Targeting Dendritic Cells to Enhance DNA Vaccine Potency

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ABSTRACT

DNA vaccination that can induce both cellular and humoral immune responses has become an attractive immunization strategy against cancer and infection. Dendritic cells (DCs) play a critical role in the induction of immune responses by DNA vaccination. However, a major problem of DNA vaccination is its limited potency, because only a very limited fraction of injected DNA molecules are taken up by DCs. In this study, we describe a novel DNA vaccination strategy to enhance uptake and presentation of antigens by DCs. Specifically, we developed a DNA vaccine based upon expression of a model hepatitis B virus (HBV) $e$ antigen fused to an IgG Fc fragment. After vaccination, the DNA are taken up by cells that produce and secrete the antigen-Fc fusion proteins. The secreted fusion proteins, in addition to inducing B cells, are efficiently captured and processed by DCs via receptor-mediated endocytosis and then presented to the MHC class II and as -I (cross-priming). The results of this study demonstrate that broad enhancement of antigen-specific CD4+ helper, CD8+ cytotoxic T-cell, and B-cell responses can be achieved by this DNA vaccination strategy. Thus, the strategy capable of inducing all arms of the adaptive immunity may provide a novel, generic design for the development of therapeutic and preventive DNA vaccines.

INTRODUCTION

DNA vaccination has become an attractive immunization strategy against tumor and infection, because it has the ability to induce both cellular and humoral immune responses. However, a major problem of DNA vaccination is its limited potency to induce immune responses. It is known that DNA applied either i.m. or intradermally is primarily taken up by muscle cells or keratinocytes, respectively (1–8). However, these transfected cells expressing the encoded proteins are unable to initiate primary immune responses. Although the mechanism by which DNA vaccination induces immune responses is still poorly understood, accumulating evidence indicates the critical role of DCs, the most potent APCs, in inducing immune responses of DNA vaccines (2–8). Thus, enhancement of antigen presentation by DCs offers an attractive strategy to increase the potency of DNA vaccines. DCs express receptors for the Fc portion of IgG (FcγRs), which mediate internalization of antigen-IgG complexes and promote efficient MHC class II-restricted antigen presentation, 1,000–10,000-fold more efficiently than fluid phase pinocytosis (9–11). FcγRs, which are expressed on DCs, are activated by the Fcγ receptor, and the secreted FcγRs activate DCs by up-regulating surface molecules and cytokines involved in antigen presentation (11). Thus, FcγRs represent a privileged antigen internalization route for efficient MHC class I- and II-restricted antigen presentation by DCs (9–11).

In this study, we develop a novel DNA vaccination strategy that relies on the enhancement of DC antigen presentation. Specifically, a DNA vaccine was constructed to express a model HBsAg fused to an IgG Fc fragment. After vaccination, DNA molecules are taken up by cells, which produce and secrete antigen-Fc fusion proteins. The secreted fusion proteins, in addition to inducing B cells, can be efficiently captured and processed by DCs via receptor-mediated endocytosis and presented to MHC class II and I (cross-priming). The results of this study demonstrate the broad enhancement of antigen-specific CD4+ helper, CD8+ cytotoxic T-cell, and B-cell responses by this DNA vaccination strategy.

MATERIALS AND METHODS

Mice and Cell Lines. Female C57BL/6 and BALB/c mice were purchased from Harlan. All mice were maintained in the animal facility at Baylor College of Medicine with approval of the Institutional Animal Care and Use Committee. The tumor cell lines EL4 (C57BL/6, H-2$^b$) and p815 (DBA/2, H-2$^d$) were purchased from the ATCC. The EL-4.HBcAg cell line was generated by transfection of the plasmid pRe/CMV-HBcAg using Lipofectin (Life Technologies, Inc.) to EL-4 cells and then selection in the presence of 1 mg/ml G418 (Life Technologies, Inc.). G418-resistant clones were subcloned and then screened for HBcAg expression by immunoprecipitation and PCR. The resultant EL4-HBcAg cells expressing HBcAg were maintained at 37°C in 5% CO2 in DMEM containing 10% heat-inactivated horse serum and 1 mg/ml G418. In addition, the EL4-MAGE-3 cell line expressing a tumor-associated antigen MAGE-3 (17) was also generated.

DNA Constructs. A plasmid encoding the full-length HBV (adw subtype) genome was obtained from ATCC. The HBV precore/core gene was found to contain a single bp deletion, which causes a frameshift at codon 79, resulting in two consecutive stop codons at 84 and 85. This gene was repaired by inserting the deleted base by PCR mutagenesis as described previously (18) and confirmed by DNA sequencing. The full-length HBsAg gene was generated by PCR amplification of the repaired HBV genome with a pair of primers [5-primer (P-A): 5'$\text{TTTCTAGAATCGATTACATGATCGATCAGA}3'$, corresponding to the nucleotide sequence 1904–2020 of the HBV genome, and 3-primer (P-B): 5'$\text{TTTCTAGAATCGATTACATGATCGATCAGA}3'$, corresponding to the nucleotide sequence 2437–2457 of the HBV genome with additional Xhol and Clal sites]. The truncated HBsAg gene with deletion of the arginine-rich, C-terminal sequence of HBeAg (amino acids 150–185), which is cleaved during viral infection, was generated by PCR amplification with a pair of primers [5'-primer (P-A): 5'$\text{GTGCCGCGCCGTCTACGAACACACAGTAGTTTCCGGAAGTGT}3'$, corresponding to the nucleotide sequence 2324–2350 of the HBV genome with an additional NotI restriction site]. The full-length HBsAg gene was generated by PCR amplification with a pair of primers [5'-primer (P-A): 5'$\text{TAAAGCCATATGCAACATCTCTGCTGCTC}3'$, corresponding to the nucleotide sequence 1904–2020 of the HBV genome with an additional HindIII restriction site, and 3'-primer (P-B): 5'$\text{TATTCTAGAATCGATTACATGATCGATCAGA}3'$, corresponding to the nucleotide sequence 2437–2457 of the HBV genome with an additional Xhol and Clal site]. The truncated HBsAg gene with deletion of the arginine-rich, C-terminal sequence of HBeAg (amino acids 150–185), which is cleaved during viral infection, was generated by PCR amplification with a pair of primers [5'-primer (P-A): 5'$\text{TAAAGCTTATGCAACATCTCTGCTGCTC}3'$, corresponding to the nucleotide sequence 1904–2020 of the HBV genome with an additional HindIII restriction site, and 3'-primer (P-C): 5'$\text{TATTCTAGAATCGATTACATGATCGATCAGA}3'$, corresponding to the nucleotide sequence 1447–1468 of the heavy chain with a Clal site]. pRe/CMV vector (Invitrogen) was used for this study.

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The abbreviations used are: DC, dendritic cell; APC, antigen-presenting cell; FcγR, Fcγ receptor; ATCC, American Type Culture Collection; HBsAg, hepatitis B virus core antigen; HBeAg, hepatitis B virus envelope antigen; mAb, monoclonal antibody; SP, spleen; LN, lymph node; RT-PCR, reverse transcription; mGM-CSF, murine granulocyte/macrophage-colony stimulating factor; FBS, fetal bovine serum; BM, bone marrow; IL, interleukin; mIL, murine IL; CMV, cytomegalovirus.
expression vector HBe-Fc, which expresses the secretory HBeAg fusion protein consisting of the truncated HBeAg fused in-frame to the IgG Fc, was constructed by a three-piece ligation of the truncated HBe fragment, IgG Fc, and HindIII/ClaI-cut pRc/CMV vector (20). The expression vector HBeAg, which expresses a secretory HBeAg protein, was constructed by inserting the HBeAg gene into the HindIII/ClaI-cut-pRc/CMV vector. The expression vector HBeAg, which expresses a cytosolic HBeAg protein, was constructed by inserting the HBeAg gene into the HindIII/ClaI-cut-pRc/CMV vector. To construct the IgG Fc expression vector, the human IgG Fc cDNA fragment was linked with a mouse VH signal leader sequence by two PCR reactions. In the first PCR reaction, the IgG Fc cDNA was used as a template for the amplification with a pair of primers (5′ primer, 5′-GACAATTCACACATGGCACCAGTGTCGTCGGTGACACG-3′; corresponding to the nucleotides 785–815 of the human VH leader sequence, and the 3′-primer P-C). The second PCR using the product of the first PCR as a template was carried out with a pair of primers (5′ primer, 5′-TAAGCTCCATGGAATCTGTTTCTTCCTTCTGCGACGTCCCAAGATGTCGTCGTCGGTGACACG-3′, corresponding to the NH2-terminal nucleotide sequence of the VH-leader sequence with additional HindIII and NdeI sites, and the 3′-primer P-C). The Fc cDNA with a leader sequence was cloned into the HindIII/ClaI-cut-pRc/CMV vector. The resulting vectors were identified by restriction enzyme analysis and confirmed by DNA sequencing.

Immunoprecipitation and Western Blot Analysis. 293T cells (ATCC) grown in 150-mm dishes were transfected with various recombinant pRc/CMV vectors using GenePORTER (Gene Therapy Systems, San Diego, CA). Two days later, the culture medium was harvested, and cells were lysed in a lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% TX-100 (Sigma), 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets (Boehringer Mannheim)]. Cell lysates and culture media were then subjected to Western blot analysis (21). Briefly, protein samples (50 µg) were fractionated by a 10% SDS-PAGE gel and transferred to a nitrocellular membrane (Bio-Rad Laboratories), which was blocked by incubation in PBS (pH 7.5) containing 5% nonfat dried milk (Carnation) and 0.1% (v/v) Tween 20 (Fisher Scientific) at room temperature. After washing with a buffer [PBS containing 0.1% (v/v) Tween 20], the membrane was incubated with rabbit anti-HBeAg antibody (Dako) diluted in a PBS buffer containing 2.5% nonfat milk and 0.1% Tween 20 (1:500) at room temperature overnight. After washing, the membrane was then incubated in the buffer with a horseradish peroxidase-labeled antirabbit IgG (Sigma; 1:12,000) at room temperature for 1.5 h. After the final washes, the membrane was visualized with an ECL-Plus chemiluminescence detection kit (Amersham Pharmacia Biotech) and exposed on Kodak film.

DNA Preparation and Immunization. DNA was isolated with an endotoxin-free purification kit (Qiagen) according to a standard protocol. DNA was resuspended in endotoxin-free PBS (Sigma) at a final concentration of 1 mg/ml. DNA was stored at −20°C and analyzed by restriction digestion before the day of immunization. Mice were injected i.m. in the quadriceps with 100 µl of DNA containing 10 µg DNA per injection. Immunization was carried out by a single intramuscular injection at the base of the tail with a 1-ml sterilized disposable syringe equipped with a 25-ga needle. The DNA solution was injected using a 1-ml sterilized disposable syringe equipped with a 25-ga needle.

CD4+ or CD8+ T cells were isolated from SP suspensions using CD4+ or CD8+ T-cell enrichment columns (R&D Systems) and cultured in RPMI 1640 supplemented with 10% FBS for 24–48 h before further analysis. Splenocytes and draining lymph nodes from immunized mice were digested with a cocktail of 0.1% DNase I (fraction IX; Sigma) and 1 mg/ml collagenase (Roche) at 37°C for 40–60 min. SP and LN DCs were positively selected with CD11c (N418) Micro-Beads (Miltenyi Biotec, Inc.) for further study.

RT-PCR. CD11c+ DCs and CD11c− cells from SPs or draining LNs of immunized mice were lysed, and total RNA was extracted with TRizol reagent (Life Technologies, Inc.). RT-PCR products were generated from the miRNA template using a pair of HBeAg- or Fc-specific primers and Titan One Tube RT-PCR kits (Roche).

HBeAg or HBsAg Pulsing of DCs. Murine bone marrow-derived DCs were isolated and cultured as described previously (22). In brief, bone marrow stem cells were cultured in RPMI 1640 supplemented with 6% FBS, 60 ng of mGM-CSF/ml, and 100 units of mIL-4/ml for 4 days. DCs were then cultured in medium containing a mixture of the recombinant HBeAg (100 µg/ml) and HBcAg (100 µg/ml) proteins or HBsAg (200 µg/ml) for an additional 4 days. Pulsed DCs were washed twice with 1× PBS at 1000 rpm for 5 min and resuspended in RPMI 1640 for further analysis.

DC Preparation and Coculture Experiments. The HBe-Fc and HBeAg DNAs were cloned into a retroviral vector, pLNCX (20). To produce retroviral vectors, packaging cells (PA317; ATCC) were cultured in 100-mm culture dishes with DMEM containing 10% heat-inactivated FBS (Life Technologies, Inc.) and transfected with 10–15 µg of retroviral vectors expressing HBe-Fc by Lipofectin (Life Technologies, Inc.). After overnight incubation, the medium was replaced with DMEM containing 5% FBS. Forty-eight h later, the culture medium containing recombinant retroviruses was harvested and filtered (0.45 µm), as described previously (20).

To generate mouse DCs, BM cells were flushed from mouse limbs, passed through a nylon mesh, and depleted of red cells with ammonium chloride. After extensive washing with RPMI 1640, cells were cultured for 40–60 min with rabbit complements (Caltbioch) and a mixture of mAbs consisting of anti-CD4, anti-CD8, anti-CD45R/B220, and anti-MHC-II (PharMingen and BioSource International) in RPMI 1640 at 37°C. After extensive washing with RPMI 1640, cells (5 × 10^5 cells/ml) in RPMI 1640 supplemented with 6% FBS, 80 ng of murine stem cell factor/ml (R&D Systems), and 20 units of mIL-6/ml (BioSource International) were plated in 12-well culture plates (2.5 ml/well), incubated overnight at 37°C, 5% CO2, and then refed with fresh medium. After 48 h of incubation, the cells were spun down, resuspended in 1.5 ml of the retrovirus supernatants, and placed onto 24-well culture plates coated with Retinectin (PanVera) at a concentration of 10–20 ng/ml. The cells were incubated at 37°C, 5% CO2 for 3–4 h. The supernatants were then replaced with 1.5 ml of RPMI 1640 supplemented with 5% FBS, 10 ng of murine stem cell factor/ml, 60 ng of mGM-CSF/ml (BioSource International), and 100 units of mIL-4/ml (R&D Systems). After the transduction, the cells were washed and cultured in Opti-MEM (Life Technologies, Inc.) containing mGM-CSF and mIL-4 for several days to allow further differentiation of DCs.

Cytotoxicity Assays. The JAM test was used to perform cytotoxicity assays (23). Mice were sacrificed several weeks after immunization. Splenocytes from immunized mice were restimulated in vitro in RPMI 1640 containing either synthetic peptide HBeAg13–27 (Chiron; 1 µM) or irradiated EL4-HBeAg cells for 4–6 days. Target cells (EL4-HBeAg cells or peptide pulsed-EL4 cells) and control cells (parental EL4 cells, EL4-MAGE3, or peptide pulsed-p815 cells) were labeled with ¹¹¹I-iodomandelic acid to a final concentration of 2 µCi/ml in culture medium. The cells were incubated for 4 h, gently washed once with 1× PBS, and resuspended to 1 × 10^5 cells/ml. Different numbers of effector cells were incubated with a constant number of target cells (1 × 10^4/well) in 96-well round-bottomed plates (200 µl/well) at 37°C. The cells in triplicate wells were harvested onto fiberglass filters using Filter Mate Harvester (Packard), and the filters were washed extensively. After drying the filters, 25 µl of MicroScint 20 (Packard) were added into each well, and radioactivity was counted with a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard). In some experiments, the restimulated effector cells were incubated with the anti-CD4 or anti-CD8 antibody (30 µg/well; PharMingen) for 30 min to deplete CD4+ or CD8+ T cells before cytotoxicity assays. The percentage of specific killing was defined as: [retained radioactivity in the absence of killers (spontaneous) − experimentally retained DNA in the presence of killers]/spontaneous × 100.
Antibody Test. Anti-HBc/eAg antibodies in the sera of immunized mice were determined by ELISA. Briefly, microtiter plates (Dynatech) coated with a mixture of recombinant HBeAg and HBcAg proteins (50 ng/well) were incubated with serially diluted sera in a blocking buffer (KPL; Gaithersburg, MD) at room temperature for 2 h. Bound antibody was detected after incubation with peroxidase-conjugated antibodies against mouse IgG (Sigma) diluted in the blocking buffer. A polyclonal anti-HBc/eAg antibody (Dako) was used as a positive control, and nonimmunized mouse sera was used as a negative control.

Adoptive Transfer of DCs. CD11c+ DCs were isolated from the spleocytes of mice immunized with different DNA constructs as described above. Isolated DCs were injected into the lateral tail veins of syngeneic naïve recipients (2 × 10^7 per mouse; six mice/group). Two to 4 weeks after the adoptive transfer, T-cell proliferation assays were performed as described above.

Statistical Analyses. All data are presented as the mean and SE. ANOVA was used to determine the levels of differences between groups. Different groups were compared by Student-Newman-Keuls test with SigmaStat 2.03 software (SPSS, Inc.). \( P \leq 0.05 \) was considered significant.

RESULTS

Construction and Expression of DNA Constructs. The Fc fragment derived from a human IgG1 was used as a cell-binding domain to enhance internalization of the model HBV nucleocapsid protein, because the human IgG1 can efficiently bind to murine DCs (24, 25). Although both HBcAg and HBeAg are encoded by the HBV pre-C/C gene, the secretory HBeAg protein is initiated at a start codon 29 residues upstream of the start codon for HBcAg (26–28). HBeAg was fused in-frame with the human IgG1 Fc fragment cDNA gene and then cloned into the pRc/CMV vector. Control vectors containing the HBeAg gene (secretory), Fc fragment gene with a leader sequence (secretory), or HBcAg gene (cytosolic) were constructed (Fig. 1A). By radiolabeling and immunoprecipitation/SDS-PAGE analyses (20), HBeAg-Fc proteins (HBe-Fc) with an estimated molecular weight of \( M_r \ 46,000 \) and HBcAg with an estimated molecular weight of \( M_r \ 21,000 \) were found to be efficiently produced and secreted from transfected cells. Both intracellular and secreted HBe-Fc were directly precipitated with protein A beads, indicating that the fusion protein retains its binding ability to protein A (Fig. 1B). The HBeAg and HBe-Fc proteins were assembled into dimers, which were, to some extent, resistant to the reducing agent (25 mM DTT). A fraction of HBe-Fc fusion proteins was cleaved, because a lower molecular weight band was reacted with the anti-HBe/c antibody.

Enhancement of Th1, CTL, and Antibody Responses by HBe-Fc DNA Vaccine. To evaluate the capacity of this strategy to enhance immune responses in vivo, C57BL/6 mice were divided into four groups, and each mouse was immunized by i.m. injection of 100 \( \mu \)g of HBcAg, HBeAg, HBe-Fc, or Fc DNA once. After 4 weeks of immunization, the mice were sacrificed, and peripheral blood, SPs, and other tissue samples were collected. We evaluated whether T cells from immunized mice would respond to antigen stimulation. Splenocytes from the mice 4 weeks after DNA immunization were restimulated with a mixture of recombinant HBeAg and HBcAg proteins or HBsAg proteins (control) for 5 days. T cells were isolated from restimulated splenocytes and then assessed with the [3H]thymidine incorporation assay. As shown in Fig. 2A, T cells from mice immunized with HBe-Fc DNA actively proliferated in response to HBeAg stimulation but not to the irrelevant HBsAg stimulation. By contrast, T cells from the mice immunized with HBeAg, HBeAg, or Fc DNA vaccine did not actively proliferate.

To determine whether CD4+ helper T-cell responses are induced, CD4+ T cells isolated from splenocytes of the immunized mice were cocultured with DCs that were pulsed with a mixture of recombinant HBeAg and HBcAg proteins, or HBsAg proteins (control). During 6 days of coculture with different ratios of T cells and DCs, CD4+ T cells from the mice immunized with HBeAg, HBcAg, or Fc did not actively proliferate, and only low levels of IL-2 and IFN-\( \gamma \) were detected in the coculture medium (Fig. 2B). By contrast, in the cocultures with CD4+ T cells from the mice immunized with HBe-Fc DNA, CD4+ T cells actively proliferated after only 48–72 h coculture, even at a 1:400 (DC:T-cell) ratio. Levels of IL-2 and IFN-\( \gamma \) in the coculture medium were substantially higher than those in the cocultures with the CD4+ T cells from the mice administered with HBeAg or HBcAg (Fig. 2B). Anti-CD4, but not anti-CD8 antibodies, dramatically blocked the production of these cytokines by the cocultured cells (Fig. 2B). An appreciable level of IL-4 was not detected in any of the experiments (data not shown). In addition, an irrelevant antigen, the recombinant HBsAg protein, was used to pulse DCs in parallel with HBeAg. The HBsAg-pulsed DCs failed to stimulate the CD4+ T cells of HBe-Fc immunized mice (Fig. 2C), demonstrating the specificity of CD4+ T-cell responses induced by HBe-Fc immunization.

To determine whether the Th1-cell response induced by HBe-Fc is attributable to the nonspecific effect of human Fc fragments, mice were i.m. injected with HBeFc plasmid (100 \( \mu \)g), a mixture of HBcAg plasmid (100 \( \mu \)g), and Fc plasmid (100 \( \mu \)g) at the same site or coinjected with HBeAg and Fc plasmids (100 \( \mu \)g each) at different sites. As shown in Fig. 2D, immunization with the mixture of HBcAg plasmid (100 \( \mu \)g) and Fc plasmid (100 \( \mu \)g) did not enhance the T-cell responses. Repeated experiments produced similar results.
These results indicate that HBe-Fc DNA can activate Th1 cells more efficiently than either HBeAg or HBcAg DNA.

To determine whether immunization with HBe-Fc can induce CTL responses, the JAM test was performed (23). Splenocytes from different immunized mice were restimulated in vitro for 4–6 days in medium either containing synthetic peptide HBcAg13–27 or irradiated EL4-HBcAg cells. The restimulated cells were then cocultivated with [3H]thymidine (1 μCi/well) for 24 h. [3H]Thymidine incorporation rates of the T cells are presented after subtracting background of radioactivity. A, CD4+ T cells were isolated from restimulated splenocytes of six immunized mice (each group) by using a CD4+ T-cell enrichment column and then cultured with irradiated HBcAg-pulsed DCs. The CD4+ T cells from the mice immunized with HBe-Fc DNA were also cocultured with HBcAg-pulsed DCs in the presence of anti-CD4 or anti-CD8 antibodies or culture medium control. The concentrations of IFN-γ and IL-2 in the medium were determined by ELISA after 48 h of coculture. B, CD4+ T cells isolated from HBe-Fc DNA immunized mice were further cocultured with HBcAg- or HBsAg-pulsed (M) DCs. C, CD4+ T cells isolated from different immunized mice were stimulated with HBcAg-pulsed DCs at a ratio of 1:200. The concentrations of IFN-γ and IL-2 in the medium were determined by ELISA after 48 h of coculture. Data represent means of three independent assays of one representative experiment of three experiments (six mice/group); bars, SE.

Fig. 2. Induction of CD4+ T-cell responses in vivo. C57BL/6 mice (six/group) were immunized with each plasmid by i.m. injection once (100 μg; 50 μg/quadricep; A–C). Mice were i.m. injected with HBeFc plasmid (100 μg), a mixture of HBeAg plasmid (100 μg), and Fc plasmid (100 μg) at the same site or cocojected with HBcAg and Fc plasmids (100 μg each) at different sites (D). Two to 4 weeks after immunization, splenocytes from sacrificed mice were restimulated with HBcAg recombinant proteins for 5 days. A, T cells were isolated from the antigen-pulsed splenocytes by using a T-cell enrichment column and then cultured with irradiated HBcAg- or HBsAg-pulsed DCs (4000 rads; 2 × 10^7 cells/well) and [3H]thymidine (1 μCi/well) for 24 h. [3H]Thymidine incorporation rates of the T cells are presented after subtracting background of radioactivity. B, CD4+ T cells were isolated from restimulated splenocytes of six immunized mice (each group) by using a CD4+ T-cell enrichment column and then cocultured in duplicate with HBcAg-pulsed DCs. The CD4+ T cells from the mice immunized with HBe-Fc DNA were also cocultured with HBcAg-pulsed DCs in the presence of anti-CD4 or anti-CD8 antibodies or culture medium control. The concentrations of IFN-γ and IL-2 in the medium were determined by ELISA after 48 h of coculture. C, CD4+ T cells isolated from HBe-Fc DNA immunized mice were further cocultured with HBcAg- or HBsAg-pulsed DCs. D, CD4+ T cells isolated from different immunized mice were stimulated with HBcAg-pulsed DCs at a ratio of 1:200. The concentrations of IFN-γ and IL-2 in the medium were determined by ELISA after 48 h of coculture. Data represent means of three independent assays of one representative experiment of three experiments (six mice/group); bars, SE.

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To determine whether HBe-Fc immunization can also induce antibody responses, we measured anti-HBeAg antibody titers in the pooled sera of mice immunized with different DNA constructs. As shown in Fig. 4, antibody levels detected in mice immunized with HBeAg or HBcAg DNA were markedly lower than those seen with HBe-Fc DNA. The specificity of the antibody responses was demonstrated by the lack of antibody against HBsAg in the immunized mice (data not shown). Taken together, the results indicate the superiority of HBe-Fc DNA in inducing CD4+ Th1 and CD8+ cytotoxic T-cell as well as B-cell responses.
Enhancement of DC Antigen Presentation by HBe-Fc DNA Vaccination. There are two main routes by which DCs obtain antigens they present. DCs take up proteins secreted or released by other cells, which are transfected by DNA vaccination. Alternatively, DCs take up the injected DNA and express the encoded proteins themselves. To assess this possibility of DC uptake of injected DNA, we used RT-PCR to look for DNA transcripts in DCs in draining LNs and in SPs. SP-DCs and LN-DCs were positively isolated using CD11c (N418) Micro-Beads (Miltenyi Biotec, Inc.). DNA transcripts were consistently detected in the LN-DCs of all mice immunized with different DNA constructs by RT-PCR (28 cycles) but were not found in the SP-DCs (Fig. 5A). However, small amounts of HBeFc/HBe/ HBe/Fc gene products were detected from SP-DCs when the DCs were transduced with HBeAg-Fc or HBeAg constructs, and ~30% DCs were transduced, as determined by flow cytometric assay. Nontransduced BM-derived DCs were cultured in the culture medium of HBe-Fc transduced DCs (HBe-Fc-DCs) that were transfected by DNA vaccination, circulated throughout the LN-DCs containing HBe-Fc DNA is probably attributable to the secretion and subsequent receptor-mediated internalization of HBe-Fc fusion proteins by DCs for enhanced antigen presentation.

Systemic Activation of DCs by HBe-Fc DNA Vaccination. Conceivably, the HBe-Fc or HBeAg proteins can be secreted from the transfected cells after DNA vaccination, circulate throughout the body, and can be captured by nontransfected APCs as well as transfected APCs. To test whether distant DCs are conditioned, a DC adoptive transfer experiment was performed in which DCs isolated from SPs of immunized mice were transferred into naive mice to assess whether the transferred DCs can prime naive T cells. Mice were immunized with the HBeAg-Fc, HBeAg, or HBeAg DNA weekly for three times and then sacrificed 1 month after the final immunization. Mouse CD11c MicroBeads (Miltenyi Biotec) are used to isolate DCs from mouse SPs. The isolated DCs with a purity of ~92% as determined by flow cytometric assay with CD11c-PE and MHC class II-FITC double staining were then injected (i.v.) into naive mice (around 2 × 10^7/mouse). Two weeks after the DC transfer, the mice were sacrificed, and T cells from the mice were isolated for further analysis. As shown in Fig. 6, the T cells from mice administered with SP-DCs of mice immunized with HBe-Fc DNA responded vigorously to HBeFc stimulation. In contrast, the T cells from mice administered with SP-DCs of mice immunized with HBeAg or HBeAg DNA did not respond effectively to HBe/cAg stimulation. This result suggests that distant DCs in the mice immunized with HBeFc are also conditioned and can induce T-cell responses.

We further tested this possibility of enhanced antigen presentation by FcγR-mediated endocytosis. HBe-Fc and HBeAg constructs were cloned into a retroviral vector, pLNCX (20, 30). Murine BM-derived DCs were transduced with HBe-Fc or HBeAg constructs, and ~30% DCs were transduced, as determined by flow cytometric assay. Nontransduced BM-derived DCs were cultured in the culture medium of HBe-Fc transduced DCs (HBe-Fc-DCs) or HBeAg transduced DCs for 4 days. The cultured nontransduced DCs were then used to stimulate CD4+ T cells from HBe-Fc DNA immunized mice. As shown in Fig. 7, the nontransduced DCs cultured in the HBe-Fc-DCs medium efficiently stimulated the CD4+ T cells. In contrast, the nontransduced DCs cultured in the HBeAg-DCs medium only weakly stimulated the CD4+ T cells. Taken together, these results suggest that DCs are inefficient to take up soluble antigens, such as HBeAg by

![Fig. 3. Induction of CTL responses in vivo. Splenocytes taken from six mice/group 4 weeks after DNA immunization were restimulated in vitro with irradiated EL4-HBcAg cells for 5 days. The restimulated splenocytes (E) were cocultured for 4 h with the 3H-labeled target cells (T), EL4-HBcAg, EL4-MAGE3, or parental EL4 (A). The restimulated splenocytes (E) were also cocultured for 4 h with the 3H-labeled HBcAg13–27-pulsed EL4 or HBcAg13–27-pulsed p815 (different gene background control) target cells (B). Percentages of target cell killing by the splenocytes from different immunized mice are shown; P < 0.05, HBe-Fc compared with other groups. Data represent the means of triplicate samples from one representative experiment of three total (six mice/group); bars, SE.](image1)

![Fig. 4. Induction of antibody responses. Mice (six/group) were immunized with different DNA constructs once, and sera were harvested at 4 and 12 weeks after DNA immunization. The HBe/cAg-specific IgG antibodies from sera of different mouse groups were determined by ELISA. The mean A450 values of pooled sera from different mouse groups were presented; bars, SE. The background A450 of normal mouse sera was <0.04.](image2)
The CD11c-positive DCs (LNDC) and negative cells (NLNDC) from the draining lymph nodes of mice immunized with different DNA constructs once were isolated 10 days after immunization. The CD11c-positive DCs (SPDC) and negative cells (NSPDC) from splenocytes of the immunized mice were also isolated. Total RNA was extracted using TRIzol reagent. RT-PCR products (28 cycles) were generated from the mRNA template with a pair of primers specific for HBe-Fc, HBeAg, HBcAg, and Fc, respectively, and the Titan One Tube RT-PCR kit (Roche). β-actin was amplified with β-actin-specific primers (R&D Systems) as RNA quality control. B, DC activation of T cells. Mice were immunized with different plasmids once and then sacrificed 4 weeks after immunization. The CD11c+ DCs were isolated from the lymph nodes of immunized mice. CD4+ T cells were isolated from pooled splenocytes of mice immunized with HBe-Fc DNA by using a CD4+ T-cell enrichment column. CD4+ T cells were then cocultured in duplicate with isolated DCs at a ratio of 1:800 (DCs: T cells). The concentration of IFN-γ in the medium was determined by ELISA after 48–72 h of coculture. Data represent the means of three independent assays of one representative experiment of three total; bars, SE.

Fig. 5. DNA uptake and antigen presentation by DCs. A, RT-PCR. The CD11c-positive DCs (LNDC) and negative cells (NLNDC) from the draining lymph nodes of mice immunized with different DNA constructs once were isolated 10 days after immunization. The CD11c-positive DCs (SPDC) and negative cells (NSPDC) from splenocytes of the immunized mice were also isolated. Total RNA was extracted using TRIzol reagent. RT-PCR products (28 cycles) were generated from the mRNA template with a pair of primers specific for HBe-Fc, HBeAg, HBcAg, and Fc, respectively, and the Titan One Tube RT-PCR kit (Roche). β-actin was amplified with β-actin-specific primers (R&D Systems) as RNA quality control. B, DC activation of T cells. Mice were immunized with different plasmids once and then sacrificed 4 weeks after immunization. The CD11c+ DCs were isolated from the lymph nodes of immunized mice. CD4+ T cells were isolated from pooled splenocytes of mice immunized with HBe-Fc DNA by using a CD4+ T-cell enrichment column. CD4+ T cells were then cocultured in duplicate with isolated DCs at a ratio of 1:800 (DCs: T cells). The concentration of IFN-γ in the medium was determined by ELISA after 48–72 h of coculture. Data represent the means of three independent assays of one representative experiment of three total; bars, SE.

pinocytosis, and that the HBe-Fc fusion proteins enhance antigen uptake and presentation by DCs, probably via FcγR-mediated endocytosis.

Role of FcγRs in Enhancement of Immune Responses Induced by HBe-Fc DNA. We further examined the role of FcγR-mediated internalization of secreted HBe-Fc by DCs in induction of T-cell responses in FcγR-knockout mice. The FcγR-knockout C57BL/6 mice (Taconic) are defective in the Fcγ RI, RII, and RIII genes and, thus, cannot internalize antigens by FcγRs-mediated endocytosis. FcγR-knockout and wild-type C57BL/6 mice (six mice/group) were inoculated with HBe-Fc DNA weekly for three times and sacrificed 1 week after the final immunization. CD4+ T cells were isolated from splenocytes of individual mice immunized with HBe-Fc DNA by using a CD4+ T-cell enrichment column (R&D Systems). CD4+ T cells were then cocultured in triplicate with HBeAg-pulsed DCs. As shown in Fig. 8, CD4+ T cells from FcγR-knockout mice consistently responded less vigorously to the antigen stimulation than those T cells from wild-type mice, as judged by cell proliferation (data not shown) and cytokine production. The mean IFN-γ concentrations of the wild-type mouse group and FcγR-knockout mouse group were 2155 pg/ml and 303 pg/ml, respectively (P < 0.05). This result indicates that FcγR-mediated internalization of secreted HBe-Fc antigens plays an important role in the enhancement of immune responses induced by HBe-Fc DNA.

DISCUSSION

DCs are critical for initiating and modulating B- and T-cell responses elicited by DNA vaccination. However, only a very limited fraction of injected DNA molecules are taken up by DCs in draining LNs (28). Even when DCs are transfected, the intracellular antigens expressed by DCs are difficult to be processed and presented to MHC class II (31). Secretory antigens cannot be efficiently presented to MHC class I and II because of the inefficiency of internalization by endocytosis.

Role of FcγRs in Enhancement of Immune Responses Induced by HBe-Fc DNA. We further examined the role of FcγR-mediated internalization of secreted HBe-Fc by DCs in induction of T-cell responses in FcγR-knockout mice. The FcγR-knockout C57BL/6 mice (Taconic) are defective in the Fcγ RI, RII, and RIII genes and, thus, cannot internalize antigens by FcγRs-mediated endocytosis. FcγR-knockout and wild-type C57BL/6 mice (six mice/group) were inoculated with HBe-Fc DNA weekly for three times and then sacrificed 1 month after the final immunization. Isolated DCs were then injected into the lateral tail vein of syngeneic naive recipients (2 × 10⁷ DCs/mouse; six mice/group). Two weeks after the adoptive transfer, splenocytes from sacrificed mice were restimulated by HBeAg recombinant proteins for 5 days. T cells were then isolated from the antigen-pulsed splenocytes by using a T-cell enrichment column and cultured with irradiated HBeAg-pulsed DCs (4000 rads; 2 × 10⁷ cells/well) and [³H]thymidine (1 μCi/well) for 24 h. Data represent the mean [³H]thymidine incorporation of three independent assays of one representative experiment of three total (six mice/group); bars, SE.

Fig. 6. Adoptive transfer of DCs to induce T-cell responses. CD11c+ DCs were isolated from the splenocytes of mice immunized with different DNA constructs weekly for three times and then sacrificed 1 month after the final immunization. Isolated DCs were then injected into the lateral tail vein of syngeneic naive recipients (2 × 10⁷ DCs/mouse; six mice/group). Two weeks after the adoptive transfer, splenocytes from sacrificed mice were restimulated by HBeAg recombinant proteins for 5 days. T cells were then isolated from the antigen-pulsed splenocytes by using a T-cell enrichment column and cultured with irradiated HBeAg-pulsed DCs (4000 rads; 2 × 10⁷ cells/well) and [³H]thymidine (1 μCi/well) for 24 h. Data represent the mean [³H]thymidine incorporation of three independent assays of one representative experiment of three total (six mice/group); bars, SE.

Fig. 7. DCs conditioned by secreted HBe-Fc. Murine BM-derived DCs were transduced with HBe-Fc and HBeAg constructs by retroviral vectors. Nontransduced BM-derived DCs were cultured in the medium of HBe-Fc-transduced DCs or HBeAg-transduced DCs for 4 days. The nontransduced DCs cultured in the medium of HBe-Fc DCs or in the medium of HBeAg DCs, and nontransduced DCs control (5 × 10⁶), were then cocultured with CD4+ T cells (2 × 10⁴) isolated from splenocytes of mice immunized with HBe-Fc DNA in duplicate for 72 h. The concentration of IFN-γ in the medium was determined by ELISA after 48–72 h of coculture. Data represent the means of three independent assays of one representative experiment of three total; bars, SE.

Fig. 8. Adoptive transfer of DCs to induce T-cell responses. CD11c+ DCs were isolated from the splenocytes of mice immunized with different DNA constructs weekly for three times and then sacrificed 1 month after the final immunization. Isolated DCs were then injected into the lateral tail vein of syngeneic naive recipients (2 × 10⁷ DCs/mouse; six mice/group). Two weeks after the adoptive transfer, splenocytes from sacrificed mice were restimulated by HBeAg recombinant proteins for 5 days. T cells were then isolated from the antigen-pulsed splenocytes by using a T-cell enrichment column and cultured with irradiated HBeAg-pulsed DCs (4000 rads; 2 × 10⁷ cells/well) and [³H]thymidine (1 μCi/well) for 24 h. Data represent the mean [³H]thymidine incorporation of three independent assays of one representative experiment of three total (six mice/group); bars, SE.

Fig. 9. Adoptive transfer of DCs to induce T-cell responses. CD11c+ DCs were isolated from the splenocytes of mice immunized with different DNA constructs weekly for three times and then sacrificed 1 month after the final immunization. Isolated DCs were then injected into the lateral tail vein of syngeneic naive recipients (2 × 10⁷ DCs/mouse; six mice/group). Two weeks after the adoptive transfer, splenocytes from sacrificed mice were restimulated by HBeAg recombinant proteins for 5 days. T cells were then isolated from the antigen-pulsed splenocytes by using a T-cell enrichment column and cultured with irradiated HBeAg-pulsed DCs (4000 rads; 2 × 10⁷ cells/well) and [³H]thymidine (1 μCi/well) for 24 h. Data represent the mean [³H]thymidine incorporation of three independent assays of one representative experiment of three total (six mice/group); bars, SE.
Fig. 8. Role of FcγRs in the induction of immune responses. The FcγR-knockout mice deficient in all of Fcγ RI, RII, and RIII and wild-type C57BL/6 mice (Taconic) were immunized with HBe-Fc DNA weekly for three times. One week after the last DNA injection, CD4+ T cells were isolated from splenocytes of individual mice immunized with HBe-Fc DNA and then cocultured in triplicate with HBeAg-pulsed DCs at a ratio of 1:400 (DCs:T cells). The concentration of IFN-γ in the culture medium of individual mice was determined by ELISA after 72 h of coculture. Data represent the means of IFN-γ in the cell culture medium of individual wild-type (■) and FcγR-knockout (□) mice from two independent assays after subtracting the background; bars, SE.

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4 Unpublished data.

DENDRITIC CELLS ENHANCE DNA VACCINE POTENCY

little or no serological evidence of active immunity against HBV (44, 45). Thus, future therapies for clearance of chronic HBV infection and establishment of immunity must involve stimulation of a CD4+ Th response.

DNA vaccination has been exploited to induce HBV-specific immune responses to clear HBV infection in mouse models (46, 47). Although comparable CTLs responses were observed in mice immunized with DNA vaccines expressing either intracellular HBeAg or secretory HBeAg, priming of CD4+ Th cells was not demonstrated (47, 48). Also, DNA expressing HBeAg or HBeAg showed no apparent difference in CTL priming efficiency (46, 47), which may reflect the inefficient internalization of HBeAg by APCs and the coexpression of HBeAg from the HBeAg construct (27). By contrast, rigorous CD4+ Th responses and CD8+ CTL responses were induced by the HBe-Fc DNA. Potent CD4+ Th responses and CD8+ CTL responses were also induced when DCs were transduced with HBeFc (30). Thus, this HBe-Fc DNA vaccine may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

ization of soluble antigens through fluid phase pinocytosis (9, 11, 31–33). In this study, we used a receptor-mediated internalization process to enhance DC antigen presentation and increase immune responses, especially CD4+ T cells. The results of this study demonstrate more efficient induction of antigen-specific CD4+ Th1, CD8+ CTL, and B-cell responses by HBeAg-Fc DNA vaccination than by HBeAg or HBeAg DNA vaccination. Although there have been attempts to target an antigen to APCs to enhance the potency of DNA vaccines, such as using CTLA4 molecules (34, 35), the vaccination strategy we describe has many unique features. It relies on the receptor-mediated endocytosis pathway, permitting fusion antigens to be efficiently captured, processed in endosomes, and presented to MHC class II by DCs to induce CD4+ Th and to directly present internalized antigens to MHC class I (cross-presentation) and to efficiently induce CTL responses (11, 13–16, 31, 36, 37). Class I antigen presentation by transduced DCs can also occur thanks to the degradation of newly synthesized proteins. The interaction of Fc with its receptors can activate DCs (9, 11, 31). Secreted fusion proteins can be taken up by transduced DCs in an autocrine manner and by untransduced DCs in a paracrine mode to augment immune responses. This strategy can elicit strong antibody responses because of efficient protein secretion from transduced cells and enhanced T-helper response (38, 39). Finally, the strategy may be adapted to any antigen or many cell-binding domains. Indeed, the superior ability of HBe-Fc to induce immune responses was also demonstrated in HBe-Fc-transduced DCs. We have applied this strategy to other tumor antigens, such as HER-2/neu and human papillomavirus E-7 antigen, to enhance DNA vaccine potency. Thus, the strategy capable of inducing all arms of the adaptive immunity may provide a novel, generic DNA vaccine design for the development of therapeutic and preventive DNA vaccines.

Chronic HBV infection is associated with a high incidence of hepatocellular carcinoma (40, 41). The host immune response to HBeAg and HBeAg appears critical in both viral clearance and clinical resolution (26). During acute HBV infection, both CD8+ CTL and CD4+ Th cells specific for HBeAg can be detected in the circulation of the infected host (26). An increasing CD4+ Th cell response to HbeAg coincides with loss of HBeAg and HBV surface antigen. In contrast, HbeAg-specific CTL and Th cell activity is not readily detected in chronic HBV infection, except during exacerbation (42, 43). In addition, during chronic HBV infection, there is often...
DENDRITIC CELLS ENHANCE DNA VACCINE POTENCY


Targeting Dendritic Cells to Enhance DNA Vaccine Potency

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