

# HEPATITIS B VACCINES

Daniel SHOIVAL, Hadassah University Hospital, Jerusalem, Israel



[Print this page](#)

## Introduction

Hepatitis B vaccines are now available for over 20 years. The World Health Organization has recommended that hepatitis B vaccination should be included in routine immunization for all children, worldwide. As a result, various immunization strategies have been developed for routine infant vaccination, prevention of perinatal transmission and catch-up vaccination for older age groups.

Knowledge of the structure and genomic organization of the hepatitis B virus (HBV), reviewed in reference 1 and 2 has led to the development of immunogenic HBV vaccines with an excellent record of safety and reactogenicity. The purpose of this short overview is to summarize the structure and properties of available licensed HBV vaccines relevant to the hepatitis B consensus conference and to discuss potential applications for some of the newly developed vaccines.

All available HBV vaccines contain the hepatitis B envelope protein. Hepatitis B surface antigen(s) (HBsAg) is composed of three related envelope proteins which are synthesized by the alternate use of three translational start codons and a common stop codon. The HBsAg proteins include a major polypeptide of 226 amino acids (aa) designated small HBs (SHBs) previously also called major surface protein, in a non-glycosylated (p24) and glycosylated (gp27) form. The middle-sized protein (MHBs), which shares the 226 aa of the p24 region at the C terminus and has an additional 55 aa residue at the N terminus, is termed pre-S<sub>2</sub> corresponding to gp33 and gp36. The large HBs protein (LHBs) contains, in addition to the S and pre-S<sub>2</sub> domains, the pre-S<sub>1</sub> domain of 119 aa (p39, gp42)<sup>1-2, 4-6</sup>. In the native envelope HBsAg particles, all the proteins SHBs, MHBs, and LHBs are covalently linked to one another by intermolecular disulphide bonds between the S domains and partially embedded in membrane lipids. A relatively high proportion of LHBs will favor formation of filamentous HBsAg particles, while presence or absence of the MHBs domain will not affect the morphology of the HBsAg particle. The envelope of the wild type complete HBV particle has a composition similar to that of secreted HBsAg filaments, with a high proportion of LHBs. Thus, a vaccine containing HBsAg filaments should be able to induce an immune response to all three-envelope antigen components.

Originally four major subtypes of the HBV envelope protein, namely *adw*, *adr*, *ayw*, and *Ayr* were serologically identified. These were further divided into several subgroups. The frequency of these subtypes is distinct in different geographic areas. To date, at least seven genotypes (A-G), coding for various subtypes are known. The antigenic *specificity* is common to all HBV subtypes, while *d/y* and *w/r* are of mutually exclusive specificity. Antibodies to the determinant provide protection against infection by any of the HBV subtypes. However, several mutants of the envelope protein have been described following active or passive immunization with HBV vaccines or hepatitis B immunoglobulin (HBIG)<sup>3</sup>.

The biologic function of the various envelope proteins and the relative significance of the immune response to each of its components (S, pre-S<sub>2</sub>, and pre-S<sub>1</sub>) are only partially understood<sup>4, 5</sup>. Induction of immune memory against HBsAg and generation of anti-HBs antibodies are essential for long-term protection against HBV. In acute HBV infection, followed by resolution of viremia, detection of anti-HBs in serum, which represents the humoral immune response to the SHBs, is delayed for several weeks. In contrast, Pre-S<sub>1</sub> and pre-S<sub>2</sub> antigens and antibodies appear and disappear early after acute infection, sometimes in a biphasic pattern<sup>15</sup>. The pre-S antigens seem to be important in inducing T-cell help for production of anti-HBs. Thus, T cell recognition requires presentation to T cells of HBV antigenic determinants, which must be processed by antigen presenting cells prior to expression on the surface of T cells in association with HLA

antigens. Milich and co-workers have characterized the T-cell mediated immune response to pre-S antigens and have shown that enhanced immunity to HBsAg can be induced using Pre-S antigens in non-responder mice resistant to SHBs antigen<sup>5, 11, 13</sup>. This observation led to development of new vaccines containing pre-S antigens also called “third-generation HBV vaccines”.

Pre-S antigen(s) seem to induce neutralizing antibodies, which block attachment, endocytosis and possibly membrane penetration of HBV into the hepatocyte. The pre-S<sub>1</sub> domain of the large envelope protein contains a 21-47 AA sequence, important for attachment to the hepatocyte. The pre-S<sub>2</sub> domain has four putative functions. It is a proteolysis sensitive site and it has a 5-16 AA sequence which can block a human serum albumin receptor-binding site. It also has an activated protein kinase binding site as well as a permeabilization site, which may be important in transfer of HBV particles into the cytosol. Pre-S antigens, and particularly pre-S<sub>1</sub>, express highly immunogenic T and B cell epitopes, a property which has potential applications in third generation vaccines<sup>4, 7, 8, 9</sup>. Furthermore, synthetic peptides derived from AA sequence 5-32 may protect chimpanzees against HBV challenge<sup>10</sup>. It was suggested that the pre-S<sub>2</sub> antigen plays a role in penetration of HBV into its target cell, which may be prevented through an adequate anti-pre-S<sub>2</sub> response<sup>9</sup>. However, the precise mechanism of how HBV enters the hepatocyte is far from understood. There is also some evidence that HBV may attach to physiologically important receptors through its Pre-S<sub>1</sub> site, reacting with interleukin 6, asialoglycoprotein, or transferrin<sup>1-15</sup>.

These combined properties seem to contribute to the enhanced immunogenicity of pre-S<sub>2</sub><sup>5,11,12,13</sup> in a glycosylated and possibly in a non-glycosylated form.

### **Measurement of humoral immune response to vaccination**

Antibody responses after exposure to HBsAg following infection with wild type HBV or after immunization are well characterized. Anti-HBs antibodies can be either subtype specific or common to all genotypes of HBsAg, for example, against the determinant, which is a neutralizing epitope present in all HBV vaccines in use. After natural infection, Pre-S<sub>1</sub> and pre-S<sub>2</sub> antibodies appear first, and before anti-HBs, but also disappear rapidly<sup>15</sup>. Their detection has been inconsistent because of varying specificities and sensitivity of the experimental assays used<sup>5, 6, 12, 15, 68, 69</sup>. Several murine monoclonal antibodies developed against HBV envelope proteins, and directed against the epitope, were shown to neutralize chimpanzee infectious doses of both *ad* or *ay* HBV subtypes. By convention, seroconversion to anti-HBs positivity is defined as detection of anti-HBs in an immunological assay at  $\geq 2.1$  standard deviations from the reading of the negative control, which is usually  $\sim 1$  mIU/ml (2.1-9 mIU/ml). Seroprotection against clinical disease is present when anti-HBs levels are  $\geq 10$  mIU/ml. Numerous studies have shown that most children and young adults will develop hundreds to several thousand mIU/ml of anti-HBs following three doses of a conventional HBV vaccine. Vaccines who develop an anti-HBs response above 2.1 and below 10 mIU/ml after three doses, given at 0, 1 and 6 months, are sometimes referred to as hyporesponders and probably are not adequately protected against challenge with HBV. Vaccines that develop an anti-HBs level between 10 and 100 mIU/ml after three doses are referred to as low responders. In the United Kingdom, seroprotection against HBV infection was recently re-defined at anti-HBs levels  $\geq 100$  mIU/ml. This approach has public health implications and may require redefinition of non-responsiveness to routine immunization. Anti-HBs levels tend to fall with time, but primed immune memory will respond to challenge with wild type virus as well as to booster inoculation with HBsAg through an anamnestic anti-HBs response. Currently, there is no reason to offer booster doses of HBV vaccines to vaccines who developed anti-HBs titers  $>100$  mIU/ml following a course immunization.

Detection of antibodies to pre-S antigens remains a research tool. Several assays were evaluated to monitor anti-pre-S<sub>2</sub> and anti-pre-S<sub>1</sub> antibodies but none of them was considered satisfactory for clinical monitoring in humans.

### **Hepatitis B Vaccines**

In the late 1970s, two vaccines against HBV were developed in the United States and France, both

containing purified HBsAg obtained from serum of HBsAg carriers<sup>16-18</sup>. These plasma-derived vaccines contained a suspension of HBsAg that had been subjected to a combination of aggressive biophysical and biochemical treatments, which led to partial disruption of the surface antigen. The final purified HBsAg was subjected to formaldehyde treatment and adsorbed to alum. A U.S. Product contained 22 nm HBsAg particles devoid of the pre-S proteins while a French HBV vaccine contained additional small and inconsistent amounts of pre-S<sub>2</sub> and pre-S<sub>1</sub> antigens in the final preparation. Later, similar vaccines were produced in Korea and China. Plasma-derived vaccines have been shown to be highly immunogenic, efficacious, and safe<sup>19-21</sup>.

Concerns as to the safety of a blood-derived product, as well as the inconsistency of a source of raw material and the availability of new recombinant DNA technology led to the development of "second-generation" recombinant vaccines produced from yeast transfected with the DNA sequence coding for the SHBs<sup>18, 21-24</sup>. Most experience available to date was gained using two recombinant vaccines, Engerix-B<sup>â</sup> (SmithKline Biologicals, Belgium) and RECOMBIVAX/ HB-Vax II<sup>â</sup> (Merck & Co., USA)<sup>22, 23</sup>. These two vaccines contain non-glycosylated SHBs p24, which must be released from within the yeast during the manufacturing process<sup>24</sup>. All licensed HBV vaccines in conventional use contain alum (Al(OH)<sub>3</sub>) as an adjuvant. Although a potent B cell stimulator, alum is ineffective in inducing a Th<sub>1</sub> response. New more potent adjuvants are under different stages of development. Such adjuvants are intended to induce a more rapid and enhanced anti-HBs response. Furthermore, Th<sub>1</sub> inducing adjuvants may be evaluated in patients already persistently infected with HBV. Although the yeast-derived particles of different vaccine manufacturers seem to have similar physical properties, their immunogenicity differs as shown in animal potency studies<sup>25</sup>. The recommended pediatric and adult dose per injection for Engerix-B<sup>â</sup> is double the dose for RECOMBIVAX<sup>â</sup> HB Vax II. So far, over a billion doses of recombinant vaccines have been administered worldwide, with an excellent record of safety and immunogenicity. Cost-benefit analyses have strongly supported the introduction of universal immunization against HBV to newborns<sup>26, 27</sup>. Indeed, over 140 countries have already introduced universal vaccination against HBV for newborns or individuals at risk. Furthermore, combination vaccines, using tetravalent diphtheria-tetanus-pertussis and hexavalent hepatitis B vaccines<sup>28</sup>, or combined A and B vaccine<sup>29</sup>, have been developed.

Despite the extraordinary efficacy of "second-generation" HBV vaccines, immunization failure may occur and can sometimes be explained by variables such as improper storage or administration, advanced age, obesity, renal failure, chronic liver disease and especially, immunosuppression. Another important factor that may affect non-responsiveness to SHBs immunization seems to be genetically determined resistance<sup>30, 31</sup>. The ability to produce antibodies in response to immunization with HBsAg is controlled by autosomal dominantly expressed HLA Class II molecules<sup>30-33</sup>. Hohler and co-workers have reported enhanced expression of DRB 1\*3, DRB 1\*7 and DRB 1\*14 in non-responders to an HBV vaccine<sup>33</sup>. In contrast, response to vaccination is related to DRB 1\*13, which seems to have a promoting effect on anti-HBs seroconversion. It was suggested that the differential association of DRB 1\*13 and DRB 1\*14 might allow identification of non-responsiveness to immunization with HBsAg. Milich and others have shown that non-responsiveness to SHBs immunization in mice can be circumvented through immunization with pre-S proteins<sup>32</sup>. Furthermore, immunization with synthetic MHBs (pre-S<sub>2</sub> or pre-S<sub>2</sub>/S) peptides, as well as with yeast and mammalian CHO cell derived pre-S<sub>2</sub>/S vaccines, may induce neutralizing antibodies and protect chimpanzees against HBV challenge<sup>10</sup>.

Yeast (and plasma) derived HBV vaccines are highly efficacious in preventing HBV and in reducing the incidence of persistent infection as well as of hepatocellular carcinoma. In a recent report, a total of 181 clinical studies were reviewed in which 24,277 individuals were immunized with Engerix B<sup>R</sup> and 8627 with Recombivax/HB-Vax II<sup>R</sup>.<sup>34</sup> The recommended pediatric dose per injection was 10 and 5 microgram for both vaccines and the adult dose was 20 and 10 microgram respectively. Seroprotection (>10 mIU/ml) was achieved in 95.8% and 94.3% respectively using the licensed 3 dose schedule at 0, 1 and 6 months.

Children and adolescents (1-19y) achieved the highest seroprotection rates, namely 98.6% and 98.8% respectively. Thus degree of non-response in children and young adults is low. Accelerated immunization schedules using two to four doses are effective although titer of anti-HBs antibodies is lower when interval between injections is reduced.

In summary, serum-derived and recombinant HBV vaccines have gained worldwide acceptance based on demonstration of efficacy, safety and cost effectiveness.

### **Do we need more immunogenic hepatitis B vaccines?**

Currently available vaccines are immunogenic, efficacious and safe. Yet there is justification for producing more immunogenic vaccines that could eventually be more efficacious in defined groups such as non-responders to yeast derived vaccines or travelers and healthcare workers who need rapid protection even after priming dose of a vaccine. The main target groups for such a vaccine should consist of genetically, overweight and age associated non-responders to conventional immunization, immunosuppressed and dialysis patients, as well as patients with chronic liver disease. A more immunogenic vaccine may also enable a reduction in the number of injections required for long-term protection against HBV, which could be administered alone or in combination with an HAV or other childhood vaccines, thus reducing the cumulative load of alum used as adjuvant in vaccines.

There is also the issue of vaccine-induced mutants. HBV-envelope variants (“escape mutants”) have been described in Italy, Singapore, Gambia and the USA, in recipients of serum derived, as well as recombinant, HBV vaccines<sup>3, 35-37</sup>. Similar mutants have also been described in liver transplant patients receiving polyclonal or monoclonal HBIG for protection against reinfection with HBV<sup>35, 38</sup>. The common reason for generation of such mutants is a single point mutation in one of the amino acids coding for a determinant of the viral surface protein (often at aa 145). It is likely that inclusion of pre-S<sub>2</sub> and pre-S<sub>1</sub> epitopes in future HBV vaccines may eliminate or reduce the generation of vaccine-associated escape mutants.

Several new experimental designs for novel HBV vaccines were reported in the last decade including:

- a. Yeast derived Pre-S/S vaccines
- b. Mammalian-cell derived pre-S/S vaccines
- c. DNA vaccines
- d. Polypeptide micelle vaccine derived from HBsAg
- e. Expression of immunogenic HBV peptides in vaccinia virus
- f. Synthetic polypeptides containing immunogenic surface or core epitopes
- g. Anti-idiotypic vaccines
- h. Oral immunization with a recombinant salmonella gene product containing HBcASg epitopes

The following discussion will concentrate on Pre S/S HBV vaccines with a focus on mammalian cell derived vaccines. Mammalian-cell derived vaccines expressing pre-S/S antigens have undergone clinical trials and three of them are already licensed in a number of countries<sup>39-67</sup>. These vaccines were developed following the pioneering studies of Neurath, Milich, Gerlich and Tiollais on the role of the pre-S antigens in neutralization and prevention of HBV<sup>8, 9, 14, 32</sup>.

## **Pre-S/S hepatitis B vaccines**

### **Yeast-Derived pre-S<sub>2</sub>/S HBV Vaccines**

Vaccines containing pre-S<sub>2</sub>/S particles expressed in yeast have been developed in Japan, the US and Belgium<sup>42-44</sup>. A preliminary study with one experimental pre-S<sub>2</sub>/S vaccine developed in the US, suggested a somewhat higher immunogenicity and good tolerability for the pre-S<sub>2</sub>/S vaccine as compared to the S vaccine, in male volunteers. A larger scale comparative trial did not confirm the preliminary observation.

More encouraging results were reported from Japan, where a yeast-derived pre-S<sub>2</sub>/S recombinant vaccine was reported to produce a faster seroconversion rate, in non-responders to conventional vaccination and in overweight Sumo wrestlers<sup>42-45</sup>. However, efforts to produce a yeast derived pre-S/S vaccine have not resulted in a licensed product in the Western Hemisphere.

### **Rationale for Developing Mammalian-Cell Derived HBV Vaccines**

Choosing a host cell for expression of a recombinant vaccine depends upon the safety, efficacy, potential for scaling-up production and cost effectiveness. Early expression systems used initially for production of recombinant HBsAg, included *E. coli*, yeast (*S. cerevisiae* or *H. polymorpha*), insect-derived Baculovirus, and vaccinia virus. The yeast has been developed to an excellent expression system for SHBsAg. It is cost-effective, easy to scale up, and its HBsAg product was shown to be well tolerated safe and immunogenic. Nevertheless, it has a number of disadvantages, including the fact that the antigen is internal and the yeast must be broken to release the recombinant product. Furthermore, the yeast is unable to provide the same post translational modifications, protein folding, macromolecular assembly, and glycosylation, as observed in infected human hepatocytes, properties which are important for inducing enhanced immunogenicity, and which are present in mammalian cells. Indeed, studies in mice and in humans suggest an immunogenic advantage of mammalian-cell derived envelope particles (pre-S<sub>2</sub>/S or pre-S<sub>1</sub>/pre-S<sub>2</sub>/S), as compared to yeast-derived non-glycosylated small surface antigen<sup>25, 40, 46-47</sup>. Available information suggests that Chinese hamster ovary cell line (CHO) and mouse-cell line derived pre-S/S hepatitis B vaccines seem to be more immunogenic on the T cell level (even with the use of alum as an adjuvant). Such vaccines were shown to generate T cell help leading to higher seroconversion rates and anti-HBs titers at lower immunogenic doses, as compared to yeast-derived SHBsAg containing products<sup>46-54</sup>. Concern has been raised as to the safety of mammalian-cell line proto-oncogenes, which theoretically may be able to induce neoplastic transformation in the vaccinee. Temin has already stated that the risk must be very low<sup>55</sup>. Current CHO derived HBV vaccines have a residual DNA content estimated at <10 pg/dose, which is 1/10 of the WHO recommended limit and one billionths of the dose shown to induce tumors in mice<sup>56</sup>. The current safety record of mammalian-cell derived biological products, and especially CHO-derived antigens, is excellent, and a number of such products are already licensed. Specifically, significant adverse effects were not reported in four clinical trials, in which 920 newborns and adults received the pre-S<sub>2</sub>/S CHO-derived vaccine<sup>57</sup> and in a number of clinical trials using either CHO or mouse derived mammalian cell Pre-S<sub>1</sub>/Pre-S<sub>2</sub>/S vaccines<sup>46, 49, 50-51, 53-54, 61-66</sup>.

### **Mammalian-cell derived Pre-S<sub>2</sub>/S HBV vaccines**

Early observations using serum-derived vaccines which contained low levels of pre-S<sub>2</sub> antigen suggested an enhanced immunogenicity as compared to recombinant vaccines<sup>58</sup>. A "third-generation" mammalian-cell derived vaccine was first developed at the Pasteur Institute in transfected CHO cells, expressing S and pre-S<sub>2</sub> antigens<sup>39</sup>. A large-scale clinical trial with the CHO-derived vaccine was conducted in French Polynesia starting in 1988<sup>57</sup>. Newborns received 3 or 4 doses of 20 mg Gen Hevac B, given at different time intervals. The vaccine was well tolerated without significant adverse events. Moreover, seroconversion rates were 88-98% after the first and second dose, respectively, implying an extraordinary immunogenicity. Out of 582 vaccinated children, four children became HBsAg carriers (most probably as a result of intrauterine infection), as compared with the theoretically expected 38 HBsAg carriers in non-vaccinated individuals. Sixteen percent of vaccinees seroconverted to anti-HBc despite vaccination, but did not develop clinical disease or persistent viral infection. This result was obtained without the coverage of hepatitis B immune globulin, and presumably these anti-HBc<sup>+</sup> children were born to HBV infected or recovered mothers, since some of them lost anti-HBc over time. The high immunogenicity of this new vaccine was also demonstrated in a study where 120 chronic uremic pre-dialysis patients received 4 doses of either 20 mg/dose of the pre-S<sub>2</sub>/S Gen Hevac Bâ vaccine or 5 mg/dose of the Pasteur plasma-derived vaccine<sup>59</sup>. The new recombinant vaccine elicited higher seroconversion rates (94%), as compared to the plasma-derived vaccine (76%) in this population of vaccine-resistant patients.

## **Mammalian-cell derived pre-S<sub>1</sub>/pre-S<sub>2</sub>/S HBV vaccines**

Two such vaccines, Bio-Hep B<sup>R</sup>/Sci B Vac<sup>TM</sup> 42, 48, 50, 53, 54, 56, 57, 62-69 and Hepacare<sup>R</sup> 43, 49, 51, 52, 55 were developed in CHO cells and in a mouse cell line respectively, both expressing pre-S<sub>1</sub>/pre-S<sub>2</sub>/S epitopes. Both vaccines were shown to induce a rapid and augmented anti-HBs response with seroconversion appearing earlier as compared to yeast derived vaccines<sup>66</sup>. Furthermore, non-response to conventional SHBs containing vaccines was bypassed as shown in a limited number of clinical trials. The role of such vaccines in protecting defined risk groups against HBV infection is under discussion. Available evidence already suggests that the high seroconversion rates observed after the priming dose with a pre-S<sub>1</sub>/pre-S<sub>2</sub>/S vaccine may enable reduction in the number of recommended doses from three to two (with a one-five month interval between injections).

## **Summary**

Yeast derived hepatitis B vaccines, containing the small HBV envelope protein SHBAg are immunogenic, safe and cost effective in prevention of hepatitis B virus infection in neonates, children and adults. Newly developed pre-S/S hepatitis B vaccines may play a role in inducing fast and augmented seroconversion rates in special risk groups.

Table 1. Development of Hepatitis B Vaccines, Worldwide

Type	Manufacturer	Envelope Antigen	Remarks
Plasma-derived, SHBs	Hepatavax-B® (Merck & Co.)	SHBs	HBsAg, 5-40 mg/dose
	Hevac B® (Pasteur)	SHBsAg, (±MHBs*)	HBsAg, 5-20 mg/dose
	KGCâ (Korea Green Cross)	SHBs <sup>1</sup>	
Recombinant, yeast derived*	RECOMBIVAXâ HB (Merck & Co., USA)	SHBs <sup>1</sup> HBsAg	HBsAg, 2.5-10 mg/dose
	Engerix-Bâ (SmithKline, Belgium)	SHBs <sup>1</sup> HBsAg	HBsAg, 10-20 mg/dose
	TGP 943â (Takeda Chem, Japan) <sup>4</sup>	SHBs <sup>1</sup> , MHBs HBsAg	10 mg/dose
Recombinant, mammalian cell derived	Gen Hevac Bâ (Pasteur, France) <sup>2</sup>	SHBs, MHBs	HBsAg +pre-S <sub>2</sub> , 20 mg/dose
	Bio-Hep-Bâ/Sci-B-Vacâ (Bio-Technology General, Israel) <sup>3,5</sup>	SHBs, MHBs, LHBs	HBsAg, pre-S <sub>2</sub> , pre-S <sub>1</sub> 2.5-10mg/dose
	AG-3â (Hepageneâ, Hepacare <sup>R</sup> ) (Medeva, UK, Evans UK) <sup>3,6</sup>	SHBs, MHBs, LHBs	HBsAg, pre-S <sub>2</sub> , pre-S <sub>1</sub> 10-20mg/dose

\*Additional yeast derived vaccines are produced in Japan, Korea, Cuba and Germany (*Hansenula polymorpha*)

<sup>1</sup> SHBs-p24

<sup>2</sup> Contain non-glycosylated and glycosylated p24, gp27, gp33, gp36

<sup>3</sup> Contain non-glycosylated and glycosylated p24, gp27, gp33, gp36, p39, gp42.

<sup>4</sup> licenced in Japan

<sup>5</sup> licenced in Israel

<sup>6</sup> licenced in W. Europe

## **REFERENCES**

1. Ganem D, Varmus HE. *The molecular biology of the hepatitis B viruses*. *Ann Rev Biochem* 1987, 56:651-693.
2. Lau JYN, Wright TL. *Molecular virology and pathogenesis of hepatitis B*. *Lancet* 1993, 342:1335-1340.
3. Oon CJ, Lim GK, Ye Z, Goh KT, Tan KL, Yo SL et al. *Molecular epidemiology of HBV vaccine variants in Singapore*. *Vaccine* 1995, 13:699-702.
4. Neurath AR, Kent SBH. *Antigenic structure of human hepatitis viruses*. In: MHV van Regenmantel and AR Neurath, eds. *Immunochemistry of viruses: The basis for serodiagnosis and vaccines*. Elsevier Science Publishers BV 1985, pp 325-365.
5. Milich DR, Thornton GB, Neurath AR, Kent SB, Michel ML, Tiollais P, Chisari FV. *Enhanced immunogenicity of the pre-S region of hepatitis B surface antigen*. *Science* 1985, 228:1195-1199.
6. Heerman KH, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH. *Large surface proteins of hepatitis B virus containing the pre-S sequence*. *J Virol* 1984 52:396-402.
7. Neurath AR, Kent SBH, Strick N, Taylor P, Stevens CE. *Hepatitis B virus contains pre-S gene-encoded domains*. *Nature* 1985 315:154-156.
8. Neurath AR, Kent SB, Parker K et al. *Antibodies to a synthetic peptide from the pre-S 120-145 region of the hepatitis B virus envelope are virus neutralizing*. *Vaccine* 1986, 4:35-37.
9. Gerlich WH, Deepen R, Heermann KH, Krone B, LU XY, Seifer M, Thomssen R. *Protective potential of hepatitis B virus antigens other than the S gene*. *Vaccine* 1990, 8:S63-S68.
10. Itoh Y, Taka E, Ohnuma H et al. *A synthetic peptide vaccine involves the product of the pre-S<sub>2</sub> region of hepatitis B virus DNA: protective efficacy in chimpanzees*. *Proc Natl Acad Sci (USA)* 1986, 83:9174-9178.
11. Milich DR, McLachlan A, Chisari FU, Kent SBH, Thornton GB. *Immune response to the pre-S1 region of the HBsAg: A pre-S1 specific T cell response can bypass non-responsiveness to the pre-S2 and S regions of HBsAg*. *J Immunol* 1986; 137:315-322.
12. Petit MA, Maillard P, Capel F, Pillot J. *Immunochemical structure of the hepatitis B surface antigen vaccine-II. Analysis of antibody responses in human sera against the envelope proteins*. *Molec Immun* 1986 23:511-523.
13. Milich DR, Hughes JL, McLachlan A, Thornton GB, Moriarty A. *Hepatitis B synthetic immunogen composed of nucleocapsid T-cell response sites and an envelope B cell epitope*. *Proc Natl Acad Sci (USA)* 1988, 85:1610-1614.
14. Michel ML, Pontisso P, Sobczak E, Malpiece Y, Streeck RE, Tiollais P. *Synthesis in animal cells of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin*. *Proc Natl Acad Sci USA* 1984, 81:7708-7712.
15. Klinkert MQ, Theilmann L, Pfaff E, Schaller H. *Pre-S1 antigens and antibodies early in the course of acute hepatitis B virus infection*. *J Virol* 1986, 58:522-525.
16. Maupas P, Chirion JP, Goudeau A, Coursaget P, Perrin J, Barin F et al. *Active immunization against hepatitis B in an area of high endemicity. Part II*. *Prog Med Virol* 1981, 27:185-201.
17. Szmuness W, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC et al. *Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high risk population in the US*. *N Eng J Med* 1980, 303:833-841.
18. Sitrin RD, Wampler DE, Ellis RW. *Survey of hepatitis B vaccines and their production processes*. In: RW Ellis, ed. *Hepatitis B Vaccine in Clinical Practice*. Marcel Dekker, New York 1993, pp 83-101.
19. Szmuness W, Stevens CE, Zang EA, Harley EJ, Kellner A. *A controlled clinical trial of the efficacy of the hepatitis B Vaccine (Heptavax B): A final report*. *Hepatology* 1981, 1:377-385.
20. Shaw FE, Graham DJ, Guess HA, Milstein JB, Johnson JM, Schatz GC et al. *Postmarketing surveillance for neurologic adverse events reported after hepatitis B vaccination: Experience of the first three years*. *Am J Epidemiol* 1988, 127:337-351.
21. McMahon BJ, Helminiak C, Wainwright RB, Bulkow L, Trimble BA, Wainwright K et al. *Frequency of adverse reactions to hepatitis B vaccine in 43618 persons*. *Am J Med* 1992, 92:254-256.
22. McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR et al. *Human hepatitis B vaccine from recombinant yeast*. *Nature* 1984, 307:178-181.
23. Andre FE. *Overview of a 5-year clinical experience with a yeast derived hepatitis B vaccine*. *Vaccine* 1990, 8:574-578.
24. Stephanie J. *Development and production aspects of a recombinant yeast vaccine*. *Vaccine* 1990,

8:S69-S73.

25. Shouval D, Ilan Y, Adler R, Deepen R, Panet A, Even-Chen Z, Gorecki M, Gerlich WH. Improved immunogenicity in mice of a mammalian cell-derived recombinant hepatitis B vaccine containing pre-S<sub>1</sub> and pre-S<sub>2</sub> antigens as compared with conventional yeast-derived vaccines. *Vaccine* 1994, 12:1453-1459.
26. Arevalo JA, Washington E. Cost effectiveness of perinatal screening and immunization for hepatitis B virus. *JAMA* 1988, 259:365-369.
27. Ginsberg GM, Shouval D. Cost-benefit analysis of a nationwide inoculation programme against hepatitis B in an area of intermediate endemicity. *J Epidemiol & Comm Health* 1992, 46:587-594.
28. Poovorawan Y, Theamboonlers A, Sanpavat S et al. The immunogenicity and reactogenicity of combined tetravalent diphtheria, tetanus, pertussis and hepatitis B vaccine in infants. In: T Oda, K Nishioka, H Suzuki, eds. *Viral hepatitis and liver disease*. Springer Verlag, Tokyo 1994, pp 526-529.
29. Thoelen S, Van Damme I, Leentvaar-Kuypers A, Leroux-Roels G, Bruguera M, Frei PC et al. The first combined vaccine against hepatitis A and B: an overview. *Vaccine* 1999, 17:1657-1662.
30. Craven DE, Awdeh ZL, Kunches LM, Yunis EJ, Deinstag JL, Werner BG et al. Nonresponsiveness to hepatitis B vaccine in health care workers: Results of revaccination and genetic typing. *Ann Int Med* 1986, 105:356-360.
31. Alper CA, Kruskall MS, Marcus-Bagley D, Craven DE, Katz AJ, Brink SJ et al. Genetic prediction of nonresponsiveness to hepatitis B vaccine. *N Eng J Med* 1989, 321:708-712.
32. Milich DR. T and B cell recognition of hepatitis B viral antigens. *Immunol Today* 1988, 9:380-391.
33. Hohler T, Meyer CU, Notghi A, Stradmann-Bellinghausen B, Schneider PM, Starke R et al. Influence of major histocompatibility complex class II and T cell V beta repertoire on response to immunization with HBsAg. *Hum Immunol* 1998, 59:212-218.
34. Coates T, Wilson R, Patrick G, et al. Hepatitis B vaccines: Assessment of the seroprotective efficacy of two recombinant DNA vaccines. *Clin thera* 2001;23:392-403
35. Hunt CM, McGill JM, Allen MI, Condreay LD. Clinical relevance of hepatitis B viral mutations. (Concise Review.) *Hepatology* 2000, 31:1037-1044.
36. Oren I, Hershow RC, Ben-Porath E, Krivoy N, Goldstein N, Rishpon S, Shouval D, Hadler SC, Alter MJ, Maynard JE, Alroy G. A common-source outbreak of fulminant hepatitis B in a hospital. *Ann Intern Med* 1989, 110:691-698.
37. Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *New Eng J Med* 1991, 324:1706-1709.
38. Shouval D, Samuel D. Hepatitis B immune globulin to prevent HBV graft re-infection following liver transplantation. (Concise Review) *Hepatology* 2000, 32:1189-1195.
39. Soulie JC, Devillier P, Santarelli J, Goudeau A, Vermeulen P, Geullier M et al. Immunogenicity and safety in newborns of a new recombinant hepatitis B vaccine containing the S and pre S<sub>2</sub> antigens. *Vaccine* 1991, 9:545-548.
40. Gorecki M, Panet A, Moav N. Development of a Pre-S<sub>1</sub>, Pre-S<sub>2</sub> and S recombinant hepatitis B vaccine in mammalian cells. In: Rizetto M, Purcell RH, Gerin JL, Verme G, editors. *Viral Hepatitis and Liver Disease. Proceedings of the IX Triennial International Symposium on Viral Hepatitis and Liver Disease*. Turin: Edizioni Minerva Medica, pp. 964-969, 1997.
41. Wagner D, Wagenbreth I, Stachan-Kunstyr R, Thoma HA, Hemmerling AE, Aik J et al. Hepatitis B vaccination of immunosuppressed heart transplant recipients with the vaccine Hepa Gene 3 containing pre-S<sub>1</sub> and pre-S<sub>2</sub> and S gene products. *Clin Invest* 1994, 72:350-352.
42. Suzuki H, Iino S, Shiraki K, Akahane Y, Okamoto H, Domoto K, Mishiro S et al. Safety and efficacy of a recombinant yeast-derived pre S<sub>2</sub>+S-containing hepatitis B vaccine (TGP-943): phase 1, 2 and 3 clinical testing. *Vaccine* 1994, 12:1090-1096.
43. Kuroda S, Fujisawa Y, Iino S, Akahane Y, Suzuki H. Induction of protection level of anti-pre S<sub>2</sub> antibodies in humans immunized with a novel hepatitis B vaccine consisting of M (pre S<sub>2</sub> + S) protein particles (a third generation vaccine). *Vaccine* 1991, 9:163-169.
44. Fujisawa Y, Kuroda S, Van Eerd PM, Schellekens H, Kakinuma A. Protective efficacy of a novel hepatitis B vaccine consisting of M (pre S<sub>2</sub> + S) protein particles (a third generation vaccine). *Vaccine* 1990, 8:192-198.
45. Yamada K, Kotani M, Eguchi T, Kobayashi M, Yamazoe K, Yanagi R, Ichikawa S, Tsuda F, Okamoto H. Efficacy of hepatitis B vaccines with and without preS<sub>2</sub>-region product in Sumo wrestlers in Japan.

*Hepatology Research* 1998, 12:3-11.

46. Koren R, Yap I, Guan R, Lolekha S, Hourvitz A, Roash Z, Atzmon Y, Bass D, Kagan L, Zychowicz HC, Czubkowska I, Gornicki J, Dagan R, Raz R, Shouval D. Safety, tolerability and immunogenicity in adults and children of a mammalian cell-derived recombinant hepatitis B vaccine, containing S, Pre-S<sub>1</sub> and Pre-S<sub>2</sub>. In: Rizzetto M, Purcell RH, Gerin JL, Verme G, editors. *Viral Hepatitis and Liver Disease. Proceedings of the IX Triennial International Symposium on Viral Hepatitis and Liver Disease*. Turin: Edizioni Minerva Medica, pp. 970–973, 1998.
47. Jones CD, Page M, Bacon A, Cahill E, Bentley M, Chatfield SN. T cell and antibody response characterization of a new recombinant Pre-S<sub>1</sub>, Pre-S<sub>2</sub> and SHBs antigen containing hepatitis B vaccine; demonstration of superior anti-SHBs antibody induction in responder mice. *Vaccine* 2000, 17:2528-2537.
48. Waters JA, Bailey C, Love C, Thomas HC. A study of the antigenicity and immunogenicity of a new hepatitis B vaccine using a panel of monoclonal antibodies. *J Med Virol* 1998, 54:1-6.
49. Zuckerman JN. Hepatitis B third generation vaccines: improved response and conventional vaccine non-response. *Vaccine* 1999, 17:330-339.
50. Yap I, Guan R, Chan SH. Study on the comparative immunogenicity of a recombinant DNA hepatitis B vaccine containing pre-S components of the HBV coat protein with non-pre-S containing vaccines. *Journal of Gastroenterology and Hepatology* 1995, 10:51-55.
51. Shouval D, Ilan Y, Hourvitz A, Mosseri R, Solomon A, Zychowicz C, Gornicki J, Czubkowska I, Madalinski K, Burczynska B, Adler R, Gorecki M, Koren R. Immunogenicity of a mammalian cell-derived recombinant hepatitis B vaccine containing pre S<sub>2</sub> and pPre S<sub>1</sub> antigens: A preliminary report. In: *Viral Hepatitis and Liver Disease* 1993, 543-546, Eds. K. Nishioka, H. Suzuki, S. Mishiro, T. Oda, Springer Verlag, Tokyo.
52. Pride MW, Bailey CR, Muchmore E, Thanavala Y. Evaluation of B and T cell responses in chimpanzees immunized with Hepagene, a hepatitis B vaccine containing Pre-S<sub>1</sub>, Pre-S<sub>2</sub> gene products. *Vaccine*, 1998, 16:543-550.
53. Raz R, Dagan R, Gallil A, Brill G, Kassis I, Koren R. Safety and immunogenicity of a novel mammalian cell-derived recombinant hepatitis B vaccine containing Pre-S<sub>1</sub> and Pre-S<sub>2</sub> antigens in children. *Vaccine* 1996, 3:207-211.
54. Yerushalmi B, Raz R, Blondheim O, Shumov E, Koren R, Dagan R. Safety and immunogenicity of a novel mammalian cell-derived recombinant hepatitis B vaccine containing Pre-S<sub>1</sub> and Pre-S<sub>2</sub> antigens in neonates. *Pediatr Infect Dis* 1997, 16:587-592.
55. Temin HM. Overview of biological effects of addition of DNA molecules to cells. *J Med Virol* 1990, 31:13-17.
56. Löwen J. Risk of tumor induction in-vivo by residual cellular DNA: Quantitative considerations. *J Med Virol* 1990, 31:50-53.
57. Moulia-Pelat JP, Spiegel A, Martin PM, Cardines R, Boutin JP, Roux JF et al. A 5 year immunization field trial against hepatitis B using CHO cell recombinant vaccine in French Polynesian Newborns. *Vaccine* 1994, 12:499-502.
58. Smit-Leijds MB, Kramer P, Heijtkink RA, Hop WC, Schlam SW. Hepatitis B vaccination of haemodialysis patients: randomized controlled trial comparing plasma-derived vaccine with and without pre S<sub>2</sub> antigen. *Eur J Clin Invest* 1990, 20:540-545.
59. Jungers P, Chauveau P, Courouce AM, Abbassi A, Devillier P, Marie FN et al. Recombinant vaccine and extracting vaccine against hepatitis B in patients with kidney insufficiency. A comparative study. *Press Medical* 1994, 23:277-280.
60. Diminsky D, Moav N, Gorecki M, Barenholz Y. Physical, chemical and immunological stability of CHO-derived hepatitis B surface antigen (HBsAg) particles. *Vaccine* 2000, 18:3-17.
61. Hourvitz A, Mosseri R, Solomon A, Yehezkeili, Y, Atsmon J, Danon YL, Koren R and Shouval D. Reactogenicity and immunogenicity of a new recombinant hepatitis B vaccine containing Pre S antigens: a preliminary report. *J Viral Hepatitis* 1996, 3:37-42.
62. Madalinski K, Zychowicz C, Gornicki J, Czubkowska I, Skarpetowski AC, Gorecki M, Koren R. *Polish Journal of Immunology* 1994 XIX:333-340.
63. Yap I, Guan R, Chan SH. Recombinant DNA hepatitis B vaccine containing pre-S<sub>2</sub> components of the HBV coat protein-a preliminary study on immunogenicity. *Vaccine* 1992, 10:439-432.
64. Yap I, Guan R, Chan SH. Recombinant DNA hepatitis B vaccine containing Pre-S components of the

*HBV coat protein-a preliminary study on immunogenicity. Vaccine 1992 10:439-432.*

65. Yap I, Guan R, Chan SH. Comparison of immunogenicity of a pre-S containing HBV vaccine with non-pre-S containing vaccines (Abstract 272). In: T. Oda, K. Nishioka, H. Susuki, eds. *Proceedings of the International Symposium on Viral Hepatitis and Liver Disease (8<sup>th</sup> Triennial Congress)* Springer Verlag, Tokyo 1993, p. 86.

Shapira MY, Zeira E, Adler R, Shouval D. Rapid seroprotection against hepatitis B following the first dose of Pre-S1/Pre-S2/S vaccine. *J Hepatology 34(1):123-127, 2001.*

67. Diminsky D, Schirmbeck R, Reimann J, Barenholz Y. Comparison between HBsAg particles derived from mammalian cells (CHO) and yeast cells (*Hansenula polymorpha*): composition, structure and immunogenicity. *Vaccine 1997, 15:637-647.*

68. Budkowska A, Dubreuil P, Maillard P, Poynard T, Pillot J. A biphasic pattern of anti-pre S response in acute hepatitis B virus infection. *Hepatology 1990, 12:1271-1277.*

69. Deepen R, Heermann KH, Thomsen UR, Gerlich WH. Assay of pre S epitopes and pre S<sub>1</sub> antibody in hepatitis B virus carriers and immune persons. *Med Microbiol Immunol 1990, 49-60.*