Prophylactic vaccination against hepatitis B: Achievements, challenges and perspectives

Article *in* Medical Microbiology and Immunology · December 2014

DOI: 10.1007/s00430-014-0373-y · Source: PubMed

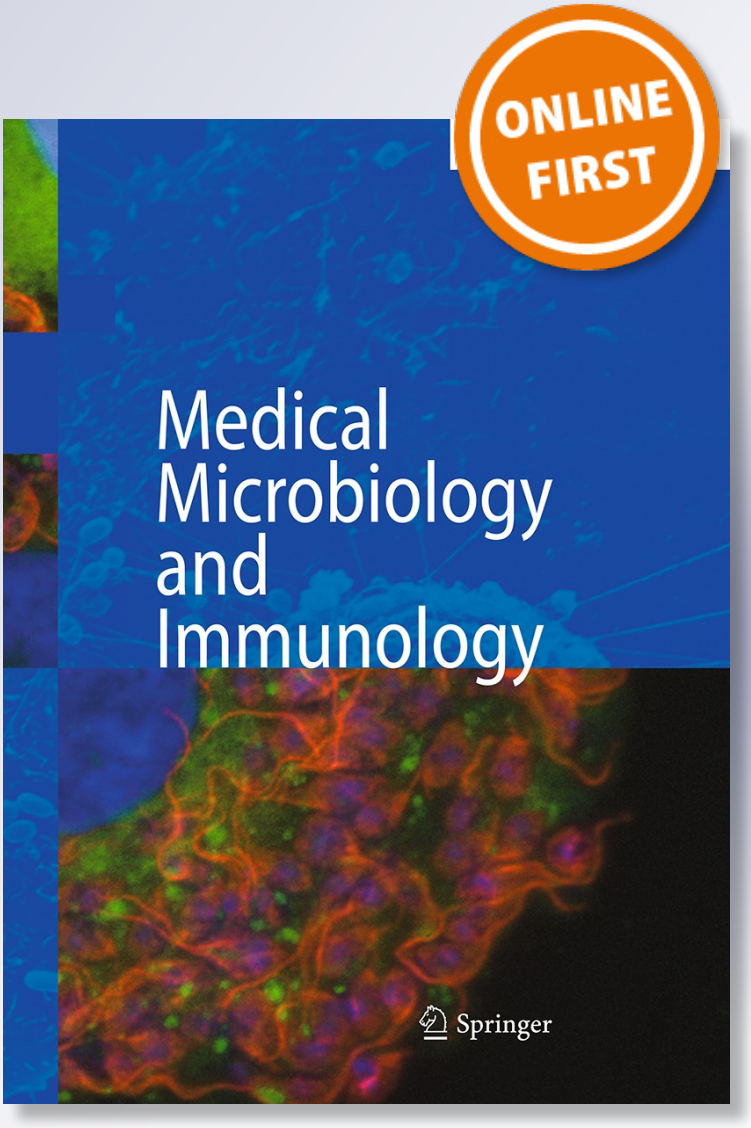
Prophylactic vaccination against hepatitis B: achievements, challenges and perspectives

Wolfram H. Gerlich

Medical Microbiology and Immunology

ISSN 0300-8584

Med Microbiol Immunol
DOI 10.1007/s00430-014-0373-y



Springer

Med Microbiol Immunol
DOI 10.1007/s00430-014-0373-y

REVIEW

Prophylactic vaccination against hepatitis B: achievements, challenges and perspectives

Wolfram H. Gerlich

Received: 14 May 2014 / Accepted: 1 October 2014

Abstract Large-scale vaccination against hepatitis B virus (HBV) infection started in 1984 with first-generation vaccines made from plasma of chronic carriers containing HBV surface antigen (HBsAg). Thereafter, it was replaced in most countries by second-generation vaccines manufactured in yeast cells transformed with gene S encoding HBsAg. Both generations of vaccines have been applied for universal neonate and early childhood vaccination worldwide and have led to a 70–90 % decrease in chronic HBV carrier rates. However, 10–30 % of newborns from HBsAg/HBeAg-positive mothers cannot be protected by passive/active vaccination alone and become chronic HBV carriers themselves. Asymptomatic occult HBV infections are frequent even in those who have protective levels of anti-HBs. Suboptimal protection may be due to heterologous HBsAg subtypes that are present in 99 % of HBV carriers worldwide. Second-generation vaccines contain partially misfolded HBsAg and lack preS1 antigen that carries the major HBV attachment site and neutralizing epitopes. Third-generation vaccines produced in mammalian cells contain correctly folded HBsAg and neutralizing epitopes of the preS antigens, induce more rapid protection, overcome nonresponse to second-generation vaccines and, most importantly, may provide better protection for newborns of HBV-positive mothers. PreS/S vaccines expressed in mammalian cells are more expensive to manufacture, but introduction of more potent HBV vaccines should be considered in regions with a high rate of vertical transmission pending assessment of health economics and healthcare priorities. With optimal vaccines and vaccination coverage, eradication of HBV would be possible.

Keywords Hepatitis B virus · HBsAg · Vaccine · PreS · Mother-to-child transmission · Escape mutant

Introductory remark

The special issue in which this review is published deals with therapeutic vaccination in chronic hepatitis B. However, therapeutic vaccination would be unthinkable without the existence of prophylactic vaccination. The lessons learnt from the current prophylactic vaccination are relevant for the concepts leading to the not yet reached goal of therapeutic vaccination. This short review is not "systematic" but tries to point out the essential steps during the evolvement of hepatitis B vaccination and shortcomings of the current concepts.

First vaccination attempts

The concept of the prophylactic vaccination against hepatitis B dates back to the late 1960s to the work of Nobel Prize winner Baruch S. Blumberg (1925–2011) [1]. He and Harvey Alter detected a peculiar human serum protein, initially named by them "Australia antigen" (AuAg), which was later also found by Alfred Prince in patients with "serum" hepatitis (for review see [2]). Blumberg also observed that persons with AuAg had occasionally hepatitis, while persons with the corresponding antibody

This article is part of the special issue "Therapeutic vaccination in chronic hepatitis B—approaches, problems, and new perspectives".

W. H. Gerlich (✉)
Institute for Medical Virology, National Reference Center for Hepatitis B and D, Justus-Liebig-University Giessen, Schubert Str. 81, 35392 Giessen, Germany
e-mail: wolfram.h.gerlich@viro.med.uni-giessen.de

Published online: 19 December 2014

seemed to be protected against hepatitis. Although the nature of the antigen and its relation to the disease were unknown at that time and the experimental evidence virtually absent, Dr Blumberg filed on October 8, 1969, a patent on the use of AuAg purified from human plasma as a hepatitis vaccine [1, p. 137]. The potential applicability of this approach was demonstrated by a series of controversial experiments and by today's standards unconceivable, conducted by the New York pediatrician Dr Saul Krugman in mentally handicapped children. Since the presence or infectivity of the hepatitis B virus (HBV) could not yet be determined in vitro or in laboratory animals at that time, he inoculated children and staff with presumably infectious serum to study the transmission of the putative infectious agent and the disease. After confirming the hypothesis of two different hepatitis agents [3] (MS1 and MS2 or hepatitis A and B, respectively) by this approach, Krugman adopted the idea of Blumberg that AuAg may be used as a protective antigen against hepatitis B. Krugman did not use purified AuAg as Blumberg had suggested, but instead has diluted an AuAg-positive serum 1:10 and boiled it for 1 min. Previous experiments revealed that this harsh heat treatment destroyed the infectivity of the MS2 containing serum but not the antigenicity of AuAg [4]. Insofar, this experiment would have been ethically acceptable, but then the investigator challenged (as in his previous studies) the vaccinated children after two injections of the hypothetical antigen with an MS2 inoculum of known infectivity and followed vaccinees whether they would develop hepatitis and/or AuAg antigenemia [5]. Krugman recorded a partial but statistically significant protective response rate of 59 %, which pointed to the fact that AuAg-positive sera contained a heat-stable protection-inducing antigen. Many researchers in the hepatitis field consider these results as the first proof of concept for successful immunization with the putative hepatitis B etiologic agent, but it remained open whether the protective antigen was indeed AuAg or something else. Soon after, Australia antigen was characterized as the surface antigen of HBV and renamed as hepatitis B surface antigen (HBsAg) (for review see [2]).

First-generation vaccines

More convincing and ethically acceptable protection experiments were performed by Robert Purcell and John Gerin at the NIH [6] and in parallel by Maurice Hilleman et al. at Merck Sharp and Dohme (MSD) [7, 8]. The two groups purified HBsAg from the plasma of chronic HBsAg carriers, treated it with formaldehyde, trypsin and high concentration of urea to destroy residual viral infectivity and used this antigen for immunization of chimpanzees. Thereafter, the investigators challenged the vaccinated animals with a ca. 1,000-fold higher dose of one 50 % infectious dose and found complete protection against HBV challenge, thereby verifying the concept of Blumberg.

Independently, the group of Philippe Maupas at Institute Pasteur (Paris) developed a similar type of plasma-derived hepatitis B vaccine and was the first to initiate a clinical trial conducted in staff and patients of hemodialysis wards [9, 10]. At that time, such facilities were plagued by frequent transmission of hepatitis B. Although these clinical trials confirmed the vaccine-induced protective effect against HBV infection, competitors criticized the study design which was non-randomized and was based on historical controls. In the meantime, MSD produced large quantities of their plasma-derived hepatitis B vaccine and started to test its efficacy in a large double-blind randomized field trial conducted by Dr Wolf Szmuness (New York Bloodbank) [11]. Szmuness had observed that male homosexuals had a high risk to acquire an HBV infection [12] and selected this group for his trial. He wrote in his land mark paper from 1980 [11]: "The reduction of incidence in the vaccinees was as high as 92.3 %; none of the vaccinees with a detectable immune response to the vaccine had clinical hepatitis B or asymptomatic antigenemia". Indeed, these results were and still are considered as a pivotal step in developing biotechnological means for prevention of HBV infection through vaccination.

There were, however, a number of safety concerns regarding the future of plasma-derived HBV vaccines. Given the fact that the MSD vaccine was produced from plasma containing hepatitis B "e" antigen (HBeAg), considered a marker for high HBV infectivity, removal and inactivation of infectious HBV was critical. The efficiency of the inactivation process of HBV particles in the crude plasma used for preparation of the vaccine had been confirmed in a small number of five chimpanzees who received high doses of the HBsAg preparations intravenously. However, the absence of infectivity in millions of vaccine doses could not be guaranteed using this costly and complex safety test. Even worse, some of the HBsAg-positive plasma donors used for vaccine production subsequently developed AIDS. The causative agent of AIDS was not yet identified in the early 1980s. Consequently, it was unknown whether the inactivation procedures used for vaccine production would inactivate the putative agent of AIDS. In an attempt to reduce the risk of transmissible etiologic agents, the group of Reiner Thomssen used only anti-HBe antibody-positive plasma for vaccine production and applied four times higher formaldehyde concentrations for inactivation of viral infectivity [13, 14]. Careful evaluation of the multi-step inactivation procedures used by MSD in chimpanzees finally demonstrated the desirable level of safety concerning inactivation of HBV as well as HIV (which was meanwhile identified) and virtually all other infectious agents known at that time [15].

Another obstacle was Blumberg's patent which was exclusively licensed to MSD. The vaccine developed by Thomssen was very efficient in more than 3,000 recipients, but it could not be produced in large scale because of this patent. The group of Henk Reesink (Amsterdam) circumvented that patent by using a **crude** heat-inactivated HBsAg preparation instead of purified HBsAg. The material contained less HBsAg than the MSD vaccine (3 vs. 20 or 40 μg) but was sufficiently safe, immunogenic and protective in a field trial also conducted in male homosexuals [16, 17].

Development of efficacious HBV vaccines for protection of individuals at risk, i.e., patients on hemodialysis or men who have sex with men (MSM) as well as health care workers, was indeed a major achievement. Yet, the highest impact of vaccination was documented in newborns to highly viremic HBV carrier mothers. Such infants usually become chronic HBV carriers with the most serious long-term consequences. Palmer Beasley had already shown that passive immunization of newborns with hepatitis B immune globulin (HBIG) containing a high anti-HBs antibody concentration could protect newborns against HBV transmission from their mothers [18]. However, passive protection afforded by HBIG is transient, while the infants remain exposed to the HBV-positive mother. Thus, Palmer Beasley developed a new concept called passive/active immunization of newborns at risk that includes simultaneous administration of HBIG for immediate protection after birth and initiation of active immunization with the new plasma-derived vaccine intended to induce long-term protection against HBV. The success of this approach was confirmed through a number of clinical trials and adopted by the WHO [19–21] but the necessity of HBIG in addition to immediate active vaccination after birth is debatable (see below). Following the success of these convincing studies, many countries introduced vaccination of persons at a high risk of HBV infection and in particular in newborns to HBV-positive mothers. Taiwan as one of the highly endemic countries for HBV was the first country to start a nation-wide vaccination campaign for all newborns in 1984.

Plasma-derived vaccines produced in the USA, France, the Netherlands and Korea were used for several years worldwide but were soon replaced by second-generation vaccines, mainly in the Western hemisphere and Australia. However, many countries with limited economic resources continued to produce their own plasma-derived vaccines, unable to afford the (initially) high cost of the so-called recombinant vaccines. Regardless of the vaccine type used, the increased uptake of HBV vaccines worldwide leads gradually to a decrease in the rate of HBsAg carriers thus reducing the potential HBsAg containing plasma pool required to maintain vaccine production.

Second-generation vaccines

Molecular biologists recognized already in the 1970s that HBV could be a perfect target for the application of then newly developed gene technology. Hepatitis B was an important infection, but the virus could not be propagated in cell culture at that time, and the viral nucleic acids and antigens necessary for diagnosis and prevention were scarce and difficult to obtain. The discoverer of the HBV DNA, William Robinson (Stanford, California) [22], wanted already to clone the viral genome in 1974, but was not allowed to do so because of an initial safety concern regarding gene technology expressed in the Asilomar conference in 1975 [23]. In 1979, three other pioneers of molecular biology, Pierre Tiollais (Paris), Kenneth Murray (Edinburgh, 1930–2013), and William Rutter (San Francisco) and co-workers succeeded almost simultaneously in a head-to-head race to clone and sequence the HBV DNA [24–26]. Sequencing revealed four open reading frames (ORF) within the small circular viral genome. The ORF encoding the HBV core antigen (HBcAg) (which reacts with anti-HBc antibodies) could be identified by expression in *E. coli* [25–27]. Surprisingly, the HBcAg expressed in bacteria formed spontaneously core particles that were very similar to core particles extracted from HBV-infected livers or from HBV particles [28]. The gene encoding HBsAg could be identified on the basis of the partial amino acid sequence [29, 30], which had been determined by Darrell Petersen and Girish Vyas (San Francisco) [31]. The gene sequence coding for the HBsAg protein was then expressed in mammalian cells transformed with cloned HBV DNA which secreted the authentic 20 nm HBsAg particles [32, 33].

HBV infection was and still is one of the major causes of hepatocellular carcinoma (HCC) worldwide. Cell lines derived from tumors excised from HBV carriers with HCC became another potential source of authentic HBsAg particles (for review see 1, 2). The PLC/PRF 5 cell line developed by Jenifer Alexander in South Africa produced and secreted HBsAg [34], and this cell line was considered for a while as a potential source material for HBsAg [35]. However, growth of mammalian cell culture is laborious and costly, and the yield of HBsAg, though quite immunogenic, was low. Furthermore, concern about the risk of using a cell source co-infected with other virus(es) or etiologic agents, e.g., SV40 or mycoplasma, was an obstacle in the exploitation of these sources.

The breakthrough came with the expression of HBsAg in genetically transformed yeast cells such as Saccharomyces cerevisiae. The product of these cells was not secreted and not glycosylated (unlike authentic HBsAg) but could be produced in large amounts per cell and extracted with preserved immunogenicity [36, 37]. Protection experiments in

chimpanzees were successful [38], and convincing field studies in newborns from highly infectious mothers proved the high protective efficacy of the new vaccine [39, 40]. Importantly, a follow-up study in Thailand suggested that neonatal vaccination was also quite effective in prevention of HBV vertical transmission in the absence of HBIG administration [41]. Availability of an almost unlimited supply of this safe and efficacious antigen paved the road to global introduction of universal vaccination of neonates against HBV.

The first major yeast-derived vaccines for clinical use were developed by Michel de Wilde for GlaxoSmithKline in Belgium (Engerix-B®) and by Pablo Valenzuela for Merck Sharp and Dohme in USA (Recombivax HB®) and are now produced under license in many countries, particularly in China or Cuba. Indian companies have developed similar vaccines (Shanvac-B and Elovac-B) expressed in the yeast species *Pichia pastoris*. These recombinant vaccines are expressed by the gene encoding antigen subtype adw2 of the small (major) HBsAg protein, but a vaccine encoding subtype adr has also been developed in Korea. In Russia yeast-derived vaccines with HBsAg subtypes adw2 or ayw2 are available.

Medical impact of the second-generation vaccines

Already in 1992, the World Health Organization (WHO) recommended to introduce universal childhood vaccination worldwide. To date, ca. 180 countries have implemented this recommendation [42]. To quote a WHO report in 2009 [43]: "As of 2008, 177 countries had incorporated hepatitis B vaccine as an integral part of their national infant immunization programmes, and an estimated 69 % of the 2008 birth cohort received 3 doses of hepatitis B vaccine. In 2006, the last year for which such data are available, approximately 27 % of newborns worldwide received a birth dose of hepatitis B vaccine."

Vaccine coverage is a precondition for prevention but does not guarantee protection. A large meta-analysis concluded that the relative risk of vaccinated newborns from HBsAg-positive mothers to develop HBV infection was still 0.28 (95 % c.i. 0.20–0.40) compared to non-vaccinated infants. No difference between plasma-derived and recombinant vaccines could be demonstrated and not all studies suggested an additional protective effect of HBIG [44]. This analysis provided clear proof for vaccine efficacy in neonates, but also emphasized the importance of coverage and access to vaccine which is still limited in some regions worldwide.

Universal vaccination projects conducted in Taiwan and Thailand generated the most convincing data on the efficacy of the hepatitis B vaccination in newborns. In both countries, the HBV carrier rate has decreased from >10 % to ca. 1 %, which raised the hope that hepatitis B could be eradicated even in countries with high endemicity [45–48]. China has also achieved a 90 % drop in HBsAg prevalence in the vaccinated age groups [49]. Most important is that the age-standardized incidence of HCC in children born to HBV-infected mothers dropped in Thailand from 0.88 per 1,000,000 in a non-vaccinated population to 0.07 in the vaccinated children ($P = 0.007$) [50]. In Taiwan, the HCC incidence decreased significantly from 0.57 to 0.17 per 100,000 person-year after the introduction of mass vaccination [51]. Similar results were reported from several other countries. Thus, the hepatitis B vaccine was the first efficient vaccine for prevention of a human cancer. The full impact of the vaccination efforts will become apparent when the vaccinated population in highly endemic regions will reach an age >40 years at a time when the HBV carriers increasingly develop cirrhosis and HCC.

The incidence of acute hepatitis B in young adults has also dramatically dropped in many regions such as in Italy where between 1991 and 2005, a 24-fold decrease in HBV infection was recorded among 15–24 year-olds as a consequence of the successful vaccination efforts in neonates and in adolescents [52].

Shortcomings of the second-generation vaccines

Non-responders

The first immunization trials reported up to 99 % seroprotection rates in healthy children or female adolescents. However, in adults ca. 5–7 % remain unprotected with anti-HBs antibody levels <10 IU/ml measured 4 weeks after the last dose of the yeast-derived HBsAg [53]. Under certain unfavorable circumstances up to 70 % remain non-responders or low responders [54]. Male gender, old age, obesity, smoking and an impaired immune system, e.g., during diabetes or hemodialysis are major factors for non-response aside from frank immunodeficiency. Vaccine producers try to improve the response to vaccination by recommendation of additional vaccine injections, higher antigen doses for recipients with probable nonresponse and by adding more active adjuvants (concerning adjuvants see article from G. Leroux-Roels). The success of these attempts is limited and shall not be discussed here. New concepts may be necessary to improve the immunogenicity on the antigen side.

Mother-to-child transmission (MTCT)

Most relevant for the long-term goal of HBV eradication is the residual rate of MTCT in ca. 10–30 % of immunized newborns to HBsAg- and HBeAg-positive mothers [44, 50, 51]. Hence, immunization failures most probably contribute to the still relatively high residual incidence of HCC

Table 1 HBV genotypes in newly infected blood donors of the American Red Cross who were HBV DNA-positive and anti-HBc-negative in relation to the vaccination status

Vaccination, Anti-HBs IU/L	HBV genotype	
	Only A	Non-A
No	17	4
Yes, <10	1	1
Yes, 10–100	1	4
Yes, >100	0	0

The 28 donors were identified among 2.14 million donors of the year 2008. The infections of the seven vaccinated donors were all asymptomatic and lead after several weeks or months to a strong increase of anti-HBs. The data are extracted from reference [66]

(0.17 vs. 0.57 per 100,000 person-years) in vaccinated Taiwanese children [51]. The reasons for these failures are not completely clear but some risk factors have been identified. Many researchers believe that intra-uterine infection may be the main reason for MTCT and that in this case vaccination is administered too late to prevent infection. Amniocentesis may also theoretically increase the rate of MTCT [55] but the majority of pregnant women in countries with a high HBV rate do not necessarily undergo such a procedure. However, there are no generally accepted data to prove in utero transmission of HBV as a major factor for the subsequent failure of immune prophylaxis and development of chronic HBV infection. Since 2012, it is known that entry of HBV into hepatocytes requires a liver-specific receptor named sodium (in German Natrium)-dependent taurocholate cotransporting polypeptide (NTCP) [56]. Its presence in fetal liver has not yet been studied, but it is plausible that this exclusively hepatic bile acid transporter is not yet expressed in fetal liver and appears after the switch to adult liver function phenotype that occurs approximately within 4 weeks after birth. This hypothesis would explain the time gap available for passive and even active immunization against HBV after maternal exposure before and/or during birth. Following this rational, anti-HBs antibodies passively administered or actively induced through vaccination would not be able to neutralize the large number of infectious HBV particles transmitted from a mother with a very high viral load. Support for this hypothesis may be deduced from the report of Ding et al. who described 37 of 167 HBeAg-positive mothers with extreme viraemia $>10^7$ IU/ml HBV DNA, of whom 12 infants became chronically infected in spite of immediate post-partum passive/active immunization, whereas MTCT did not occur in children born to mothers with lower viraemia [57].

Recent studies from China and elsewhere suggest that treatment of HBV carrier mothers with a very high viral load with a nucleos(t)ide analog (NUC) such as lamivudine or telbivudine during the last trimester of pregnancy reduces the rate of vertical transmission and vaccination failure [58, 59]. Although telbivudine is somewhat more potent than lamivudine, long-term treatment with either drug may induce resistance. In future, tenofovir may be the drug of choice to reduce the risk of MTCT in highly viremic mothers because it does not induce drug resistance and is not toxic to the unborn [60]. This approach requires, however, screening of all non-immune pregnant women for HBV early enough before delivery and subsequent determination of the viral load in infected women followed by antiviral therapy in women with a viral load $>10^7$ IU/ml HBV DNA. Recent surveys indicate that this is not done even in developed countries [61, 62].

Escape mutants

MTCT with an HBV variant carrying one escape mutation G145R in the S gene has been discovered in an Italian infant in spite of immediate passive/active immunization with subsequent persisting high anti-HBs level [63]. HBV escape mutants have also been reported in vaccinated HBsAg carriers from Taiwan where perinatal transmission of escape mutants to infants born to HBsAg-positive mothers varied between 23 and 28 % as compared to only 8 % in the pre-vaccination era. Yet, at present the clinical significance of such mutants seems marginal [64, 65]. Indeed escape mutants are not a frequent reason for breakthrough HBV infections in vaccinated persons and the vaccination is not a major driving force for their selection. Among six vaccinated blood donors in the USA who acquired a new HBV infection, only one had selected an escape mutant (G145R) which was pre-existent as minor component in the infecting partner [66]; see also Table 1. In contrast, escape variants are often selected by long-term passive immunization with HBIG as it is applied in HBV-infected liver transplant recipients [67].

The main reservoir of escape mutants are persons with occult HBV infection with or without anti-HBs or anti-HBc. The term occult refers to the fact that these HBV carriers do not have detectable HBsAg in serum but HBV DNA may be present in serum or liver tissue; for review see [68, 69]. Normally, these HBV carriers have very low levels of viraemia and are not infectious for contact persons. Blood donations from these occult carriers are, however, infectious for the recipients and may cause fatal hepatitis B [70]. Furthermore, the low levels of an escape mutant may increase to $>10^7$ IU/ml HBV DNA when the occult HBV carrier is treated by immunosuppressive agents such as the anti-CD20 antibody rituximab [69].

Chimpanzees vaccinated with recombinant second-generation vaccines were protected against a challenge with the G145R mutant [71]. However, the naturally occurring escape mutants from reactivated occult HBV infections

may harbor up to 16 mutations in the S gene [69] and may no longer be sensitive to neutralization by vaccine-induced anti-HBs. An HBV-like virus from bats with a similar number of different amino acids in the S gene compared to human wild-type HBV could infect human hepatocytes but was not neutralized by human vaccine-induced anti-HBs [72]. A systematic study on the protective power of the current hepatitis B vaccines against heavily mutated HBV strains would be desirable.

Subtype specificity of the vaccine

A possibly relevant drawback of the most widespread HBV vaccines is that they represent only the HBV subgenotype A2 that prevails in the USA and Northern Europe, while >99 % of the HBV carriers worldwide have other (sub)genotypes. Serological subtypes have been recognized soon after the identification of HBsAg as the HBV surface protein and led to the definition of a common antigen determinant *a* and two allelic subtype determinants *d* or *y*, and *w1-4* or *r* [73]. Later, sequencing of numerous HBV strains worldwide suggested distinguishing genotypes A to I that differ by at least 8 % of nucleic acid sequence and subgenotypes that differ by at least 3.5 %. Furthermore, recombinants exist between the (sub)genotypes. For a recent review of this rapidly evolving field see reference [74]. The existence of the HBsAg subtypes was addressed in the early protection experiments with chimpanzees [6, 7], and cross-protection was observed. A field study also suggested that the plasma-derived adw vaccine from MSD protected against an HBV strain with ayw [75]. The cross-protection in chimpanzees was confirmed for the recombinant yeast-derived vaccine (subgenotype A2) that protected newborns from HBeAg-positive mothers in Thailand where genotype B is prevalent [40]. While results of these studies were convincing, it should be considered that cross-protection was determined soon after the immunization when the vaccinees still had high anti-HBs titers.

Overall, worldwide experience suggests that there is a high degree of cross-protection between HBsAg subtypes or genotypes. There is, however, evidence that protection against the homologous subtype or genotype is significantly better than against heterologous HBV strains. An amazing example is the overall shift from genotype B to C in infants who experienced a breakthrough after perinatal passive/active vaccination in Taiwan. Non-immunized children from HBV-positive mothers ($N = 141$) had 17 % genotype C and 82 % genotype B, but immunized children ($N = 107$) had 42 % genotype C [76]. Genotype B has the same HBsAg subtype adw2 as the hepatitis B vaccine with subgenotype A2, whereas genotype C has the HBsAg subtype adr.

Rapid post-partum anti-HBs production in response to immunization is essential to prevent MTCT. The initial anti-HBs response to a plasma-derived vaccine in adults was predominantly subtype specific [77], and there is no reason to believe that this would be different in newborns receiving a yeast-derived vaccine. The subtype specificity of experimental HBsAg vaccines expressed in the yeast Hansenula polymorpha has been studied with HBsAg subtypes adw2, ayw3 and adr in animals. It was found that about two-third of the anti-HBs response was subtype specific [78].

A significant genotype effect was observed in US blood donors who were vaccinated but developed transient viraemia without detectable HBsAg [66]. The majority of the US blood donors are vaccinated, but only 44 % of the donors have anti-HBs levels >10 IU/L and are believed to be protected. Newly acquired HBV infection was detected in 28 out of a total of 2.13 million blood donors during 2008 based on detection of HBV DNA but in the absence of anti-HBc (Table 1). The majority of these donors were not vaccinated (21/28) and had genotype A (16/21), which is predominant in USA. Among the seven vaccinated donors, only two had genotype A, the other five had B, C, two times D and F, which means that non-A genotypes were significantly enriched in the breakthrough infections ($\chi^2 = 6.6$, $P < 0.01$). An escape mutant was found only in one infected donor who had genotype C and G145R. All vaccinated but infected donors had anti-HBs <100 IU/L, two were anti-HBs-negative at the time of detection. All these vaccinated donors remained asymptomatic but were HBV DNA-positive for several weeks or even months before they developed a strong rise in anti-HBs levels. No infections were observed in donors with anti-HBs >100 IU/L, but only 21 % of the donors had such levels [66].

The author observed in four previously vaccinated patients with acute or chronic hepatitis B genotype D, in three cases a wild-type strain (unpublished). One case was different because he had been silently infected with the rare subgenotype D4 after vaccination. The wild-type consensus sequence of this subgenotype has seven exchanges in the HBsAg loop (amino acids 100–170 of HBsAg) compared to the vaccine. The strain after reactivation during lymphoma therapy had 4 additional escape mutations [79]. It appears plausible that a leaky protection by the vaccine was due to significant sequence differences between the vaccine and the infecting strain and allowed for selection of variants with additional escape mutations.

The HBV genotype F with HBsAg subtype adw4 is most divergent from the vaccine genotype A. Although F is mostly found in Latin America and relatively rare in Europe, it was found in two well-documented European cases of vaccine breakthrough with acute hepatitis B in presence of detectable anti-HBs [80, 81].

Asymptomatic infections of vaccinated persons

It is known since the 1980s that vaccinated persons may acquire an HBV infection, but in most cases the infection remains asymptomatic and self-limited. A study in 635 MSM vaccinated with a plasma-derived vaccine showed that anti-HBs titers disappeared relatively fast and that those without anti-HBs were often transiently infected but only 1 in 34 infections led to viraemia or clinical hepatitis [82]. More recently, occult HBV infection has been found in children or adults who were most likely infected by MTCT. In Taiwan, 11 % of vaccinated young children with anti-HBs were found to be HBV DNA-positive [83]. In India, 42 % of children from HBsAg-positive mothers vaccinated at birth and with a full cycle of booster doses had occult HBV infection at the age of 2 years, mostly with genotype D [84]. A study from the Qidong province in China found in young adults who were vaccinated at birth, 42 % with anti-HBc as a marker of previous or ongoing infection with HBV, and 76 % of these were reported to have an occult HBV infection [85]. This latter finding appears exceedingly high and needs confirmation. In Thailand, it was also shown that protection became incomplete within the first 20 years of life with 23 % prevalence of markers for transient inapparent HBV infection, i.e., anti-HBc or an anti-HBs rise without additional vaccination [86]. These estimates are based on the detection of HBV-specific antibodies, but recently it has been reported that inapparent HBV infections frequently occurring in vaccinated health care workers do not induce a detectable anti-HBc response but only T cell immunity against the HBV core and polymerase antigen [87]. However, chronic HBV infections or clinical hepatitis were virtually never noted. Thus, the great majority of health policy makers considers clinically silent virus breakthroughs irrelevant or even welcomes them as a kind of natural booster. Yet, these infections may pose a risk regarding the safety of blood or organ donations with occult HBV infection [66]. Furthermore, it appears that such silent infections may persist in an occult form in the liver and may reactivate with serious consequences if the person is treated with immunosuppressive agents. The problem is aggravated by the fact that reactivated occult HBV strains have usually a different HBV genotype and often carry several escape mutations against which the current vaccines may be ineffective [69, 79, 88, 89]. Recently, Lai et al. reported an increasing seroprevalence of anti-HBc and/or HBsAg as well as HBV DNA in an older group of vaccinees immunized 18–21 years before. The overall prevalence of HBV variants rose from 0.45 % in vaccinees born after 1984 to 2.63 % in those >18 years old [90]. Thus, in the coming decades, immunity of the populations vaccinated early in childhood may gradually fade so much that—as a worst case scenario—symptomatic and chronic HBV infection will re-occur more often.

Conceptual flaws of the first- and second-generation vaccines

The greatest drawback of the first- and second-generation vaccines was that they were designed on a highly incomplete theoretical basis. The exact protein composition of the HBV particles and the receptor(s) of the virus were not known at that time. Only the major HBsAg protein P24 and its glycosylated form GP27 were dependably identified as components of the HBsAg particles [31]. The then newly developed SDS polyacrylamide gel electrophoresis generated besides P24 and GP27 very diffuse and controversial patterns of larger polypeptides [91–94] which, however, disappeared if the HBsAg was treated for purification with proteases. The HBs antigenicity seemingly was not decreased by this treatment [95]. Thus, many researchers believed that the removed components were adsorbed serum proteins [96] and not components of HBV. Consequently, the plasma-derived vaccine from MSD was treated with protease which removed the preS domains [16], and the second-generation vaccines were made exclusively with the gene S encoding P24 [36, 37].

Low pH during purification or heat treatment for inactivation and certain other not well defined steps altered the structure of plasma-derived HBsAg vaccine in a way that lead to blurred P24 and GP27 bands in SDS electrophoresis and aggregates, but the conformational HBsAg epitopes seem to be maintained as shown for the second WHO International Standard HBsAg which was initially produced as the Dutch hepatitis B vaccine [97]. Furthermore, unwanted activation of (auto?) proteolysis removed the larger preS-containing components (see below) of HBsAg almost completely from the Dutch vaccine [97] and the French vaccine (W.G. unpublished) although they were not treated with proteases. The German vaccine [13, 14] was purified with more gentle methods, but it was made from anti-HBe-positive donors and its starting plasma contained very little of these larger components.

A further potential weakness of most plasma-derived vaccines was that they were treated with formaldehyde. This aldehyde reacts with the lysine residues at position 122, 141 and 160 that are essential parts of the vaccine HBsAg determinants adw2. This treatment is unnecessary for the yeast-derived vaccines and not applied to Engerix-B.

A potential *advantage* of all plasma-derived and mammalian cell-derived vaccines over the yeast-derived vaccines is that the HBsAg is folded to its native conformation, whereas a major part of the yeast-derived antigen is misfolded or unfolded. High-titered antibodies from

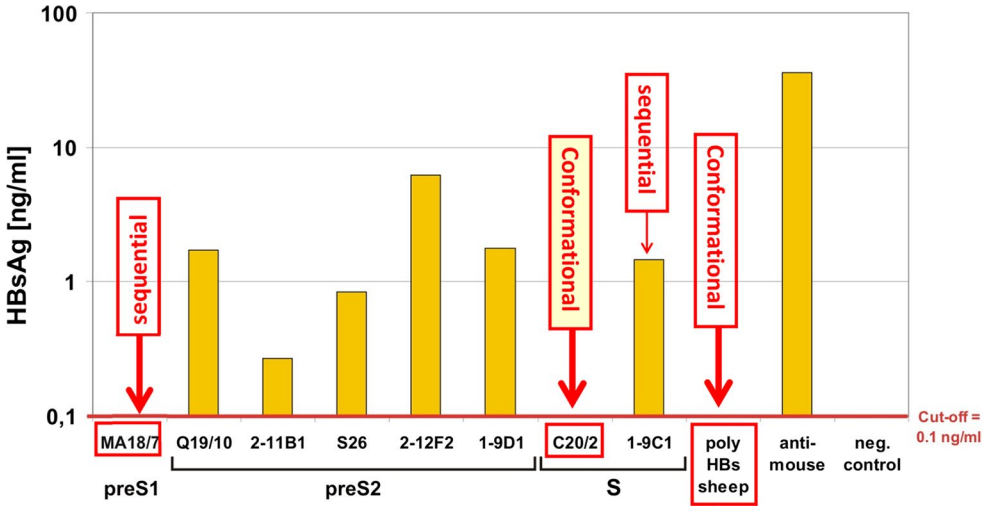


Fig. 1 Neutralization of HBV infectivity by selected antibodies against the S, preS2 and preS1 domains of the HBV surface proteins. A highly infectious HBV inoculum was pre-incubated with the antibodies and added to susceptible primary Tupaia hepatocyte cultures. After 12 days, the newly produced HBsAg was measured. Absent HBsAg (*arrows*) was a sign of complete neutralization. The designations MA18/7 etc., indicate different mabs mentioned in the text. "poly HBs sheep" refers to a polyvalent immunoglobulin from sheep immunized with plasma-derived native HBsAg, anti-mouse refers to the assay without HBV antibody but with an anti-mouse immunoglobulin. Data from reference [99]

persons vaccinated with plasma- and/or mammalian cell-derived HBsAg or from HBV convalescents do not react with SDS-denatured P24 or GP27 in Western blots (W. Gerlich, unpublished), and they do not react with partial peptides encoded by the S gene [98]. The opposite is true for antibodies from persons who received the yeast-derived vaccine suggesting that a major part of the HBsAg in the yeast-derived vaccine has an unnatural conformation. It is unlikely that antibodies against the un- or misfolded P24 are protective because this form of HBsAg does not exist on the natural infectious HBV particles. Neutralization experiments (see Fig. 1) showed that conformational anti-HBs antibodies (C20/2 and polyvalent sheep anti-HBs) neutralized very well, whereas a monoclonal antibody (mab) 1-9C1 reacting with native *and* denatured S-HBs protein did not [99]. A mutational analysis of the epitope recognized by the neutralizing mab C20/2 revealed that 14 amino acids within the HBsAg loop spanning from position 119 to 149 were essential for its assembly (C. Sureau, personal communication, see Fig. 2), thus illustrating the highly conformational nature of the HBs determinant *a*.

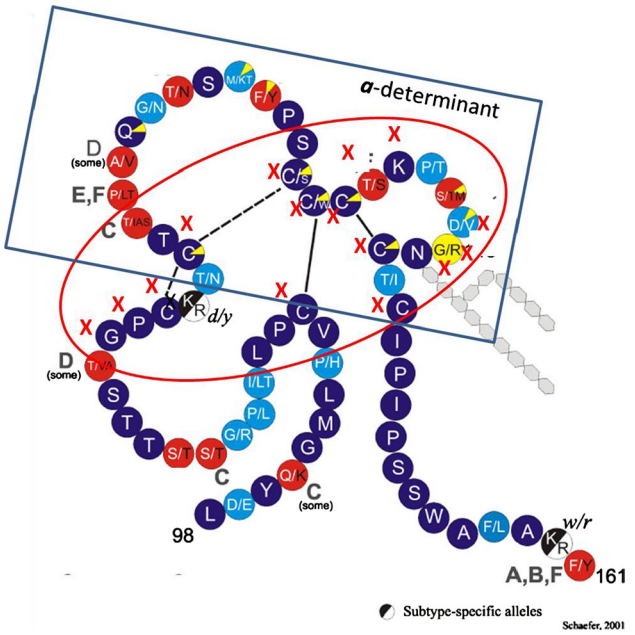


Fig. 2 Two-dimensional model of the HBsAg loop from amino acids 98–161 showing the conformational epitope of HBV infectivity neutralizing mab C20/2 (*oval line*) and the region forming the so-called determinant *a* (*box*). Mutations of the amino acids marked by X impair or destroy the reactivity of that epitope. Shown is a consensus sequence in the one-letter code with the putative cysteine cross-links (from S. Schaefer). The mab was generated in the laboratory of the author, the mutational analysis was done in the laboratory from C. Sureau (Tours, France)

Third-generation vaccines

The transition from the first- to the second-generation HBV vaccines can be clearly justified as replacement of the

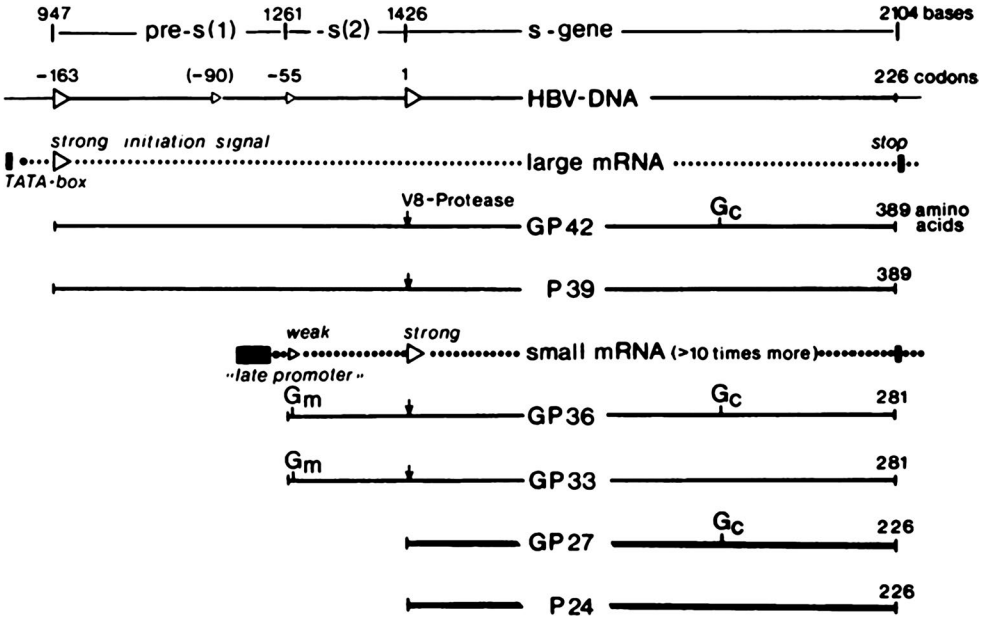


Fig. 3 Reprint of the genetic map from reference [103] presenting the transcription, translation and posttranslational modification of the three HBs protein pairs from S ORF of HBV genotype D. Gm and Gc give the position of the N-linked glycans. *Large* and *small triangles* indicate strong and weak translational initiation signals. The numbering of the codons relates to the start codon of the S-HBs protein

so-called "natural source" of HBV-infected human plasmas by an antigen produced in transformed host cells, typically yeast cells. In contrast, the rational for developing third-generation vaccines is more complex.

Identification of the preS domains as surface antigens of HBV

Precondition for the further development of hepatitis B vaccines was a better understanding of the virus structure and function. DNA sequence analysis of several HBV strains in the late 1970s revealed that the gene coding for the major HBsAg protein P24 and its glycosylated form GP27, covered only the last 226 codons of a larger ORF which spanned over 400 or 389 codons (depending on the HBV genotype) [24–30]. The meaning of this additional protein-coding sequence was not understood for several years. It was termed simply pre-S to reflect the fact that this part of the S ORF was upstream of the S gene. It was, however, speculated that the product of the entire ORF would be a precursor of the mature HBsAg [100]. While this speculation was wrong, the solution came stepwise from re-analysis of the protein composition of HBV-associated particles using improved techniques of SDS gel electrophoresis and silver staining of the gels. The HBsAg proteins are very weakly stained by the standard stain Coomassie blue, whereas silver stains the HBsAg proteins very strongly [101]. Furthermore, a purification technique was used which prevented spontaneous proteolysis and allowed separation of the HBV and HBsAg subviral particles. The small round 20-nm HBsAg particles from HBeAg-positive HBV carriers contained in addition to P24 and GP27 a protein pair GP33 and GP36 that was co-carboxyterminal to P24 and GP27, respectively, but contained an aminoterminal extension [101]. Partial proteolysis and glycosidase treatment showed that this extension consisted of the 55 amino acids encoded upstream of the S gene with an N-linked glycan at Asn 4 [102]. Analysis of the filamentous form of subviral HBsAg particles and purified HBV particles identified an additional protein pair P39 and GP42 that was shown to be encoded by the entire S ORF. The intensity of the silver staining suggested that HBV particles and filaments contained 10–20 times more of the large protein pair than the small round particles from the same donor. The mab MA18/7 raised against purified HBV particles was instrumental in identification of this protein pair in Western blots [103]. Thereafter, the nomenclature large (L), middle (M) and small (S) HBs protein was suggested at the first International Meeting on the Molecular Biology of Hepatitis B viruses in 1985. The existence of two protein pairs containing different portions of the preS sequence made it necessary to distinguish the preS1 domain with 108 or 119 N-terminal amino acids present only in the L protein from the preS2 domain present both in L and M protein. It should be kept in mind, however, that preS1 and preS2 form one continuous preS domain in L protein and that the pres2 sequence of L protein has no N-linked glycan. The three protein pairs are generated from different mRNAs and are differently modified after translation as shown in Fig. 3.

PreS2 as an additional vaccine component

Soon, both the preS2 and the preS1 domains gained attention from the scientific community. The relatively small preS2 domain was interesting as a vaccine component for

two reasons: One reason was that it was assumed to mediate host-specific attachment of HBV to hepatocytes. Even before the MHBs protein was identified, it was recognized that HBsAg particles from HBeAg-positive HBV carriers bound polymerized human serum albumin (pHSA) [104]. Furthermore, it was shown that pHSA had an affinity to hepatocyte membranes and this suggested that pHSA would bring HBV to its target cell [105]. Machida et al. found that pHSA bound to GP33 of HBsAg (named by them p31) and confirmed that GP33 contained the preS2 sequence. They could also show that the binding was species-specific because polymerized serum albumin, only from humans or chimpanzees but not from cattle or other non-primates, bound to preS2, which seemed to explain the narrow host range of HBV for higher primates [106]. PreS2 was inappropriately termed pHSA "receptor," and antibodies against preS2 were supposed to neutralize infectivity of HBV. The second reason to include the preS2 domain in a hepatitis B vaccine was the observation that it seemed to be immunodominant over the S gene product and enhanced the T cell response in certain mouse strains [107].

Immunization of chimpanzees with the aminoterminal part of preS2 conjugated to a carrier protein [108] or the entire preS2 peptide [109] induced protection in absence of HBsAg or anti-HBs. Rabbit antisera against preS2 peptide (1–26) neutralized an HBV inoculum infectious for chimpanzees [110]. Michel et al. succeeded to express high levels of M- and S-HB-containing particles in transformed animal cells [111], which served as basis for a recombinant vaccine (Gen Hevac B from Institute Pasteur) containing the preS2 domain [112]. Gen Hevac B was approximately as immunogenic as the plasma-derived Hevac B [111, 113] but not superior to various yeast-derived S gene-encoded vaccines [114, 115]. Finally, this more expensive vaccine has not found large distribution. PreS2-containing vaccine candidates have also been produced in transformed yeast cells, but proteolysis [116] and too strong glycosylation [117] of the preS2 domain were problems encountered.

The main reason that preS2-containing vaccines (without preS1) were after all not successful is related to the fact that the role of preS2 in the life cycle of HBV turned out to be minor and not so promising as a basis for induction of protective immunity as originally believed. The binding of pHSA to preS2 could not be confirmed as major attachment or uptake mechanism for HBV. M-HBs protein [118] and even the preS2 domain itself which is also part of the L-HBs protein [119] are not essential for replication of HBV and M-HBs-negative variants are found in patients with chronic hepatitis B and are infectious [120]. Although immunization with preS2 peptides induced protection of chimpanzees against experimental HBV infection [108–110], not all mab to preS2 antigens neutralized HBV infectivity for susceptible hepatocyte cultures [99] (see also Fig. 1). Partial neutralization was achieved with those mabs which inhibited binding of pHSA strongly, i.e., mabs that bound to peptide preS2(3–17) [121].

Functions of the preS1 domain

The finding that the HBV envelope contained two protein pairs partially encoded by the preS genome region [99–101] was soon confirmed, in particular by A.R. Neurath first for M-HBs [122] and thereafter for L-HBs [123]. Neurath was the first to point to the significance of the preS domains as potential vaccine components [121] and was soon able to show that antibodies both against preS2(1–26) [108] and preS1(21–47) [124] neutralized HBV infectivity in chimpanzees. Furthermore, he reported that a solid phase-immobilized preS1(21–47) peptide (sequence numbers for genotype A,10–36 for D) mediated a species and organ-specific binding of human hepatocyte suspensions in vitro [125]. A follow-up of these interesting results was difficult for many years because the only cell culture system for the measurement of HBV infectivity was primary human or chimpanzee hepatocytes from fresh surgically obtained livers. As a consequence, efforts to identify the authentic receptor(s) and the exact attachment sites for HBV were incomplete, misleading and futile [126] until the bile acid transporter NTCP was identified as receptor for preS1 [56] and heparan sulfate proteoglycan as definitive receptor for the antigen determinant *a* in S-HBs protein [127]. A precondition for this late success was the generation of two additional cell culture systems for the study of HBV infectivity, namely primary hepatocyte cultures from the small primate-related animal Tupaia belangeri [99] and the re-differentiated hepatoma cell line HepaRG [128]. Using the Tupaia hepatocytes, D. Glebe could show that the prototype mab MA18/7 directed to preS1(20–23), genotype D, and a well-characterized mab C20/2 against the determinant *a*-inhibited infectivity of HBV completely, whereas several preS2-specific mabs or a sequence specific S-HBs mab (1-9C1) did not [99] (Fig. 1). The importance of the sequence preS1(2–48) peptide (genotype D) was corroborated by its strong inhibitory power on the infection of Tupaia hepatocytes [129] and of the HepaRG cell line [130].

L-HBs expression in yeast cells

The detection of the preS antigens on the surface of HBV particles and particularly the fact that preS1 was enriched on the infectious virions compared to small subviral particles should have prompted the rapid translation into development of new vaccine projects. However, it turned out that industry was very hesitant to support even a patent application (W. G., personal experience). One reason may

have been that second-generation vaccines were just been introduced and were very successful. Another reason may have been that the preS-containing proteins were not easy to express. Toxicity, proteolysis and ectopic glycosylation were problems in yeast cells [116, 117, 131]. Nevertheless, attempts were undertaken, e.g., by the GSK precursor firm SKB to remove disturbing sequence elements and add only those preS1 and preS2 sequences to the S protein, which were believed to confer protection after immunization. Such a preS-containing vaccine was evaluated in field trials. According to the published results, the preS-containing vaccine was not superior to the pure S vaccine in young healthy adults [132] or in poor responders to the S vaccine [133]. It has to be noted that this conclusion was mainly based on the level of anti-HBs, i.e., antibody to the S-HBs protein, and not on real protection rates against proven exposure in a controlled trial.

L-HBs expression in mammalian cells

Using transfected mammalian cells, it was noted already in 1983 that expression of the full-length S ORF led to strongly impaired secretion of the HBs proteins compared to the expression of the gene encoding only for S-HBs [134]. It was recognized that the preS1 domain contained cellular retention signals, which prevented secretion of subviral HBsAg particles with too much L-HBs protein [135, 136]. With the selection of the right combination of the cell line, in this case HeLa cells, and a heterologous promoter, it was possible to obtain secreted HBsAg filamentous particles with a relatively high content of full-length L-HBs [136]. The immunogenicity of this recombinant "third-generation antigen" was compared with plasma-derived HBsAg filaments and small HBsAg particles in mice, and the specificity of the induced antibodies was analyzed [137]. The antibody spectrum induced by plasma-derived or recombinant filaments was similar and contained conformational S antibodies, sequential anti-preS2 antibodies and partly conformational preS1 antibodies. The authors of the paper from 1987 concluded: "The results suggest that recombinant HBsAg filaments containing both subtypes ad and ay may be optimal hepatitis B vaccines." An additional advantage of the L-HBs antigen is that it enhances also the spectrum of potential T epitopes [107] and can help to overcome H2-linked nonresponsiveness to S-HBsAg [138].

PreS1-containing vaccines

A slightly different approach was followed by an Israeli company (BioTechnology General Ltd, Israel), which expressed the entire S ORF (genotype A2, HBsAg adw2) in Chinese hamster ovary (CHO) cells (see also article from D. Shouval, H. and M. Roggendorf in this issue). The secreted particles were similar to native small round HBsAg particles derived from plasma in that they contained all three HBs protein pairs correctly folded and glycosylated with S-HBs as major component, moderate amounts of M-HBs and a few percent L-HBs. Mice immunized with this vaccine candidate (Bio-Hep-B) developed detectable amounts of anti-preS1 and anti-preS2 antibodies and needed only one 0.13 µg as 50 % immunogenic dose to develop detectable anti-HBs in a standard assay compared to >0.81 µg for the two available yeast-derived S vaccines [139]. The vaccine was licensed in Israel and in some countries in East Asia and is still available as Sci-B-Vac in Israel where it is used for universal vaccination of neonates as well as in non-responders to conventional yeast-derived vaccines [140].

A second mammalian cell-derived vaccine candidate (Hep-B-3) was developed by the group of Hans A. Thoma initially for the German firm Hexal. They had three different gene constructs introduced in their mouse-derived production cell line: an S-HBs (A2, adw2) gene, an M-HBs (D2, ayw3) encoding gene and a chimeric gene where the preS1(3–47) was inserted after the start codon of SHBs (adw2). Thus, this vaccine combined theoretically all requirements for an optimal vaccine mentioned in this paper: a major HBV genotype (D), correctly folded and glycosylated HBs proteins, and protective preS1 and preS2 epitopes in one particle. The vaccine was tested in two small clinical trials. Immunization of heart transplant recipients after transplantation was attempted (because of an outbreak of hepatitis B in a heart transplant unit) but virtually unsuccessful [141], whereas immunization of hemodialysis patients was very promising [142]. Thereafter, the vaccine was licensed to the former British vaccine producer Evans (later Medeva) and named first Hepagene, then Hepacare. The anti-preS1 antibody response was relatively weak, but the anti-HBs and T cell responses in chimpanzees were very good [143]. This vaccine had been given to >10,000 recipients in large trials [144] and received official approval in Europe. Its market perspectives were considered promising [145], but Medeva was taken over by the US Company Powderject and the vaccine was shelved and no longer produced.

Applications of Sci-B-Vac and Hepacare

Both vaccines were not intended to replace the yeast-derived vaccines because they were more costly to produce. They were, however, recommended for HBV vaccine-naïve health care workers who require rapid protection against HBV. Both vaccines were shown to be superior for this application due to a faster seroprotection rate following one and two doses as compared to yeast-derived vaccines [146, 147]. As an example, it was reported [148]: "Following

primary immunization, seroprotection occurred in 6, 39, 53 and 60 % in the Bio-Hep-B group at weeks 1, 2, 3 and 4, compared with 0, 12, 18 and 12.5 % in the Engerix-B vaccinees, respectively." Furthermore, the two vaccines should be given to non-responders or low responders to the yeast-derived vaccine. To quote a comparative study with Hepacare and Engerix B conducted in 915 non-responders [148]: "This study demonstrated that in healthcare workers who had responded inadequately to at least a full course of immunization (median, 5 doses), a single 20-microgram dose of a new triple-antigen vaccine induced protective antibody level in more vaccinees ($P = 0.002$) and increased the average antibody titer (GMT) in those protected successfully to a greater degree ($P < 0.001$) than a further attempt with a current vaccine (Engerix-B)." Similar results were found for Sci-B-Vac [149], and the influence of the HLA background on the anti-HBs response was confirmed in human recipients [149].

Sci-B-Vac in newborns

Bio-Hep-B/Sci-B-Vac has been applied in newborns from non-infected mothers with very good success [150]: "After the first dose, a significantly higher proportion of neonates seroconverted in the Bio-Hep-B group than in the Engerix-B group (83 % vs. 34 %; $P < 0.001$); this difference in seroresponse was even more pronounced for those achieving seroprotective concentrations (>10.0 mIU/ml) after the first dose: 54 % vs. 7 %, respectively ($P < 0.001$). Geometric mean concentrations were significantly higher at all points in the Bio-Hep-B group." Further studies proved that the vaccinated newborns develop anti-preS antibodies in addition to anti-HBs and that recognition of preS1 epitopes was associated with a better anti-HBs response [151–153]. The combined action of neutralizing anti-HBs and anti-preS1 is expected more reliably to block infection of hepatocytes and prevent occult infection with the subsequent selection of escape mutants.

Most important seems to be the rapid response to vaccination with the PreS1/PreS2/S vaccine in newborns from HBV-infected mothers. Formal studies on the protection rate in this special group have not been published for third-generation vaccines although they would be most urgently needed. An unpublished study was conducted in Vietnam in a relatively large cohort of newborns to HBsAg-positive mothers immunized with three 5 microgram doses of Sci-B-Vac without co-administration of HBIG. According to the vaccine manufacturer records, none of the babies became HBsAg-positive at 1 year post-partum (D. Shouval, personal communication). A trilateral vaccine study conducted in newborns to HBsAg-positive mothers was initiated in Israel and Palestine with financial and scientific support from the German Research Foundation (DFG, guided by D. Glebe, Giessen). The aim was to compare the protection rate by vaccination either with Engerix B or with Sci-B-Vac, particularly in newborns from HBsAg-positive mothers. Both groups received in addition passive HBIG prophylaxis at birth. The researchers guided by Rifaat Safadi (Jerusalem) reported as interim result on the follow-up of the newborns from HBsAg-positive mothers [154]: "All 60 newborns who received Engerix-B vaccine were evaluated at 1 year, as were 28 of 60 in the Sci-B-Vac group. All 28 Sci-B-Vac recipients tested negative for HBsAg, while 10 % of Engerix-B recipients tested HBsAg-positive." This borderline statistically significant observation—if confirmed in the final evaluation—would give an important impetus for the use of improved third-generation vaccines in newborns from HBsAg-positive mothers and may help to overcome the still observed residual MTCT without antiviral therapy of the mother.

Other vaccine concepts

Virtually every possible expression system including plants and many unusual modes of delivery, e.g., by the nasal route [155], have been explored to induce immunity to HBV proteins in animals or occasionally even in human subjects. Examples with very good induction of broadly neutralizing antibodies in mice are non-dividing vectors derived from Semliki Forest virus, which express a well-balanced ratio of S and pres1 antigens [156]. Such alternative approaches may be justified for the development of therapeutic vaccination, but for large-scale prophylactic vaccination more conservative approaches are probably more appropriate. Relatively simple ways to improve current prophylactic vaccines have been pointed out above and would not need revolutionary changes.

Inclusion of HBV core antigen (HBcAg) as a highly immunogenic and easy to produce HBV antigen could be considered [157] because it is an excellent T cell antigen and may provide intermolecular T cell help for stimulation of B cells recognizing preS- and S-specific epitopes [158]. Furthermore, the tip of the spikes in HBcAg particles is an excellent locus to present B cell epitopes [157]. Using this system, the most suitable preS1 epitopes for induction of neutralizing antibodies have been mapped and highly protective antisera obtained [98]. Production and addition of these chimeric core particles to a conventional S-HBs vaccine may turn out to be a viable option.

Perspectives

Vaccination against hepatitis B is one of the success stories in modern medicine. Depending on vaccine coverage, many countries have reduced the rate of new chronic HBV

infections by a factor of three to ten. This great achievement was made possible by concerted global efforts from WHO [42–44], GAVI, UNICEF, World Bank and the Bill Gates foundation together with worldwide national public health authorities who promoted control of HBV infection and its complications at an affordable expense. This success is in fact so encouraging that experts have started to discuss the possibility to eradicate at least the human HBV genotypes [45–47]. However, to envisage this goal seriously, the current vaccines should be improved. Currently predominating HBV vaccines manufactured in yeasts with partially misfolded epitopes and a less prevalent HBsAg subtype are not optimal. Immunization with these vaccines needs a cycle of three to four injections over 6–12 months, which is often not completed. Nevertheless, many completely vaccinated adults do not reach dependable protection and too many newborns from highly viremic mothers become chronically infected in spite of immediate passive/active immunization after birth. The occult HBV infections in vaccinated and exposed individuals deserve attention because they may reactivate under immune deficiency and harbor escape mutants. Obvious improvements of the current vaccines would be inclusion of HBsAg subtypes homologous to the predominant HBV strains, inclusion of neutralizing preS1 and preS2 epitopes, correctly folded HBsAg epitopes and addition of T helper epitopes, e.g., from HBcAg. More active adjuvants with an acceptable low level of adverse effects may be helpful. With improved vaccines, eradication of HBV within several decades seems to be at reach.

Acknowledgments The author thanks Dieter Glebe, Michael Roggendorf and Daniel Shouval for critical reading and fruitful collaboration over decades.

Conflict of interest None.

Ethical standard Not applicable.

References

1. Blumberg BS (2002) Hepatitis B—the hunt for a killer virus. Princeton University Press, Princeton. ISBN 0-691-00692-X
2. Gerlich WH (2013) Medical virology of hepatitis B: How it began and where we are now? Virol J. 10:239
3. Krugman S, Giles JP, Hammond J (1967) Infectious hepatitis. Evidence for two distinctive clinical, epidemiological, and immunological types of infection. JAMA 200:365–373
4. Krugman S, Giles JP, Hammond J (1970) Hepatitis virus: effect of heat on the infectivity and antigenicity of the MS-1 and MS-2 strains. J Infect Dis 122:432–436
5. Krugman S, Giles JP, Hammond J (1971) Viral hepatitis, type B (MS-2 strain). Studies on active immunization. JAMA 217:41–45
6. Purcell RH, Gerin JL (1975) Hepatitis B subunit vaccine: a preliminary report of safety and efficacy tests in chimpanzees. Am J Med Sci 270:395–399
7. Hilleman MR, Buynak EB, Roehm RR, Tytell AA, Bertland AU, Lampson GP (1975) Purified and inactivated human hepatitis B vaccine: progress report. Am J Med Sci 270:401–404
8. Buynak EB, Roehm RR, Tytell AA, Bertland AU 2nd, Lampson GP, Hilleman MR (1976) Development and chimpanzee testing of a vaccine against human hepatitis B. Proc Soc Exp Biol Med 151:694–700
9. Maupas P, Goudeau A, Coursaget P, Drucker J, Bagros P (1976) Immunisation against hepatitis B in man. Lancet 1(7974):1367–1370
10. Maupas P, Goudeau A, Coursaget P, Drucker J, Bagros P (1978) Hepatitis B vaccine: efficacy in high-risk settings, a two-year study. Intervirology 10:196–208
11. Szmuness W, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC, Sadovsky R, Morrison JM, Kellner A (1980) Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. N Engl J Med 303:833–841
12. Szmuness W, Much I, Prince AM, Hoofnagle JH, Cherubin CE, Harley EJ, Block GH (1975) On the role of sexual behavior in the spread of hepatitis B infection. Ann Intern Med 83:489–495
13. Thomssen R, Gerlich W, Böttcher U, Stibbe W, Legler K, Weinmann E, Klinge O, Pfeifer U (1982) Preparation and testing of a hepatitis B vaccine. Dtsch Med Wochenschr 107:125–131 German
14. Thomssen R, Gerlich WH, Böttcher U, Legler K, Ritter S, Stibbe W, Weinmann W, Klinge O, Pfeifer U (1983) Safety and potency aspects in the preparation of an experimental HBsAg vaccine. Dev Biol Stand 54:23–31
15. Tabor E, Buynak E, Smallwood LA, Snoy P, Hilleman M, Gerety RJ (1983) Inactivation of hepatitis B virus by three methods: treatment with pepsin, urea, or formalin. J Med Virol 11:1–9
16. Coutinho RA, Lelie N, Albrecht-Van Lent P, Reerink-Brongers EE, Stoutjesdijk L, Dees P, Nivard J, Huisman J, Reesink HW (1983) Efficacy of a heat inactivated hepatitis B vaccine in male homosexuals: outcome of a placebo controlled double blind trial. Br Med J (Clin Res Ed). 286:1305–1308
17. Lelie PN, Reesink HW, Niessen J, Brotman B, Prince AM (1987) Inactivation of 10^{15} chimpanzee-infectious doses of hepatitis B virus during preparation of a heat-inactivated hepatitis B vaccine. J Med Virol 23:289–295
18. Beasley RP, Hwang LY, Lin CC, Stevens CE, Wang KY, Sun TS, Hsieh FJ, Szmuness W (1981) Hepatitis B immune globulin (HBIG) efficacy in the interruption of perinatal transmission of hepatitis B virus carrier state. Initial report of a randomised double-blind placebo-controlled trial. Lancet 2(8243):388–393
19. Maupas P, Chiron JP, Barin F, Coursaget P, Goudeau A, Perrin J, Denis F, Mar ID (1981) Efficacy of hepatitis B vaccine in prevention of early HBsAg carrier state in children. Controlled trial in an endemic area (Senegal). Lancet 1(8215):289–292
20. Prozesky OW, Stevens CE, Szmuness W, Rolka H, Harley EJ, Kew MC, Scholtz JE, Mitchell AD (1983) Immune response to hepatitis B vaccine in newborns. J Infect 7(Suppl 1):53–55
21. Stevens CE, Toy PT, Tong MJ, Taylor PE, Vyas GN, Nair PV, Gudavalli M, Krugman S (1985) Perinatal hepatitis B virus transmission in the United States. Prevention by passive-active immunization. JAMA 253:1740–1745
22. Robinson WS, Clayton DA, Greenman RL (1974) DNA of a human hepatitis B virus candidate. J Virol 14:384–391
23. Berg P, Baltimore D, Brenner S, Roblin RO, Singer MF (1975) Summary statement of the Asilomar conference on recombinant DNA molecules. Proc Natl Acad Sci USA 72:1981–1984
24. Charnay P, Pourcel C, Louise A, Fritsch A, Tiollais P (1979) Cloning in Escherichia coli and physical structure of hepatitis B virion DNA. Proc Natl Acad Sci USA 76:2222–2226

25. Burrell CJ, Mackay P, Greenaway PJ, Hofschneider PH, Murray K (1979) Expression in Escherichia coli of hepatitis B virus DNA sequences cloned in plasmid pBR322. Nature 279:43–47
26. Pasek M, Goto T, Gilbert W, Zink B, Schaller H, MacKay P, Leadbetter G, Murray K (1979) Hepatitis B virus genes and their expression in E. coli. Nature 282:575–579
27. Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P (1979) Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature 281:646–650
28. Cohen BJ, Richmond JE (1982) Electron microscopy of hepatitis B core antigen synthesized in E. coli. Nature 296:677–679
29. Valenzuela P, Gray P, Quiroga M, Zaldivar J, Goodman HM, Rutter WJ (1979) Nucleotide sequence of the gene coding for the major protein of hepatitis B virus surface antigen. Nature 280:815–819
30. Charnay P, Mandart E, Hampe A, Fitoussi F, Tiollais P, Galibert F (1979) Localization on the viral genome and nucleotide sequence of the gene coding for the two major polypeptides of the hepatitis B surface antigen (HBs Ag). Nucleic Acids Res 7:335–346
31. Peterson DL, Roberts IM, Vyas GN (1977) Partial amino acid sequence of two major component polypeptides of hepatitis B surface antigen. Proc Natl Acad Sci USA 74:1530–1534
32. Dubois MF, Pourcel C, Rousset S, Chany C, Tiollais P (1980) Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. Proc Natl Acad Sci U S A 77:4549–4553
33. Moriarty AM, Hoyer BH, Shih JW, Gerin JL, Hamer DH (1981) Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector. Proc Natl Acad Sci USA 78:2606–2610
34. Alexander JJ, Bey EM, Geddes EW, Lecatsas G (1976) Establishment of a continuously growing cell line from primary carcinoma of the liver. S Afr Med J 50:2124–2128
35. McAleer WJ, Markus HZ, Bailey FJ, Herman AC, Harder BJ, Wampler DE, Miller WJ, Keller PM, Buynak EB, Hilleman MR (1983) Production of purified hepatitis B surface antigen from Alexander hepatoma cells grown in artificial capillary units. J Virol Methods 7:263–271
36. Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD (1982) Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 298:347–350
37. Harford N, Cabezon T, Crabeel M, Simoen E, Rutgers A, De Wilde M (1983) Expression of hepatitis B surface antigen in yeast. Dev Biol Stand 54:125–130
38. McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR (1984) Human hepatitis B vaccine from recombinant yeast. Nature 307:178–180
39. Stevens CE, Taylor PE, Tong MJ, Toy PT, Vyas GN, Nair PV, Weissman JY, Krugman S (1987) Yeast-recombinant hepatitis B vaccine. efficacy with hepatitis B immune globulin in prevention of perinatal hepatitis B virus transmission. JAMA 257:2612–2616
40. Poovorawan Y, Sanpavat S, Pongpunlert W, Chumdermpadetsuk S, Sentrakul P, Safary A (1989) Protective efficacy of a recombinant DNA hepatitis B vaccine in neonates of HBe antigen-positive mothers. JAMA 261:3278–3281
41. Poovorawan Y, Sanpavat S, Pongpunglert W, Chumdermpadetsuk S, Sentrakul P, van de Papelière P, Safary A (1992) Long term efficacy of hepatitis B vaccine in infants born to hepatitis B e antigen-positive mothers. Pediatr Infect Dis J 11:816–821
42. Lavanchy D (2012) Viral hepatitis: global goals for vaccination. J Clin Virol 55:296–302
43. World Health Organization (2009) Hepatitis B vaccines. Wkly Epidemiol Rec 84:405–420
44. Lee C, Gong Y, Brok J, Boxall EH, Gluud C (2006) Effect of hepatitis B immunisation in newborn infants of mothers positive for hepatitis B surface antigen: systematic review and meta-analysis. BMJ 332(7537):328–336
45. Chen D-S (2010) Toward elimination and eradication of hepatitis B. J Gastroenterol Hepatol 25:11–25
46. Wait S, Chen DS, Kaohsiung J (2012) Towards the eradication of hepatitis B in Taiwan. Med Sci 28:1–9
47. Thursz M, Njie R, Lemoine M (2012) Hepatitis: global eradication of hepatitis B–feasible or fallacy? Nat Rev Gastroenterol Hepatol 9:492–494
48. Alter HJ (2012) To have B or not to have B: vaccine and the potential eradication of hepatitis B. J Hepatol 57:715–717
49. Liang X, Bi S, Yang W, Wang L, Cui G, Cui F, Zhang Y, Liu J, Gong X, Chen Y, Wang F, Zheng H, Wang F, Guo J, Jia Z, Ma J, Wang H, Luo H, Li L, Jin S, Hadler SC, Wang Y (2009) Epidemiological serosurvey of hepatitis B in China—declining HBV prevalence due to hepatitis B vaccination. Vaccine 27:6550–6557
50. Wichajarn K, Kosalaraksa P, Wiangnon S (2008) Incidence of hepatocellular carcinoma in children in Khon Kaen before and after national hepatitis B vaccine program. Asian Pac J Cancer Prev 9:507–509
51. Chang MH, You SL, Chen CJ, Liu CJ, Lee CM, Lin SM, Chu HC, Wu TC, Yang SS, Kuo HS, Chen DS, Taiwan Hepatoma Study Group (2009) Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. J Natl Cancer Inst 101:1348–1355
52. Mele A, Tosti ME, Mariano A, Pizzuti R, Ferro A, Borrini B, Zotti C, Lopalco P, Curtale F, Balocchini E, Spada E, National Surveillance System for Acute Viral Hepatitis (SEIEVA) Collaborating Group (2008) Acute hepatitis B 14 years after the implementation of universal vaccination in Italy: areas of improvement and emerging challenges. Clin Infect Dis 46:868–875
53. Coates T, Wilson R, Patrick G, André F, Watson V (2001) Hepatitis B vaccines: assessment of the seroprotective efficacy of two recombinant DNA vaccines. Clin Ther 23:392–403
54. Wolters B, Junge U, Dziuba S, Roggendorf M (2003) Immunogenicity of combined hepatitis A and B vaccine in elderly persons. Vaccine 21:3623–3628
55. Yi W, Pan CQ, Hao J, Hu Y, Liu M, Li L, Liang D (2013) Risk of vertical transmission of hepatitis B after amniocentesis in HBs antigen-positive mothers. J Hepatol 60:523–529
56. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 1:e00049
57. Ding Y, Sheng Q, Ma L, Dou X (2013) Chronic HBV infection among pregnant women and their infants in Shenyang, China. Virol J 10:17
58. Han L, Zhang HW, Xie JX, Zhang Q, Wang HY, Cao GW (2011) A meta-analysis of lamivudine for interruption of mother-to-child transmission of hepatitis B virus. World J Gastroenterol 17:4321–4333
59. Deng M, Zhou X, Gao S, Yang SG, Wang B, Chen HZ, Ruan B (2012) The effects of telbivudine in late pregnancy to prevent intrauterine transmission of the hepatitis B virus: a systematic review and meta-analysis. Virol J 9:185
60. Pan CQ, Mi LJ, Bunchorntavakul C, Karsdon J, Huang WM, Singhvi G, Ghany MG, Reddy KR (2012) Tenofovir disoproxil fumarate for prevention of vertical transmission of hepatitis B virus infection by highly viremic pregnant women: a case series. Dig Dis Sci 57:2423–2429

61. Godbole G, Irish D, Basarab M, Mahungu T, Fox-Lewis A, Thorne C, Jacobs M, Dusheiko G, Rosenberg WM, Suri D, Millar AD, Nastouli E (2013) Management of hepatitis B in pregnant women and infants: a multicentre audit from four London hospitals. BMC Pregnancy Childbirth 13:222
62. Giles ML, Grace R, Tai A, Michalak K, Walker SP (2013) Prevention of mother-to-child transmission of hepatitis B virus (HBV) during pregnancy and the puerperium: current standards of care. Aust NZ J Obstet Gynaecol 53:231–235
63. Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, Zuckerman AJ, Thomas HC (1990) Vaccine-induced escape mutant of hepatitis B virus. Lancet 336(8711):325–329
64. Hsu HY, Chang MH, Ni YH, Chiang CL, Chen HL, Wu JF, Chen PJ (2010) No increase in prevalence of hepatitis B surface antigen mutant in a population of children and adolescents who were fully covered by universal infant immunization. J Infect Dis 201:1192–1200
65. Shouval D, Locarnini S (2012) Increased prevalence of HBV envelope mutants in Taiwan: an emerging public health risk or a false alarm? Gastroenterology 143:290–293
66. Stramer SL, Wend U, Candotti D, Foster GA, Hollinger FB, Dodd RY, Allain J-P, Gerlich W (2011) Nucleic acid testing to detect HBV infection in blood donors. New Engl J Med 364:236–247
67. Ghany MG, Ayola B, Villamil FG, Gish RG, Rojter S, Vierling JM, Lok AS (1998) Hepatitis B virus S mutants in liver transplant recipients who were reinfected despite hepatitis B immune globulin prophylaxis. Hepatology 27:213–222
68. Raimondo G, Allain JP, Brunetto MR, Buendia MA, Chen DS, Colombo M, Craxì A, Donato F, Ferrari C, Gaeta GB, Gerlich WH, Levrero M, Locarnini S, Michalak T, Mondelli MU, Pawlotsky JM, Pollicino T, Prati D, Puoti M, Samuel D, Shouval D, Smedile A, Squadrito G, Trépo C, Villa E, Will H, Zanetti AR, Zoulim F (2008) Statements from the Taormina expert meeting on occult hepatitis B virus infection. J Hepatol 49:652–657
69. Gerlich WH, Bremer C, Saniewski M, Schüttler CG, Wend UC, Willems WR, Glebe D (2010) Occult hepatitis B virus infection: detection and significance. Dig Dis 28:116–125
70. Allain JP, Mihaljevic I, Gonzalez-Fraile MI, Gubbe K, Holm-Harritshøj L, Garcia JM, Brojer E, Erikstrup C, Saniewski M, Wernish L, Bianco L, Ullum H, Candotti D, Lelie N, Gerlich WH, Chudy M (2013) Infectivity of blood products from donors with occult hepatitis B virus infection (OBI). Transfusion 53:1405–1415
71. Ogata N, Cote PJ, Zanetti AR, Miller RH, Shapiro M, Gerin J, Purcell RH (1999) Licensed recombinant hepatitis B vaccines protect chimpanzees against infection with the prototype surface gene mutant of hepatitis B virus. Hepatology 30:779–786
72. Drexler JF, Geipel A, König A, Corman VM, van Riel D, Leijten LM, Bremer CM, Rasche A, Cottontail VM, Maganga GD, Schlegel M, Müller MA, Adam A, Klose SM, Borges Carneiro AJ, Stöcker A, Franke CR, Gloza-Rauscha F, Geyer J, Annan A, Adu-Sarkodie Y, Oppong S, Binger T, Vallod P, Tschapka M, Ulrich RG, Gerlich WH, Leroy E, Kuiken T, Glebe D, Drosten C (2013) Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes. Proc Natl Acad Sci U S A 110:16151–16156
73. Couroucé-Pauty AM, Soulier JP (1974) Further data on HBs antigen subtypes—geographical distribution. Vox Sang 27:533–549
74. Kramvis A (2014) Genotypes and genetic variability of hepatitis B virus. Intervirology 57:141–150
75. Szmuness W, Stevens CE, Harley EJ, Zang EA, Alter HJ, Taylor PE, DeVera A, Chen GT, Kellner A (1982) Hepatitis B vaccine in medical staff of hemodialysis units: efficacy and subtype cross-protection. New Engl J Med 307:1481–1486
76. Wen WH, Chen HL, Ni YH, Hsu HY, Kao JH, Hu FC, Chang MH (2011) Secular trend of the viral genotype distribution in children with chronic hepatitis B virus infection after universal infant immunization. Hepatology 53:429–436
77. Legler K, Strohmeyer H, Ritter S, Gerlich WH, Thomssen R (1983) Kinetics, subtype specificity and immunoglobulin class of anti-HBs induced by hepatitis B vaccine. Dev Biol Stand 54:179–189
78. Heijtink RA, Bergen Pv, Melber K, Janowicz ZA, Osterhaus AD (2002) Hepatitis B surface antigen (HBsAg) derived from yeast cells (Hansenula polymorpha) used to establish an influence of antigenic subtype (adw2, adr, ayw3) in measuring the immune response after vaccination. Vaccine 20:2191–2196
79. Feeney SA, McCaughey C, Watt AP, El Agnaf MR, McDougal N, Morris K, Wend UC, Gerlich WH, Coyle PV (2013) Reactivation of occult hepatitis B virus infection following cytotoxic lymphoma therapy in an anti-HBc negative patient. J Med Virol 85:597–601
80. Tacke F, Amini-Bavil-Olyaee S, Heim A, Luedde T, Manns MP, Trautwein C (2007) Acute hepatitis B virus infection by genotype F despite successful vaccination in an immune-competent German patient. J Clin Virol 38:353–357
81. O'Halloran JA, De Gascun CF, Dunford L, Carr MJ, Connell J, Howard R, Hall WW, Lambert JS (2011) Hepatitis B virus vaccine failure resulting in chronic hepatitis B infection. J Clin Virol 52:151–154
82. Hadler SC, Francis DP, Maynard JE, Thompson SE, Judson FN, Echenberg DF, Ostrow DG, O'Malley PM, Penley KA, Altman NL et al (1986) Long-term immunogenicity and efficacy of hepatitis B vaccine in homosexual men. N Engl J Med 315:209–214
83. Mu SC, Lin YM, Jow GM, Chen BF (2009) Occult hepatitis B virus infection in hepatitis B vaccinated children in Taiwan. J Hepatol 50:264–272
84. Pande C, Sarin SK, Patra S, Kumar A, Mishra S, Srivastava S, Bhutia K, Gupta E, Mukhopadhyay CK, Dutta AK, Trivedi SS (2013) Hepatitis B vaccination with or without hepatitis B immunoglobulin at birth to babies born of HBsAg-positive mothers prevents overt HBV transmission but may not prevent occult HBV infection in babies: a randomized controlled trial. J Viral Hepat 20:801–810
85. Xu L, Wei Y, Chen T, Lu J, Zhu CL, Ni Z, Huang F, Du J, Sun Z, Qu C (2010) Occult HBV infection in anti-HBs-positive young adults after neonatal HB vaccination. Vaccine 28:5986–5992
86. Poovorawan Y, Chongsrisawat V, Theamboonlers A, Leroux-Roels G, Kuriyakose S, Leyssen M, Jacquet J-M (2011) Evidence of protection against clinical and chronic hepatitis B infection 20 years after infant vaccination in a high endemicity region. J Viral Hepat 18:369–375
87. Werner JM, Abdalla A, Gara N, Ghany MG, Rehermann B (2013) The hepatitis B vaccine protects re-exposed health care workers, but does not provide sterilizing immunity. Gastroenterology 145:1026–1034
88. Westhoff TH, Jochimsen F, Schmittel A, Stöffler-Meilicke M, Schäfer J-H, Zidek W, Gerlich WH, Thiel E (2003) Fatal hepatitis B virus reactivation by an escape mutant following rituximab therapy. Blood 102:1930
89. Awerkiew S, Däumer M, Reiser M, Wend UC, Pfister H, Kaiser R, Willems WR, Gerlich WH (2007) Reactivation of an occult hepatitis B virus escape mutant in an anti-HBs positive, anti-HBc negative lymphoma patient. J Clin Virol 38:83–86
90. Lai MW, Lin TY, Tsao KC, Huang CG, Hsiao MJ, Liang KH, Yeh CT (2012) Increased seroprevalence of HBV DNA with mutations in the s gene among individuals greater than 18 years old after complete vaccination. Gastroenterology 143:400–407

91. Gerin JL, Holland PV, Purcell RH (1971) Australia antigen: large-scale purification from human serum and biochemical studies of its proteins. J Virol 7:569–576
92. Gerlich W, May G (1973) Hepatitis-associated antigen: purification and properties. Zentralbl Bakteriol Orig A 224:49–60 German
93. Chairez R, Hollinger FB, Brunschwig JP, Dreesman GR (1975) Comparative biophysical studies of hepatitis B antigen, subtypes adw and ayw. J Virol 15:182–190
94. Shih JW, Gerin JL (1977) Proteins of hepatitis B surface antigen. J Virol 21:347–357
95. Millman I, Loeb LA, Bayer ME, Blumberg BS (1970) Australia antigen (a hepatitis-associated antigen): purification and physical properties. Exp Med 131:1190–1199
96. Neurath AR, Prince AM, Lippin A (1974) Hepatitis B antigen: antigenic sites related to human serum proteins revealed by affinity chromatography. Proc Natl Acad Sci USA 71:2663–2667
97. Schüttler CG, Wend UC, Faupel FM, Lelie PN, Gerlich WH (2010) Antigenic and physicochemical characterization of the 2nd International Standard for hepatitis B virus surface antigen (HBsAg). J Clin Virol 47:238–242
98. Bremer CM, Sominskaya I, Skrastina D, Pumpens P, El Wahed AA, Beutling U, Frank R, Fritz HJ, Hunsmann G, Gerlich WH, Glebe D (2011) N-terminal myristoylation-dependent masking of neutralizing epitopes in the preS1 attachment site of hepatitis B virus. J Hepatol 55:29–37
99. Glebe D, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH (2003) Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. J Virol 77:9511–9521 Erratum in: J Virol 2005 79:14470
100. Tiollais P, Charnay P, Vyas GN (1981) Biology of hepatitis B virus. Science 213(4506):406–411
101. Stibbe W, Gerlich WH (1982) Variable protein composition of hepatitis B surface antigen from different donors. Virology 123:436–442
102. Stibbe W, Gerlich WH (1983) Structural relationships between minor and major proteins of hepatitis B surface antigen. J Virol 46:626–628
103. Heermann KH, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH (1984) Large surface proteins of hepatitis B virus containing the pre-s sequence. J Virol 52:396–402
104. Imai M, Yanase Y, Nojiri T, Miyakawa Y, Mayumi M (1979) A receptor for polymerized human and chimpanzee albumins on hepatitis B virus particles co-occurring with HBeAg. Gastroenterology 76:242–247
105. Trevisan A, Gudat F, Guggenheim R, Krey G, Dürmüller U, Lüond G, Düggelin M, Landmann J, Tondelli P, Bianchi L (1982) Demonstration of albumin receptors on isolated human hepatocytes by light and scanning electron microscopy. Hepatology 2:832–835
106. Machida A, Kishimoto S, Ohnuma H, Miyamoto H, Baba K, Oda K, Nakamura T, Miyakawa Y, Mayumi M (1983) A hepatitis B surface antigen polypeptide (P31) with the receptor for polymerized human as well as chimpanzee albumins. Gastroenterology 85:268–274
107. Milich DR, Thornton GB, Neurath AR, Kent SB, Michel ML, Tiollais P, Chisari FV (1985) Enhanced immunogenicity of the pre-S region of hepatitis B surface antigen. Science 228(4704):1195–1199
108. Itoh Y, Takai E, Ohnuma H, Kitajima K, Tsuda F, Machida A, Mishiro S, Nakamura T, Miyakawa Y, Mayumi M (1986) A synthetic peptide vaccine involving the product of the pre-S(2) region of hepatitis B virus DNA: protective efficacy in chimpanzees. Proc Natl Acad Sci USA 83:9174–9178
109. Emini EA, Larson V, Eichberg J, Conard P, Garsky VM, Lee DR, Ellis RW, Miller WJ, Anderson CA, Gerety RJ (1989) Protective effect of a synthetic peptide comprising the complete preS2 region of the hepatitis B virus surface protein. J Med Virol 28:7–12
110. Neurath AR, Kent SB, Parker K, Prince AM, Strick N, Brotman B, Sproul P (1986) Antibodies to a synthetic peptide from the preS 120–145 region of the hepatitis B virus envelope are virus neutralizing. Vaccine 4:35–37
111. Michel ML, Pontisso P, Sobczak E, Malpièce Y, Streeck RE, Tiollais P (1984) Synthesis in animal cells of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin. Proc Natl Acad Sci USA 81:7708–7712
112. Tron F, Degos F, Bréchot C, Couroucé AM, Goudeau A, Marie FN, Adamowicz P, Saliou P, Laplanche A, Benhamou JP et al (1989) Randomized dose range study of a recombinant hepatitis B vaccine produced in mammalian cells and containing the S and PreS2 sequences. J Infect Dis 160:199–204
113. Jungers P, Chauveau P, Couroucé AM, Devillier P, Excler JL, Bailleux F, Saliou P (1994) Immunogenicity of the recombinant GenHevac B Pasteur vaccine against hepatitis B in chronic uremic patients. J Infect Dis 169:399–402
114. Eyigün CP, Yilmaz S, Gül C, Sengül A, Hacibektasoglu A, Van Thiel DH (1998) A comparative trial of two surface subunit recombinant hepatitis B vaccines vs a surface and PreS subunit vaccine for immunization of healthy adults. J Viral Hepat 5:265–269
115. Cavuslu S, Birinci I, Oncul O, Emekdas G, Erdemoglu A (2002) The comparison of different recombinant hepatitis-B vaccines. Internet J Infect Dis 2(2)
116. Imamura T, Araki M, Miyanohara A, Nakao J, Yonemura H, Ohtomo N, Matsubara K (1987) Expression of hepatitis B virus middle and large surface antigen genes in *Saccharomyces cerevisiae*. J Virol 61:3543–3549
117. Kniskern PJ, Hagopian A, Burke P, Schultz LD, Montgomery DL, Hurni WM, Ip CY, Schulman CA, Maigetter RZ, Wampler DE et al (1994) Characterization and evaluation of a recombinant hepatitis B vaccine expressed in yeast defective for N-linked hyperglycosylation. Vaccine 12:1021–1025
118. Bruss V, Ganem D (1991) The role of envelope proteins in hepatitis B virus assembly. Proc Natl Acad Sci USA 88:1059–1063
119. Ni Y, Sonnabend J, Seitz S, Urban S (2010) The pre-s2 domain of the hepatitis B virus is dispensable for infectivity but serves a spacer function for L-protein-connected virus assembly. J Virol 84:3879–3888
120. Fernholz D, Galle PR, Stemler M, Brunetto M, Bonino F, Will H (1993) Infectious hepatitis B virus variant defective in pre-S2 protein expression in a chronic carrier. Virology 194:137–148
121. Sobotta D, Sominskaya I, Jansons J, Meisel H, Schmitt S, Heermann KH, Kaluza G, Pumpens P, Gerlich WH (2000) Mapping of immunodominant B-cell epitopes and the human serum albumin-binding site in natural hepatitis B virus surface antigen of defined genosubtype. J Gen Virol 81:369–378
122. Neurath AR, Kent SB, Strick N (1984) Location and chemical synthesis of a pre-S gene coded immunodominant epitope of hepatitis B virus. Science 224(4647):392–395
123. Neurath AR, Kent SB, Strick N, Taylor P, Stevens CE (1985) Hepatitis B virus contains pre-S gene-encoded domains. Nature 315(6015):154–156
124. Neurath AR, Seto B, Strick N (1989) Antibodies to synthetic peptides from the preS1 region of the hepatitis B virus (HBV) envelope (env) protein are virus-neutralizing and protective. Vaccine 7:234–236
125. Neurath AR, Kent SB, Strick N, Parker K (1986) Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 46:429–436
126. Glebe D, Urban S (2007) Viral and cellular determinants involved in hepadnaviral entry. World J Gastroenterol 13:22–38 Review

127. Sureau C, Salisse J (2013) A conformational heparan sulfate binding site essential to infectivity overlaps with the conserved hepatitis B virus a-determinant. Hepatology 57:985–994
128. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C (2002) Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci USA 99:15655–15660
129. Glebe D, Urban S, Knoop EV, Cag N, Krass P, Grün S, Bulavaite A, Sasnauskas K, Gerlich WH (2005) Mapping of the hepatitis B virus attachment site by use of infection-inhibiting preS1 lipopeptides and tupaia hepatocytes. Gastroenterology 129:234–245
130. Gripon P, Cannie I, Urban S (2005) Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein. J Virol 79:1613–1622
131. Janowicz ZA, Melber K, Merckelbach A, Jacobs E, Harford N, Comberbach M, Hollenberg CP (1991) Simultaneous expression of the S and L surface antigens of hepatitis B, and formation of mixed particles in the methylotrophic yeast, Hansenula polymorpha. Yeast 7:431–443
132. Leroux-Roels G, Desombere I, De Tollenaere G, Petit MA, Desmons P, Hauser P, Delem A, De Grave D, Safary A (1997) Hepatitis B vaccine containing surface antigen and selected preS1 and preS2 sequences. 1. Safety and immunogenicity in young, healthy adults. Vaccine 15:1724–1731
133. Leroux-Roels G, Desombere I, Cobbaut L, Petit MA, Desmons P, Hauser P, Delem A, De Grave D, Safary A (1997) Hepatitis B vaccine containing surface antigen and selected preS1 and preS2 sequences. 2. Immunogenicity in poor responders to hepatitis B vaccines. Vaccine 15:1732–1736
134. Laub O, Rall LB, Truett M, Shaul Y, Standring DN, Valenzuela P, Rutter WJ (1983) Synthesis of hepatitis B surface antigen in mammalian cells: expression of the entire gene and the coding region. J Virol 48:271–280
135. Persing DH, Varmus HE, Ganem D (1986) Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. Science 234(4782):1388–1391
136. Marquardt O, Heermann KH, Seifer M, Gerlich WH (1987) Cell type specific expression of preS1 antigen and secretion of hepatitis B virus surface antigen. Arch Virol 96:249–256
137. Heermann KH, Kruse F, Seifer M, Gerlich WH (1987) Immunogenicity of the gene S and Pre-S domains in hepatitis B virions and HBsAg filaments. Intervirology 28:14–25
138. Milich DR, McNamara MK, McLachlan A, Thornton GB, Chisari FV (1985) Distinct H-2-linked regulation of T-cell responses to the pre-S and S regions of the same hepatitis B surface antigen polypeptide allows circumvention of nonresponsiveness to the S region. Proc Natl Acad Sci USA 82:8168–8172
139. Shouval D, Ilan Y, Adler R, Deepen R, Panet A, Even-Chen Z, Gorecki M, Gerlich WH (1994) Improved immunogenicity in mice of a mammalian cell-derived recombinant hepatitis B vaccine containing pre-S1 and pre-S2 antigens as compared with conventional yeast-derived vaccines. Vaccine 12:1453–1459
140. Rendi-Wagner P, Shouval D, Genton B, Lurie Y, Rümke H, Boland G, Cerny A, Heim M, Bach D, Schroeder M, Kollaritsch H (2006) Comparative immunogenicity of a PreS/S hepatitis B vaccine in non- and low responders to conventional vaccine. Vaccine 24:2781–2789
141. Wagner D, Wagenbreth I, Stachan-Kunstyr R, Thoma HA, Hemmerling AE, Flik J (1994) Hepatitis B vaccination of immunosuppressed heart transplant recipients with the vaccine Hepa Gene 3 containing pre-S1, pre-S2, and S gene products. Clin Investig 72:350–352
142. Haubitz M, Ehlerding G, Beigel A, Heuer U, Hemmerling AE, Thoma HA (1996) Clinical experience with a new recombinant hepatitis-B vaccine in previous non-responders with chronic renal insufficiency. Clin Nephrol 45:180–182
143. Pride MW, Bailey CR, Muchmore E, Thanavala Y (1998) Evaluation of B and T-cell responses in chimpanzees immunized with Hepagene, a hepatitis B vaccine containing pre-S1, pre-S2 gene products. Vaccine 16:543–550
144. Young MD, Schneider DL, Zuckerman AJ, Du W, Dickson B, Maddrey WC, US Hepacare Study Group (2001) Adult hepatitis B vaccination using a novel triple antigen recombinant vaccine. Hepatology 34:372–376
145. Jones T (2002) Hepagene (PowderJect). Curr Opin Investig Drugs 3:987–991
146. Shapira MY, Zeira E, Adler R, Shouval D (2001) Rapid seroprotection against hepatitis B following the first dose of a Pre-S1/Pre-S2/S vaccine. J Hepatol 34:123–127
147. Young MD, Rosenthal MH, Dickson B, Du W, Maddrey WC (2001) A multi-center controlled study of rapid hepatitis B vaccination using a novel triple antigen recombinant vaccine. Vaccine 19:3437–3443
148. Zuckerman JN, Zuckerman AJ, Symington I, Du W, Williams A, Dickson B, Young MD, UK Hepacare Study Group (2001) Evaluation of a new hepatitis B triple-antigen vaccine in inadequate responders to current vaccines. Hepatology 34:798–802
149. Krawczyk A, Ludwig C, Jochum C, Fiedler M, Heinemann FM, Shouval D, Roggendorf M, Roggendorf H, Lindemann M (2014) Induction of a robust T- and B-cell immune response in non- and low-responders to conventional vaccination against hepatitis B by using a third generation PreS/S vaccine. Vaccine 32:5077–5082
150. Yerushalmi B, Raz R, Blondheim O, Shumov E, Koren R, Dagan R (1997) Safety and immunogenicity of a novel mammalian cell-derived recombinant hepatitis B vaccine containing Pre-S1 and Pre-S2 antigens 46 in neonates. Pediatr Infect Dis J 16:587–592
151. Madaliński K, Sylvan SP, Hellstrom U, Mikołajewicz J, Dzierzanowska-Fangrat K (2004) Presence of anti-preS1, anti-preS2, and anti-HBs antibodies in newborns immunized with Bio-Hep-B vaccine. Med Sci Monit 10:10–17
152. Sylvan SP, Madalinski K, Hellström UB (2009) Anti-preS responses influence the anti-HBs response in newborns after vaccination with the third generation Sci-B-Vac vaccine. Vaccine 28:446–451
153. Hellström UB, Madalinski K, Sylvan SP (2009) PreS1 epitope recognition in newborns after vaccination with the third-generation Sci-B-Vac vaccine and their relation to the antibody response to hepatitis B surface antigen. Virol J 6:7. doi:10.1186/1743-422X-6-7
154. Saed N, Hakim M, Nijim Y, Safadi R et al (2014) Sci-B-Vac is superior to Engerix-B for preventing HBV vertical transmission. In: 49th European Association for the Study of the Liver International Liver Congress (EASL 2014), London, April 9–13, 2014. Abstract O121 J Hepatol 60, pp S1–S50
155. Aguilar A, González CA, Cinza Z, Cabrera J, Veliz G, Moreno SR et al (2007) Phase I clinical trial in healthy adults of a nasal vaccine candidate containing recombinant hepatitis B surface and core antigens. Int J Infect Dis 11:394–401
156. Niedre-Otomere B, Bogdanova A, Skrastina D, Zajakina A, Bruvere R, Ose V, Gerlich WH, Garoff H, Pumpens P, Glebe D, Kozlovska T (2012) Recombinant Semliki forest virus vectors encoding hepatitis B virus small surface and pre-S1 antigens induce broadly reactive neutralizing antibodies. J Viral Hepat 19:664–673
157. Pumpens P (2001) Grens E HBV core particles as a carrier for B cell/T cell epitopes. Intervirology 44:98–114
158. Milich DR, McLachlan A, Thornton GB, Hughes JL (1987) Antibody production to the nucleocapsid and envelope of the hepatitis B virus primed by a single synthetic T cell site. Nature 329(6139):547–549