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Immunology

DNA-mediated immunization to the hepatitis B surface antigen in mice: Aspects of the humoral response mimic hepatitis B viral infection in humans

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ABSTRACT **Intramuscular injection of plasmid DNA expression vectors encoding the three envelope proteins of the hepatitis B virus (HBV) induced humoral responses in C57BL/6 mice specific to several antigenic determinants of the viral envelope. The first antibodies appeared within 1–2 weeks after injection of DNA and included antibodies of the IgM isotype. Over the next few weeks, an IgM to IgG class switch occurred, indicating helper T-lymphocyte activity. Peak IgG titers were reached by 4–8 weeks after a single DNA injection and were maintained for at least 6 months without further DNA injections. The antibodies to the envelope proteins reacted with group- and subtype-specific antigenic determinants of the HBV surface antigen (HBsAg). Expression vectors encoding the major (S) and middle (preS2 plus S) envelope proteins induced antibodies specific to the S protein and the preS2 domain, and preS2 antibodies were prominent at early time points. In general, the expression vectors induced humoral responses in mice that mimic those observed in humans during the course of natural HBV infection.**

DNA-mediated immunization refers to the induction of an immune response to antigen expressed *in vivo* subsequent to the introduction of DNA carrying the protein coding sequences and the regulatory elements needed to express them (for review, see ref. 1). An important feature of DNA-based immunization is the *in situ* production of the expressed protein(s), mimicking in this respect a viral infection. This endogenous synthesis should allow presentation of antigens by class I molecules of the major histocompatibility complex (MHC) and thus result in the induction of $CD8^+$ cytotoxic T lymphocytes (CTL) (2). Therefore, the potential of DNA-mediated immunization to partially mimic viral infection promises the efficacy of live attenuated vaccines without the risk of inadvertent infection (3). Another attractive and important feature is the ease of designing expression vectors including sequences chosen to induce a desired immune response. There have been several reports of animal models in which pure recombinant plasmid DNA was used to induce immune responses to proteins of pathogens, including influenza A (4–9), human immunodeficiency virus type 1 (10, 11), bovine herpes virus (12), rabies virus (13), malarial parasites (14), and, in our own earlier work, hepatitis B virus (HBV) (15, 16). In these studies, DNA-based immunization was shown to induce a broad range of immune responses, including neutralizing antibodies, CTL, T-cell proliferation, and (where evaluation was possible) protection against challenge with the pathogen.

The structural gene encoding the HBV envelope proteins carrying the surface antigen determinants (HBsAg) has a single open reading frame containing three in-frame ATG start codons that divide the gene into three coding regions known as preS1, preS2, and S (proceeding in a 5′ → 3′ direction) (see refs. 17 and 18 and Fig. 1). The three different-sized polypeptides produced are known as the major (S), middle (preS2 plus S), and large (preS1 plus preS2 plus S) envelope proteins, and these spontaneously assemble into subviral particles. Protection against HBV infection in humans can be achieved by inducing antibodies to the viral surface antigenic determinants.

In this paper we compare the fine specificity of the humoral response obtained after DNA-mediated immunization using several plasmid vectors encoding the different HBV envelope proteins. We find that the humoral immune response induced by the *in situ* production of the protein antigen(s) in mice can mimic aspects of that which occurs during natural infection in humans.

MATERIALS AND METHODS

HBV Envelope Plasmid Expression Vectors. The plasmid pCP10 (19) was the source of the envelope coding sequences and the 3′ untranslated sequences that include the viral poly(A) signal. In three of the plasmids (pCMV-S, pCMV-S1.S2.S, and pCMV-S2.S) the HBV sequences were placed under the transcriptional control of the human cytomegalovirus (CMV) immediate early promoter, whereas the fourth construct (pHBV-S2.S) used the endogenous HBV promoter elements situated in the preS1 protein coding region of the envelope gene (see Fig. 1).

Plasmid pCMV-S. This construct, which has been described previously (15), expresses only the S region of the HBV envelope gene.

Plasmid pHBV-S2.S. A 2.4-kb *Bgl* II–*Bgl* II restriction fragment from pCP10 (containing the preS1, preS2, and S coding sequences) was cloned into the *Bam*HI site of a modified pSK Bluescript vector. The endogenous HBV promoter within the preS1 region drives expression of the major and middle HBV envelope proteins.

Plasmid pCMV-S1.S2.S. A 2.6-kb fragment was removed from the pHBV-S2.S vector using the Bluescript *Bgl* II and *Bss*HII sites and cloned between the *Bam*HI site of the polylinker of pcDNA3 (Invitrogen) and the *Bss*HII site within the neomycin gene of the vector. This generated plasmid pCMV-S1.S2.S.

Plasmid pCMV-S2.S. The pCMV-S2.S vector was generated by digesting pCMV-S1.S2.S with *Kpn* I and *Sau* I, treated with

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Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; mIU, milli-international units; CMV, cytomegalovirus; MHC, major histocompatibility complex.

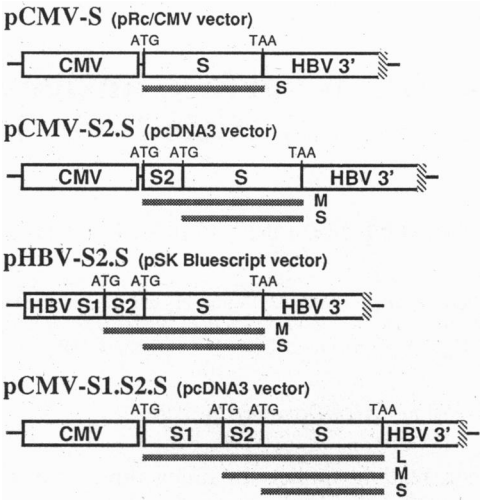

FIG. 1. Schematic diagram of the HBV envelope expression vectors. The preS1, preS2, and S sequences are indicated along with the three in-frame ATG initiator codons and the TAA stop codon. The expected protein products are indicated as thick gray lines below the respective coding sequences and represent the major surface protein (S) as well as the middle (M) and large (L) envelope proteins.

S1 nuclease, and ligated, thus deleting most of the preS1 sequences.

The plasmid DNA used for cell culture transfection and *in vivo* injection was prepared using Qiagen DNA purification columns (Diagen, Hilden, Germany) as described (15).

***In Vitro* Transfection with HBV Envelope Expression Vectors.** *Transfection of cell lines.* Mouse L cells and C2-18 mouse myoblasts were transiently transfected as described (20, 21). At various times after transfection, aliquots of culture supernatants were removed for determination of the amount of HBsAg particles secreted.

Detection of HBV envelope proteins by ELISA. The HBsAg particles secreted from transfected cells or those that remained intracellular (recovered by three rounds of freezing and thawing of the harvested cells) were quantitated using a commercial ELISA kit (Monolisa AgHBs, Diagnostics Pasteur, Marnes la Coquette, France). The proportion of the different envelope proteins in particles produced from the various plasmids was determined with a specific sandwich ELISA assay using different monoclonal antibodies (mAbs) bound to the solid phase. These included a group-specific anti-S (112A26 from J. P. Bourgeois, Diagnostics Pasteur), an anti-preS2 (F-124 from A. Budkowska, Pasteur Institute; ref. 22), and an anti-preS1 (MA18/7 from W. Gerlich, University of Göttingen, Göttingen, Germany). Peroxidase-labeled anti-S mAbs 6-16-A15 and 144A2 (also from J. P. Bourgeois), which do not react with the previous three, were used as probes. Supernatant recovered from cultures of cells transfected with pSVS plasmid (23) was used as a positive control for the presence of the preS1 and preS2 epitopes.

DNA-Based Immunization of Mice. In vivo *gene transfer.* Each expression vector was injected into 5-day regenerating tibialis anterior muscles of groups of eight 6- to 8-week-old (19–21 g) male mice of the C57BL/6 strain (*H-2*^{b}) as described (24).

Measurement of in vivo *antibody production.* At various times following gene transfer, pooled sera were tested for reactivity against a panel of antigens. Since group and subtype determinants of HBsAg are conformational (25), whole particles rather than peptides were used as the solid phase for the ELISA. Particles of a different (*adw*) or same (*ayw*) subtype were used to determine group (*a*) and group plus subtype (*y*) specific titers, respectively (26). The preS2-specific response was determined as the difference between results obtained using particles with and without the preS2 domain. The preS2 domain had been removed from the middle protein of Chinese hamster ovary-derived particles (23) by treatment with trypsin (1 μg/ml) as described (27). Antibodies to the preS regions of the HBV envelope were also quantitated using synthetic peptides corresponding to amino acid sequence 12–49 (preS1) or 120–145 (preS2) of the *ayw* subtype or sequence 94–117 (preS1) of the *adw* subtype.

RESULTS

Expression of HBV Envelope Proteins Following Transfection of Cells in Culture. Particles composed of HBV envelope proteins were synthesized and secreted from mouse L cells or differentiated C2 myotubes transiently transfected with each of the expression vectors (Fig. 2 *Upper*). With L cells, the greatest accumulation of particles in the culture supernatant was obtained with the pHBV-S2.S vector and the least was with pCMV-S1.S2.S (5000 ng/ml and 40 ng/ml, respectively, at day 14). With C2 myotube cultures, pCMV-S gave the greatest secretion, whereas pCMV-S1.S2.S and pCMV-S2.S gave similar results, although none of the secreted particles contained the large protein (see below).

For those vectors encoding the preS domains, it was important to verify the presence of the expected HBV envelope proteins in the resulting particles (see Fig. 1). Analysis by solid-phase sandwich ELISA confirmed that the preS2 sequence was present in particles secreted by L or C2 cells transfected with pCMV-S2.S, pHBV-S2.S, or pCMV-S1.S2.S plasmid, although each of these produced a different relative proportion of preS2 antigen (Fig. 2 *Lower*). Interestingly, preS1 determinants were detected on particles secreted by L cells but not C2 cells transfected with pCMV-S1.S2.S DNA, even though particles extracted from C2 cells did contain preS1 antigen (results not shown).

Humoral Response to HBV Envelope Proteins in Mice Immunized with DNA Expression Vectors. *Response to the major (S) envelope protein.* Antibodies to HBsAg, which were first detected 1 week after injection of the pCMV-S expression vector, increased to peak ELISA antibody titers of $>10^4$ by 4 weeks and high levels were maintained for at least 6 months despite the absence of further DNA injections (Fig. 3*A*). HBsAg-specific IgM predominated at 1 week after DNA injection, but a class shift to IgG isotypes was observed over the following 2 weeks. The humoral response to the group-specific epitopes clearly preceded that directed to the subtype-specific determinants using this expression vector (compare titers with *ad* and *ay* particles, Fig. 3*A*).

Response to the middle (M) envelope protein. Inclusion of preS2 coding sequences (pCMV-S2.S) resulted in a much stronger early antibody response. At 1 week ELISA, titers for IgM and IgG responses were about 10-fold greater than that seen with pCMV-S (Fig. 3*B*). At this time IgM antibodies predominated and these were almost exclusively preS2-specific. Antibodies to the S domain (group- and subtype-specific) were not detected until 2 weeks after DNA injection. They did not peak until 8 weeks and the peak level was only half that obtained with pCMV-S.

Response to the large (L) envelope protein. With inclusion of preS1 coding sequences (pCMV-S1.S2.S), antibodies appeared about 1 week later than with the other two CMV-based expression vectors (Fig. 3*C*) and, although peak levels were not attained until 12 weeks, they were nevertheless comparable to those obtained with pCMV-S. As with the pCMV-S2.S vector, anti-preS2 immunoglobulins predominated at early times. Antibodies to HBsAg were directed to the group- and subtype-specific determinants.

Response with the endogenous HBV promoter. The pHBV-S2.S vector containing the endogenous HBV promoter expressed the major and middle envelope proteins and resulted in the slowest but strongest humoral response (Fig. 3*D*).

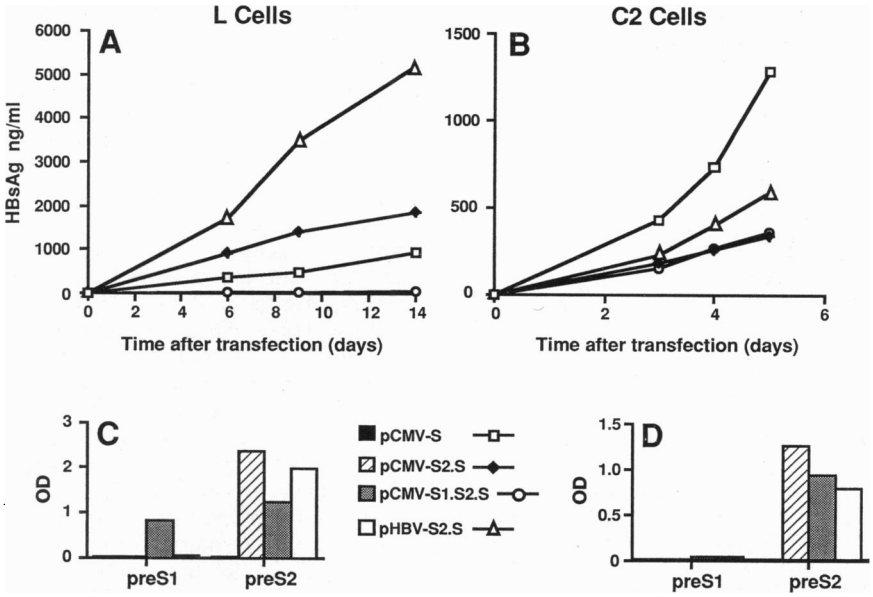

FIG. 2. Expression of HBsAg after transient transfection in cell culture. (*Upper*) The secretion of HBsAg was measured in cultures of mouse L cells (*A*) or the mouse C2 myogenic cell line (*B*) transfected with the different HBV envelope-expressing plasmids. The C2 cells underwent fusion within 2 days after transfection and thus myotubes were present at the time the supernatant was sampled. (*Lower*) The relative amounts of preS2 and preS1 sequences in the HBsAg particles secreted from transiently transfected L cells (*C*) and C2 cells (*D*) were determined. HBsAg particles at a concentration of 25 ng/ml were analyzed for preS2 and preS1 antigenicity by a solid-phase sandwich ELISA method. The results are expressed as OD_{492} units after correction for background.

Antibodies were first detected at 3 weeks and appeared to be directed almost solely against the HBsAg carried by the major protein since similar titers were obtained whether the sera were allowed to react against particles with or without preS2 determinants. However, antibodies to preS2 sequences could be found using synthetic peptides (see below). Maximum anti-HBsAg titers were more than twice as high as those obtained with pCMV-S, and they attained nearly 10^5.

Presence of antibodies to preS regions. Synthetic peptides were used to directly measure the relative proportions of preS2- or

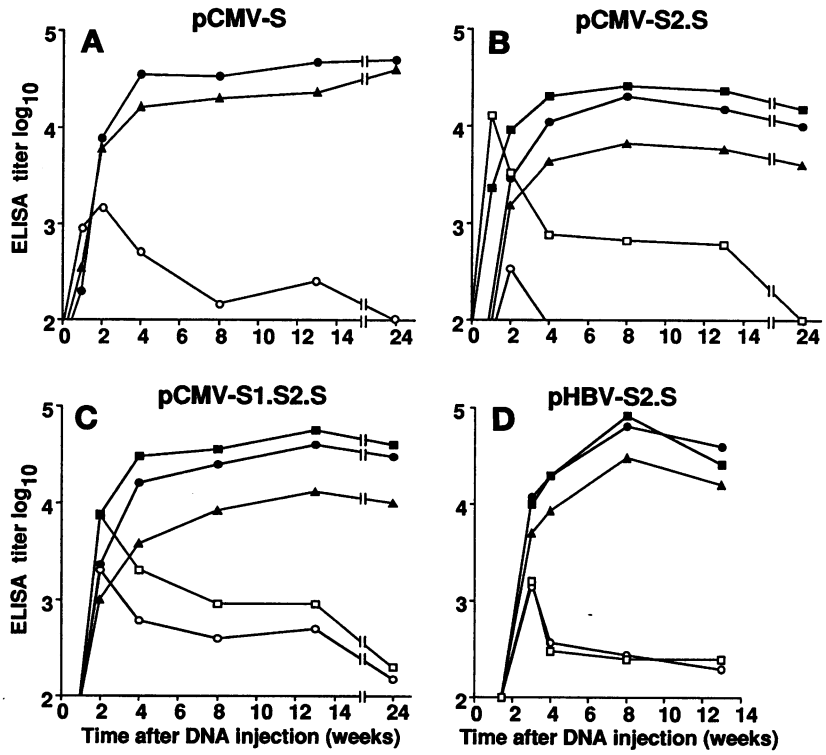

FIG. 3. Kinetics of IgM and IgG anti-HBs antibodies in mice immunized with different HBV envelope-expressing plasmids. Sera were taken at different times after a single DNA injection and pools were made from all sera ($n = 8$) taken at a single time point. The fine specificity of the antibodies was determined using S-containing HBsAg of a homologous (*ay*, ○, ●) or heterologous (*ad*, ▲) subtype as well as HBsAg containing the middle (preS2 plus S, □, ■) protein of the *ay* subtype. The bound antibodies were detected in the second step by the addition of peroxidase-labeled goat anti-mouse IgG (closed symbols) or anti-mouse IgM (open symbols). End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of nonimmune serum (or of pooled sera from mice immunized with an irrelevant DNA) with a cutoff value of 0.050.

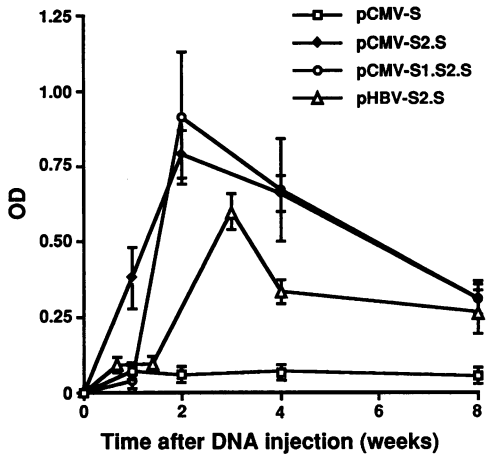

FIG. 4. Kinetics of appearance of anti-preS2 antibodies in sera from groups of mice (n = 8) injected with different HBV envelope-expressing plasmids. Sera were used individually at a 1:100 dilution and the binding to a synthetic preS2 peptide was measured. Bound antibodies were revealed by peroxidase-labeled goat anti-mouse immunoglobulins. Results are expressed as mean ± SEM of the OD at 492 nm.

preS1-specific antibodies induced by the different HBV envelope expression vectors. Antibodies to amino acids 120–145 were detected in the serum of pCMV-S2.S-immunized mice as early as 1 week after DNA injection (Fig. 4). The level of antibodies peaked at 2 weeks and then declined steadily over the next 2 months. In mice immunized with pCMV-S1.S2.S and pHBV-S2.S, antibodies to the preS2 peptide were not detected until 2–3 weeks after DNA injection. Despite their later appearance, they reached levels similar to those which appeared earlier with the pCMV-S2.S vector.

No anti-preS1-specific antibodies were detected using peptides in mouse serum at any time after injection of any of the vectors (results not shown). This is consistent with the cell culture data because, although L and C2 cells transfected with pCMV-S1.S2.S synthesized particles with preS1 polypeptides, only the L cells actually secreted particles with preS1 determinants.

Correlation with clinically defined antibody levels. The antibody levels attained in individual mice were also expressed in milli-international units per ml (mIU/ml) (see Table 1). One week after DNA injection, all mice injected with pCMV-S had seroconverted to a titer of at least 10 mIU/ml [a level that is recognized to be protective against infection in humans (41)] and all those injected with pCMV-S2.S had >100 mIU/ml, although this was largely preS2-specific (compare Fig. 3*B*). In contrast, most mice injected with pCMV-S1.S2.S or pHBV-S2.S failed to seroconvert until after 2 weeks, but by 3 months high levels of antibody had been induced.

DISCUSSION

We have evaluated the humoral immune response obtained after DNA-mediated immunization using four different plasmid DNA vectors for the *in vivo* expression of various HBV envelope proteins. These studies were carried out in mice of the C57BL/6 strain, which are of a haplotype ($H\text{-}2^b$) known to respond well to S and preS2 components of the viral envelope (28). The results presented here show that after a single intramuscular injection of DNA, all vectors induced a strong and sustained antibody response that resembles that of natural HBV infection in terms of the fine specificity, immunoglobulin class, and relative kinetics of the antibodies induced. The group- and subtype-specific determinants are conformational (25) and yet are also well recognized by the antibodies generated after intramuscular injection of DNA expression vectors. These data represent evidence that the envelope proteins encoded by the plasmid DNA have adopted a conformation similar to that of the proteins present during natural infection. This conclusion, drawn from serological evidence, is important because it validates the use of DNA-based *in vivo* synthesis of the antigen for immunization purposes.

All four vectors produce high levels of antibodies specific for group and subtype determinants of the S domain such as appear during resolution of natural infection (29). Antibody levels reach ELISA titers of 10^4–10^5 by 3 months after a single DNA injection and persist at those levels for at least 6 months without further DNA injections. The three plasmid vectors that include preS2 coding sequences induce preS2-specific antibodies, although to different levels, and these antibodies generally appeared earlier than those against the S domain of the envelope proteins. This is similar to the course of the immune response seen in early phases of HBV infection, which is characterized by an immunodominance of the preS2 over the S region (30). The humoral response to preS2 is particularly strong and precocious in C57BL/6 mice when using the pCMV-S2.S vector.

The IgM to IgG class shift, which is also typical of natural infection, indicates that specific helper T cells are probably involved. The T-cell-dependent nature of the humoral response to HBV envelope proteins is well established (see ref. 31); however, neither the phenotype nor the location of the antigen-presenting cells (APCs) that may be involved in T-cell stimulation after DNA injection has been determined. Helper T-cell function is generally an indication of antigen presentation by MHC class II molecules but it is unlikely that this takes place on muscle cells, which do not normally express class II (32). Professional APCs of the leukocyte lineage, such as interstitial dendritic cells in the muscle tissue, could efficiently present the very small quantities of antigen produced (on the order of nanograms; see ref. 15). Thus, with the DNA approach, the kinetics of antigen secretion combined with the residual inflammation from the intramuscular injection could provide favorable conditions for antigen uptake by the dendritic cells and transport to lymph nodes (33, 34). Other cell types might play a role in class II antigen presentation, including macrophages and lymphocytes as well as non-leukocytes such as myoblasts, which have also been shown to express MHC class II (35). Myoblasts are quiescent mononuclear cells found in small numbers in mature muscle and they

Table 1. Anti-HBV immune response in mice injected with the different expression vectors

Vector	Time of 100% seroconversion	Mean titer, mIU/ml		
		1 week	3 months	6 months
pCMV-S	1 week	48 ± 11	937 ± 337	1022 ± 398
pCMV-S2.S	1 week	439 ± 80	466 ± 307	267 ± 168
pCMV-S1.S2.S	2 weeks	1 ± 1	517 ± 188	461 ± 274
pHBV-S2.S	20 days	0 and 3 ± 3*	1725 ± 572	ND

Anti-HBs antibodies were quantitated using a commercial kit (Monolisa anti-HBs, Diagnostics Pasteur, Marnes la Coquette, France) and values were determined relative to the standard provided. Mean titers are expressed in mIU/ml. The threshold for seroconversion was defined as ≥10 mIU/ml. The data are presented as mean ± SEM (n = 8). ND, not determined.

*The two values for the pHBV-S2.S vector were determined at 5 and 10 days, respectively.

proliferate in response to muscle fiber injury such as occurs with the cardiotoxin pretreatment and intramuscular injection used here. Even though muscle fibers are reformed by the time the DNA is injected (36), residual proliferating myoblasts might facilitate the immune response.

It is known from human and primate vaccination studies that antibodies to HBsAg are alone sufficient to confer protection against viral infection. In this regard, a single injection of DNA is able to induce high levels of antibodies to HBsAg in mice that are sustained for at least 6 months. If a comparable response can be attained with DNA in humans, this would offer clear advantages over the current protein vaccines, which usually involve a series of three or four injections given over a 6- to 12-month period. The rapid and strong antibody response to the preS2 region is important for vaccine design since inclusion of the preS2 domain may be beneficial. During natural HBV infection, the presence of preS2 antibodies is a marker of virus clearance from the liver, whereas they are absent in people who progress to a chronic carrier state (37, 38). The preS2 epitopes alone can induce a protective immune response since vaccination of chimpanzees with a synthetic preS2 peptide provides immunity against challenge with HBV (39, 40). Finally, the very rapid appearance of antibodies achieved using the DNA-based immunization may prove particularly beneficial for vaccination against perinatal transmission of the virus, such as occurs in areas where HBV infection is endemic.

DNA-mediated immunization may therefore allow rational design of DNA expression vectors to induce a particular type of immune response. Since changes in the antigen composition and coding sequences can be made and evaluated more rapidly than for recombinant proteins, this approach could also lead to the development of new generations of vaccines based either on plasmid DNA or on novel recombinant proteins.

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