Clinical Cancer Investigation Journal-ISSN: 2278-0513

Hepatitis B Vaccines Based On preS1, preS2 and S Domains

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Abstract

Hepatitis B virus (HBV) remains a global health problem, one of the leading causes of liver cirrhosis and carcinogenesis. Vaccination is the only effective way to fight HBV, as the prevalence rate of the virus in children under 5 years of age dropped to 1.3 from 4.7 by the end of 2018 following the implementation of a global vaccination program in 189 countries. Despite the admirable impact of the global vaccination program, the vaccine continues to be non-responsive or hypo-responsive in some susceptible and specific groups. Due to the absence of a standard treatment protocol for the complete elimination of the virus from the body, the design of therapeutic vaccines or those with both therapeutic and prophylactic roles in patients with chronic HBV is of interest to researchers in this field. In this regard, the focus is on fragments with greater immunogenicity and the use of more efficient adjuvant compounds. The use of other protein fragments such as Pres1, Pres2, and S has also been more successful in designing both therapeutic and prophylactic vaccines. Different adjuvants targeting humoral and cell-mediated immune pathways, such as CpG oligonucleotide motifs, are used alongside different fragments of viral coat protein now in different phases of clinical and preclinical trials.

Keywords: *Hepatitis B Vaccines, preS2HBsAg, Hepatitis B virus, preSHBsAg*

Introduction

More than 50 years have passed since the hepatitis B virus (HBV) was identified by Blumberg and colleagues (1). HBV was first detected in the blood sample of a patient with hemophilia who was an Australian native, hence the name Australian antigen (2). The virus has infected 240 million people to date, and geographical differences have led to differences in various serotypes and genotypes (A-J), as well as differences in clinical manifestations, virus persistence, and response to treatment (3). The prevalence of HBV is over 80% in hyperendemic regions and including countries such as Africa, Central Asia, and the Amazon region, and it is moderate to low (2-7%) in European countries and Australia (1). An estimated one million people die each year from HBV-related diseases worldwide (4). Although it is a self-limited disease in most adults, 5% of them remain clinically symptomatic or asymptomatic carriers (2). Due to the high pathogenicity of HBV, which is 50 to 100 times higher than HIV (human immunodeficiency virus) infection, it can progress disease to cirrhosis and hepatocellular carcinoma. In addition, only about 10% of infants survive the disease, and about 90% experience chronic hepatitis (5, 6). The environmental resistance of the virus, which survives up to 7 days outside the host body, and the ability to spread from various sources that are often found in body secretions and fluids such as blood, urine, sweat, breast milk, sexual secretions, and cerumen, highlight the need for effective vaccination (7). Accordingly, efforts have been made to develop vaccines from the earliest years of virus identification, which continue despite the 85 to 95% effectiveness depending on the area of the outbreak and (the type of vaccine) of the existing recombinant vaccine (8, 9). There is no effective treatment for people with HBV infection. Interferon (IFN) therapy and hepatitis B immune globulin (HBIG) injection can help control the virus to some extent in people with chronic hepatitis (10).

Gemology of molecularly important and effective fragments in vaccine fabrication based on preS1, preS2, and S proteins

HBV has the smallest genome among DNA viruses, with double-stranded and circular DNA, part of which is single-stranded. Genome size varies in different genotypes, approximately 3.2 kb. Among 10 different genotypes, genotype D has the smallest genome size (3182 kb) and genotype G has the largest size (3248 kb) (11). The virus genome exists in two forms:

1- **Virion:** The genome has a relaxed circular DNA (cDNA) of which only part is double-stranded. One of the strands is complete and is known as the minus strand. This form of the genome contains all the genetic information but is not suitable for replication. The other strand is the positive (plus) strand, which is incomplete. After infection of the liver cells, the positive strand is completed (11-13).
2- **Covalently-closed-circular DNA (cccDNA)**, is found in hepatocytes and is the main platform for replication. This form itself contains Pregenomic RNA (Pg RNA), the transcription of which is a signal to initiate unique viral polyadenylation downstream of the genome. Upon exposure to the host cell cytoplasm, this fragment is encapsulated in the capsid and released along with viral polymerase and minus strand by reverse transcriptase (RT) (13-15).

The RT is attached to the viral genome and has ribonuclease activity, and is employed to transcribe the viral genome; The frequency of mutations in the virus genome is high due to the high error of this enzyme (13). Four open reading frames (ORFs) organize the viral genome. The longest ORF encodes the viral polymerase (Pol). The expression of these four transcripts is guided by enhancer 2/core promoter, large surface antigen promoter (L), major surface antigen promoter (S), and enhancer 1/X gene promoter, respectively (2, 3, 11).

(16)The HBV envelope proteins (or viral coat proteins, VCPs) include LHBs (HBsAg), MHBs (HBsAg), and SHBs (HBsAg) (11, 15).

The gene encoding this protein is from nucleotide 947 to 2104 and has the most overlap with the Pol gene, with three start codons (17). The first start codon leads to the production of mRNA with a molecular weight of 2.4 kb, eventually resulting in the synthesis of a protein with 389-400 amino acids known as Large protein (HBsAg) containing the preS1, preS2, and S domains (11, 15, 18, 19). This protein is in two glycosylated forms at the amino acid position 146 of S fragment with a molecular weight of 42 kDa and a non-glycosylated form with a molecular weight of 32 kDa (12, 20). Most studies on this virus have shown that the Pre-S1 domain is required for binding to the cellular receptor and initiating infection because of N-terminally myristoylated preS1 domain and acting as an anchor and role in virus establishment between the luminal membrane and cytoplasm (in the hydrophobic region of the host cell membrane) (12, 19-21). This fragment is of interest in the design of therapeutic vaccines (22).

The second start codon produces mRNA with a molecular weight of 2.1 kb, which eventually synthesizes the Middle protein (HBsAg), including the preS2 domain and the S domain with a total of 281 amino acids (16, 19). This protein is found in three forms: with a glycosylated site at amino acid 146 in the S region with a molecular weight of 33 kDa, with two glycosylated sites at amino acid 146 in the S region, and amino acid 4 in the preS2 region with a molecular weight of 36 kDa and a non-glycosylated form with a molecular weight of 30 kDa, found in virions and subviral particles (16, 19). The presence of a glycosylated site in the preS2 region is essential for both protein secretion and immunogenicity of M protein (18). O-glycosylation site is found in genotype D at position threonine 37 (O-glycosylated by Neu5Ac (a2–3) Gal (b1–3) GalNAca-or Gal (b1–3) GalNAca-units at Thr-37) and is no conserved in different genotypes (17, 20, 23, 24).

Shen et al. (1989) succeeded in expressing M protein in yeast *Hansenula polymorpha* by modulating methanol-inducible promoters and cited this expression system as a viable alternative to protein production (25).

The presence of glycosylation sites in M protein makes the expression of this protein difficult. Hamsa et al. (1994) proposed a new expression system for the synthesis of this protein. They employed the yeast *Yarrowia lipolytica* for expression, which is a non-methanolic yeast that has been used experimentally in the production of recombinant proteins (26). Hamsa et al. replaced the dominant "a" of HBsAg with a leader peptide and expressed the resulting fusion protein in yeast, which ultimately collected the highest protein level in the stationary phase, accounting for 2.35% of the yeast intracellular material (27). Intracellular production of products is difficult to purify and extract, and any process may interfere with the basic structure of the protein and impair protein function.

Similarly, Azizi et al. (2006) inserted the immunodominant "a" of HBsAg (amino acid 149-111) into HBcAg (amino acid 139-122) and were able to express the vector carrying the chimeric protein gene made in human embryonic kidney cells (2). This study provided a design for a DNA vaccine containing immunogenic fragments against HBV. This study did not address the stability of the chimeric protein produced because most vaccines based on DNA and gene structures are unable to stimulate long-term antibody responses, and this is a major problem with DNA vaccines. However, subunit or inactivated and attenuated vaccines have considerable potency in this regard (2).

In the development of gene vaccines, the use of DNA vaccines containing the M protein gene fragment in the Vaccina vector was proposed by Kutinova et al. in 1990(27). In a brief report, they stated that the Vaccina vaccine carrying the M protein could be expressed in mammalian cells (20, 27).

The design of a DNA vaccine carrying the M protein gene was re-introduced by Liu et al. in 2015(20, 28). In this project, they evaluated different mutations of N- and O-glycosylation sites in terms of immunogenicity and stated that the presence of glycosylation site in the S2 region of M protein is effective and necessary in designing DNA vaccines to enhance immunogenicity (20).

The third start codon results in the production of a small protein (S-protein) with 261 amino acids, which can be found in two forms: with a glycosylation site at amino acid position 146 with a molecular weight of 27 kDa and without a glycosylation site with a molecular weight of 24 kDa (15, 19). Common vaccines use S-protein with one glycosylation site (8). The resultant immune response is induced in the hydrophilic region located in the residues 99 to 170 (23). This anti-HB response for the mentioned epitopic region plays a role in the induction of preventive immunity (29). This protein also has a conserved region called "a" from 122 to 146, which is common to all genotypes(4). This conserved region is an antigenic domain and is important in antibody neutralization and cross-immunity of vaccines (6).

The PreS2 region alone contains 55 amino acids, of which amino acids 1 to 45 have more pronounced immunogenicity than the S protein alone and overlap with the spacer region of Pol protein. The preS1 region, with amino acids 108-119, shows similarities to the constant region of the IgA heavy chain, suggesting the possibility of binding sites for HBV in cells bearing IgA receptors. The use of preS regions in third-generation vaccines and dendritic cell receptor-targeted vaccines is effective in controlling hepatitis B infection in transgenic mice. Jing et al. (2016) fused the preS region with streptavidin protein, a molecule with a high affinity to bind to dendritic cell receptors, whose expression in *E. coli* was associated with high antibody titers of IgG2a and IgG1 classes in non-transgenic mice (30). They also suggested the role of the above-designed vaccine in controlling HBV infection in transgenic mice. This study did not provide any comparisons with conventional vaccines to confirm the research results (30).

Among the various HBV surface protein fragments, protein M has recently received attention. This protein is synthesized from an mRNA with a molecular weight of 2.1 kb and contains the domains S2 and S with a total of 281 amino acids and has three forms (glycosylated with one site, glycosylated with 2 sites, and non-glycosylated) (31). S2 region plays an important role in both the secretion of HBV surface antigen and its immunogenicity due to its N-glycosylated site at Asn4(12). In addition, preS1 and preS2 fragments are effective in rapidly elevating antibody levels against the virus surface antigen. Because parts of preS2 are evolutionarily conserved, they are a suitable target for therapeutic and diagnostic research (20, 21, 32). The use of these fragments alone or in combination with the S region has been evaluated in various studies and is currently used in third-generation vaccines and several research vaccines.

The S region with 261 amino acids is important in the development of vaccines and diagnostic kits due to its "a" region from position 122 to 146 in this section, which has been conserved in various genotypes (15, 19). Circulating M protein (preS2/S) is often filamentous and the virus nucleocapsid (virion) often contains this protein (16). Therefore, M protein with S and S2 fragments in expanding and strengthening the specific responses of B and T cells is an appropriate target for the development of new therapeutic and prophylactic vaccines (33). Mancini et al. (2004) designed DNA vaccines based on small (S) and medium (S2/S) proteins without the use of any adjuvant that was very potent in clearing HBV and is currently in phase I clinical trial (34).

Adjuvants alongside viral coat proteins as HBV vaccine platforms

Depending on the manufacturing process in each company and the target population for vaccination, the level of antigenic protein varies at each dose of HBsAg vaccine products (35). This level is commonly used in vaccines and varies between 5 and 40 µl, depending on the age of the target population (less antigen in infants, children, and adolescents), recombinant HBsAg, and aluminum phosphate or aluminum hydroxide adjuvants (36). The use of aluminum salts, which is common in many vaccines, generates insoluble particles in the body and gradually releases its associated antigens, inducing innate immunity over time (35). Newer second- and third-generation vaccines reduce the concentration of antigenic fragments and focus on selecting adjuvants that are more effective in inducing immune responses (37).

Adjuvant systems used in conjunction with recombinant HBV antigen components include liposomal class (ASO1B, ASO1E), oil-in-water emulsion class (ASO2A, ASO2B, ASO2V), α-tocopherol and squalene in oil-in-water emulsion (ASO3) class, mixed with aluminum phosphate/hydroxide in sodium chloride and water (ASO4) class, or class of immune-stimulating sequences such as non-CpG methylated motifs and oligodeoxynucleotide (ODN) sequences that target both innate and adaptive immunity and induce memory responses (2, 6).

Since 1984, the use of oligodeoxynucleotides containing immunomodulatory motifs (CpG-ODNs) that act similar to Th1 and enhance immunity has been proposed as an adjuvant for the production of vaccines (36). The CpG ODNs enhance

the immune response of vaccines against a large number of pathogens, including anthrax, Leishmania, influenza virus, measles virus, lymphocytic choriomeningitis virus, Orthopoxvirus, HBV surface antigen, and tetanus toxoid, by using antibody titers for specific antigens and multiplying them several times (35). The CpGs are agonists of TLRs and directly activate pDCs and B-cells, helping to induce innate and adaptive immune systems (35, 38). TLR expression decreases in most viral infections (38). Therefore, the infectious agent can rid itself of antigen-presenting systems and disrupt the subsequent processes of the immune system, thus causing the spread of the disease. As for HBV, studies have shown reduced TLR9 expression in the face of the virus (34). TLR9 induces B cells to produce IL-6, IL-12, and the chemokine CXCR3. In addition, TLR9 is involved in IgM secretion by expressing IL-6 (39). The CpG-activated B-cells show an increase in their Fc receptor expression, along with costimulatory molecules, class II MHCs, CD40, CD80, and CD86(40). While increasing the expression of these molecules, TLR9 increases the production of IL-1, IL-6, IL-12, IL-18, and TNF-a. TLR9 alone is sufficient to induce human memory B cells to express antibodies (10, 36, 40).

The idea of using CpG DNA to enhance immunogenicity against HBV surface antigens in mice was proposed by Davis et al. in 1998 (10). According to their findings, the antibody titers in mice immunized with HBsAg and both CpG ODN plus alum were 35 times higher than the titers in mice immunized with alum alone(18). The CpG ODN was also found to give a strong Th1 response mainly in combination with IgG2a and CTL and retained this property even when mixed with alum. High expression of co-stimulatory molecules on the antigen-presenting cells and derivation of B-cell isotypes and its promotion to the production of cytokines were other achievements of this study (10, 40).

The HBV vaccine with CpG adjuvant in orangutans was studied by Davis et al. in 1999(35). This study stated that the addition of CpG ODN to the HBV vaccine mainly increases serum levels and anti-HBs antibody titers, and CpG was proposed as a useful substance in boosting the vaccine against hepatitis B and other diseases (35, 40).

Mac Culis and Davis (2004) pursued the idea of adding CpG ODN to study the process of mucosal immunogenicity against HBsAg in mice in 1998 and 1999 (36). In a study, HBsAg, CpG, and cholera toxin (CT) were given to mice by intranasal injection (41). Examination of the immunogenicity process revealed that CpG plus CT was able to initiate processes in which Th1s were induced, and it was found that the antibody response was 10 times higher than when the adjuvant was used alone (35).

Cooper and Davis (2004) used CPG7909 as an immunostimulatory TLR9 agonist ODN in combination with the ENGERIX-B vaccine in healthy adults for a phase I/II study (10). This study describes the success of the vaccine with CpG in control and test groups to increase the antibody titer in less time. This study suggested that the best dose of CpG to increase immunity was 0.5 mg, which was increased from 4 weeks onwards, and its stability was determined up to 48 weeks.

Cooper and Davis (2008) tested their proposed vaccine on HIV patients (42). It was found that HIV patients receiving the HBV vaccine combined with CpG7909 showed higher anti-HBs antibody titers than those receiving the conventional vaccine without CpG. They recommended the new vaccine to populations with weakened immune systems (10).

The new HBV subunit vaccine has been in phase III clinical trial in humans since 2004. HEPLISAV™ Co. is responsible for this and states that the new vaccine, in addition to its higher immunogenicity, can act in a shorter time and at a lower dose (two doses) than the conventional Engerix-B vaccine (43). However, it has shown more side effects at the injection site. The vaccine designed by the company uses another type of TLR-9 agonist called CPG1018. This vaccine expresses the HBsAgS fragment in Hansenula yeast (43).

Another compound used in adjuvant vaccines is monophosphoryl-lipid A (MPLA), which is present in the Fendrix® vaccine with aluminum phosphate (Adjuvant System 04 or AS04). MPL is derived from gram-negative lipopolysaccharide and is more potent than alum in stimulating immune responses (44).

History of prophylactic HBV vaccines in the world and the rationale for their development

The history of the HBV vaccine dates back more than 40 years ago (1). After Blumberg introduced the virus in 1965, four years later the first vaccine against it was made by Millman and Blumberg using plasma derived from infected patients (2). The HBV vaccine is also the first anti-cancer vaccine because it can help prevent liver cancer (45). Chronic hepatitis B and C account for 80% of liver cancers worldwide, the second most common cause of cancer deaths (45). Therefore, a vaccine that can have a protective effect against HBV infection may also help prevent liver cancer.

First-generation HBV vaccines

This generation is known as plasma-derived vaccines. In a pioneering approach, Kurgman et al. were able to eliminate HBV infectivity by boiling the plasma of its carriers and could induce antibodies (anti-HBs) and passive immunization by injecting HBsAg-containing plasma (30). They showed that HBV patients were likely to naturally generate products that could help make the vaccine. In later years, with the development of antigen purification methods, Blumberg and Millman tested anti-HBs in chimpanzees through active immunization with human immunoglobulins, with very

satisfactory results, and its injection in children, although incomplete, induced immunoprotection against HBV (9).

These vaccines were developed following the development of electron microscopy, the observation of 22-nm subviral particle (SVP) and 42-nm virion in the blood of HBV patients, the development of HBsAg purification methods such as isopycnic, rate-zonal separation, adsorption on colloidal silica, gel filtration and differential polyethylene glycol (PEG), and also the formalin inactivation of virions due to the risk of infection, as well as the realization that chimpanzees are also susceptible to HBV (19). The first plasma-derived vaccines were licensed in the United States in 1981 and in France in 1982 (15, 30).

Serological subtypes were identified after the introduction of HBsAg as a surface protein and eventually led to the recognition of common antigen determinant "*a*" and allelic subtype (11). Determinants "*d*" or "*y*" and "*w1-4*" or "*r*". Although cross-reactivity occurs between diverse subgenotypes, cross-protection is achieved when the anti-HBS titer is high immediately after immunization with the vaccine (23).

The majority of SVPs in these HBsAgS vaccines, depending on the purification method, may also contain small amounts of HBsAgM, such as HevacB®, which contained 1-2% HBsAgM. Most of the subtypes in these vaccines were "*ay*" and "*ad*" according to the blood of plasma donors (11, 15).

Heptavax-B® was the first commercial plasma-derived heat-inactivated HBV vaccine developed by Merck, Germany, which did not contain preS2 due to its one-step treatment with proteases (8). The SVPs in this vaccine were HBsAg S and contained the "*ad*" subtype (4). Other plasma-derived vaccines included Hepaccin-B (Korea) and GCC VAC (Osaka), which mainly contained HBsAgS (6, 19).

However, in 1991, due to the contamination of such vaccines with chronic infections and the potential presence of other blood-borne pathogens, such as non-A, non-B hepatitis, and HIV, production ceased and efforts for safer vaccines continued(19). Over the next few years, second-generation HBV vaccines based on genetic engineering (or DNA Recombinant) were developed and pioneered in the vaccination program of more than 170 countries (9).

Second-generation HBV vaccines

Following the introduction of HBsAgS as one of the VCPs in the plasma of infected individuals and its use in the development of plasma-derived vaccines and with the development of recombinant DNA technologies, the S gene was successfully cloned in bacteria (19). This move led to the development of second-generation vaccines (15). Because HBsAgS synthesized in bacteria is not similar to what is assembled in the human body during a natural infection, studies have been performed on eukaryotic systems such as *Saccharomyces cerevisiae*, *Hansenolla polymorpha,* and *Picha pastures* (46). The HBsAg generated in *S. cerevisiae* is similar to the 22-nm particles formed during human infection. Although yeast-derived SVPs have a lower antigenic effect than human plasma-derived SVPs, the antigens produced by this method we're able to show an effective anti-HBs immune response in chimpanzees and subsequently in clinical trials on healthy volunteer groups and specific groups with diverse age groups (11). The recombinant vaccine subtypes are predominantly "*adw*" and cross-react with "*ADR*" and "*new*" subtypes (47).

Eventually, these vaccines replaced plasma-derived vaccines following the US Food and Drug Administration's approval of recombinant vaccines in 1981, although China continued to include first-generation vaccines in its vaccination program until 2000 (6).

Common vaccines produced in *S. cerevisiae* using the "*adw*" subtype are EngerixB (Belgium) and RecombivaxHB (Germany), which are currently licensed in many countries, especially China and Korea. The antigen used in these vaccines is HBsAgS (2, 4).

India has developed a similar vaccine called ShanvacB and ElovacB, which is expressed in *P. pastures*. The *adw2* subtype is a small or major HBsAg protein antigen (15).

Yeast-derived vaccines with *adw2* and *ayw2* subtypes are also available in Russia (9).

Successful seroprotection is achieved in second-generation vaccines when the anti-HBS antibody titer reaches ≥10 mIU/ml after three doses of injection. This result in healthy people may even increase to several times this amount (46).

Disadvantages of second-generation vaccines

1- Perhaps the biggest drawback of the HBV vaccine in the world is that it contains HBV subtype A2, which is prevalent in Northern Europe and the United States, while 99% of the world's HBV carriers are from other genotypes, and cross-seroprotection occurs when the antibody titer is produced in the shortest possible time and at the maximum amount to prevent infection with other virus genotypes (48). Therefore, efforts to produce the HBV vaccine following the common conditions and genotypes in each country are now in focus.

2- The second-generation vaccines in some groups are associated with non- or hypo-responsiveness, some of the reasons for which are as follows; I) Slow protection so that protection in the prevention of the disease is possible during three doses and six months (49), and thus if a person is exposed to the virus during this period, the desired protective effect will not be applied; II) low protection in hypo-responder or non-responder populations (48, 49). The present vaccine will result in low rates of seroprotection for people over 40 years of age (50), diabetics (47), people with chronic hepatitis B, HIV, HCV (51), as well as people with celiac disease (52),

hemodialysis and immunosuppressive diseases, infants born to HBsAg carrier mothers (53), patients with chronic liver disease or after liver transplantation, intravenous drug users, patients with chronic renal failure, and non- or hypo-responsiveness to vaccines (48). In addition, some HLAs, such as HLA-A11, HLA-A24, HLA-CW6, HLA-DR, (HLA-DRB1 * 04X, DRB1 * 0401X, DRB1 * 11/13, and DRB1 * 0401X0201) repel the vaccine. Some of these HLAs are common in patients with diabetes and celiac disease (DQ8 and DQ2) (49).

Some single nucleotide polymorphisms (SNPs) occurring in the genes of some cytokines such as IL-2, IL-4, and IL12B cause non- or hypo-responsiveness to the vaccine (10, 11). In HIV patients, due to the hypo-responsiveness to CD4, there is a poor response to the HBV vaccine. HCV patients also have problems in regulating the IL-12 and IL-23 genes due to disruption of T cell immunoglobulin mucin-domain-3, resulting in impaired response to the HBV vaccine (4, 38, 54).

3- Because the vaccine is available in three doses, the application and completion of the vaccination period in countries with economic problems are disrupted and only 30% of the people in such countries can complete the vaccination period (22).

4- Immunization failure can also occur due to variables such as improper storage and intragluteal injection (1, 6).

5- Another factor influencing the ineffectiveness of common second-generation vaccines is that HBsAg alone is a weak immunogen and requires an adjuvant to boost efficacy, and sometimes suppresses immune responses even if it is highly present in the body (42). The ability to produce anti-HBs antibodies in response to HBsAg immunization is controlled by Class II HLA molecules with autosomal dominant expression (6). Millich et al., followed by Schwall et al., have shown that non-responsiveness to immunization with small HBsAg (SHB) in mice can be circumvented through immunization with Pre-S proteins (36). In addition, immunization with synthetic middle Pre-S2 HBV peptide (MBs) and also with yeast and mammalian CHO cell-derived Pre-S2 vaccines may induce neutralizing antibodies and protect chimpanzees versus HBV (30).

In addition to the above, several studies have shown that 5-15% of healthy individuals, for unknown reasons, after completing the vaccination period, fail to mount adequate antibody levels, and this percentage cannot be ignored due to the risky nature of this disease and the absence of an effective treatment strategy (9, 47, 48, 55).

Third-generation HBV vaccines

Another type of vaccine is now available, known as third-generation vaccines, although they have not been generalized and are currently used more in research. This group of vaccines has been introduced under other names, including genetic vaccines, RNA vaccines, DNA vaccines, and nucleic acid vaccines, which is the direct use of plasmid DNA containing the gene encoding the protein for the prevention and treatment of HBV. Due to the above-mentioned problems of second-generation vaccines, the development of third-generation vaccines considers combining preS1 and preS2 sequences with or without the S domain (HBsAgL, HBsAgM) to enhance protective efficacy (23, 28, 29, 54, 56). The ability to express HBsAgS subunits in yeasts used in second-generation vaccines and mammalian cell lines such as Chinese hamster ovary (CHO) cells and mouse intestinal cells to form SVPs during downstream processes and to reconstruct native antigenic structures has made it possible to develop successful prophylactic vaccines (57).

The importance of the preS1 domain for virus penetration and assembly has been revealed. The preS1 and preS2 sequences stimulate B cell epitopes to produce protective antibodies (54). These two sequences are also able to elicit immune responses based on T cells that the S domain alone cannot activate (39). The use of platforms carrying these two sequences has generated anti-HBs responses in non-responders to standard second-generation vaccines (32).

One of the yeast-based vaccines consisting of both HBsAg and HBsAgM is TGP943, which is expressed in *S. cerevisiae* and has the "*ADR*" subtype (6). This platform has been able to show a protective effect in chimpanzees and anti-preS2 antibodies in humans. Anti-S and anti-preS2 responses are seen in 50% of non-responders to the standard vaccine (11).

The AG3^{TM} (Hepcare) vaccine, which uses the preS1, pres2, and S fragments (HBsAgS, HBsAgM, HBsAgL) and is derived from the "*adw*" subtype and expressed in the mouse cell line, was able to cover 76% of non-responders and elicit the appropriate antibody response in them (8). Another type of third-generation vaccine is called Sci-B-Vac with the "*adw*" subtype, which results in protective antibody response in 20 to 21% of non-responders and hypo-responders, and has been able to elicit antigen protection up to 50% earlier than second-generation vaccines (11). The vaccine contains the complete HBsAgS, HBsAgM, and HBsAgL genes with glycosylated isoforms expressed in CHO. The vaccine was also designed for infants and had an acceptable immune response.

Another third-generation vaccine expressed in CHO is Gen Hevac®, which has an HBsAgS to HBsAgM ratio of 80: 20 and uses the "*ayw*" subtype (15). The vaccine has also been able to increase specific antibodies by more than 90% in people aged 18-40 (11).

The main problem with third-generation vaccines is their production in mammalian cell lines such as CHO, which makes production costs difficult (46). Given the high production cost of these vaccines, the efficacy of these vaccines seems to be lower than expected (33). Widespread and general use of these vaccines is conditional on reducing their production costs.

Due to the mentioned limitations, the use of these vaccines is recommended in a very limited way only in high-risk groups as well as in travelers who travel to the native areas of HBV (22).

Recently, the idea has been suggested that Pre-S/S vaccines can be effective not only for prevention but also for intervention in persistent HBV infection (2). However, the future of such vaccines depends on their reinforcement, which we will discuss in the following sections.

Production of the HBV vaccine in some crops, such as potatoes, rice, and bananas, has had limited success, and all of this has remained in the laboratory (29).

Global perspectives on therapeutic vaccines based on preS1, preS2, and S proteins

The key to the design of vaccines is the multifaceted function of the humoral and cell-mediated immune systems. Simultaneous cooperation of innate and adaptive immunity to reduce the viral load, create hepatic immunotolerance, and eliminate the dysfunction of T cells is a way to promote the effectiveness of the vaccine (39).

As mentioned, the presence of preS1 is essential as a binding factor for liposomes to hepatocytes (58). Unlike prophylactic vaccines, which aim to produce antibodies against HBsAg and produce $CD4^+$-based responses, therapeutic vaccines aim to eliminate immunological markers such as HBsAg, HBeAg, and HBV DNA in vaccinated individuals and focus more on $CD8^+$ responses (39). The use of HBIG or a combination of nucleotide analogs and interferons has been somewhat effective in treatment (6). HBsAg levels before injecting vaccines greatly affect their effectiveness (33).

Recombinant therapeutic vaccines

One of the vaccines in this group is CVI-HBV002, containing two S and M proteins along with an L-pampa adjuvant, which is in phase II clinical trial for the treatment of chronic HBV patients. The efficacy of this vaccine was dependent on the patient's HBsAg level and the viral load, so patients with lower HBsAg levels had a better response to the vaccine, leading to further reductions in this antigen and some cases antigen deletion (6, 33). One of the reasons for the ineffectiveness of therapeutic vaccines could be the nature of their single antigen because the production of multiple antibodies in acute infections can be more successful in advancing the immune system to eliminate or reduce the virus. For example, the use of HBcAg alongside HBsAg is seen in the GS-4774 vaccine (59). In addition to the above domains, the X fragment of the virus has been used for further protection in a variety of genotypes (14). The response rate varied from low to moderate in healthy individuals. Despite the reduction in rates in people with chronic HBsAg infection, the effectiveness of this vaccine was not promising (59).

Therapeutic DNA vaccines

These vaccines have been tested alone or in combination with other vector-based therapeutic vaccines or treatment strategies such as nucleotide analogs (54). However, they perform poorly in improving the performance of CD8s and are unable to eliminate hypo-responsiveness in chronic infections (54).

Adenoviral vector-based therapeutic vaccines

The vaccine in this group is TG1050, which contains three fragments of the virus, including core, polymerase, and HBsAg, and the outer part that carries these three fragments is the human adenovirus vector (60). The vaccine is being tested as a single dose or prime-boost in phase I clinical trials. In vitro studies found that the highest T response was related to core antigens, followed by polymerase and surface antigens. Decreased HBsAg titers after one year in volunteers with chronic infections gave hope for the vaccine, although the vaccine has not been tested in healthy individuals (60).

One of the disadvantages of these vaccines in humans is the possibility of producing adenoviral antibodies that can interfere with the immune process against HBV, a challenge that has been addressed in similar studies.

Therapeutic vaccines based on lipopeptide epitopes

One of the vaccines in this class is CY-1889, which contains HBcAg (18–27) epitope (core antigen) and HLA-A2 domain. This core antigen can increase the levels of IF-Y and IL-2 cytokines (61). It also intensifies HBV-specific cytotoxic T responses that have been effective in clearing the virus and reducing HBsAg and HBcAg levels in transgenic mice. In addition, it has increased CD8 responses in people with acute HBV infection but has not had a significant effect on HBV-DNA (61). Limitations of this vaccine include specific alleles of HLA that limit its effectiveness on other epitopes and may cause immune evasion in different individuals.

CONCLUSIONS

The World Health Organization has declared 2030 as the end of HBV infection as a threat to public health (6). The strategies proposed to achieve this ambitious goal are based more on vaccination at birth, and efforts to do so have resulted in a 90% reduction in new infections and a 65% reduction in HBV-related deaths (8). However, implementing such health services on the global scale and receiving vaccine doses at birth is less than desirable in areas with limited and remote resources, and people in many hyperendemic regions receive only one in three doses because of limited access to the vaccine (1). Therefore, developing vaccines with lower doses (at least two doses) and reaching the protective titer of antibodies as soon as possible is one of the ways to raise the aims of global vaccination. Although the prophylactic vaccine is very effective and safe in controlling the disease, many factors can affect its effectiveness, from observing the principles of the cold chain to the individual and genetic conditions of people receiving the vaccine.

In addition, chronic hepatitis B is incurable and it is now one of the goals of therapeutic vaccines to establish a stable, even incomplete, immunity. Therefore, the development of a vaccine that can simultaneously cover both therapeutic and prophylactic effects can be effective in achieving the goals of the World Health Organization.

One of the goals of the researchers is to use different HBV fragments along with other antigenic components and new adjuvants that can induce both humoral and cell-mediated immune systems simultaneously and appropriately. Although outcomes of this goal have been narrow in human clinical trials, advances in existing technologies can hopefully help achieve this goals.

Financial support

No financial

Conflict of interest

The authors declare no conflict of interest associated with the present manuscript.

Compliance with ethical standards

All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

Ethical statement

None

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