg epitopes were not mutated, the entire genome sequence was divergent between several clones and showed numerous nonsynonymous mutations. This suggests a strong selection pressure by the immune system. The unusually rapid appearance of a high anti-HBs titer (>1,000 IU/l) is in line with that assumption.

Table 1. HBV transmissions by occult infected blood donors; summary of data from Germany and Denmark 2004–2005

5 donors
HBV DNA 9–240 ge/ml, no anti-HBs
4 donors had several mutations of the HBs loop
Only 1 donor had wild-type HBsAg
55 recipients, 68% anti-HBc-positive.
10 possible transmissions, asymptomatic
22 probable transmissions, asymptomatic
3 fatal hepatitis B cases; cofactors: immunosuppression, sepsis
No normal acute hepatitis B after transfusion

OBI after Resolution of Hepatitis B

Today we have to assume that OBI develops both after apparent and unapparent infections. The good side of OBI may be that it maintains immunity by persistent antigen presentation. The bad side is that some of the weakly replicating virus may spill over to blood or is transmitted by liver donation. What makes things worse is that replication occurs in the presence of neutralizing antibodies and, thus, may lead to escape mutants (fig. 3). Anti-HBs blocks the intercellular spread of the virus, but it cannot block replication of intracellular virus. Due to reverse transcription, HBV genome replication is inaccurate, a mutated HBsAg may escape recognition, and the mutated viruses may spread within the liver. In 1990, Carman et al. [16] described an escape mutation from glycine to arginine at position 145 (G145R), which they found in vaccinated babies from HBV-infected mothers. Figure 4a shows a model of the HBs antigen loop and the position of that frequent escape mutation.

Persistent OBI in Blood Donors

We looked for escape mutations in blood donors with OBI (table 1). All of them were anti-HBc-positive, anti-HBs-negative and, by definition, HBsAg-negative. We found 5 donors who had low levels of HBV DNA, and in four of these we found several escape mutations [13].

We could retrospectively examine 55 recipients of donations containing mutated HBV. Thirty-two of them were anti-HBc positive. They had no HBsAg or HBV DNA and none of them seemed to have had overt hepatitis. It was not known whether these recipients were already anti-HBc-positive before transfusion. However, the prevalence of anti-HBc is only 7% in Germany. Thus, we estimated that there were 10 possible and 22 probable transmissions by the donations from donors with OBI, with all of them asymptomatic. However, 3 further recipients suffering from some kind of immunosuppression at the time of transfusion died of liver failure. In the first case, the donor and recipient had a virus with 6 mutations in the HBsAg loop, 4 of which were in the so-called *'a'* determinant. The next donor and recipient pair had only 3 mutations all outside of the *'a'* determinant, but one altered the HBsAg subtype determinant *'w'* to an unknown specificity. One donor and recipient pair had exclusively wild-type virus.

Fortunately, detection of OBI in a blood donor does not only depend on the detection of transmission to a recipient with a fatal outcome. One case was found by NAT screening. An HBV quasi-species was found which was so divergent that direct sequencing of the PCR product failed to detect the mutations. After cloning we found 6 positions partially mutated.

Another means to detect OBI is the test for anti-HBc, which was introduced in Germany in 2006. Using this, we identified one long-term donor who had low viremia which could be reliably detected only after ultracentrifugation of the virus. Looking back at the recipients of previous donations revealed 5 of 13 red cell and all 11 plasma recipients as HBV DNA- or HBsAg-negative, but anti-HBc-positive. None of them had suffered from apparent hepatitis. The donor had numerous mutations in the HBs antigen loop and a dynamic quasi-species. Seven mutations were present in all clones and samples, but 5 disappeared or emerged during follow-up.

A false OBI blood was identified in a further donor. She was HBsAg-negative in the screening test, but strongly positive in a second assay. She also had, however, 20–40 IU/l of anti-HBs. Direct sequencing of the HBV DNA

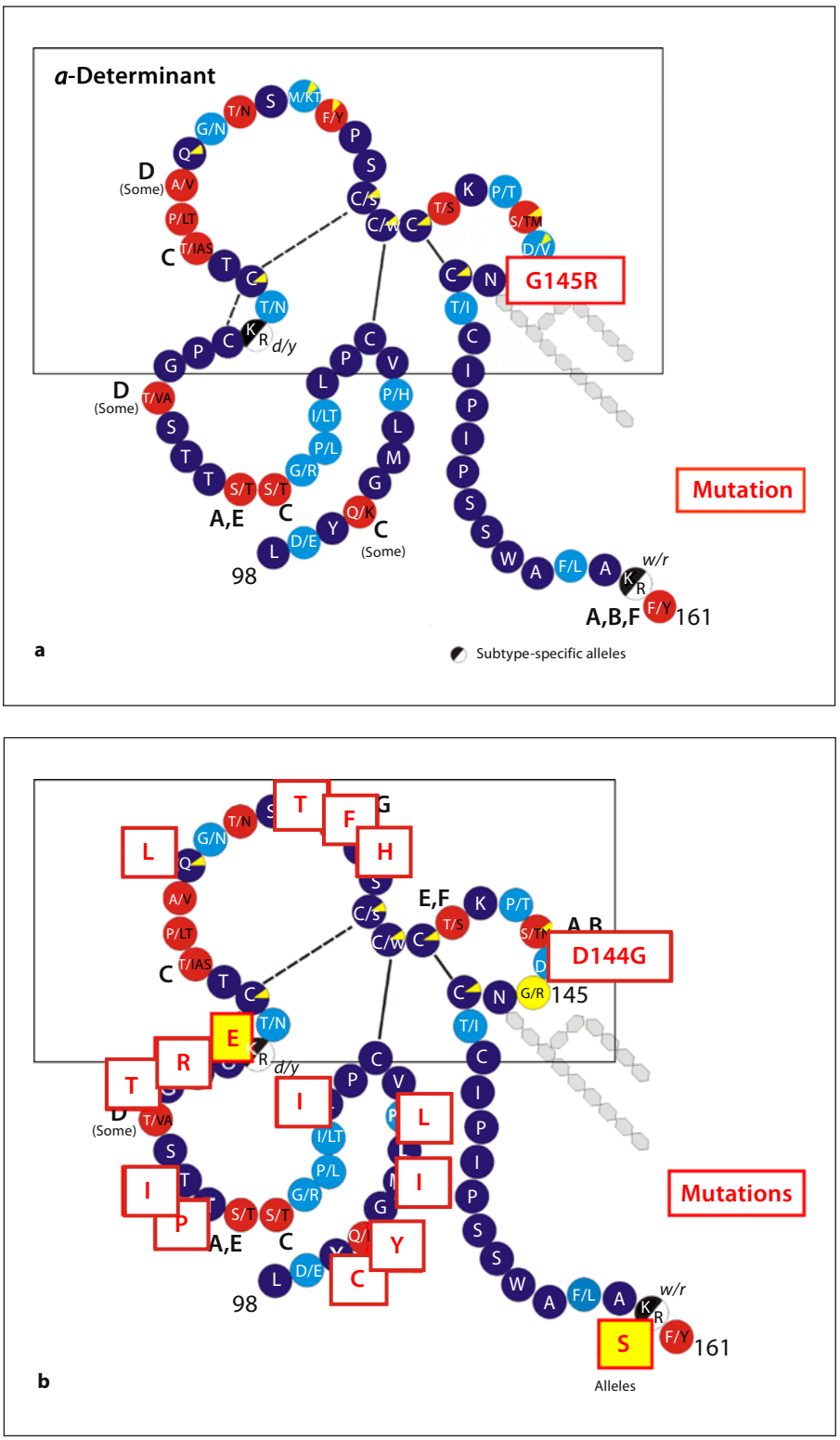

Fig. 4. Escape mutations in the HBsAg loop. The topology of the two-dimensional model and the cross-linking by disulfide bonds is speculative. The amino acids are shown in the one-letter code from position 98 to 161. Dark blue = conserved positions; light blue = variable; red = genotype specifics indicated by the capital letters (colors in online version). **a** Wild-type sequence with the first identified mutation of glycine to arginine at position 145 [16]. **b** Mutations found in 11 clones derived from a patient with reactivated acute hepatitis B.

identified the mutation D144G, which had caused the failure of the first test. Interestingly, 3 of 4 corresponding recipients were anti-HBc-negative and not infected, with the fourth anti-HBc-positive, but the pretransfusion status was not known. In a second donation 1 year later, 4 new mutations were found, but the exchange G144 (which had caused the diagnostic escape) had reverted to D in 18 of 19 clones and was no longer detectable by direct sequencing.

Sequencing of cloned genomes showed a very heterogeneous quasi-species and a subtype change. Among 19 clones, we found 11 different sequences just for the HBs antigen loop. Such heterogeneity is not found in an HBV carrier without active immune defense. Furthermore, the virus obviously tried to reduce the excess of subviral particles. In most clones the start codon of the small HBs protein was mutated without interruption of the ORF for the large HBs protein which is essential for HBV.

Sequencing of S-ORF clones from blood donors with true or false OBI led to following conclusions:

- The variability is 8–22 times higher in OBI than in normal HBV carriers. This has been confirmed for European [17], but not African blood donors [18].
- The HBs antigen loop is hypervariable, probably due to neutralizing antibodies, even in the absence of detectable anti-HBs.
- There are more and other mutations than in vaccine escape.
- The mutants are infectious and stable.
- The attachment site in preS1 is less variable and possibly a better target for diagnostics and vaccines.

Several recent studies have shown the surprisingly high yield of sensitive blood donor screening for HBV DNA [e.g. 14, 19–21], which is occasionally found even in individuals with anti-HBs. Although it has been shown in experimentally inoculated chimpanzees that anti-HBs-positive blood from a donor with well detectable HBV DNA was not infectious [22], a more recent report showed that a low titer of anti-HBs does not exclude transmission [23].

Reactivation of OBI under Immunosuppression

Reactivation of OBI is relatively infrequent in solid organ transplantation or therapy of solid tumors, but more frequent in lymphoma and leukemia therapy or after bone marrow and stem cell transplantation [see 24 for a review].

Table 2. Serology and escape mutations in 9 cases of reactivated occult HBV infections

Cause of immune suppression	Immune suppression			Escape mutations	
	before	under/after			
	anti-HBc/s	anti-HBc/s	HBsAg[a]	HBs loop[b]	PreS1[c]
Kidney transplant	+/+	+/–	+/–	3	0
Liver transplant	+/+[d]	+/–	+[e]	5	0
Lymphoma	+/+	+/+	+/–	5	0
Leukemia	(+/+)	+/+	+/–	10	2
Lymphoma	+/+	+/–	–/–	16	2
Lymphoma	–/+[f]	–/+	+/–	3	0
Lymphoma 1st	–/+[f]	–/–	+/–	4	2
Lymphoma 2nd		–/–	+/–	2	2
Bone marrow transpl.	+/+	–/–	+/+	0	0
Stem cell therapy	+/+	+/+	+	0	1

[a] HBsAg AxSym or other tests. [b] HBsAg loop aa 99–170. [c] Attachment site aa 20–59. [d] From HBIG. [e] Breakthrough after 10 years. [f] No vaccination known.

Reactivation goes clinically unnoticed for a long time under immunosuppression. After stem cell transplantation, it may even lead to a kind of immunotolerance with loss of anti-HBc [25]. It can be fatal, however, after immune reconstitution. To make things worse, it is often caused by escape mutants [26–28].

Table 2 lists 9 cases which we have analyzed [W.H.G., unpubl.]. The patients had anti-HBc and/or anti-HBs before therapy. Some lost the antibodies during therapy. HBsAg was not reliably detectable by all assays because of escape mutations which were sometimes very numerous in the HBs antigen loop. The preS1 attachment site was significantly less mutated.

Reactivation may come in many disguises. A kidney transplant recipient needed dialysis due to organ failure 10 years after transplantation. He was negative in the HBsAg screening assay, but was accidentally retested (because of false positivity due to a washing error) with a second assay and found to be positive. He had a very high viremia, and five S gene mutations. Before transplantation, he had been anti-HBc- and anti-HBs-positive. Thus, his reactivation and high viremia had not been noticed for 10 years. The mutation at P120Q was the reason for the diagnostic escape in the screening test.

Another patient seemed to have mild acute hepatitis B because anti-HBc IgM was very high. Viremia and

HBsAg were also very high. It was surprising, therefore, that this patient simultaneously had 240 IU/l of anti-HBs. It was speculated that he had been immunized and later infected on travel by an escape mutant. It turned out, however, that he had chronic leukemia. The HBV genotype (A2) was that of his home country. Fourteen mutations in the S gene proved that his HBV previously had been selected by strong antibody pressure during OBI and that the seemingly new infection was caused by a reactivated escape mutant.

In a similar but more severe case, acute reactivated hepatitis became clinically apparent with ALT levels of 2,000 U/l and 64,000 copies/ml of HBV DNA after completion of lymphoma therapy. All HBsAg assays were negative. The mutation K122E caused the total diagnostic escape, but there were 15 mutations more in the HBsAg loop (fig. 4b). All clones had the same sequence. Therefore, this extremely altered mutant was viable and stable. The patient had been anti-HBc- and anti-HBs-positive before therapy and had obviously harbored the occult variant in the liver. A similar case with heavily mutated HBsAg was published recently by Miyagawa et al. [28].

The first known reactivated OBI case with fatal outcome was reported by us in 2003 [26]. Here, the virus had only 7 mutations in the HBsAg loop, but still the majority of HBsAg assays failed. The patient had recovered 15 years before from acute hepatitis B and was anti-HBc- and anti-HBs-positive before immunosuppression. Initially, he received an aggressive lymphoma therapy which suppressed both T and B cell immunity and obviously allowed outgrowth of the pre-existing occult escape variant, traces of which were retrospectively detected in a serum sample taken before lymphoma therapy. Two months after the switch to the B cell-specific immunosuppressant rituximab, the patient developed acute liver failure, probably due to the recovered population of HBV-specific cytotoxic T cells.

In summary, persistent OBI may develop after unapparent or apparent hepatitis B. By definition, HBsAg is undetectable because its level is too low or it is mutated, or both. OBI is maintained by the intrahepatic reservoir of cccDNA and restricted by the immune system to replication at low level. Diagnosis is difficult, but important, because the virus can be transmitted by blood or liver donation and can reactivate. Reactivation occurs in cases of strong immunosuppression and usually leads to high viremia without clinical symptoms. Nevertheless, the infection may remain undetected because of escape mutations if only HBsAg is tested for. The future will show whether the vaccine protects against such escape mutants. These mutants are highly pathogenic when immune reconstitution occurs. The observations described above and in many other publications suggest that the following measures could help to prevent or control reactivation:

- Anti-HBc [10] and anti-HBs should be determined before immunosuppression. Sometimes anti-HBs without previous vaccination is the only HBV marker detectable [27].
- HBsAg and HBV DNA should be monitored during therapy. HBsAg may be missed due to escape mutations or masking by anti-HBs [26, 27].
- Pre-emptive HBV therapy could be given if HBV DNA is already detectable before immunosuppression [24].
- Monitoring for ALT as suggested by a consensus conference [10] is insufficient. Once hepatitis has developed, it is too late to stop hepatocyte destruction by activated cytotoxic T memory cells.

This personal opinion, however, is not covered by current state of the art consensus papers.

OBI in Vaccinated Blood Donors

It has long been known that vaccination against HBV of an already exposed individual may fail and even lead to escape mutants [16]. Pre-existing anti-HBs, however, at titers of >10 IU/l induced by hepatitis B vaccine have been shown to protect quite reliably against HBV infection and virtually always against HBV disease or persistence [29]. Highly sensitive screening of vaccinated blood donors for anti-HBc seroconversion or HBV DNA revealed, however, that the protection is not as complete as believed, and may still allow for low-level HBV replication and persistence. A vaccinated thrombapheresis donor had >1,000 IU/l of anti-HBs, but seroconverted after 3 years of follow-up to anti-HBc positivity [30]. Retesting of archived samples with another anti-HBc test showed that the donor had even seroconverted 2 years earlier. Traces of HBV DNA were detected in the plasma samples kept for quarantine and showed that the virus was of a genotype different from the genotype of the HBsAg used for vaccination (A2). Furthermore, it contained the escape mutation P120T. Fortunately, all of the surviving 23 HBV susceptible recipients remained HBsAg- and anti-HBc-negative, probably due to the high anti-HBs titer and the very low virus load of <10 copies/ml.

In a recent large study [31], the American Red Cross in 2008 tested 3.7 million donations with NAT (Ultrio from Chiron Novartis, detection limit 166 IU/ml). With 9 HBV

DNA-positive, but HBsAg- and anti-HBc-negative donors, the NAT yield was low, but 3–5 times higher than expected. Surprisingly, 6 of the 9 donors had been vaccinated and had low levels of anti-HBs (3–100 IU/l). Thus, the yield of NAT-only positive donors was higher in vaccinated than in unvaccinated donors. None of the vaccinated donors developed elevated ALT levels, but they had HB viremia for up to 137 days and anti-HBc appeared very slowly. The preS/S DNA sequences were amplified and cloned, with several clones sequenced. The vaccinated donors with protective levels of anti-HBs (>10 IU/l) were infected with non-A genotypes, while the 4 donors with less or no anti-HBs were infected with genotype A2, which is predominant in the USA and is the vaccine genotype (table 3). The HBV strains isolated from 3 of the vaccinated donors were essentially wild-type, whereas quasi-species and escape mutations (K122E, T143A and G145R) were found in 3 other donors. These findings suggest:

- Low anti-HBs levels induced by vaccination protect against hepatitis B disease and chronic infection, but favor occult infections.
- The genotype A2 vaccine protects better against A2 than against other genotypes.
- By favoring HBsAg-negative HBV infections, HB vaccine may possibly reduce the viral safety of blood donations, unless donors are also screened for HBV DNA with very sensitive assays.

In conclusion, anti-HBs titers should be controlled in vaccinated persons who are at increased risk or in whom HBV infection would be particularly detrimental, and the aim should be for titers of >100 IU/l. In addition, inclusion of the major HBV genotypes in the vaccine may be considered. Another option would be the inclusion of neutralizing preS epitopes which are less prone to immune escape. Since the preS peptides are not very immunogenic by themselves, these epitopes have been presented on the very immunogenic HBV core particles [32]. This type of antigen induced very high titers of neutralizing antibodies in mice [Bremer et al., submitted], and may be considered a candidate for a new generation of hepatitis B vaccine.

Table 3. HBV genotype distribution in unvaccinated, or partially protected, NAT-only positive donors

Anti-HBs titer, IU/l	HBV genotype	
	A2 only	Non-A2
Unvaccinated or vaccinated, <10	4	0
10–100, vaccinated	0	5

Significant, $\chi^2 = 9.0$; $p = 0.0027$.

Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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