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A single HBsAg DNA vaccination in combination with electroporation elicits long-term antibody responses in sheep ☆

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Abstract

Vaccines continue to be the most cost effective method to reduce the burden of disease in both human and animal health. However, there is a need to improve the duration of immunity following vaccination, since maintenance of protective levels of antibody in serum or the ability to rapidly respond upon re-exposure (memory) is critical if vaccines are to provide long-term protective immunity. The purpose of this experiment was to test the duration of antibody responses and the ability to generate anamnestic responses following a single immunization with a DNA vaccine encoding hepatitis B surface antigen (HBsAg) delivered by a variety of routes. Sheep immunized with the conventional HBsAg subunit vaccine (Engerix-B) as well as sheep immunized with a HBsAg DNA vaccine, combined with electroporation, generated significant antibody responses that were sustained for 25 weeks after primary immunization. At 25 weeks, all experimental groups received a secondary immunization with the HBsAg subunit vaccine. Sheep that received a primary DNA immunization, in combination with electroporation, mounted an anamnestic response similar to the cohort immunized with the HBsAg subunit vaccine. In contrast, animals immunized with DNA vaccines administered without electroporation elicited no detectable memory response. The presence of immune memory was significantly correlated with the induction of a prolonged primary immune response. Thus, a single DNA vaccination, in combination with electroporation, approached the efficacy of the commercial subunit vaccine in the maintenance of long-term protective serum antibody titres and immune memory.

Keywords: DNA vaccine; Electroporation; HBsAg; Immune memory; Sheep

1. Introduction

Vaccines are one of the most valuable tools for reducing morbidity and mortality caused by infectious disease. There are, however, still many infectious diseases that cause tremendous suffering worldwide because no vaccine is currently available. In addition, there are effective vaccines available for some diseases, such as the recombinant hepatitis B surface antigen (HBsAg) subunit vaccine, but cost restricts vaccine usage. The next generation DNA vaccines, however, have the potential to eliminate many of the limitations of current vaccine technologies. DNA vaccine technology is a simple concept based on relatively simple design and production technologies [1]. Another advantage of DNA vaccines over conventional protein vaccines is the low cost of producing a highly purified product. Furthermore, DNA vaccine distribution is not dependent upon maintaining a cold chain so the vaccines can be easily distributed, especially in developing countries. DNA vaccination works by using host cells as protein factories to produce the plasmid encoded antigen. The translated protein is then processed and presented by the immune system in a manner similar to that which occurs following a natural infection. This elegant concept has been demonstrated successfully for a wide variety of vaccine antigens and has been effective in preventing both infectious diseases and cancer in mouse models [2].

Unfortunately, translating the success of DNA vaccines from mice to agriculturally important species [3] and humans [4] has not been very successful. This is illustrated by the fact that the

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only licensed DNA vaccine currently available is a vaccine for West Nile virus in horses [5]. One potential reason for the poor translation of DNA vaccine technology from mice to target species is the low level of gene expression following plasmid administration. A variety of approaches have been tested to improve the efficiency of plasmid delivery, including polymer and liposome formulations [6], biolistic delivery [7], and electroporation [8]. Currently electroporation is the most effective method for delivering plasmid DNA and has been reported to increase gene expression 10- to100-fold in tissues such as skin and muscle [9] and enhance immune responses in mice [11], pigs [12], and sheep [13]. Electroporation works by permeabilizing cell membranes through the creation of temporary pores and increasing plasmid distribution throughout the tissues [10]. Furthermore, electroporation induces an acute, localized infiltration of immune cells [14,15] with an up-regulation of cytokines, heat shock proteins and other co-stimulatory molecules [16]. Therefore, the acute inflammatory response induced by electroporation works in concert with increased gene expression to enhance immune responses to plasmid encoded antigens [14].

It has been hypothesized that DNA vaccines may be able to elicit sustained memory due to long-term expression of antigen produced by plasmids. In mice, DNA immunization has been able to maintain immune memory for at least 150 days [17]. There have been, however, few studies of the capacity of DNA vaccines to maintain immune memory in large animal species. In a fetal lamb model, memory was demonstrated to be present at least 3 months after birth [18]. Using a fetal pig model with an HBsAg DNA vaccine, immune memory was demonstrated for at least 4 months after birth [19]. Although a variety of methods are used to assess immune memory the best approach is to analyze immune responses following an immune boost with a protein antigen. For this study, sheep were chosen as a large animal model to determine if a DNA vaccine encoding HBsAg could elicit immune memory 6 months after primary immunization.

2. Materials and methods

2.1. Animals

Experiments were performed with 2–3 month old, female or castrated male Suffolk-cross lambs that were housed in a single outdoor pen. All experiments were conducted following the guidelines of the Canadian Council on Animal Care. Animals were randomly assigned to 6 treatment groups ($n=6$ or 7 per group) (Table 1).

2.2. Vaccines

The HBsAg encoding plasmid (pHBsAg) pMCG-16-HBsAg was a generous gift from H. L. Davis and R. Weeratna (Loeb Research Institute, Ottawa, Ontario, Canada) and contained the open reading frame of the HBV S gene flanked by the CMV promoter upstream and downstream with a bovine growth hormone poly(A) region [20]. In addition, the plasmid contained 16 copies of a murine CpG immunostimulatory motif (1826). Murine CpG motif 1826 does not have detectable immunostimulatory activity in sheep [21]. HBsAg-plasmid was grown in *E. coli* DH5α (New England Biolabs, Mississauga, ON), purified using the EndoFree™ Plasmid Giga Kit (Qiagen, Mississauga, ON), re-suspended in endotoxin-free Dulbecco's PBS (Sigma-Aldrich, St. Louis, MO), and stored at − 20 °C. Endotoxin levels, assessed using the QCL-1000 Limulus Amebocyte Lysate Kit (BioWhittaker Inc., Walkersville, MD), were <0.01 EU/ml, which is below the minimal threshold required to induce non-specific immune activity [22]. The HBsAg subunit Engerix-B vaccine (SmithKline Beecham Pharma, Oakville, ON) was used at the pediatric dose of 0.5 ml.

Table 1
Experimental groups and immunization protocol

Group/name	*n*	Immunization at day 0		Immunization (week 25)	
		Route	Vaccine	Route	Vaccine
1 Control	6	–	PBS	IM [a]	Engerix-B
2 Protein	7	IM [a]	Engerix-B	IM [a]	Engerix-B
3 ID	7	ID [b]	500 μg pHBsAg	IM [a]	Engerix-B
4 IM	7	IM [a]	500 μg pHBsAg [c]	IM [a]	Engerix-B
5 Electro+IM	7	IM [a]	500 μg pHBsAg [c], electroporation [d]	IM [a]	Engerix-B
6 Electro+IM(2X)	7	IM [a]	1 mg pHBsAg [c], electroporation [d]	IM [a]	Engerix-B

[a] All IM injections were in the right semimembranosus muscle.
[b] Each lamb received 100 μg plasmid/100 μl delivered to five sites between the central ridges on the convex site of one ear.
[c] Two adjacent sites in each semimembranosus muscle were injected with either 250 or 500 μg of plasmid in 500 μl of saline (for a total of 500 or 1000 μg in a volume of 1 ml).
[d] Electroporation parameters: 6 pulses (200 V/20 ms, 5 Hz).

2.3. Electroporation and Immunization

Animals were sedated by intravenous injection of 12.5 mg/kg Pentothal (Abbott Labs) and then injected in two adjacent sites in the right semimembranosus muscle with either 250 or 500 μg of pHBsAg in a 500 μl volume (a total of 500 or 1000 μg in 1 ml). Immediately afterward, an electrode with six 23-gauge needles arranged equidistant in a 1 cm diameter circle (Genetronics, San Diego, CA) was placed directly over each injection site and inserted to a depth of 1 cm into the muscle. Six pulses (200 V/20 ms, 5 Hz per pulse) were applied to each injection site (Babiuk et al., 2002). For the intradermal immunization, sheep were injected with 100 μg pHBsAg in 100 μl at five sites between the central ridges on the convex site of the right ear. For the Engerix-B immunization, sheep were injected in the right semimembranosus muscle with 500 μl of Engerix-B. Lambs were immunized on experimental day 0 and 25 weeks later as shown in Table 1. For the primary immunization, the Control group received phosphate buffered saline (PBS). Twenty-five weeks following the primary immunization, all animals, including the Control group, were injected IM with 500 μl of Engerix-B vaccine.

2.4. Serum HBsAg ELISA

Serum samples were collected in Vacutainer®STT™ tubes (BD Sciences, Franklin Lakes, NJ) and stored at − 20 °C until analyzed. Anti-HBsAg antibodies were measured using the AUSAB EIA Diagnostic Kit and expressed in milli-international units (mIU)/ml using the AUSAB Quantification Panel following instructions provided by the manufacturer (Abbott Laboratories, Abbott Park, IL). In humans, a protective HBsAg-specific titre is considered to be greater than 10 mIU/ml [23].

2.5. Statistics

Primary antibody responses were analyzed using the Kruskal–Wallis test for non-parametrically distributed samples followed by Dunn's comparison of (1) selected pairs to compare each treatment group against the control group, and (2) multiple groups to compare outcomes of different treatments (GraphPad Prism 4.0, GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Electroporation enhances HBsAg primary antibody responses

To determine whether different vaccine antigens and different DNA vaccine delivery modalities would influence primary immune responses and subsequent immune memory, sheep were immunized as described in Table 1. Individual animal HBsAg-specific titres were determined 25 weeks following the primary immunization (Fig. 1). None of the control animals had detectable HBsAg-specific antibody titres in their sera. The cohort immunized with Engerix-B subunit vaccine displayed significant HBsAg-specific titres with 6 of 7 animals having protective titres (>10 mIU/ml). In contrast, only 1 of 7 sheep immunized intradermally (ID) or IM with pHBsAg, without electroporation, developed a detectable and protective HBsAg-specific titre. A significant difference was observed when DNA immunization was combined with electroporation. This vaccination protocol, regardless of DNA vaccine dose, resulted in 6 of 7 sheep developing detectable HBsAg-specific titres at 25 weeks post-immunization. Doubling the dose of DNA vaccine, however, increased the number of animals with protective HBsAg-specific titres in 4 of 7 animals (Electro+IM) to 6 of 7 animals (Electro+IM[X2]).

3.2. Electroporation generates an anamnestic response to Engerix-B

Duration of immunity is a critical component of vaccine efficacy that is not analyzed in most immunization studies. A common experimental protocol is to use a short vaccination schedule with the primary immunization followed within 1 month or less by a secondary immunization and immune protection is often evaluated within 2 to 3 weeks after the secondary immunization. This approach may demonstrate the induction of immunity, but the duration of immune memory and

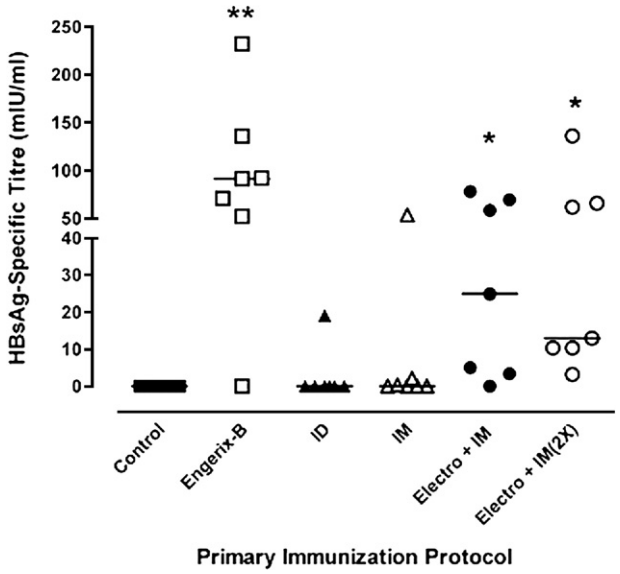

Fig. 1. HBsAg-specific antibody responses at 25 weeks after primary immunization. HBsAg-specific antibody responses were measured using the AUSAB EIA kit and quantified in mIU/ml. Individual animal antibody responses are presented for each group. The experimental groups were: no immunization (Control); IM injection of 500 μl Engerix-B (Engerix-B); 500 μg pHBsAg injected intradermally (ID); 500 μg pHBsAg injected intramuscularly (IM); 500 μg pHBsAg injected IM with electroporation (Electro+IM); and 1000 μg pHBsAg injected IM with electroporation (Electro+IM[X2]). Statistical differences among treatment group were analyzed using the Kruskal–Wallis test followed by Dunn's comparison of groups. Statistical differences relative to the control group are shown as *($p<0.05$) and **($p<0.01$). There were no significant differences between the Engerix-B group and either of the electroporation groups.

immune protection is completely ignored. The present study analyzed the duration of immune memory following primary immunization by boosting all groups with Engerix-B vaccine at 25 weeks following the primary immunization (Table 1). HBsAg-specific antibody titres were then determined in sera at 2 and 4 weeks following the secondary immunization (Fig. 2).

Immune memory was analyzed by comparing the magnitude of HBsAg-specific antibody responses following a protein boost of all groups relative to a primary immunization of the naive Control group. Based on the mean antibody titre in the Control group plus two standard deviations a cut-off level was established and any response above this level was considered to represent an anamnestic response. Control animals developed a primary immune response to HBsAg characterized by detectable antibody titres in 3 of 6 animals at 2 weeks post-immunization and low levels of HBsAg-specific antibody titres in all animals at 4 weeks post-immunization (Fig. 2B). Animals receiving DNA vaccines administered intradermally or intramuscularly without electroporation developed similar antibody responses at 2 and 4 weeks post-immunization which were suggestive of a primary immune response. In contrast, animals receiving two Engerix-B immunizations mounted an anamnestic immune response characterized by high HBsAg-specific antibody titres in 6 of 7 animals at 2 and 4 weeks (Fig. 2), following the secondary immunization. Animals receiving DNA, in conjunction with electroporation, also mounted rapid

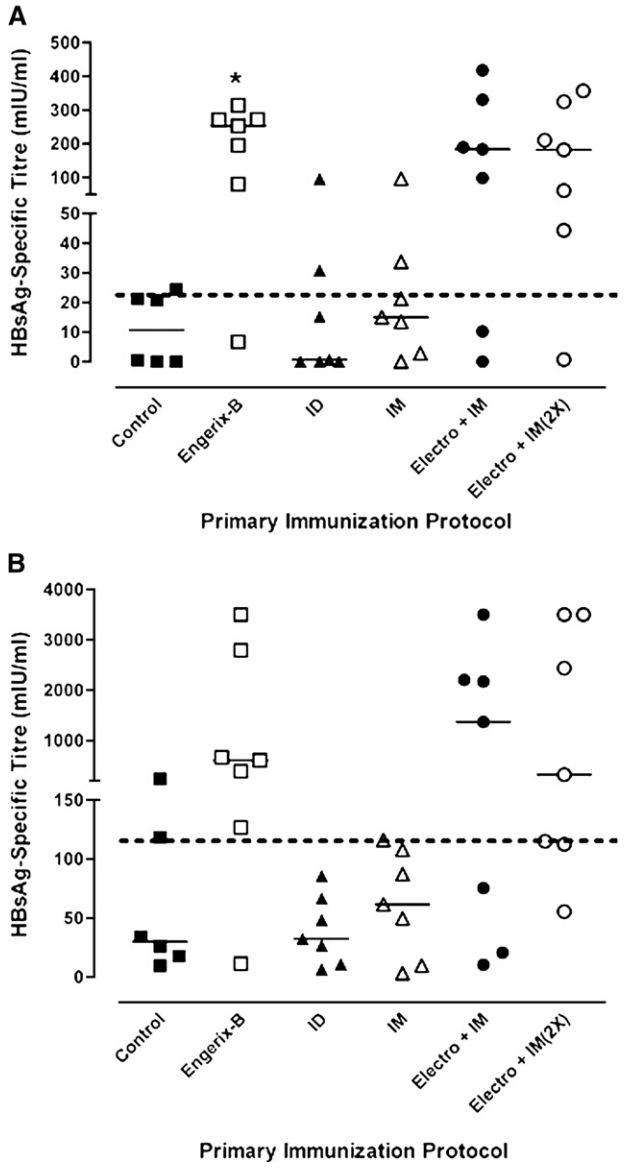

Fig. 2. HBsAg-specific antibody responses following a secondary Engerix-B immunization. Experimental groups were as defined in Fig. 1 and all groups received a 500 µl IM injection of Engerix-B vaccine on week 25. HBsAg-specific antibody responses were measured using the AUSAB EIA kit and quantified in mIU/ml. Individual animal antibody responses are presented for each group at (A) 2 weeks and (B) 4 weeks after secondary immunization. Statistical differences among groups were analyzed using the Kruskal–Wallis test followed by Dunn's comparison of groups. Statistical differences relative to the control group are shown as *($p<0.05$). The horizontal dashed line represented the mean plus two standard deviations of values for the Control group and values greater than this line represent probable anamnestic responses.

and elevated HBsAg-specific antibody responses following the secondary immunization with Engerix-B vaccine in 5 of 7 animals (Electro + IM) and 6 of 7 animals (Electro + IM[X2]).

This investigation demonstrated that a single immunization with a DNA vaccine, when combined with electroporation, elicited an immune response in a large animal model that could be considered protective for at least 25 weeks. Furthermore, immune memory was apparent following a secondary immunization with the commercial HBsAg subunit vaccine. This booster vaccination resulted in a very rapid increase in antibody titres to a level comparable to that observed following two immunizations with the commercial HBsAg subunit vaccine. Thus, the efficacy of the DNA vaccine administered with electroporation approached the efficacy of the commercial HBsAg subunit vaccine. The apparent lack of immune priming following a single DNA immunization, in the absence of electroporation, was surprising since an HBsAg DNA vaccine administered without electroporation was able to prime HBsAg antibody responses in pigs [12]. There was, however, only a two week interval between DNA priming and protein boost in this previous study [12]. A single DNA immunization, in the absence of electroporation, may induce a transient immune response with short-lived immune memory which may explain the absence of a detectable anamnestic response at 25 weeks following intradermal or intramuscular DNA immunization without electroporation. The persistence of HBsAg-specific antibody titres for 25 weeks following a single DNA immunization, in combination with electroporation, would be consistent with an increased level of DNA transfection and more prolonged period of DNA expression [14].

In this study, the amplitude of the HBsAg-specific antibody response at 25 weeks following a single immunization was significantly ($p<0.001$) correlated with the amplitude of the secondary immune response (Fig. 3). Thus, immune memory appeared to correlate with the duration of the primary immune response and there was no difference in the immune memory which persisted for 25 weeks following a single injection of a conventional protein vaccine or a DNA vaccine administered with electroporation. The prolonged immune response to a single protein or DNA immunization may be explained, in part, by the unusual nature of the HBsAg which self-assembles to form a virus-like particle (VLP). Antigen presented as a VLP may have superior immunogenicity over soluble, secreted monomeric or

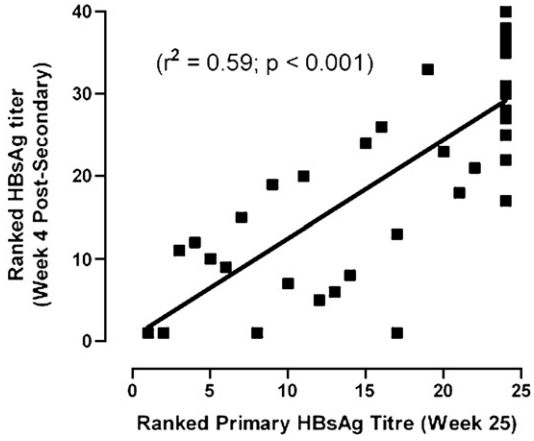

Fig. 3. Regression analysis of ranked values for HBsAg-specific antibody titres at 25 weeks post-primary immunization and 4 weeks following secondary immunization with Engerix-B. Antibody titres at each time point were numerically ranked relative to all other animals which had received a primary immunization ($n=35$). Ranking was performed from lowest (value = 1) to highest (value = 35) and animals with the same antibody titre received the same ranking. Individual points represent the paired rankings at 25 weeks post-primary immunization and 4 weeks following secondary immunization for each animal.

dimeric equivalent proteins [24,25]. The highly repetitive nature of HBsAg VLPs may facilitate T-cell independent B cell expansion as was reported for hepatitis B core antigen [26]. In addition, VLPs may facilitate attachment to antigen presenting cells and transport of endocytosed vesicles to the endosomal compartment for increased processing of antigen onto MHC-I molecules [27]. This route of antigen presentation results in the preferential induction of cytotoxic T lymphocytes (CTLs) by VLPs and may also contribute to prolonged immune memory since CTLs have been shown to persist in the absence of further antigenic stimuli for up to 2 years [28]. Thus, the present results are encouraging since they demonstrate that DNA vaccines which encode an appropriate antigen can elicit long-term immune responses of a magnitude considered to be protective, if delivered effectively by using electroporation. Further improvements in electroporation technology should lead to a more practical application of electroporation than is currently available. These improvements might include using fewer pulses, decreasing the number of needles on the electrode, using the injection needle as an electrode in the electroporation device [29], and creating a handheld battery powered electroporation device.

It is important to note that the animals in the groups immunized with the HBsAg protein (Engerix-B) or pHBsAg in combination with electroporation (Electro+IM and Electro+IM[X2]), that did not respond following primary immunization (Fig. 1) were the same three animals which failed to develop an anamnestic response following boosting with Engerix-B (Fig. 2). If these non-responder animals were removed from their respective treatment groups, then the secondary immune responses of these three groups were highly significant ($p<0.001$) when compared to the Control group. The presence of these non-responder animals indicates that the induction of an immune response to the HBsAg is a complex process. Furthermore, the low frequency of non-responders also meant that it was not possible to determine if the DNA vaccine or the HBsAg subunit vaccine was more effective for the induction of a primary immune response and immune memory. We can only conclude, based on the limited number of animals in the current study, that when delivered appropriately the pHBsAg vaccine was able to replicate the induction of protective immunity and immune memory observed with a commercial protein-based vaccine.

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