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DNA-Mediated Immunization of Mice with Plasmid Encoding HBs Antigen

In order to develop an experimental DNA vaccine for the prevention and treatment of hepatitis B virus infection, hepatitis B virus surface antigen (HBsAg) DNA was subcloned into an *E. coli*-eukaryotic cell shuttle vector and was expressed in the Baculovirus expression system. Intramuscular, intradermal, and intraperitoneal injections of 30 μg of the plasmid DNA expressing HBsAg induced humoral and cellular immune responses in ICR mice. The first IgG antibodies were detected after ten days and specific IgG antibody titers peaked after two months of a single intramuscular DNA injection. Anti-HBs antibody titers gradually increased and peaked at four months following intradermal DNA injection, and in case of intraperitoneal injection they peaked at seven months. Generation of HBs-specific helper T lymphocytes was also investigated through the production of interleukin-2 by T helper cells. Boosting effects of HBs DNA were investigated without much results. In general, DNA-mediated HBs immunization induced humoral and cellular immune responses in mice that appears to simulate immune responses in human during the course of HBV vaccination.

Key Words : *Vaccines; DNA; Hepatitis B surface antigens*

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INTRODUCTION

Hepatitis B virus (HBV) infection is endemic in many parts of the world. The most common mode of transmission is from chronically infected mothers to their newborns; neonates exposed in this fashion usually develop chronic hepatitis B infections. The number of hepatitis B virus carriers worldwide has been estimated at over 250 million (1). Although HBV vaccine has been successful in preventing new infections (2), a large proportion of the carriers die annually from long-term sequelae of chronic hepatitis such as cirrhosis and hepatocellular carcinoma (1). Thus, the development of effective therapeutic protocols for these carriers remains as a challenging problem in the HBV-infested regions of the world.

An interesting idea in vaccine development has been the subunit vaccine. The strategy of subunit vaccine has been developed to introduce only selected components of a virus to the immune system. Gene vaccine, or the use of antigen-encoding DNAs as vaccines, represents a new approach to the development of subunit vaccines (3). A remarkable feature of DNA-mediated immunization procedure is that in situ expressed small quantities of antigens can give rise to strong and broad-based immune responses. It has been shown that HBs (Hepatitis B virus surface antigen) DNA vaccination induces a potent anti-HBs antibody response (4) and a MHC class I-restricted cytotoxic T cell response in mice (5).

This study was designed to determine several factors affecting antibody responses of HBs DNA vaccination in ICR (Institute of Cancer Research) mice. We tested the minimal quantities of HBs DNA and several routes of immunization that can give rise to the expression of specific anti-HBs IgG antibodies. We also examined the boosting effect of HBs DNA, and interleukin-2 (IL-2) level was measured in an in vitro HBs-specific antigen presenting cell (APC)-lymphocyte coculture system.

MATERIALS AND METHODS

HBs DNA synthesis and cloning

Viral DNA was extracted from chronic hepatitis B virus carrier serum by NaOH extraction method (6). We designed HBs primers to amplify the DNA encoding middle HBsAg (preS2 and S, 848 bp, GeneBank Accession No. A00678) and PCR amplification was performed by using HBs oligonucleotide primers (Bioneer, Taejun, Korea): HBs-s (5'-CC ATG CAG TGG AAT TCC ACA ACA-3') and HBs-as (5'-TTA AAT GTA TAC CCA AAG ACA AA-3'). A T-HBs plasmid was constructed by ligating the HBs PCR product into the pT7Blue (R) vector (Novagen, U.S.A.) and HBs DNA were subcloned into a mammalian expression vector, pcDNA3 (Invitrogen, U.S.A.) by disrupting the *Bam*HI and *Xba*I site of T-HBs and ligating the insert into the pcDNA3 vector. The recombinant pcDNA3-HBs DNA was used for immunizing the mice. HBs inserts in both the T-HBs and pcDNA3-HBs plasmids were identified by 1.5% agarose gel electrophoresis of the plasmids after *Bam*HI-*Hind* III or *Bam*HI-*Xba*I digestion. We also performed DNA sequencing of the plasmids to verify that the correct inserts are ligated into the proper reading frame. HBs DNA were also subcloned into pBlueBacHisC (Invitrogen), a Baculovirus expression vector for the expression of recombinant HBs protein in *Sf21 (Spodoptera frugiperda)* insect cell culture system (Invitrogen). The pBlueBacHisC-HBs plasmid was constructed by disrupting the *Bam*HI and *Hind* III site of T-HBs and ligating the HBs insert into the pBlueBacHisC vector.

Immunization of mice

Three groups of ICR mice (male, 8 week-old) were injected with 30 μg of pcDNA3-HBs DNA in 60 μL of PBS by intramuscular (IM, quadriceps, n=15), intradermal (ID, base of tail, n=6), and intraperitoneal (IP, n=15) routes, respectively. Plasmid DNA was prepared with the use of Wizard Maxiprep kit (Promega, U.S.A.) and was dissolved in PBS (Gibco BRL, U.S.A.) to a concentration of 0.5 mg/mL. IM injection was done by using a 28 G needle attached to a 1 mL tuberculin syringe (Boin Medica, Korea). Immunized mice were killed during the period of 10 to 250 days after the single primary injection of DNA, depending on the routes of immunization. Mice sera were collected and frozen at -20℃, until an enzyme linked immunosorbent assay (ELISA) was performed. Five mice from the IM injected group received another IM injection of 30 μg of pcDNA3-HBs for boosting, 15 days after the primary immunization, which was administered to the opposite site of the previously injected thigh. Mice sera were collected on day 30 and 60 following the first injection. Different doses of HBs DNA were tested to examine the dose-response relationship. Three groups of 10 mice were given 30 μg, 100 μg, and 200 μg of HBs DNA each. The DNA was prepared in PBS at a concentration of 0.3 mg/mL, 1.0 mg/mL, and 2.0 mg/mL each, and was injected by the IM routes. Anti-HBs antibody titer was determined by ELISA at 10, 30, and 60 days after the immunizations.

Anti-HBs humoral immune response

Anti-HBs IgG antibody production in the sera of IM, ID, and IP immunized mice and HBs DNA-boosted mice were measured by ELISA method. ELISA plates were coated overnight at 4℃ with 1 μg/mL of HBV preS2 antigen (a.a. 120-145, Sigma, H-7395, U.S.A.) dissolved in 50 μL of carbonate buffer (pH 9.6). Blocking was done by incubating the plates for 2 hr at room temperature with 200 μL of blocking solution (3% BSA dissolved in 0.05% Tween20 (T)-PBS). Mice sera were serially 2-fold diluted from 1:50 to 1:1,600 in the antibody buffer (1% BSA solution in 0.05% Tween20 (Sigma)-PBS) and 100 μL of the diluted sera were applied to the plates which were incubated for 2 hr at 37℃. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (diluted to 1:1,000 in antibody buffer, Tago 2390, Burlingame, CA, U.S.A.) was used as a secondary antibody, and the plate was further incubated for 2 hr at 37℃. Substrate solution (4 mM ortho-phenylenediamine (OPD, HyBRL Screen 9505A3, U.S.A.) in 50 mM sodium phosphate, 20 mM citric acid, pH 5.0) was applied for 30 min at room temperature and absorbance at 490 nm was read by the ELISA reader (BioRad, U.S.A.). Antibody titer was expressed as the reciprocal of the last dilution of the test sera yielding a value above that of the pooled preimmune sera which was set as the blank.

HBs-specific induction of IL-2 by HBs DNA-boosted splenocytes

Splenocytes were collected from HBs DNA-boosted mouse on day 60 by Ficoll/Hypaque gradient centrifugation, and were incubated overnight at 37℃, 5% CO_2 in complete RPMI1640 (Gibco BRL) at a concentration of 1.0×10^6 cells/mL. Peritoneal macrophages were washed out of the peritoneum of ICR mice and were suspended in complete RPMI1640 at a concentration of 1.0×10^6 cells/mL. 1.0×10^5 cells of peritoneal macrophages were plated in a 96 well microtiter plate, and were stimulated overnight with 2 μg of HBV preS2 antigen (Sigma, H-7395) per well at 37℃, 5% CO_2. The next day, the supernatant of HBs antigen-primed macrophages was removed and 1.0×10^5 cells of overnight cultured splenocytes were added to each well and were cocultured with peritoneal macrophages. After 16, and 64 hr of coculture, the supernatant was collected and IL-2 concentration was measured by mouse Inter-

leukin-2 ELISA kit (Genzyme, Cambridge, MA, U.S.A.). The IL-2 concentrations in the samples were determined from a standard curve obtained by plotting the concentrations of IL-2 standards versus their resulting absorbances.

RESULTS

Cloning of HBs DNA

HBs DNA encoding middle HBsAg were amplified from the viral DNA extracted from chronic HBV carrier serum. The PCR product was cloned into a pT7Blue (R) vector (Novagen). We subcloned the HBs insert of the T-HBs plasmid into a eukaryotic expression vector, pcDNA3 (Invitrogen) and carried out PCR analysis and enzyme digestion of the recombinant plasmid to verify the presence of the insert (Fig. 1). In order to determine the ability of the HBs DNA to be expressed properly in the eukaryotic system, we produced recombinant HBs protein in the Baculoviral expression system. Recombinant Baculovirus was produced by homologous recombination and was purified by repeated plaque cloning. Immunodot blotting of the *Sf21* insect cell culture supernatant infected with the recombinant virus demonstrated the presence of HBs protein which was precipitating with high titer anti-HBs antibody containing human carrier serum (data not shown).

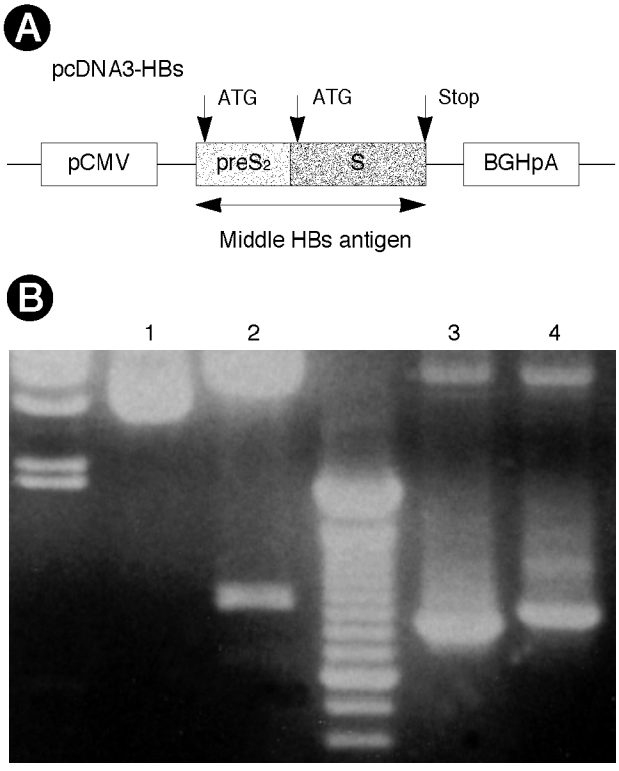


Fig. 1. Construction of the pcDNA3-HBs plasmid. A: HBs-expressing pcDNA3 plasmid is schematically drawn. B: Subcloning of HBs DNA into pcDNA3 vector. Lane 1, pcDNA3-HBs plasmid. The cloned HBs DNA insert is shown by enzyme digestion of pcDNA3-HBs with *Bam*HI and *Xba*I(lane 2), by PCR amplification with HBs primers (lane 3), and by PCR amplification with T7 promoter and HBs-as primers (lane 4).

Anti-HBs antibody response

Anti-HBs antibody titer of collected mice sera was assessed by ELISA. The antibody titer after a single IM inoculation of 30 μg of HBs DNA ranged between 989 and 1,339, with slight increase at day 60, and day 100 after immunization (Fig. 2A). As for the route of inoculation, intradermal as well as intraperitoneal immunization induced immune responses similar to intramuscular injection of HBs DNA. The antibody titer was in the range of 1,015 and 1,342 when injection was done intradermally (Fig. 2B). Intraperitoneal administration induced a wider range of fluctuation in antibody titer, which was between 796 and 1,673. But anti-HBs antibody were detected as long as 250 days after a single intraperitoneal injection of 30 μg of DNA (Fig. 2C). The anti-HBs antibody titers of both HBs DNA-boosted and non-boosted mice remained at similar levels over the period of 30 and 60 days after the first immunizations. Boosting of 30 μg of HBs DNA on day 15 did not seem to be of any benefit to the anti-HBs antibody production (Fig. 3).

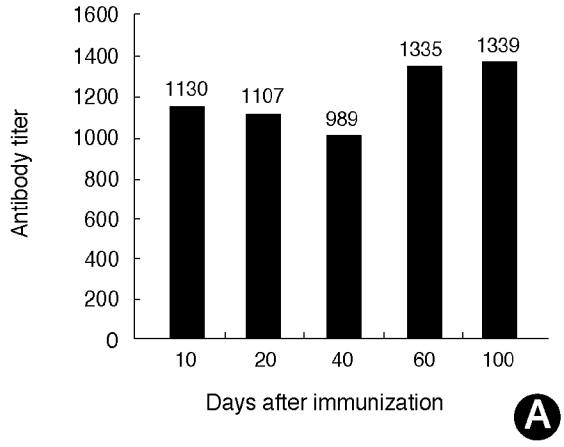
With different dosages of HBs DNA, 30 μg, 100 μg, and 200 μg, respectively, 200 μg of DNA gave the highest antibody titer at day 30, whereas 100 μg of DNA recorded higher titers of antibody than 30 μg of DNA at all times measured. However, the antibody responses did not show any great enhancement with increasing amount of DNA (Fig. 4).

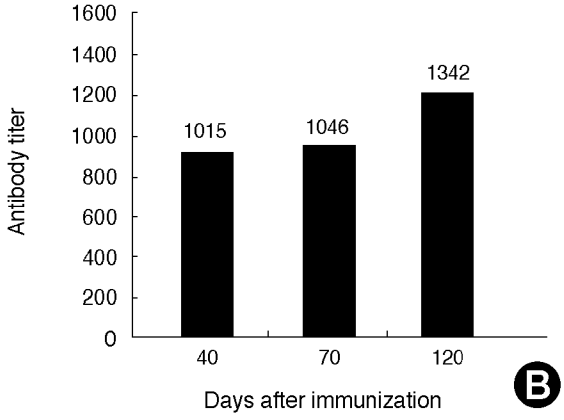
IL-2 induction in HBs DNA-boosted splenocytes

We measured the IL-2 concentration in the coculture system of HBsAg-primed peritoneal macrophages and the splenocytes collected from HBs DNA-boosted mice. IL-2 level was higher in the HBs immunized mice, suggesting a proliferative T cell response. They were 51 pg/mL and 154 pg/mL after 16 hr and 64 hr of coculture, respectively. The control mice which received the vector alone, showed 18 pg/mL and 58 pg/mL of IL-2, respectively (Fig. 5).

DISCUSSION

We evaluated anti-HBs antibody responses after the single application of HBs DNA into mouse tissues in relation to immunization routes, dosage of DNA, and boosting effects of HBs DNA. Our results show that an intramuscular (IM),





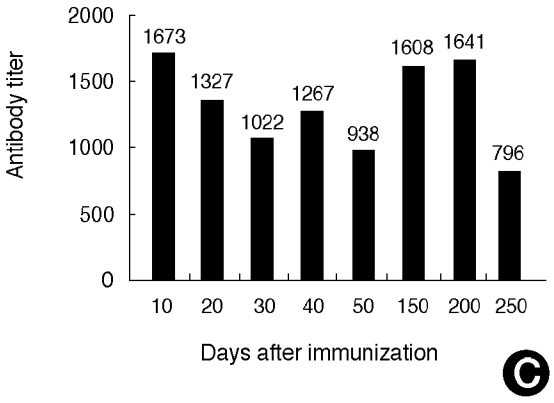


Fig. 2. Serum anti-HBs antibody titer of IM (A), ID (B), and IP (C) immunized mice. Antibody titer is expressed as the reciprocal of the last dilution yielding a value above that of the pooled preimmune sera.

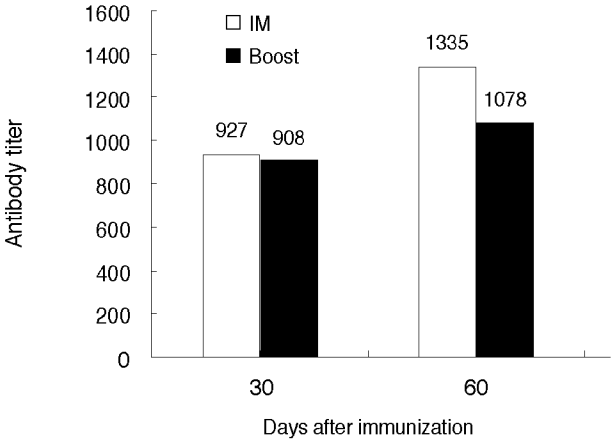


Fig. 3. Serum anti-HBs antibody titer of mice intramuscularly injected with 30 μg of HBs DNA is compared with the titer of mice boosted with 30 μg of HBs DNA on day 15.

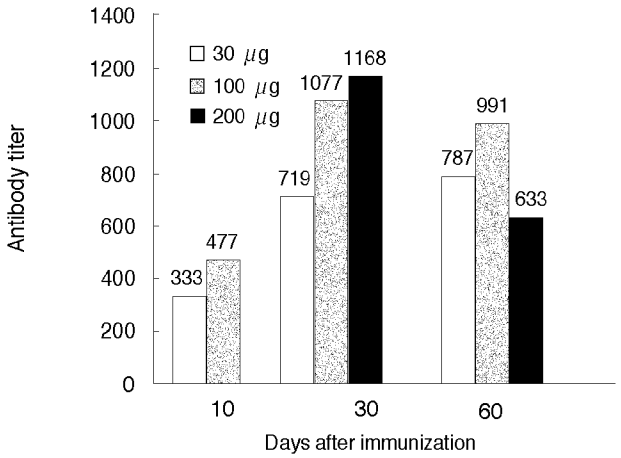


Fig. 4. Serum anti-HBs antibody titer of mice immunized with 30 (□), 100 (▨), or 200 (■) μg of HBs DNA.

intradermal (ID), and intraperitoneal (IP) inoculation of a HBs DNA-carrying eukaryotic expression vector induces strong and sustained anti-HBs IgG antibody responses in mice.

In any transfection procedure, the efficiency of plasmid delivery and expression can vary as a function of cell type,

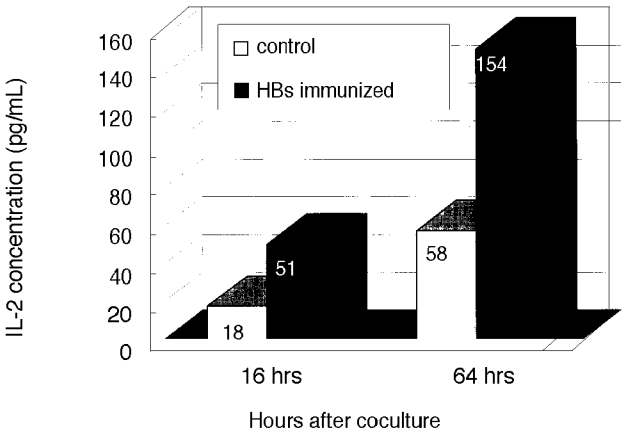


Fig. 5. HBs-specific induction of IL-2 by HBs DNA immunized splenocytes, cocultured with HBs-primed peritoneal macrophages.

density and condition, and states of the DNA (7, 8). Previous studies have revealed that the choice of promoter, preinjection with hypertonic sucrose or cardiotoxins, and the dose and volume of injection influence the efficiency of gene transfer and the stability of expression in DNA-mediated immunizations (9, 10). In this study we used the pcDNA3 vector (Invitrogen) in which the cloned gene expression was under the control of a CMV promoter. Although we have focused on 1 μg of DNA per weight (gram) of mouse in most of the experiments, in order to evaluate the scalability to human dosage, we tested 30 μg, 100 μg, and 200 μg of DNA in IM injections. We found out that 100 μg of DNA worked best in our system, whereas 30 μg of DNA which we thought might be applicable to human vaccination trials, were sufficient to induce a relatively high level of anti-HBs antibody production in ICR mice. These results suggest that we could use mg/kg scales of DNA in human vaccination programs. Anti-HBs IgG antibody synthesis was proportional to the inoculated DNA concentrations to some extent. Therefore, it may be necessary to improve transfection frequencies by modifying the expression plasmids or by using chemical agents such as lipofectin in the future.

We pretreated neither hypertonic sucrose nor cardiotoxin prior to IM immunizations. Boosting the 30 μg of HBs DNA 15 days after primary injection did not seem to affect the antibody titer much. This result contrasts the report by Davis et al. (11) who demonstrated 10 to 200-fold increase in anti-HBs antibody levels by boosting with a second injection of DNA. The authors also showed a boosting effect with a second injection of recombinant HBsAg. The reasons for these discrepancies are not clear for us at present, however one possibility may be that they have used genetically known high responder strain to HBsAg, such as C57BL/6 and BALB/c mice, and the strain we used here belong to low responder (3). 30 μg of DNA is a relatively small amount of DNA compared to 100 μg that has been used in most experiments. Therefore, another possibility is that a single boosting of only 30 μg of DNA on day 15 might not have great influence on the anti-HBs immune responses in our system.

The routes of DNA inoculation were not critical factors for effective antibody synthesis in mice. Consistent with recent demonstrations that intradermal (12), intramuscular (4), parenteral, mucosal, and gene-gun inoculations (13) can induce good humoral and cellular immunity in mice and chicken, we observed equally efficient anti-HBs antibody synthesis among IM, ID, and IP inoculated mouse groups. In most of the investigations (4, 12), antibody synthesis appeared to last for 6 to 17 months suggesting the possibility that very low numbers of transfected cells can survive for a long period of the time and that antigen presenting cells (APCs) may even take up released foreign DNAs from dying transfected cells. In our study we observed antibody synthesis longer than eight months in the case of IP immunization. It might imply that peritoneal macrophages may play a role in the uptake and presentation of injected antigen. The ID immunized group of mice secreted a high titer of antibody as the IM and IP immunized groups. A previous study demonstrated that ID immunization with less than 10 μg of DNA encoding influenza nucleoprotein (NP) could induce anti-NP specific antibody and cytotoxic T lymphocyte responses, as well as virus neutralization activity against influenza viral challenge (12). Thus we need further studies on ID, and IP administrations with lesser amount of HBs DNA, and have to demonstrate cytotoxic T cell responses and viral neutralization activities.

The switch of the IgM to IgG class implies that specific T cells helped in the switch. The T cell dependent nature of the HBsAg has been established by the previous investigators (5). Involvement of T cells and APCs has been confirmed in this work by the HBsAg-specific lymphocyte proliferation assay. The authors showed the proliferation of HBsAg-specific T cells by measuring the IL-2 level in the supernatant of the HBs-primed APC-lymphocyte coculture system. Further studies involving the proliferative helper T cell assay and cytotoxic T lymphocyte response assay need to be performed in order to demonstrate the cellular immune responses directly in this DNA vaccination model.

Adjuvanticity of double-stranded polynucleotides has already been recognized for some time (14). Recently, another group identified a specific immunostimulatory DNA sequence (CpG sequence, e.g. 5'-AACGTT-3') required for an effective DNA vaccination which was a segment of ampicillin resistance marker of the plasmids (15). The pcDNA3 plasmid utilized by the authors also contains such palindromic immunostimulatory DNA sequences which may have contributed to an effective anti-HBs antibody synthesis in our

study.

It is known from human and primate vaccination studies that antibodies of HBsAg are sufficient to confer protection against viral infection. By inoculation of HBs DNA followed by boosting at 6 and 24 weeks, Prince and his colleagues were able to induce high levels of antibodies of HBsAg in chimpanzees (16). If a similar response can be attained with HBs DNA in humans, this would offer some advantages over the current protein vaccines. During natural HBV infection, the presence of anti-preS$_2$ antibodies is considered a marker of virus clearance from the liver, whereas they are absent in patients who progress to a chronic carrier state. Therefore, further investigations are necessary to identify the cause of this immunological defect found among chronic HBV carriers. Nevertheless, it is clear that the DNA vaccination approach has potential as an investigative tool for the design of new generations of vaccines for chronic carriers.

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