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Immunology

DNA vaccine for hepatitis B: Evidence for immunogenicity in chimpanzees and comparison with other vaccines

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ABSTRACT **Vaccination of two chimpanzees against hepatitis B virus (HBV) by intramuscular injection of plasmid DNA encoding the major and middle HBV envelope proteins induced group-, subtype- and preS2-specific antibodies. These were initially of IgM isotype, and then they were of IgG (predominantly IgG1) isotype. The chimpanzee injected with 2 mg of DNA attained >100 milli-international units/ml of anti-HBs antibody after one injection and 14,000 milli-international units/ml after four injections. A smaller dose (400 μg) induced lower and transient titers, but a strong anamnestic response occurred 1 year later. Comparison with responses in 23 chimpanzees receiving various antigen-based HBV vaccines suggests that the DNA approach is promising for prophylactic immunization against HBV.**

Hepatitis B virus (HBV) remains an important worldwide health problem, with an estimated 250 million chronic carriers who face increased risk of developing cirrhosis and hepatocellular carcinoma (1). The prospects for control of infection and disease depend on the availability of safe, effective, and affordable vaccines.

Although both humoral and cell-mediated immunity may result from natural HBV infection, antibodies alone are sufficient to confer protection, and the exact role of cytotoxic T lymphocytes is not known. After natural infection, antibodies are detected against the surface antigen of the HBV viral envelope (HBsAg; anti-HBs) and the viral core protein (HBcAg; anti-HBc/anti-HBe). There is a very clear role for anti-HBs in conferring protective immunity, and all licensed vaccines used in humans to date have been designed to elicit this. The common clinical standard for anti-HBs antibody levels is milli-international units (mIU)/ml, and in humans, a level of 10 mIU/ml is considered sufficient to confer protection (2). The role for anti-HBc in conferring protective immunity is less clear. Although HBcAg is highly immunogenic and immunization with HBcAg alone has been shown to protect chimpanzees against challenge with live HBV (3, 4), high titers of maternal anti-HBc fail to protect infants of chronically infected mothers from infection (4).

The structural gene for the HBV envelope protein is a single long open reading frame containing three inframe ATG start codons (dividing the gene into three domains designated preS1, preS2, and S from 5′ to 3′) and a single stop codon. The different sized polypeptides produced are known as small or major (S), middle (M = preS2 + S), and large (L = preS1 + preS2 + S). The envelope of the infectious 42 nm HBV (Dane) particle contains all forms, but with a predominance of S. The serum of infected individuals also contains large numbers of smaller (22 nm) empty subviral particles composed solely or predominantly of S (5). B- and T-cell epitopes are found on both S and preS domains. The S domain encodes the primary protective B-cell epitope (group-specific determinant *a*) and two other determinants, (*d* or *y* and *w* or *r*), resulting in four subtypes: *adw*, *adr*, *ayw*, and *ayr* (1).

The first HBV vaccines to be used in humans involved injection of empty 22-nm subviral particles purified from the plasma of chronic carriers (6–9). The second (and current) generation of HBV vaccines consists of similar subviral particles, which are produced as recombinant proteins in stably transfected eukaryotic cell lines (10–12). Despite their high efficacy, subunit HBV vaccines are not widely used in developing areas of the world, where they are most needed, owing to the high cost of production. In addition, plasma-derived subunit vaccines are poorly accepted because of persistent (but unfounded) concerns about their safety (13).

Various attempts have been made to develop HBV vaccines that are less expensive but as efficacious as the subunit vaccines. Unfortunately, approaches such as recombinant live viral vectors (14, 15) and synthetic peptides (16, 17) did not prove sufficiently effective. Thus for worldwide application there is still a need to develop an efficacious but inexpensive vaccine against HBV.

A novel approach to immunization is the induction of immune responses against an antigenic protein expressed *in vivo* from an introduced gene. DNA vaccines are attractive, because endogenous antigen synthesis induces $CD8^+$ major histocompatibility complex class I-restricted cytotoxic T lymphocytes, such as are obtained with live viral vaccines but rarely with whole killed pathogen or subunit vaccines. Furthermore, antigen synthesis over a sustained period may be advantageous for the immune system and as such might help overcome low responsiveness and eliminate or reduce the requirement for booster injections. Several animal models using recombinant plasmid DNA coding for specific viral, bacterial, or parasitic antigens have been reported (18, 19), and in most cases a full-range of immune responses was obtained including antibodies, cytotoxic T lymphocytes, T-cell help, and (where evaluation was possible) protection against challenge.

We have previously demonstrated, in mice, genetic immunization against HBV based on plasmid DNA vectors expressing HBsAg (20, 21). A single intramuscular injection of DNA resulted in more rapid, stronger, and longer-lasting humoral and cellular immune responses than those obtained by injection of recombinant HBsAg (20, 21). Thus it was desirable to evaluate the DNA approach for safety and potential for protective immunity in nonhuman primates, which are more relevant for vaccination of humans. The chimpanzee is similar to man with respect to susceptibility to HBV infection and antibody titer required for protection, and in fact it is the only animal that can be challenged with HBV (22). Here we describe genetic vaccination of two chimpanzees against HBV and show that the DNA approach can induce serum levels of

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Abbreviations: HBV, hepatitis B virus; mIU, milli-international unit(s); S, small- or major-sized polypeptide; M, middle-sized polypeptide; L, large-sized polypeptide.

anti-HBs as high as those induced with recombinant subunit vaccines.

MATERIALS AND METHODS

DNA HBsAg-Expression Vector. The HBsAg-expression vector that encodes the S and M (preS2 + S) forms of the HBV (*ayw* strain) envelope protein under the control of the cytomegalovirus (CMV) immediate early promoter (pCMV-S2.S) has been described (21). DNA purified by anion exchange chromatography (Qiagen, Hilden, Germany) was redissolved in endotoxin-free sterile phosphate-buffered saline (Sigma) for injection.

DNA-Based Vaccination of Chimpanzees. The two chimpanzees (CH 1545, male, 9.8 kg, 18 months; and CH 1547, female, 7.3 kg, 16 months) were maintained at Bioqual (Rockville, MD), where they were pair-housed in a glass biocontainment suite, where they had visual contact with other chimpanzees (23). Each chimpanzee was injected with 400 μg (CH 1545) or 2 mg (CH 1547) of DNA on four separate occasions (0, 8, 16, and 27 weeks) using the Biojector needleless injection system (Bioject, Seattle, WA) or a 1-cc insulin syringe fitted with a 30-gauge needle, as described in Table 1. To test for an anamnestic response, the chimpanzees were further boosted at 52 weeks by intramuscular injection in the thigh with 10 μg of recombinant S protein (*adw* subtype) obtained as a commercial subunit vaccine (SmithKline Beecham, Rixensart, Belgium). Animals were followed for 72 weeks after the initial DNA injection.

Detection of Anti-HBs. The chimpanzees were bled weekly for the duration of the study, and serum titers of anti-HBs were determined for individual samples (mean of triplicate assays) with three different commercial kits as follows: Monolisa anti-HBs kit (Sanofi, Montreal), AUSAB-EIA kit (Abbott), and Hepanostika anti-HBs kit (Organon Teknika–Cappel). Assays were carried out according to the manufacturer's instructions and titers were expressed in mIU/ml by comparison with World Health Organization-defined standards (Monolisa Anti-HBs "Standards," Sanofi, Montreal).

Serum anti-HBs antibody titers were also determined (in triplicate) by endpoint dilution ELISA assay using particles of recombinant S protein of a different (*ad*) or same (*ay*) subtype (Abbott) to detect group (*a*) and group plus subtype (*a*+*y*) specific antibodies, respectively, or recombinant S plus M proteins of the same subtype (*ayw*) (Pasteur-Merieux Serums and Vaccines, Val de Reuil, France) to detect group plus subtype anti-S plus anti-preS2 antibodies. Antibodies to the preS2 region of the HBV envelope were also quantified using a synthetic peptide corresponding to amino acid sequence 120–145 of the preS2 region (*ayw* subtype). For each antigen, 96-well polystyrene plates (Corning) were coated with 0.1 μg of recombinant particles/well (100 μl at 1 μg/ml in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6), incubated overnight at room temperature, rinsed five times with PBS-Tween (PBS with 0.05% Tween-20), blocked for 1 hr with 10% fetal calf serum (FCS) in carbonate buffer, and rinsed again. Ten-fold serial dilutions of sera diluted in PBS-Tween-FCS (PBS-Tween with 10% FCS) were then added, and the plates were incubated at 37°C for 1 hr and rinsed. Different isotypes of anti-HBs antibodies were assayed by addition of horseradish peroxidase-labeled mouse monoclonal anti-human IgG, IgM, IgG1, IgG2, IgG3, or IgG4 (Southern Biotechnology Associates, Birmingham, AL) diluted 1:2000 in PBS-Tween-FCS and incubated at 37°C for 1 hr. After rinsing, bound antibodies were detected by reaction with *o*-phenylenediamine dihydrochloride (OPD; Sigma) at 1.6 mg/ml in 0.05 M sodium citrate buffer (pH 5.0) with 0.001% H_2O_2 and quantified by reading absorbance at 450 nm. Titers were expressed as the dilution that yielded twice background, with a cutoff OD of 0.05.

To ensure that inadvertent HBV infection was not responsible for anti-HBs titers, serum was also tested for anti-HBc and levels of liver enzymes such as alanine aminotransferase.

Antibody titers in sera obtained from 37 other vaccinated or control chimpanzees (Table 2) just before challenge with live HBV were reassayed for anti-HBs by the Monolisa Anti-HBs kit and end-point dilution ELISA as described above. The details of those studies are described in the listed references.

Table 1. Protocol for DNA vaccination of chimpanzees

Procedure	CH 1545	CH 1547
DNA prime (0 weeks) and DNA boost 1 (8 weeks)	Biojector (No. 2 for deltoid, No. 3 for quads), each site 0.5 ml at 0.2 mg/ml	Biojector (No. 2 for deltoid, No. 3 for quads), each site 0.5 ml at 1 mg/ml
DNA boost 2 (16 weeks)	Preinjected with 25% sucrose (1 ml in deltoid, 2 ml in quads); syringe and needle, each site 0.5 ml at 0.2 mg/ml	Preinjected with 25% sucrose (1 ml in deltoid, 2 ml in quads); syringe and needle, each site 0.5 ml at 1 mg/ml
DNA boost 3 (27 weeks)	Right side, biojector (No. 5 for deltoid, No. 7 for quads), each site 0.5 ml at 0.2 mg/ml; left side, as for boost 2	Right side, biojector (No. 4 for deltoid, No. 5 for quads), each site 0.5 ml at 1 mg/ml; left side: as for boost 2.

RESULTS

Humoral Response to HBV DNA Vaccine. Immunization of chimpanzees with HBsAg-expressing plasmid DNA induced specific anti-HBs antibodies (Fig. 1). The higher dose of DNA (2 mg) induced significant titers (>100 mIU/ml) of anti-HBs after the initial injection of DNA. Although these fell somewhat with time after the prime and first boost, and much less so after the second and third boosts, they never went below the 10 mIU/ml level considered adequate to confer protection. By only 1 week after the first boost, remarkably high titers of nearly 10,000 mIU/ml were detected. In contrast, the lower dose (400 μg) failed to stimulate detectable levels of antibody until after the first boost. Thereafter, low levels of antibody (<60 mIU/ml) could be detected in the serum by 4 weeks after each boost, but these then fell to lower levels by 8 weeks after boost.

Comparison of Commercial Kits to Detect Anti-HBs. Assay of serum samples from CH 1547 with three different commercial kits detected different antibody titers for the same samples, even when standardized against the same World Health Organization mIU standards. This reflects the different antibody specificities detected, which were against one or more of the group, subtype, and preS2 determinants (Fig. 2). Despite the absolute differences between kits, similar patterns were obtained with all three kits, with the exception that the Hepanostika kit sometimes failed to detect antibodies in samples where titers of <100 mIU/ml had been measured with the other two kits.

Fine Specificity of Humoral Response. Results of end-point dilution ELISA assays of serum samples from CH 1547 are depicted in Fig. 3. The initial antibodies, detected 3 weeks after injection of HBsAg-encoding DNA, were of both IgM and IgG isotypes, and these were predominantly against the group (*a*) determinant of the S protein. By 1 week after the first DNA boost, most antibodies were of the IgG isotype. Most of these were against the preS2 domain, because ten-fold higher titers were detected with HBsAg particles containing M+S envelope proteins than with those composed solely of S. The early immunodominance of the preS2 domain was confirmed by ELISA assay using a preS2 peptide for the solid phase. Anti-preS2 IgG end-point dilution titers peaked at nearly 10^{-5} 1 week after the first boost, following which they fell to and

Table 2. Strategies for antigen-based vaccination of chimpanzees against hepatitis B virus

Chimp No.	Vaccine	HBV (*ayw*) challenge	Ref.
Vaccinated (n = 24)			
965, A-164, A-166	Peptide S, linear 49 (aa 110–137) plus alum	MS2 strain	16
993	Peptide S, linear 49 (aa 110–137) plus pertussis	MS2 strain	16
A-133, A-136	Peptide S, linear 49a (aa 125–137) plus alum	MS2 strain	16
A27	Peptide S, linear 49 (aa 110–137) plus FIA	MS2 strain	16
A-36, A-64	Peptide S, cyclic 49 (aa 110–137) plus alum	MS2 strain	16
1178, 1231	Peptide preS plus alum	MS2 strain	17
1475, 1479	Recombinant yeast-expressed S subunit vaccine (SKB)	AS strain	24
88A04, 1486	Recombinant yeast-expressed S subunit vaccine (MSD)	AS strain	24
974, 984	Recombinant monkey kidney cell-expressed S subunit vaccine	MS2 strain	25
66, 67	Recombinant S-expressing vaccinia vector (WT)	MS2 strain	15
1072	Recombinant S(*adw*)-expressing vaccinia vector (Vac)	MS2 strain	13
1373, 1374, 1375	Recombinant S(*adw*)-expressing adenoviral vector	MS2 strain	14
Controls (n = 13)			
A-98	Control vaccinia (WT)	MS2 strain	15
1376	Control adenoviral vector	MS2 strain	14
n = 3	None	AS strain	24
n = 3	None or placebo	MS2 strain	6, 13
n = 5	None	MS2 strain	26

FIA, Freund's incomplete adjuvant; MSD, Merck Sharp & Dohme (West Point, PA); SKB, SmithKline Beecham (Rixensart, Belgium); and WT, wild-type.

were maintained at a level of $\approx 10^{-3}$. Antibodies against the S protein reached titers of $>10^{-4}$, with both group-specific (anti-*a*) and subtype-specific (anti-*y*) antibodies being detected, although the latter became less prevalent over time. The IgG antibodies were predominantly of the IgG1 isotype (Fig. 3) with no significant levels of IgG2, IgG3, or IgG4 being detected (data not shown).

Response to Injection of Recombinant S Protein. To determine whether an anamnestic response could be induced, particularly in CH 1545, which no longer had detectable anti-HBs, a commercial subunit vaccine containing 10 μg of recombinant S particles was injected 52 weeks after initial DNA immunization (i.e., 25 weeks after the last DNA boost), and this was found to induce a rapid and strong elevation of anti-HBs antibody titers in both chimpanzees (Figs. 1–3). CH 1545, attained titers of 50 and 280 mIU/ml by 2 and 4 days, respectively, indicating a strong anamnestic response. Antibody titers in CH 1547, which had the strong and sustained response to the DNA vaccine, increased from ≈3,000 mIU/ml to 32,000 mIU/ml within 1 week after protein injection and to nearly 190,000 mIU/ml by 4 weeks. The antibodies induced by the protein boost were solely against the group specific epitope (*a*) of the S polypeptide, as evidenced by the equivalence of ELISA titers for anti-S(*a*), anti-S(*ay*), and anti-preS2 + S(*ay*) (Fig. 3). Such a selective memory response was expected, because the protein subunit vaccine consisted solely of S particles of a heterologous (*adw*) strain.

Comparison of DNA Vaccine to Other HBV Vaccines. Sera obtained just before challenge with live HBV from 23 chim-

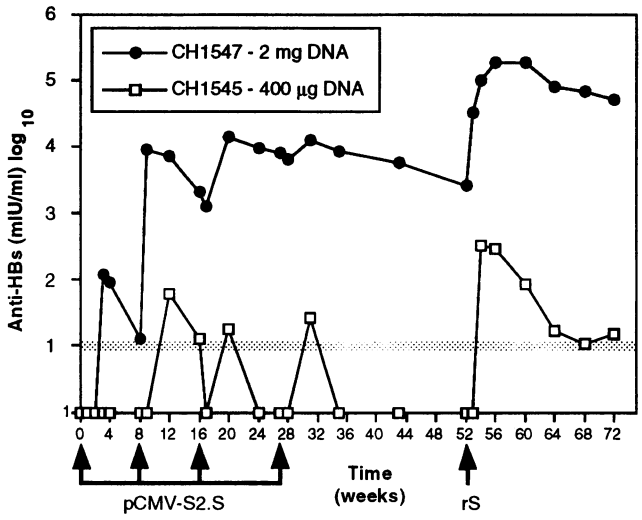

FIG. 1. Kinetics of appearance of anti-HBs antibodies in two chimpanzees immunized against HBV by intramuscular injection of HBsAg-expressing plasmid DNA (pCMV-S2.S). DNA boosts were given at 8, 16, and 27 weeks, and a recombinant S protein boost (10 μg) was given at 52 weeks after the initial DNA injection. Total anti-HBs antibodies were detected by Monolisa Anti-HBs kit (Sanofi). Each point represents the mean of values from triplicate assays expressed in mIU/ml, based on World Health Organization-defined standards. An antibody titer of 10 mIU/ml is considered protective in humans and chimpanzees.

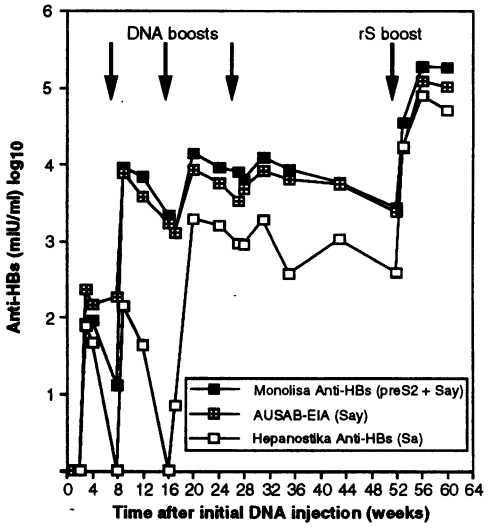

FIG. 2. Kinetics of appearance of anti-HBs antibodies in CH 1547 after DNA-based immunization with HBsAg-expression vector (pCMV-S2.S). DNA (2 mg) was injected at weeks 0, 8, 16, and 27, and recombinant S protein (10 μg) was injected at 52 weeks. Total anti-HBs antibodies were detected by three different commercial kits. Monolisa Anti-HBs kit (Sanofi) detects anti-S(*ay*) plus anti-preS2, AUSAB-EIA (Abbott) detects anti-S(*ay*), and Hepanostika Anti-HBs (Organon Teknika–Cappel) detects anti-S(*a*). Each point represents the mean of values from triplicate assays expressed in mIU/ml, based on World Health Organization-defined standards.

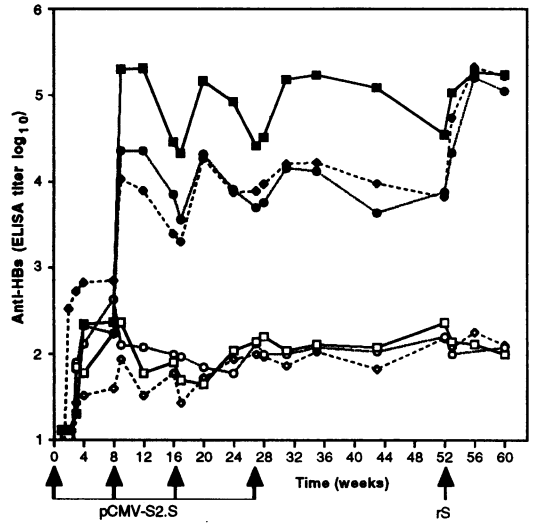

FIG. 3. Kinetics of appearance of IgM and IgG anti-HBs antibodies in sera taken from CH 1547 at various times after DNA-based immunization with plasmid DNA expressing the small (S) and middle proteins (S + pre-S2) of the *ayw* subtype. The DNA (2 mg of pCMV-S2.S) was injected at weeks 0, 8, 16 and 27 and recombinant S protein (*ad* subtype, 10 μg) at 52 weeks. The fine specificity of the antibodies was determined using S-containing HBsAg particles of a homologous (*ay*, ○, ●) or heterologous (*ad*, ◇, ◆) subtype as well as particles containing the middle (S + pre-S2, □, ■) protein of the *ay* subtype. The bound antibodies were detected in a second step by the addition of peroxidase-labeled mouse anti-human IgM (open symbols) or anti-human IgG (closed 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 with a cutoff value of 0.05. Significant levels of IgG2, IgG3, or IgG4 were not detected.

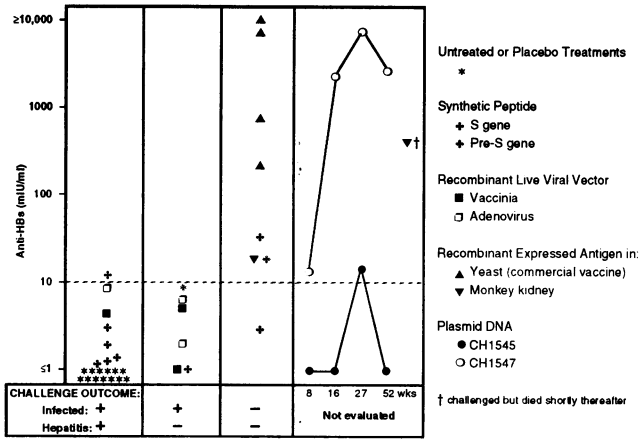

FIG. 4. Outcome following live HBV challenge of chimpanzees after vaccination with various HBV vaccines and of untreated or control-treated chimpanzees. Anti-HBs titers in sera taken immediately before challenge were determined in triplicate using the Monolisa Anti-HBs kit (Sanofi). Each point represents the mean titer (of triplicate assays) for one chimpanzee expressed in mIU/ml based on World Health Organization-defined standards. Prechallenge titers from individual chimpanzees are divided into three columns based on (*i*) whether they became infected and contracted hepatitis, (*ii*) became infected but did not develop hepatitis, or (*iii*) did not become infected or develop hepatitis. Animals were considered infected if they exhibited viremia and appearance of anti-HBc antibodies. Infected animals were considered to have hepatitis if serum alanine aminotransferase was elevated to >40 IU/ml. The fourth column shows anti-HBs antibodies in chimpanzees that were not evaluated for outcome of challenge. This includes the two DNA-vaccinated chimps at the time they were boosted with HBs-expressing DNA or with recombinant HBs protein. CH 1545 (closed circles) received 400 μg of DNA at 0, 8, 16, and 27 weeks whereas CH1547 (open circles) received 2 mg of DNA at these times, and both received 10 μg of HBsAg protein at 52 weeks.

panzees vaccinated by various other means (Table 2) showed a wide range of anti-HBs titers when assayed by the Monolisa Anti-HBs kit (Fig. 4). Antibody titers (mIU/ml; mean ± SEM) obtained for the different methods of immunization were as follows: 3 ± 1 (n = 6) for live recombinant viral vector (vaccinia or adenovirus); 10 ± 2 (n = 11) for peptide (S or preS2) plus adjuvant; and 5,843 ± 1,615 (n = 6) for recombinant S protein plus adjuvant. None of the untreated or placebo-treated chimpanzees (n = 13) had detectable anti-HBs.

The outcome of challenge with live HBV of the *ayw* strain was strongly correlated with the anti-HBs titers regardless of the method of immunization (Fig. 4). All animals with a titer of <1 mIU/ml became infected as evidenced by viremia and development of anti-HBc, and each animal developed hepatitis B, as evidenced by elevated serum alanine aminotransferase (>40 IU/ml). Of the 14 chimpanzees with an anti-HBs titer of 1 to 10 mIU/ml, 7 showed evidence of infection and hepatitis, 6 were infected but didn't develop hepatitis, and only 1 was protected from infection. Of the eight chimpanzees with a titer >10 mIU/ml, all were protected from infection except for one, which had the lowest anti-HBs titer at 12 mIU/ml. These findings agree closely with the critical protective level of 10 mIU/ml determined for humans by the Centers for Disease Control (2).

DISCUSSION

DNA-Based Vaccination of Chimpanzees. To our knowledge, this is the first report of a test of a DNA vaccine in chimpanzees. The results show that the HBsAg synthesized *in vivo* induces antibodies that can recognize the different known conformational epitopes of both the S and preS2 components of the HBV envelope protein. In addition, the IgM to IgG class shift indicates that T-cell help (Th) was induced, and the predominance of IgG1 indicates that this was Th2 (27). This is somewhat different than the response in mice, where DNA-based immunization against HBsAg induces both IgG1 and IgG2a (H.L.D. and C. L. Brazolot Millan, unpublished results) and a Th1 cytokine profile, that is secretion of IL2 and γ-interferon, but not IL4, IL5, or IL10 (C. Leclerc and R. G. Whalen, personal communication).

The antibody titers obtained in CH 1547 (female) with 2 mg of DNA are particularly impressive, reaching levels 10 and 1000 times greater than those normally required to confer protection after prime and first boost with DNA, respectively. The weaker response in CH 1545 (male) was probably due to the 5-fold smaller dose, although other factors, such as individual variation in efficiency of gene transfer and immune response to HBsAg, may play a role. For example, it has been observed that the immune response to recombinant HBsAg is often weaker in male than female humans (28).

The connective tissue cytoarchitecture of a muscle may impede the diffusion of injected substances (29), and a more highly developed connective tissue probably accounts for the finding that gene transfer is less efficient in primate than mouse muscle (30). Different methods of DNA injection were attempted to improve the gene transfer and thus improve the immune response. The first two injections of DNA were carried out with the Biojector needleless injection system, which we have previously shown to be superior to needle and syringe for genetic immunization of rabbits (31). The third administration of DNA was with needle and syringe following pretreatment of the injection site with hypertonic sucrose, a technique which we have found to improve the efficiency of gene transfer in mouse muscle (29). From the results with CH

1545 (weak and transient responses were induced by each injection of DNA), it appears that there wasn't a significant advantage of one method over the other. However, this cannot be concluded with any certainty based on findings in a single animal, and it should be evaluated further in other primates.

The humoral response induced by the initial intramuscular injection of HBsAg-encoding DNA was clearly weaker and shorter-lived than that which has been observed previously in mice (32), but this may be largely dose-related. In mice, strong and long-lasting immune responses were obtained with 100 μg of DNA (5 mg of DNA per kg of body weight) (20) but only weak responses with 1 μg of DNA (50 μg of DNA per kg of body weight) (C. L. Brazolot Millan, A. Lobo, and H.L.D., unpublished results). When dose is taken into consideration, the chimpanzees, which received 40 or 300 μg of DNA per kg of body weight, had a response similar to that in mice.

Better results might have been obtained in the chimpanzees with higher doses of DNA; however for economic and safety reasons, it would be preferable to use smaller doses for immunization of humans. It might be possible to increase the efficiency of gene transfer, the level of gene expression, and/or the immune response to the expressed antigen through vector design, the use of formulated DNA, and/or other routes of DNA administration such as intradermal injection (33) or epidermal bombardment with DNA-coated gold particles (34). Further experimentation with nonhuman primates will be necessary to evaluate these possibilities.

DNA Versus Other Vaccination Strategies. Early vaccination strategies typically involved administration of the whole pathogen, either in a killed or live-attenuated form. A number of "new" approaches to vaccine development have been proposed in recent years. These have included recombinant vaccines, vaccines based on synthetic peptides of putative neutralization epitopes of target viruses, and "anti-idiotype" vaccines. The recombinant vaccines have consisted of subunit vaccines containing antigens expressed in prokaryotic or eukaryotic expression systems, as well as recombinant viral sequences carried in live heterologous viral vectors. All these approaches appeared promising at first. Indeed, candidate vaccines for HBV were even able to protect chimpanzees against hepatitis following challenge with virulent virus. However, only vaccines containing expressed viral antigens have proven to be successful in the long run. Even these have had limited application, and only one recombinant vaccine, that for HBV, has been licensed. In fact, the last two licensed vaccines for viral diseases, those for hepatitis A and chicken pox, were a "classical" inactivated whole virus vaccine and a live attenuated vaccine, respectively.

The newest of the new approaches to vaccine development is the use of naked DNA. As with other vaccine approaches, DNA-based vaccines yielded promising results in mice and other small animals, and it seemed likely that protection, albeit possibly marginal and transient, could be achieved with this approach in chimpanzees. The two chimpanzees in the present study were not challenged with live HBV; however we attempted to predict the level of "protection" by comparing DNA-based vaccination with results of extensive tests of other vaccine approaches by two of us (R.H.P. and J.L.G.) under standard conditions (Table 2). There was a strong correlation between titer of anti-HBs and protection for the 23 vaccinated and 13 control chimpanzees that had been challenged with live HBV (*ayw*). Titers >10 mIU/ml protected against infection in all cases but one (at 12 mIU/ml), 1–10 mIU/ml allowed infection but protected against hepatitis in about half the cases, and <1 mIU/ml didn't protect against infection or hepatitis. Comparison of the different approaches clearly shows that HBsAg subunit vaccines stimulated the development of anti-HBs that was orders of magnitude higher in titer than the viral vector or peptide approaches but that the DNA vaccine stimulated antibody in one chimpanzee to the same level as the most successful of the subunit vaccines. It appears that CH 1547 would likely have been protected against infection after only a single injection of DNA and clearly would have been protected at any time after the first boost. On the other hand, CH 1545 would probably not have been protected against infection, except possibly for a brief period following the third boost, but based on the the rapid and strong anamnestic response to the subunit vaccine, it is very likely that, in the event of infection, hepatitis would not have developed. These observations and extrapolations also provide a benchmark against which other vaccine approaches can be compared, at least with respect to hepatitis B vaccines.

Commercial Kits to Detect Anti-HBs. Because anti-HBs antibody titers correlate so well with protection, it is important that assay methods used clinically are accurate at or near the critical 10 mIU/ml level. In this context, the results of the present study are relevant to the testing of human sera. Each of the three kits evaluated detected different combinations of B-cell epitopes and thus yielded different titers for the same samples of sera. It may be argued that the Monolisa Anti-HBs kit provides falsely high values by detecting anti-preS2 antibodies; however, these do contribute to protection, because they can protect chimpanzees against HBV infection even in the absence of anti-S antibodies (17, 35, 36). Of greater concern was the finding that the Hepanostika Anti-HBs kit detected little or no anti-HBs in sera where titers of 10–1000 mIU/ml had been detected with the other two tests. This is not likely to be due solely to the narrower specificity of antibody detection, because anti-$a+y$ titers were rarely more than 10 times greater than anti-a titers with the end-point dilution ELISA assays. There are also practical aspects that can be considered in comparing the three kits. For example, the Monolisa Anti-HBs and the Hepanostika Anti-HBs both use 96-well plates and can be used with regular plate washers and readers. In addition, they both have removable components and thus allow assay of a minimum of four or eight samples, respectively. In contrast, the AUSAB-EIA uses a 20-well one-piece format that requires special equipment.

Prospects for an HBV DNA Vaccine for Humans. The results of the present study show that DNA-based vaccination is capable of inducing potent humoral immune responses in chimpanzees, although further work is required to evaluate dose response and to optimize the direct gene transfer and gene expression so that smaller and fewer doses of DNA can be used. It will also be necessary to address safety concerns, such as the potential risk of insertional mutagenesis or induction of tolerance or autoimmunity. Although safety issues were not directly assessed in the present study, no abnormalities were detected in the weekly assays of serum (e.g., routine hematology and liver enzymes), nor were any other untoward reactions noted.

Nevertheless, DNA vaccines may have a more promising future than many other approaches if a reproducible response can be achieved. In addition to their use for prophylaxis, HBsAg-expressing DNA vaccines might have therapeutic applications for HBV chronic carriers. Immunization with HBsAg-expressing DNA has been shown to break B- and T-cell tolerance in HBsAg-transgenic mice (M. Mancini, M. Hadchouel, H.L.D., R. G. Whalen, P. Tiollais, and M.-L. Michel, unpublished results).

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