Enhancement of immune responses to an HBV DNA vaccine by electroporation

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Abstract

These studies document the ability of electroporation-based DNA vaccination to induce multi-specific CTL responses to hepatitis B virus (HBV) DNA vaccination in normal mice and marked immune responses to multivalent HBV DNA immunization in larger animal species. These results suggest that electroporation-mediated HBV DNA vaccination is worth pursuing as a treatment for chronic HBV infection.

1. Introduction

Chronic HBV infection exposes to a high risk of morbidity and mortality. It is generally believed that restoration of high-magnitude, vigorous, multi-specific anti-HBV CTL responses in these patients might help achieve durable remission or cure of the disease [1]. DNA vaccines, with their ability to induce T cell responses could be very effective. To this end electroporation (EP), a potent method for *in vivo* gene delivery [2], is a promising method to potentiate the delivery of DNA vaccines [3]. This study examines key feasibility aspects of EP-based DNA immunization against HBV including the ability to 1) induce multi-specific CTL responses, and 2) stimulate immune responses in multiple species.

2. Materials and methods

Immunization procedure

The pCMV-M plasmid DNA [4] containing the gene for the medium form of HBV surface antigen (HBsAg) was obtained from Aldevron (Fargo, ND). The pVax-HBVc plasmid was made at Ichor by inserting the gene coding for the HBV core antigen (HBcAg) (synthesized by Retrogen, San Diego, CA) into the pVax backbone (Invivogen, Carlsbad, CA). Plasmids were delivered i.m. using Ichor's unique TriGrid™ EP technology. The TriGrid™ electrode array for i.m. delivery is comprised of four electrodes arranged in two equilateral triangles to form a diamond shape surrounding a central injection needle (Figure 1A). Integration of the means for agent delivery and the means for electric field propagation into a single device assures that induction of the EP effect occurs at the site of agent distribution and allows complete injection of the vaccine and application of the electric pulses in a single step. In this manner, i.m. delivery of plasmid DNA is achieved in an effective and reproducible manner. Figure 1B illustrates the integrated TriGrid™ applicator used in rodents. Figure 1C depicts the Integrated TriGrid™ EP system appropriate for use in larger experimental animals, as well as human studies.

Assessment of immune responses

Antibody responses were assessed by ELISA. Briefly, serial dilutions of serum samples were added to 96 well plates coated with 150ng/well recombinant HBsAg (Aldevron) or HBcAg (Viral Therapeutics, Ithaca, NY). Biotinylated anti-rabbit IgG (KPL, Gaithersburg, MD), streptavidin-horseradish peroxidase conjugate (Zymed, South San Francisco, CA) and SureBlue TMB microwell peroxidase substrate (KPL) were used for detection. Antibody titer was calculated as the reciprocal of the sample dilution yielding an OD_{450} of 0.600.

CTL responses were assessed using an intracellular cytokine staining kit (BD Pharmingen, San Diego, CA). The HBsAg peptides L^d/S_{28-39} (IPQSLDSWWTSL) or $D^d/S_{201-209}$ (WGPSLYSIL) (New England Peptide, Inc., Fitchburg, MA) were used for short-term *in vitro* cell restimulation. Cells were stained for surface expression of CD8 and for intracellular expression of IFN-γ using antibodies from BD Pharmingen. Flow cytometry analysis was conducted on a Coulter Elite flow cytometer (Coulter Electronics, Hialeah, FL) at the Cytofluorometry Laboratory of the VA Hospital in San Diego. One-sided Student's *t* test was used for statistical analysis of the data assuming a lognormal distribution [5].

3. Results

Induction of multi-specific CTL responses

Frequencies of CTL specific for the immunodominant L^d/S_{28-39}, and the subdominant $D^d/S_{201-209}$ HBsAg epitopes [6] were assessed in splenocytes from Balb/c mice immunized with EP or by direct injection of DNA. Controls received an empty vector with EP. Expression of IFN-γ, a major effector in induction of virus clearance from infected hepatocytes [7], was used as the endpoint. Mice immunized with direct DNA injection displayed CTL responses to the immunodominant epitope (Figure 2A) but no induction of CTL responses to the subdominant epitope (Figure 2B). In mice immunized with EP, CTL responses to the immunodominant epitope were significantly enhanced ($p<0.05$) as compared to mice immunized by direct injection. Moreover, the EP-based vaccine significantly ($p<0.01$) induced CTL responses against the subdominant epitope. Assessment of surface expression of CD107a, a degranulation marker used to enumerate CD8 T cells with cytotoxic capability

[8], indicated that approximately 30-35% of the IFN-γ-producing CD8 cells also expressed CD107a (data not shown).

A dosing analysis using the pCMV-M plasmid indicated that CTL responses to both the immunodominant (Figure 3A) and the subdominant (Figure 3B) HBsAg epitopes were markedly enhanced (3-10 fold) in the EP group as compared to the no-EP group with vaccine doses ranging from 2 to 50 μg. Parallel dosing experiments using the pVax-HBVc plasmid also showed EP-mediated enhancement of CTL responses against HBcAg (data not shown).

Induction of immune responses in larger species

TriGrid™ EP-based immunization was assessed in rabbits immunized with both pCMV-M and pVax-HBVc. Anti-HBsAg and anti-HBcAg antibody responses were used as endpoints. One group of rabbits was immunized with direct injection of 2.5mg of each plasmid. Another group was immunized with EP-based delivery of 2.5 mg of each plasmid. A third group was immunized with EP-based delivery of 0.5 mg of each plasmid, a dose that nears, on a body mass basis, vaccine doses used in clinical trials. Responses to immunization in the direct injection (no EP) group differed between the two antigens: while anti-HBsAg titers were not significantly different from preimmune levels (Figure 4A), anti-HBcAg titers were significantly induced ($p<0.001$) relative to preimmune levels (Figure 4B). Delivery of the same vaccine dose by EP significantly induced ($p<0.001$) responses to HBsAg relative to preimmune levels (Figure 4A), and significantly enhanced ($p<0.01$) responses to HBcAg as compared to the no EP group (Figure 4B). All animals immunized with the lower vaccine dose with EP also responded with elevated antibody titers to both antigens. Responses to both antigens were not significantly different between the high dose and low dose EP groups, thereby suggesting that responses to an EP-based vaccine might be achieved at relatively low doses.

4. Discussion

DNA vaccination against HBsAg by direct DNA injection induced CTL responses against the L^d/S_{28-39} immunodominant HBsAg epitope but no detectable CTL responses against the $D^d/S_{201-209}$ subdominant epitope,

consistent with previous observations that this epitope is efficiently suppressed by the L^d/S_{28-39} immunodominant epitope [6]. EP significantly enhanced CTL responses to the L^d/S_{28-39} epitope, and also induced marked responses to the $D^d/S_{201-209}$ epitope. CTL responses to the $D^d/S_{201-209}$ epitope previously reported in spleens after direct DNA vaccine injection ranged from undetectable to approximately 0.2% frequency, as assessed by IFN-γ intracellular cytokine staining [6]. CTL responses to this epitope after EP-based vaccination are clearly of higher magnitude (5-20 fold higher). Overall, this suggests that EP-based vaccination has the potential to overcome immunodominance and induce multi-specific CTL responses. This is relevant to therapeutic vaccination against HBV since CTL responses are narrowly focused in chronic HBV patients, a fact which is believed to contribute to viral persistence [1]. Induction of a broader repertoire of responses is thought to be essential to induce clearance of the virus. The ability of EP-based vaccination to induce multi-specific responses with a variety of potential effector functions as shown here for HBV in mice could be advantageous for vaccination against a variety of pathogens.

Potentiation of immune responses to HBV antigens in normal mice and rabbits is encouraging. The effect of EP-based vaccination will be examined next in the context of an animal model of chronic HBV disease. The choice model for chronic HBV infection uses woodchucks chronically infected with the woodchuck hepatitis virus [9]. It has generally been more difficult to induce immune responses to DNA vaccines in larger animals than in mice [10]. Initial feasibility of TriGrid™ EP-based DNA immunization in larger animals was established in rabbits, which approximate the size of woodchucks. EP-based DNA vaccination in rabbits induced robust immune responses to HBsAg and HBcAg, two key HBV antigens likely to be included in a therapeutic HBV vaccine. The fact that most of the rabbits immunized with direct DNA injection remained at preimmune levels in response to HBsAg immunization is illustrative of the difficulties in scaling DNA vaccines in larger experimental animals. The ability of EP-based vaccination to induce responses in a dose efficient manner provides encouragement that the technology may overcome an important hurdle hindering the clinical development of DNA vaccines.

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Figure legends

Figure 1: TriGrid™ electroporation technology for the intramuscular delivery of DNA vaccines

A. Schematic representation of the TriGrid™ array for intramuscular delivery. Electrodes are arranged in a diamond shaped array. The long axis of the diamond is placed in parallel with the direction of the muscle fibers to correspond with the fluid distribution characteristic of an intramuscular injection, thereby ensuring co-localization of the electric fields and the agent delivered.

B. TriGrid™ rodent applicator. The injection syringe and electrode array are integrated (inset). Electrodes are connected to a pulse stimulator, which generates the electric signals necessary to enhance the intramuscular delivery of the vaccine

C. Integrated TriGrid™ EP system for use in large experimental animal and human studies. The system comprises a *Pulse Stimulator* (not shown), which controls the administration sequence and generates the electric signals necessary to enhance the intramuscular delivery of the vaccine; an *Integrated Applicator*, which consists of a reusable hand-held device that contains mechanisms to automatically implant the electrodes and administer the vaccine; and a single use *Application Cartridge* that encloses the electrode array and accommodates an off the shelf syringe and features automatic sharps protection.

Figure 2: Induction of multi-specific CTL responses following DNA immunization against HBsAg of normal mice

Mice were immunized with (◊) or without EP (□) using a 50µg DNA dose in a volume of 20µl in one *cranialis tibialis* (CT) muscle. Control mice (Δ) were immunized by EP-based injection of the vector backbone without HBsAg gene ("empty vector"). Vaccination was administered three times at four weeks intervals. Three weeks after the last immunization, animals were sacrificed. Following short-term (5 hours) restimulation with the immunodominant L^d/S_{28-39} (A) or subdominant $D^d/S_{201-209}$ (B) peptide (10 µg/ml), cells were stained with CyChrome-labeled anti-CD8, permeabilized with saponin, then stained with phycoerythrin-labeled anti-IFN-γ.

Figure 3: Dose response assessment of CTL responses

Mice were immunized by injection of 20 µl DNA in a CT muscle with (◊) or without EP (□) at days 0 and 28 using the indicated doses of DNA. Mice were sacrificed at day 49. Spleens were pooled by treatment groups (N=4). Following short-term restimulation with the immunodominant $L^d/S_{28\text{-}39}$ (A) or subdominant $D^d/S_{201\text{-}209}$ (B) peptide (10 µg/ml), cells were stained with FITC-labeled anti-CD8, permeabilized with saponin, then stained with phycoerythrin-labeled anti-IFN-γ. Frequencies of CD8 cells expressing IFN-γ were assessed by flow cytometry analysis.

Figure 4: Assessment of immune responses in rabbits

New Zealand White rabbits were injected with 500 µl of the pCMV-M plasmid in one quadriceps muscle and 500 µl of the pVax-HBVc plasmid in the contralateral quadriceps muscle. Rabbits were immunized with EP (filled symbols) or with direct injection of DNA (empty symbols) using DNA doses of 2.5 (◊) or 0.5 (Δ) mg of DNA. Rabbits were immunized at 0 and 4 weeks. For assessment of antibody titers, blood was harvested by central auricular artery bleed. Anti-HBsAg titers (A) were assessed at week 6. Anti-HBcAg titers (B) were assessed at week 4. Preimmune levels are indicated as (X).

Figure 1

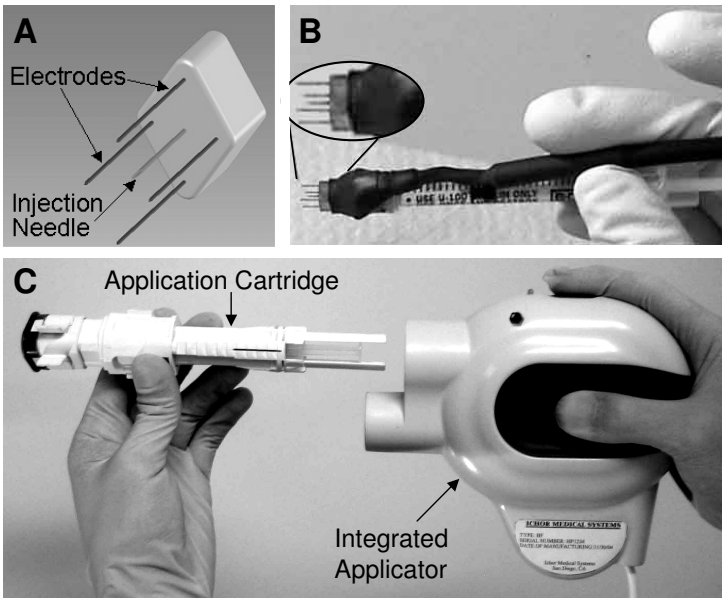

Figure 2

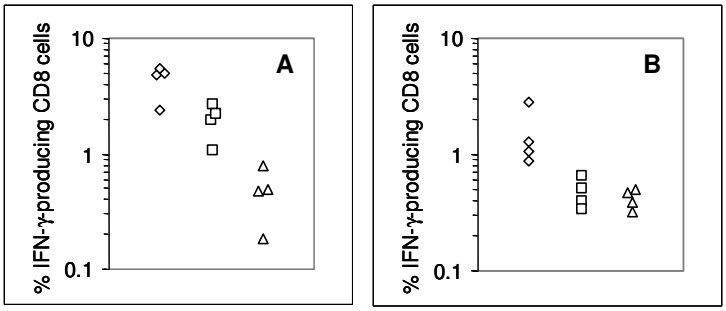

Figure 3

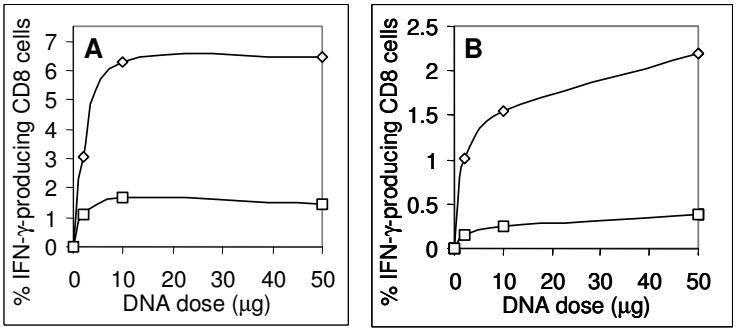

Figure 4