Targeting Dendritic Cells to Enhance DNA Vaccine Potency

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ABSTRACT

DNA vaccination can induce both cellular and humoral immune responses and 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 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 HBeAg 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-2b) and p815 (DBA/2, H-2d) were purchased from the ATCC. The EL-4 HBeAg cell line was generated by transfection of the plasmid pRe/CMV-HBeAg 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 HBeAg expression by immunoprecipitation and PCR. The resultant EL4-HBeAg cells expressing HBeAg 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 HBeAg gene was generated by PCR amplification of the repaired HBV genome with a pair of primers (5’-primer (P-A): 5’-TTAAGCITATGCACTTTTTACCTCTGGCTTATCTC-3’ corresponding to the nucleotide sequence 1904–2020 of the HBV genome with an additional HindIII restriction site, and 3’-primer (P-B): 5’-TTTCTAGAATCTTACATTTGGAGACAGG-3’, corresponding to the nucleotide sequence 2437–2457 of the HBV genome with an additional XhoI and ClaI site). The truncated HBeAg gene with deletion of the arginine-rich, C’-terminal sequence of HBeAg (amino acids 150–185), which is cleaved during viral infection, was inserted by PCR amplification with a pair of primers (5’-primer: P-A and 3’-primer: 5’-GGTCGGCGGCGCTTACAAACACAGAGTAGTTTCCGGAAGTGTT-3’, corresponding to the nucleotide sequence 2324–2350 of the HBV genome with an additional NotI restriction site). The full-length HBeAg gene was generated by PCR amplification with a pair of primers (5’-primer: P-A and 3’-primer: 5’-TGTCGGCGGCGCTTACAAACACAGAGTAGTTTCCGGAAGTGTT-3’, corresponding to the nucleotide sequence 2324–2350 of the HBV genome with an additional HindIII restriction site, and the primer P-B). The human IgG Fc fragment cDNA was generated by PCR amplification with the plasmid pEE6/CLL-1 containing human IgG heavy chain cDNA (19) as a template. The pair of primers for the PCR reaction are: 5’-primer 5’-TAAAGCITATGCACTTTTTACCTCTGGCTTATCTC-3’ (P-A), corresponding to the nucleotide sequence 1901–1932 of the HBV genome with an additional HindIII restriction site, and the primer P-B).

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expression vector HBc-Fc, which expresses the secretory HBcAg fusion protein consisting of the truncated HBcAg fused in-frame to the IgGFc, was constructed by a three-piece ligation of the truncated HBc fragment, IgGFc, and HindIII/ClaI-cut pRC/CMV vector (20). The expression vector HBcAg, which expresses a secretory HBcAg protein, was constructed by inserting the HBcAg gene into the HindIII/ClaI-cut-pRC/CMV vector. The expression vector HBcAg, which expresses a cytotoxic HBeAg protein, was constructed by inserting the HBeAg gene into the HindIII/ClaI-cut-pRC/CMV vector. To construct the IgGFC expression vector, the human IgGFc cDNA fragment was linked with a mouse VH signal leader sequence by two PCR reactions. In the first PCR reaction, the IgGFc cDNA was used as a template for the amplification with a pair of primers (5′ primer, 5′-GACAGCTCCAGATGATGTCTGTCGGTGGAATCCTACGCACGTGGTGTCGTGCAACGAC-3′, corresponding to the region 1303–895 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′-TTAAGCTTATATGGAATCTGATCCCGTTTCTCCTGCTGTCACGAGCTTCCAGATGATGTCTGTCGGTGGAATCCTACGCACGTGGTGTCGTGCAACGAC-3′, corresponding to the NH2-terminal nucleotide sequence of the VH leader sequence with additional HindIII and Ndel 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 resultant 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 precipitated with a mixture of recombinant HBeAg (100 µg/ml) and HBcAg (100 µg/ml) or HBsAg (200 µg/ml) for an additional 4 days. Pulsed DCs were washed twice with 1× PBS at 100 rpm for 5 min and resuspended in RPMI 1640 for further analysis.

**DC Preparation and Cocculture 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 or HBc/Ag 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 mice limbs, passed through a nylon mesh, and depleted of red cells with ammonium chloride. After extensive washing with RPMI 1640, cells were incubated for 40–60 min with rabbit complement (CaliBiochem) 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 × 105 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 ml of the retrovirus supernatants, and placed onto 24-well culture plates coated with Retronectin (Pentra) 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 HBcAg13–27 (Chiron; 1 µM) or irradiated EL-4 HBeAg cells for 4–6 days. Target cells (EL-4-HBeAg cells or peptide pulsed-EL4 cells) and control cells (parental EL-4 cells, EL-4-MAGE3, or peptide pulsed-p815 cells) were labeled with 51Cr and used as targets in a 5-h assay at an E:T ratio of 100:1. The percentage of specific killing was determined as: [retained DNA 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 unimmunized mouse sera was used as a negative control.

Adoptive Transfer of DCs. CD11c+ DCs were isolated from the spleenocytes of mice immunized with different DNA constructs as described above. Isolated DCs were injected into the lateral tail veins of syngeneic naive recipients (2 × 10⁶ 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 ≤ 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 Mₐ 46,000 and HBeAg with an estimated molecular weight of Mₐ 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/e 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 μg 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 analyzed 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, HBeAg, or Fc did not actively proliferate, and only low levels of IL-2 and IFN-γ 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-γ in the coculture medium were substantially higher than those in the cocultures with the CD4+ T cells from the mice administered with HBeAg or HBeAg (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 exclude the possibility that the enhanced T-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 μg), a mixture of HBeAg plasmid (100 μg), and Fc plasmid (100 μg) at the same site or coinjected with HBeAg and Fc plasmids (100 μg each) at different sites. As shown in Fig. 2D, immunization with the mixture of HBeAg plasmid (100 μg) and Fc plasmid (100 μg) 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. 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-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 or/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.
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 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 (about 2 x 10^6/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 HBe/cAg stimulation. In contrast, the T cells from mice administered with SP-DCs of mice immunized with HBeAg or HBcAg DNA did not respond effectively to HBe/cAg stimulation. This result suggests that distant DCs in the mice immunized with HBeAg 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.

**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 HBc/eAg-specific IgG antibodies from sera of different mouse groups were determined by ELISA. The mean A_450 values of pooled sera from different mouse groups were presented; bars, SE. The background A_450 of normal mouse sera was <0.04.

**Fig. 5.** A: Analysis of LN-DCs isolated from the mice immunized with different DNA constructs to stimulate CD4^+ T cells. As shown in Fig. 5B, LN-DCs isolated from the mice immunized with HBe-Fc DNA more efficiently activated CD4^+ T cells than LN-DCs from the mice immunized with HBeAg or HBcAg DNA. The superior potency of 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 HBcAg 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 (about 2 x 10^6/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 HBe/cAg stimulation. In contrast, the T cells from mice administered with SP-DCs of mice immunized with HBeAg or HBcAg DNA did not respond effectively to HBe/cAg stimulation. This result suggests that distant DCs in the mice immunized with HBeAg are also conditioned and can induce T-cell responses.

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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 (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 × 10^6 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 × 10^5 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 HBcAg constructs by retroviral vectors. Nontransduced BM-derived DCs were cultured in the medium of HBe-Fc-transduced DCs or HBcAg-transduced DCs for 4 days. The nontransduced DCs cultured in the medium of HBe-Fc- or HBcAg-transduced DCs or in the medium of HBcAg-DCs, and nontransduced DCs control (5 × 10^5), were then cocultured with CD4+ T cells (2 × 10^5) 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. Data represent the means of three independent assays of one representative experiment of three total; bars, SE.

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 RIi 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 are 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 (2–8, 29). 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 and presentation by DCs, probably via FcγR-mediated endocytosis.

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 RIi 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 are 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.
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 then cotransduced with HBeAg-pulsed DCs at a ratio of 1:400 (DCs:T cells). The concentration of IFN-γ in the cell 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.

![Fig. 8. Role of FcγRs in the induction of immune responses. The FcγR-knockout mice deficient in all of Fcγ RI, RII, and III 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 cell 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.](image)

**REFERENCES**


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**Fig. 8. Role of FcγRs in the induction of immune responses. The FcγR-knockout mice deficient in all of Fcγ RI, RII, and III 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 cell 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.**

Unpublished data.

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.


Targeting Dendritic Cells to Enhance DNA Vaccine Potency

Zhaoyang You, Xue Huang, Jenny Hester, et al.

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