Boosting Immune Response to Hepatitis B DNA Vaccine by Coadministration of Prothymosin α-Expressing Plasmid

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DNA vaccines induce protective humoral and cell-mediated immune responses in several animal models. However, compared to conventional vaccines, DNA vaccines usually induce poor antibody responses. In this study, we report that coadministration of a hepatitis B virus (HBV) DNA vaccine with prothymosin α as an adjuvant improves antibody responses to HBV S antigen. We also observed higher seroconversion rates and higher antibody titers. Prothymosin α appears to increase the number and affinity of hepatitis B surface antigen-specific, gamma interferon-secreting T cells and to enhance cellular immune response to the PreS2S DNA vaccine. Interestingly, administering the DNA separately from the prothymosin α plasmid abrogated the enhancement of DNA vaccine potency. The results suggest that prothymosin α may be a promising adjuvant for DNA vaccines.

Immunization with naked DNA can be defined as the in vivo delivery of an antigen-encoding expression vector into a given tissue for the purpose of the induction of immune responses. This novel immunization approach results in de novo production of correctly folded and glycosylated protein antigens and in certain respects mimics the actions of live attenuated or recombinant virus vaccines. As a consequence, this mode of immunization may mimic natural infection and induce protective immune responses. In animal models, such genetic immunization induces both humoral and cellular immune responses against a range of viral pathogens, such as hepatitis B virus (31), influenza A virus (37, 43), herpes simplex virus (5, 27, 28), rabies virus (48), and human immunodeficiency virus type 1 (6, 45). In spite of the existence of safe and efficacious vaccines, hepatitis B virus (HBV) infection is one of the most common infectious diseases, with an estimated 350 million chronic HBV carriers worldwide (12, 22). Patients with chronic hepatitis B are at high risk of developing liver cirrhosis, which is associated with a high rate of mortality due to the development of hepatocellular carcinoma or noncarcinomatous complications of cirrhosis (portal hypertension and liver failure) (19). In addition, these chronically HBV-infected carriers represent a reservoir of HBV. Prophylactic immunization against HBV infections can be achieved with recombinant subunit HBV vaccines (10, 30, 44); however, a small number of individuals do not develop protective immunity even after more than the recommended three hepatitis B surface antigen (HBsAg) inoculations. The incidence of nonresponse in immunocompetent young adults is around 5% but increases up to 30% with age of vaccination or even more in immunocompromised individuals (32). Other problems arising from the use of current HBV vaccines are escape variants with mutations within HBsAg and the requirement of at least three injections to achieve protection. For control of HBV infections and liver disease, efficacious but inexpensive DNA vaccines may thus be promising tools (18). Recent reports have demonstrated the efficacy of DNA-based HBV vaccines. Immunization of animals with plasmid DNA vectors encoding the small or middle viral envelope proteins elicits a stronger and longer-lasting humoral and cell-mediated immunity than does immunization with recombinant small and middle envelope protein particles, which are presently in use as vaccines (9). Moreover, DNA-mediated immunization has been shown to break immune tolerance in an HBV transgenic mouse model, indicating that DNA vaccines may also be beneficial for immunotherapy of chronic HBV infections (26).

DNA vaccines can induce cytotoxic T lymphocyte (CTL) responses that do not usually occur after immunization with protein subunit vaccines. But compared with conventional vaccines, DNA vaccines are often less potent in the induction of antibody responses. Alternative forms of immunization for DNA vaccines have been reported to increase the immunogenicity of encoded antigens. These approaches include particle bombardment using DNA-coated gold beads (40); coadministration of DNA vaccines with plasmid DNA expressing cytokines (21, 47), chemokines (42), or costimulatory molecules (7, 20, 29); formulation of DNA with cationic lipids (14, 41); or adding experimental adjuvants, such as monophosphoryl lipid A (38).

Prothymosin α is a highly acidic polypeptide, originally isolated from the rat thymus (15), and is the putative precursor of thymosin α1, an immunomodulatory peptide derived from thymus (25). Prothymosin α is reported to be more effective than thymosin α1 in protection against Candida albicans infection in mice (16). Because of its immunostimulating effects in cell-mediated immunity, prothymosin α is a promising therapeutic agent for immunodeficient and cancer patients. We found that the potency of DNA vaccines encoding HBsAg can be increased substantially with coadministration of the HBV DNA vaccine and a plasmid encoding prothymosin α.

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FIG. 1. Flag-HBV PreS2S, Flag-HBV PreS2S-prothymosin α, and Flag-prothymosin α were expressed in P815 cells. P815 cells were transfected with Flag-PreS2S, Flag-PreS2S-prothymosin α, Flag-prothymosin α, or pcDNA3/flag alone. Twenty-four to 48 h after incubation, the cells were lysed in LAC buffer, and the supernatant was separated by SDS-PAGE and analyzed by immunoblotting with an anti-Flag Western blot.

MATERIALS AND METHODS

Animals. Female BALB/c and C57BL/6 mice (6 to 8 weeks old) were purchased from the Animal Center of Academy of Medical Science, Beijing, People’s Republic of China. All studies were approved by the Institutional Animal Welfare Committee.

Plasmid expressing prothymosin α. The gene encoding prothymosin α was amplified from the plasmid by using the following primers: the forward primer sequence was 5'-CCGGATCCATGATGACACTGAGATACC-3' and the reverse primer sequence was 5'-CCGGAATTCCTACTCCCTGCG TCTTTGCT-3' (the bold nucleotides indicate the restriction endonuclease recognition site). The primers contained a flanking sequence recognized by restriction endonuclease enzymes BamHI and EcoRI, respectively, for the convenience of cloning manipulation. The PCR fragment and plasmid vector pcDNA3/flag (containing a Flag tag; Invitrogen, CA) were cleaved by BamHI and EcoRI (New England BioLabs, MA). The cleaved products were ligated using T4 ligase. Escherichia coli DH5α cells were transformed using the ligated products, and the correct recombinant plasmid was confirmed by endonuclease cleavage followed by DNA sequencing.

Construction of HBV DNA vaccines (PreS2S) and prothymosin α-linker-PreS2S constructs. DNA fragments containing HBV PreS α, S gene, and S genes were amplified by PCR using the adw genome-containing plasmid (a generous gift from Zongde Chen from Zhengzhou University) as a template with the following primers. The forward primers were S1, 5'-GAAGATCTCCGGCCA GTGACTGGAAT-3' (BglII), and S2, 5'-CCCAAGCTTTGGGCAATGCTGAT-3' (HindIII). The reverse primer was S3, 5'-GGGGGCCCACTGG ATGGATGGAGGAA-3' (ApaI). Nucleotides indicate the restriction endonuclease recognition site. The PCR fragments of S1 and S2 and S3 were cloned into plasmid vector pcDNA3/flag by using BglII-ApaI (New England BioLabs) or HindIII-ApaI (New England BioLabs) and the forward primer sequence was 5'-ATGAAGTTAGGGAA-3', the reverse primer sequence was 5'-GGATCCATGATGACACTGAGATACC-3', amplified from the plasmid by using the following primers: the forward primer sequence was 5'-ATGTCTGATGCAGCTGTAGATACC-3'. The correct recombinant plasmid was confirmed by endonuclease cleavage followed by DNA sequencing.

HBsAg protein antigen. The HBsAg used here was derived from recombinant yeast containing the gene for the adw subtype of HBsAg as prepared in the Temervac-HB (Beijing Tianjian Biological Products Co., Ltd.). In certain experiments, the HBsAg was formulated with an aluminum hydroxide adjuvant at 10 μg HBsAg per 1 mg aluminum per ml.

HBsAg-specific antibody assay. Serum samples were collected by tail bleeding at different times, and the presence of HBsAg-specific antibody was analyzed by enzyme-linked immunosorbent assay according to the manufacturer’s protocol (Sino-American Biological Co., Ltd., Luoyang, People’s Republic of China).

DNA immunization in mice. BALB/c mice (10 mice per group) were immunized with the indicated vaccine in 100 μl of sterile phosphate-buffered saline (PBS) via intramuscular (i.m.) injection into the left-thigh quadriceps muscle at a 2-week interval.

Transient expression of the proteins. P815 (mastocytoma) cells were maintained in RPMI 1640 (GIBCO) with 10% fetal bovine serum in a 6-well tissue culture plate at 37°C in a 5% CO2 humidified atmosphere and then transfected with Flag-PreS2S, Flag-PreS2S-prothymosin α, Flag-prothymosin α, or pcDNA3/flag vector by using Lipofectamine 2000 (Invitrogen, CA). For each transfection, 10 μg of plasmid and 15 μl of Lipofectamine 2000 were mixed in 0.2 ml of OPTI-MEM I reduced serum medium and incubated for 30 min. The DNA-Lipofectamine complexes were overlaid onto the cells. After incubating for 12 h, the medium was replaced with normal medium. After 24 to 48 h of incubation, the cells were collected in LAC buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 9 mM CHAPS [3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate], 25% [vol/vol] glycerol) containing 0.5 mM dithiothreitol, 2.5 mM EDTA, 5 μg/ml aprotinin, and 0.2 μM phenylmethylsulfonyl fluoride and lysed by sonication. The supernatant was collected and analyzed as follows: proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore) followed by blocking for 2 h in PBS containing 5% (wt/vol) nonfat dry milk at room temperature. For detection of HBsAg or prothymosin α-HBsAg, the membranes were incubated with horse-radish peroxidase-conjugated anti-Flag antibody (A8592; Sigma, MO) at a dilution of 1:5,000. The blots were visualized by enhanced luminol reagent and oxidizing reagent (Renaissance; NEN, MA).

Enzyme-linked immunosorbent assay (ELISPOT) assay for cytokine production. Spleen cells from mice 1 week following the booster immunization were subjected to an assay of secreted gamma interferon (IFN-γ) in vitro by restimulation with antigenic peptides. Briefly, 96-well polyvinylidene difluoride-plates (U-CyTech, B.V., The Netherlands) were coated with antibody to IFN-γ, washed three times with PBS, and then blocked with RPMI 1640 medium containing 1% heat-inactivated fetal bovine serum (FBS). Cells were cultured at 5 × 10^6 per well in 0.1 ml of medium for restimulation with defined peptides corresponding to known HBsAg T-cell epitope S28-29 (IPGSDLWTSYL; 1, 4, 24). After 18 to 24 h of incubation at 37°C, the plates were washed 10 times with PBS containing 0.005% Tween 20 and then incubated with detector antibodies (biotinylated anti-mouse IFN-γ). The plates were washed 10 more times before the addition of GABA (a-labeled antiboantit antibodies). After three washes with PBS-Tween and three washes with PBS, spots were developed with Activator I mixed with Activator II. Spots were counted using a stereomicroscope.

Cytotoxic T lymphocyte assay. BALB/c mice were immunized i.m. with 100 μg of PreS2S mixed with 100 μg of pcDNA3 or 100 μg of prothymosin α and boosted at the same dosage by the same route 21 days after the first inoculation. Mice immunized with 100 μg of pcDNA3 vector were employed as negative control. Spleens were harvested from immunized mice 3 weeks after the final immunization. Single-splenocyte suspensions from each mouse were prepared separately in RPMI 1640 (GibcoBRL) supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine (GibcoBRL), antibiotics, and 5 × 10^-4 M 2-mercaptoethanol (Invitrogen, CA) and restimulated in vitro with specific peptides (HBsAg26-39, 10 μg/ml). Effector cells were pooled 6 days after restimulation and then used in a standard 51Cr release assay. Peptide-pulsed P815 cells (H-2d, mastocytoma cell line) were used as target cells against hepatitis B surface antigen-specific effector cells. The P815 cells were preincubated with 50 μg of specific peptides for 60 min at room temperature and then labeled with 51Cr for another 60 min at 37°C and 5% CO₂. These cells were three washed three times with RPMI 1640 medium containing 2% (vol/vol) FBS and resuspended at a concentration of 5 × 10^5/ml in RPMI 1640 medium containing 10% (vol/vol) FBS. Serial dilutions of effector cells were incubated with 51Cr-labeled target cells (5 × 10^4) for 4 h in a 200-ml volume in round-bottom 96-well plates at the indicated effector/target cell ratio. After 4 h of incubation, 100 μl of supernatant was removed and examined for 51Cr release in a gamma counter (Perkin-Elmer, MA). The percent specific 51Cr release was calculated with the following equation: (experimental counts – spontaneous counts)/(total counts – spontaneous counts) × 100. Net percent specific lysis was defined as the difference between the percent specific chromium release of the peptide-unpulsed target and that of the target cell pulsed with specific CTL peptide. Spontaneous release of 51Cr from labeled cells was determined by incubating target cells with medium alone and was less than 15% of the total release in all experiments. The total release of radioactivity was obtained by lysing the target cells with 5% Triton X-100. Lysis at each effector/target cell ratio was determined in triplicate and repeated at least twice.

RESULTS

Transient expression of the antigen and prothymosin α. After P815 cells were transfected with recombinant plasmids (PreS2S and PreS2S-prothymosin α), the supernatants of lysed cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The protein expressed was analyzed by immunoblotting with anti-Flag. As shown in Fig. 1, PreS2S, PROTHYMOSIN α BOOSTS RESPONSE TO HBV DNA VACCINE 1365

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prothymosin α, and the fusion protein of PreS2S-prothymosin α were all expressed in P815 cells. As control, the P815 cells not transfected with recombinant plasmids were negative for any HBsAg antigen and prothymosin α.

**Humoral response to HBsAg in mice injected with DNA vaccines.** Anti-HBs antibody response was monitored in the sera of mice injected with a mixture of 100 μg of pcDNA3 and 100 μg of PreS2S, a mixture of 100 μg of prothymosin α and 100 μg of PreS2S, or 100 μg of PreS2S-prothymosin α in the left-thigh quadriceps muscle (i.e., under conditions inducing the strongest CTL response) (see Fig. 6). As shown in Fig. 2, after booster injection, inoculation with PreS2S-prothymosin α generated the highest responses in both BALB/c and C57BL/6 mice strains; a four- to fivefold increase in antibody titers was observed when PreS2S was coadministered with prothymosin α compared to PreS2S alone (P < 0.01). Similarly, mice inoculated with PreS2S-prothymosin α generated greater responses than those inoculated with PreS2S alone (Fig. 2A and B). To investigate whether the adjuvant effect of prothymosin is anti-HBs specific or causes a polyclonal B-cell activation, serum total immunoglobulin G (IgG) was assessed, and an increase (less than onefold) was observed. It indicates that the adjuvant effect of prothymosin α is partially anti-HBs specific, and a polyclonal B-cell activation also contributes to the effect (Fig. 2C). CD4 and CD8 T cells were also analyzed by flow cytometry; a slight increase of CD4 T cells and a decrease of CD8 T cells were observed, but the difference is not statistically significant.

**Predominance of anti-HBs antibodies of the IgG2a isotype in response to the adjuvanted DNA vaccine.** To determine whether the prothymosin α adjuvant changes the isotype profile of the response to DNA immunization, responses to the HBs DNA vaccine PreS2S formulated with or without prothymosin α were quantified. The result (Fig. 3) shows that the response to the PreS2S vaccine formulated with prothymosin α was three- to fivefold higher in the anti-HBs titers of both IgG1 (P < 0.05) and IgG2a isotypes; furthermore, the ratio of IgG1 to IgG2a showed that IgG2a antibodies predominated. Thus, prothymosin α can increase the overall anti-HBs response to the PreS2S DNA vaccine while maintaining a TH1 isotype profile.

**Prothymosin α increases the anti-HBs seroconversion rate.** As shown in Fig. 4, all BALB/c and C57BL/6 mice immunized with PreS2S-prothymosin α generated anti-HBs antibodies 4 weeks after booster immunization; the anti-HBs seroconversion rate was 100%. Similarly, seven of eight mice BALB/c mice or C57BL/6 mice immunized with PreS2S-prothymosin α generated anti-HBs antibodies at the same time. In contrast, only three of the eight BALB/c mice (37.5%) and two of eight
C57BL/6 mice immunized with PreS2S alone generated anti-HBs antibodies. These results indicate that prothymosin α significantly increases the anti-HBs seroconversion rate when coadministered with PreS2S DNA vaccine.

Prothymosin α-expressing plasmid boosts cytokine-secreting T-cell responses to the HBs DNA vaccine. An ELISPOT assay was employed to determine the number of cells secreting IFN-γ upon in vitro restimulation with antigenic peptides. As shown in Fig. 5, both BALB/c and C57BL/6 mice immunized with DNA encoding PreS2S and prothymosin α generated ~3-fold more IFN-γ ELISPOTs than did mice immunized with PreS2S alone (P < 0.01). Furthermore, the affinity of T cells appeared to be increased, because detectable responses could be elicited with 5- to 10-fold-lower concentrations of peptide in mice immunized with PreS2S-prothymosin α than in mice immunized with PreS2S alone. Interestingly, the adjuvant also improved the readability of the ELISPOTo: both the size and intensity of spots were increased for IFN-γ T-cell responses to the HBV DNA vaccine. Interestingly, the level of ELISPOT background in mice vaccinated with coadministration of prothymosin was higher (mean ± SE, 10.45 spot-forming cells [SPF] ± 3.17 SPF) than that without coadministration of prothymosin (mean ± SE, 7.65 SPF ± 4.39 SPF), although it was not statistically significant (P > 0.05).

Prothymosin α boosts the CTL activity induced by PreS2S. To assess whether the CTL-stimulating activity of a DNA vaccine can be modulated by the simultaneous expression of prothymosin α, BALB/c mice (six per group) were inoculated i.m. with PreS2S-prothymosin α plasmid or PreS2S plasmid alone. The splenocytes from each group of mice were analyzed for CTL activity 21 days after the booster inoculation. As shown in Fig. 6, mice coimmunized with PreS2S and prothymosin α

FIG. 4. Anti-HBs seroconversion rates were increased by coadministration of prothymosin α. Eight BALB/c mice (A) or eight C57BL/6 mice (B) in each group were immunized with 100 μg of Flag-PreS2S formulated with 100 μg Flag-prothymosin α (▲) or pcDNA3 vector (□), 100 μg Flag-PreS2S-prothymosin α (×), or 200 μg pcDNA3 vector as control (●). The mice were boosted once on day 14. Anti-HBs antibodies were determined 4 weeks after the first immunization.

FIG. 5. Prothymosin α boosts HBs peptide-specific cytokine responses to Flag-PreS2S. Six BALB/c mice (top) or C57BL/6 mice (bottom) in each group were immunized with 100 μg PreS2S with 100 μg Flag-prothymosin α (▲) or 100 μg pcDNA3 vector (□) on days 0 and 21 or 200 μg pcDNA3 vector as a control (●). Spleen cells were harvested 8 days after boost immunization, and IFN-γ-producing cells were assayed by ELISPOT assay. The results are demonstrated as the mean ± SE of SPF per 10^5 spleen cells in each mouse.
total prothymosin has been detected in human serum, representing 10% of the
no secretory pathway for prothymosin
111 amino acids with an unusual primary structure. Although
In this study, we found that prothymosin
boost injection and assayed for specific cytolytic activity against P815
cells pulsed with the peptide S28-39. The results represent mean spe-
pression of prothymosin
exhibited greater cellular immune responses to HBsAg than those immunized with PreS2S alone; as determined by CTL
activity of the splenocytes. Therefore, coexpression of prothymosin increased the cellular immune responses induced by the PreS2S DNA vaccine, which may result in a higher protec-
tive efficacy.

**DISCUSSION**

Despite recombinant HBV protein being used successfully as a vaccine for a long time, chronic viral hepatitis is still a significant health problem in China and other developing countries (8, 12, 49). People in these countries are at risk of developing chronic hepatitis leading to liver cirrhosis and hepatocellular carcinoma. Up to now, vaccination is the main approach in the prevention of HBV infection (11, 13, 23, 36).

DNA immunization represents a novel means of expressing antigens in vivo for the generation of both humoral and cellular immune responses. Direct injection in either skeletal muscles or skin has demonstrated the ability of either to take up and express DNA-encoded sequences without any specific deliv-
ery system (39, 46). Thus, i.m. injection of plasmid DNA has been widely used for DNA vaccination. However, the apparent potency of a DNA vaccine, as measured by antibody response, is often poorer than that of a corresponding protein-based vaccine.

To develop vaccines with clinical relevance, numerous ways have been evaluated to increase the potency of DNA vaccines, including coadministration with experimental adjuvants such as saponins, cytokine genes, CpG motifs, and prothymosin α. In this study, we found that prothymosin α significantly increases the potency of the HBV DNA vaccine.

Prothymosin α is a highly acidic and small protein of only 111 amino acids with an unusual primary structure. Although no secretory pathway for prothymosin α has been described, it has been detected in human serum, representing 10% of the total prothymosin α content in blood (34). Rat prothymosin α was demonstrated early on to be protective against *Candida albicans* infection in mice (17) and to stimulate in vivo the release of migration inhibitory factor (MIF) (33) to a degree 10 to 20 times higher than its derivative thymosin α1. In an experimental tumor model, animals pretreated with prothymosin α prolonged their survival in 40 to 60% of cases. Prothymosin α induces tumoricidal peritoneal macrophages, natural killer (NK) cells, lymphokine-activated killer activity in splenocytes, production of interleukin-2 and tumor necrosis factor alpha, and tumor-specific cytotoxic (CD8+) and helper (CD4+) T-cell activation when administered simultaneously with tumoral cells (2, 3, 35).

In this report, we show that mice vaccinated with the plasmid encoding the middle protein of HBsAg (PreS2S) elicited hu-
moral and cellular immune responses. Coadministration of the plasmid encoding prothymosin α boosts specific humoral and cellular immune responses elicited by PreS2S in mice. Coexpression of prothymosin α and PreS2S as a fusion protein can also boost the specific humoral and cellular immune responses elicited by PreS2S in mice. Specifically, prothymosin α increases the seroconversion rate of anti-HBs antibodies in mice immunized with PreS2S-prothymosin α or PreS2S-prothymo-
sin α gene fusions compared to the rate that results from PreS2S alone. Therefore, prothymosin α may be a promising adjuvant for DNA vaccines.

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