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Enhanced magnitude and breadth of neutralizing humoral response to a DNA vaccine targeting the DHBV envelope protein delivered by *in vivo* electroporation

Ghada Khawaja [a,b], Thierry Buronfosse [b,c], Catherine Jamard [b], Sylviane Guerret [d], Fabien Zoulim [a,b], Alain Luxembourg [f], Drew Hannaman [f], Claire Evans [f], Daniel Hartmann [a,e], Lucyna Cova [a,b,*]

[a] *Université Lyon 1, France*
[b] *INSERM U1052, Centre de Recherche en Cancérologie de Lyon (CRCL), France*
[c] *VetAgro-Sup, Marcy l'Etoile, Lyon, France*
[d] *Novotec, Lyon, France*
[e] *ISPB – Faculty of Pharmacy, Lyon, France*
[f] *Ichor Medical Systems, San Diego, USA*

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ABSTRACT

We explored in the duck hepatitis B virus (DHBV) model the impact of electroporation (EP)-mediated DNA vaccine delivery on the neutralizing humoral response to viral preS/S large envelope protein. EP enhanced the kinetics and magnitude of anti-preS response compared to the standard needle DNA injection (SI). Importantly, EP dramatically enhanced the neutralizing potency of the humoral response, since antibodies induced by low DNA dose (10 μg) were able to highly neutralize DHBV and to recognize ten antigenic regions, including four neutralization epitopes. Whereas, SI-induced antibodies by the same low DNA dose were not neutralizing and the epitope pattern was extremely narrow, since it was limited to only one epitope. Thus, EP-based delivery was able to improve the dose efficiency of DNA vaccine and to maintain a highly neutralizing, multi-specific B-cell response, suggesting that it may be an effective approach for chronic hepatitis B therapy at clinically feasible DNA dose.

Introduction

Viral persistence in patients chronically infected with hepatitis B virus (HBV) is typically associated with weakness or impairment of specific immune responses, whereas clearance of infection is known to be dependent of appropriate immune responses to viral antigens (Rehermann and Nascimbeni, 2005). The humoral immune response, by generating protective antibodies, contributes to the neutralization of circulating viral particles and the prevention of viral spread within the host. In addition, HBV-specific T-cell responses clear viral infection from hepatocytes, initially through type 1 inflammatory cytokine (IFN-γ, TNF-α) secretion which noncytopathically abolishes HBV gene expression and then, through the production of cytotoxic molecules leading to the apoptosis of hepatocytes (Chisari et al., 2010). In this regard, DNA vaccination able to stimulate both humoral and cellular arms of the immune response, has been proposed as a potentially promising strategy for chronic hepatitis B therapy (Michel et al., 2011). A DNA-based vaccine targeting HBV envelope proteins was shown to induce potent immune responses particularly with a Th1 cytokine profile, leading to the suppression of viral replication in transgenic mice (lineage E36) (Mancini et al., 1996). However, translating the success of DNA vaccines from mice to larger species, such as agriculturally important animals and humans, has not been very successful. It appears that DNA uptake and expression as well as recruitment of antigen-presenting cells to the injection site are less efficient in larger species compared to rodents (Luxembourg et al., 2007). Electroporation (EP)-mediated delivery of DNA vaccines has been demonstrated to clearly outperform the standard needle injection (SI) of plasmid DNA (pDNA), *via* increasing cellular permeability, which results in high level protein expression and improved immunogenicity to DNA vaccines targeting different viruses in a number of animal models including large animal species and non human primates (Ahlen et al., 2007; Capone et al., 2006; Livingston et al., 2010; Luckay et al., 2007; Otten et al., 2004; Rosati et al., 2008; van Drunen Littel-van den Hurk et al., 2008, 2010). For HBV, it has been reported that EP-based delivery dramatically enhances humoral and cellular immune responses to DNA vaccines targeting HBV envelope and/or core antigens, in several animal species such as mice (Chen et al., 2011; Kim et al., 2008; Luxembourg et al., 2006, 2008b; Peng et al., 2007; Widera et al., 2000), rabbit (Luxembourg et al., 2006, 2008b), sheep (Babiuk et al., 2007), pigs (Babiuk et al., 2002, 2004), cattle (van Drunen Littel-van den Hurk et al., 2008), rhesus-macaques (Zhao et al., 2006) and recently in the woodchuck model (Liu et al., 2011). In these studies, cellular immune responses to HBV antigens

* Corresponding author at: UMR Inserm 1052, 151 cours Albert Thomas, 69424, Lyon Cedex 03, France. Fax: +33 4 72 68 19 71.
E-mail address: lucyna.cova@inserm.fr (L. Cova).

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following DNA EP were widely investigated. EP was found to increase the antigen-specific T cell frequency by several-fold and to induce multiple T-cell effector functions, including IFN-γ production and cytotoxic T lymphocyte (CTL) activity as compared to standard needle injection (SI). EP-based DNA delivery was also shown to induce a broader repertoire of cellular responses against immunodominant and subdominant T-cell epitopes for a given vaccine (Kim et al., 2008; Luxembourg et al., 2006). Concerning the humoral response, it has been shown to be enhanced by several-fold using EP-based delivery system. However, neutralizing potency of generated antibodies to HBV DNA vaccines was not yet investigated due to the lack of robust cell system allowing *in vitro* analysis of HBV infectivity.

In this regard, the closely related duck HBV (DHBV) provides a very useful model to investigate the breadth of humoral response induced by EP-based delivery of DNA vaccine targeting hepadnavirus envelope proteins since generated antibodies can be studied *in vitro* in primary duck hepatocyte (PDH) cultures for their ability to neutralize DHBV infectivity (Cova and Zoulim, 2004; Rollier et al., 1999). Two major carboxy-terminal proteins have been identified within the DHBV envelope: a large (preS/S: L, 36 kDa) and a small (S, 18 kDa) surface protein, both required for DHBV infectivity. We and others have previously mapped the major DHBV neutralizing epitopes within the N-terminal preS domain of the DHBV L protein using monoclonal antibodies (Chassot et al., 1993; Yuasa et al., 1991). We have also characterized at the amino acid level the major B-cell epitopes on the DHBV preS protein involved in the immune response of adult ducks to DHBV (Chassot et al., 1994). Moreover, we have previously demonstrated in this model that immunization of naïve ducks with standard intramuscular (i.m.) injection (SI) of pDNA encoding the DHBV preS/S L envelope protein induced a strong, specific and long-lasting humoral response that was able to neutralize DHBV in experimentally infected hepatocytes and to protect neonatal ducklings from viral infection (Rollier et al., 1999).

In the present study, we have investigated whether EP-based delivery could further increase the magnitude and breadth of humoral response to the DNA vaccine expressing DHBV L protein. In addition to the kinetics and the dose–response efficiency, we also examined the specificity of generated antibodies, and more importantly, the neutralizing efficacy of the anti-preS antibody response. Moreover, an important aspect of this study concerns the first comparison, at the amino acid level, of B-cell epitope specificity of the humoral response induced by pDNA administrated either by EP or SI. This study in the DHBV model reveals the benefits of EP-based pDNA delivery on the biological properties of generated antibodies.

Results

Enhanced preS antigen expression and acute inflammatory cell infiltration following in vivo DNA EP

To study the effect of EP-based DNA delivery in animals on preS protein expression and the local inflammatory response at the site of injection, immunohistological examination was carried out on tissue from the injection sites sampled 6 days after administration of pCI-preS/S plasmid either by SI or EP. As expected, following EP of physiological saline solution (control group), an inflammatory response and preS expression were not detected at the injection site (Fig. 2 panels A, D, G). Similarly, the SI of 300 μg of pCI-preS/S did not cause any detectable tissue damage (Fig. 2C). A local inflammatory response was undetectable (Fig. 2F) and preS protein expression was not detected at day 6 post immunization (Fig. 2I). By contrast, the combination of pCI-preS/S injection with *in vivo* EP induced an acute local inflammation as illustrated in Fig. 2B. Importantly, the inflammatory infiltrate in the electroporated muscle contained a large proportion of CD3+ T cells (Fig. 2E). In addition, DNA EP led to more sustained preS protein expression in muscle fibers as compared with SI since at day 6 post-DNA EP, the expression of preS was still detectable at the injection site (Fig. 2H). Thus, *in vivo* EP delivery of the pCI-preS/S DNA vaccine increased and prolonged DHBV preS antigen expression, enhanced local inflammation and infiltration of CD3+ T cells, and resulted in limited local tissue damage (data not shown).

Kinetics of anti-preS antibody response in duck following SI or EP DNA delivery

To examine the potential of EP to enhance immune responses against the DHBV L envelope protein, ducks were immunized with 300 μg of pCI-preS/S plasmid using *in vivo* EP or SI, at weeks 0, 4, 8 and 30, whereas a control group received an empty pCI plasmid.

		Groups	DNA doses	Intramuscular injection
(A)	1st study	1	300 μg pCI-PreS/S	EP
		2	300 μg pCI-PreS/S	SI
		3 (control)	300 μg pCI (empty vector)	EP or SI
(B)	2nd study	DNA + EP	10 or 50 or 150 μg pCI-PreS/S	EP
		DNA	10 or 50 or 150 μg pCI-PreS/S	SI
		Control	150 μg pCI	EP or SI

Fig. 1. DNA immunization of ducks against DHBV L protein. (A) Study 1: Animals were randomly assigned into two groups, which received 300 μg of pCI-preS/S plasmid encoding the DHBV large envelope protein either by EP or by SI. The control group received the same dose of empty pCI plasmid. (B) Study 2: To evaluate the effect of DNA dose, animals were assigned randomly into six groups which received different amounts of pCI-preS/S plasmid (ranging from 10 to 150 μg) delivered either by EP or by SI. Control ducks received empty pCI plasmid. (C) Design of experimental protocol. DNA injections were performed at weeks 0, 4, and 8. Ducks from the 1st study received an additional delayed boost on week 30. The humoral response was followed during the entire immunization protocol and was continued for 4 weeks after the last immunization.

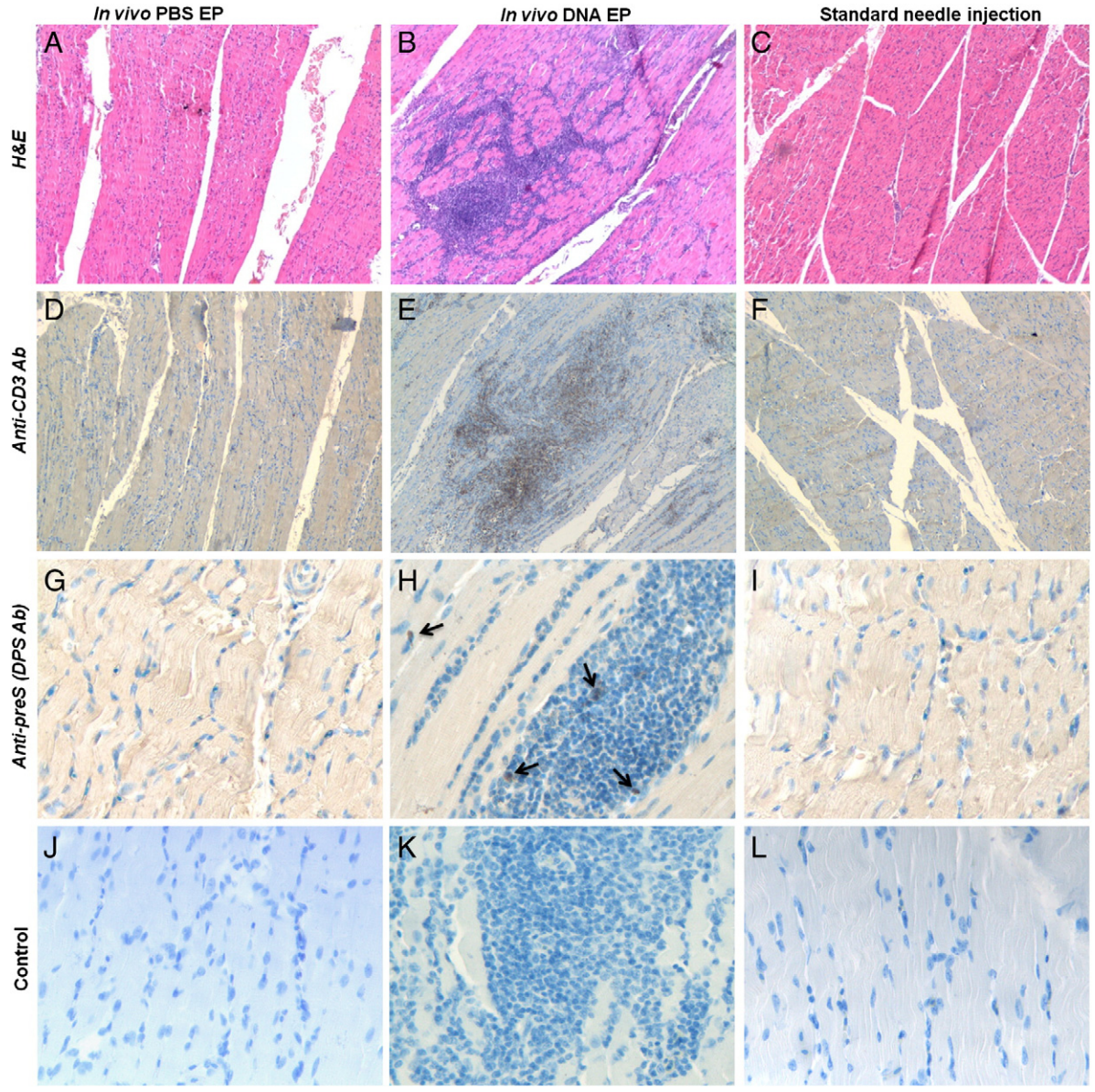

Fig. 2. Immunohistopathology of injection sites. Ducks were immunized intramuscularly with (A,D,G) phosphate buffered saline, (B,E,H) 300 μg pCI-preS/S plasmid by *in vivo* EP or (C,F,I) by SI and sacrificed at day 6 post injection for immunohistological analysis. Muscle sections (5 μm) were stained with H&E (A,B,C) or with an antibody to CD3 (D,E,F) or preS protein (G,H,I) on day 6 following immunization. The lower panel (J–L) represents control staining obtained with anti-rabbit conjugates in the absence of primary anti-preS antibody. Magnification ×10 (A, B, C, D, E, F) ×40 (G, H, I, J, K, L).

As shown in Fig. 3, the control duck group did not develop a detectable anti-preS response. In contrast, starting 4 weeks after the first DNA immunization with pCI-preS/S, a humoral response was detected with a similar pattern in duck groups immunized either by EP or SI. This response was further enhanced after the second DNA administration with the EP group showing a more rapid rise in anti-preS response, which reached significantly higher titers at week 5 that were 16-fold greater ($p<0.05$) as compared with the SI group (Fig. 3). In spite of apparently higher mean antibody titers observed for the EP group, starting after the third DNA administration, there was no significant difference in antibody titers between the EP and the SI group at weeks 16 and 24. By contrast, following the final immunization, ducks immunized by pDNA EP showed significantly higher anti-preS titers ($p<0.05$) (titers peaking to $10^{4.5}$) compared with the SI group (titers peaking to $10^{3.9}$). Taken together, these results suggest that *in vivo* EP is a potent delivery method that enhances the magnitude and maintenance of antibody responses to a DNA vaccine. We next assessed whether these features were maintained over a broad range of DNA doses. To investigate the dose–response to DNA delivered with EP, ducks were immunized with 10, 50 and 150 μg of pCI-preS/S plasmid either by EP or SI at weeks 0, 4 and 8. As shown in Fig. 4, when DNA was delivered by EP, the induced anti-preS titers were higher as compared with those induced by SI for the same DNA dose. This difference was statistically significant ($p<0.05$) after the second and the last boost (week 5 and 9 respectively). Interestingly, the delivery of only 50 μg of DNA by EP led to a higher magnitude of anti-preS response, with a significant difference ($p<0.03$) after the second boost, as compared with anti-preS response induced by a 3-fold higher (150 μg) DNA dose administrated by needle conventional injection. More importantly, even the lowest DNA dose (10 μg) delivered by EP resulted in the induction of high anti-preS titers comparable to those elicited by a 15-fold higher dose of DNA administrated by SI. Thus, EP-based delivery dramatically enhanced the dose efficiency of the DNA vaccine.

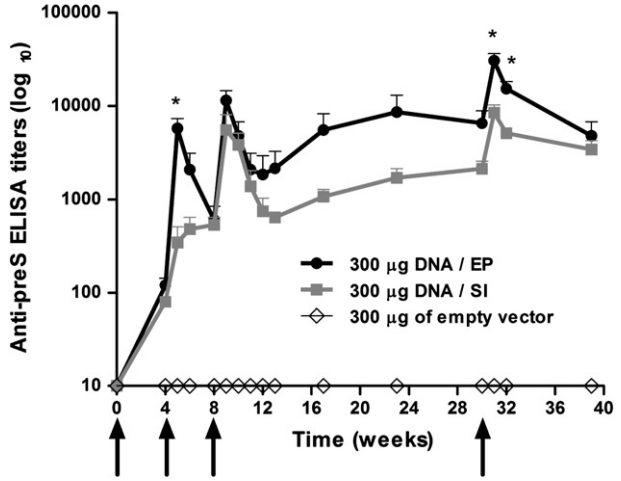

Fig. 3. Kinetics of humoral response elicited by DNA immunization against DHBV L protein. The graph shows the mean anti-preS responses for each duck group immunized with pCI-preS/S delivered by *in vivo* EP or by SI or with empty pCI vector administrated by EP (controls). Detection of anti-preS end point titers, expressed as log 10, was performed using a direct ELISA test. Black arrows indicate DNA injections. Statistically significant differences of the EP group relative to the SI group are shown * ($p<0.05$) using a two-tailed Mann–Whitney test assuming a Gaussian approximation.

PreS/S protein recognition by generated antibodies

We tested pooled sera from each group, at the same anti-preS antibody titer, for their ability to recognize the DHBV L envelope protein (preS/S). Sera from the DNA EP group and from the SI group were able to recognize in immunoblotting the p36 DHBV L protein from concentrated DHBV particles in a similar manner as the specific anti-DHBV L monoclonal antibody used as a positive control (Fig. 5). Interestingly, the antibodies induced in the group immunized by pDNA EP showed a better recognition of the major form (p36) of the DHBV L protein (Fig. 5) as compared with antibodies induced by SI. In addition, only antibodies elicited by EP were able to recognize the minor form (p28) of the DHBV L protein. These data suggest that antibodies generated by *in vivo* DNA EP could have a higher avidity for the DHBV L envelope protein form than antibodies generated following SI.

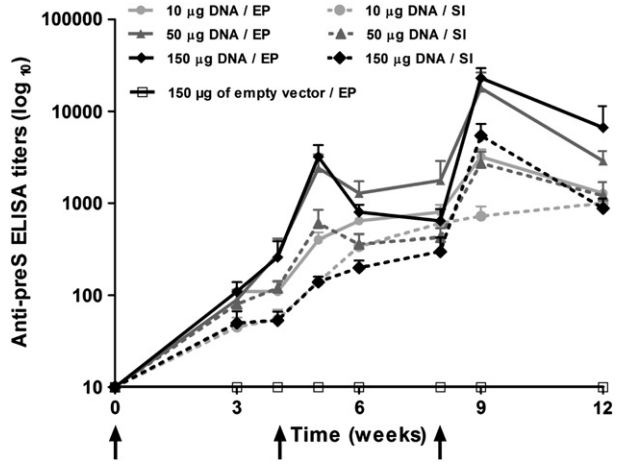

Fig. 4. DNA dose effect on kinetics of humoral response elicited either by *in vivo* EP or conventional injection. The mean anti-preS responses for each duck group immunized with pCI-preS/S at doses of 10, 50 or 150 µg either delivered by EP or SI are represented by plain (___) and dotted (— — — —) lines, respectively. Antibody levels are expressed as the $\log_{10}$ of mean end point titers obtained in ELISA test with the SD indicated for each group at each time point. Black arrows indicate DNA injections.

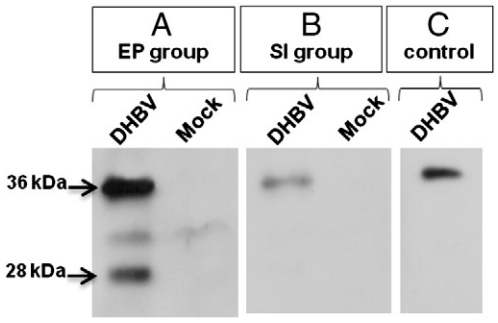

Fig. 5. Recognition of DHBV L protein by immunoblotting. Pooled sera from ducks immunized with 300 µg of pCI-preS/S by EP or SI were brought back to the same anti-preS titer for testing their specificity regarding the preS/S protein. DHBV proteins concentrated from LMHD2 supernatants were probed with pooled sera from A) DNA EP-immunized group, B) DNA SI-immunized group and with C) mouse monoclonal anti-preS antibody (Chassot et al., 1993) used as positive control.

Enhancement of neutralizing humoral response by in vivo DNA EP

Next, we asked whether the delivery of high or low pDNA doses by EP could modulate the neutralizing efficacy of anti-preS responses to DHBV in PDH cultures. Therefore, we compared the neutralizing efficacy of sera from duck groups immunized with two different doses (150 or 10 µg) of pCI-preS/S plasmid administrated either by EP or by SI.

As shown in Fig. 6, all dilutions (1/4, 1/8, 1/16, 1/32, 1/64) of sera from ducks immunized with high dose DNA (150 µg) by EP were able to completely neutralize DHBV infectivity. Based on the total area under the curve of DHBV levels in cell supernatants, a decrease of 100% of released virus was observed in PDH infected with virus preincubated with any dilution of sera (1/4 to 1/64) from the high dose EP group as compared with mock-preincubated inoculum. In contrast, in sera from ducks immunized by SI with the same DNA dose, 100% of neutralizing efficacy was obtained only when the sera were diluted to a minimal dilution of 1/4. The neutralizing capacity of these sera decreased gradually when further diluted to reach 77% of efficacy at maximal dilution (1/64) whereas sera from the EP group were significantly ($p=0.003$) more effective and remained 100% neutralizing.

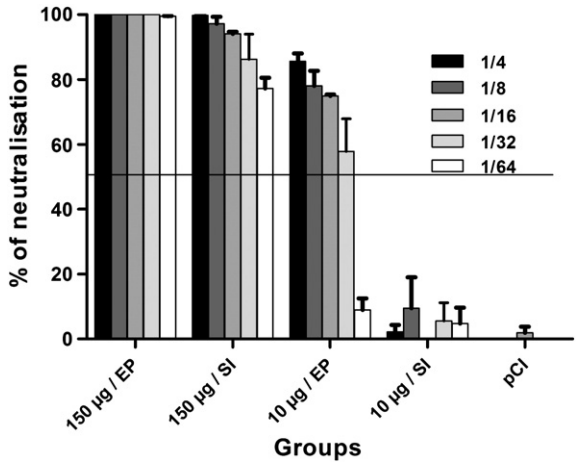

Fig. 6. *In vitro* neutralizing activity of sera from DNA-immunized ducks. Primary duck hepatocyte cultures (PDHs) were infected with DHBV-positive inoculum that was preincubated either with culture medium (mock) or different dilutions of pooled sera from ducks immunized with 150 or 10 µg of plasmid DNA by *in vivo* EP or SI, brought to anti-preS titers of 5120 and 640, respectively. Five dilutions of each pool of sera (1/4, 1/8, 1/16, 1/32, 1/64) collected at week 9 were prepared and incubated with DHBV inoculum prior to PDH inoculation. The release of viral particles was quantified in PDH supernatant by dot-blot hybridization. The area under the curve of released DHBV DNA levels in supernatants at days 3–6, which reflects total viral secretion, was used to calculate the percentage of neutralization.

For low dose DNA (10 μg), only sera from the EP group were able to neutralize the DHBV infectivity up to 88%, whereas sera from the SI group were not at all neutralizing (Fig. 6). Thus, higher anti-preS titers generated in ducks immunized with 10 μg of pDNA by EP correlated with a higher neutralizing capacity, whereas lower anti-preS titers generated in the SI group were not neutralizing (Figs. 4 and 5). These results indicate a good correlation between the two responses. Taken together, these results suggest that delivery of pDNA by EP dramatically improved the neutralizing humoral response induced with a low DNA dose.

Enhancement of the preS antigenic repertoire recognized by antibodies generated by EP-based DNA delivery

To better understand the differences in neutralizing efficacy between antibodies induced by different DNA doses (150, 10 μg) administrated either by EP or SI, we compared, by peptide scanning (pepscan) analysis, the antigenic regions (ARs) of preS protein recognized by these antibodies. Duck sera from all immunized groups, taken at week 12 (Fig. 4), were screened for their reactivity with overlapping 9-mer peptides spanning the entire DHB preS region (peptides 1 to 77) (Table 1). The scans in Fig. 7 illustrate the reactivity obtained with sera from duck groups immunized with 150 μg or 10 μg of pDNA either by EP or SI (panels A, B C and D respectively). The scans obtained with sera of ducks within each group showed some differences in the individual variation in peptide recognition and in the intensity of peptide reactivity. Despite some differences in the individual bird to bird responses, sera from ducks immunized with 150 μg of pCI-preS/S by EP recognized 10 major antigenic regions : AR1 (aa 9–31), AR2 (aa 25–43), AR3 (aa 37–51), AR4 (aa 53–65), AR5 (aa 61–79), AR6 (aa 81–95), AR7 (aa 89–107), AR8 (aa 113–129), AR9 (aa 131–149) and AR10 (aa 145–159), as shown in Fig. 7A. Moreover, antibodies induced by EP were highly reactive to AR7. The reactivity spectrum of sera from ducks immunized with the same DNA dose (150 μg) delivered by SI differed slightly (Fig. 7B). In fact, these sera recognized all the ARs except AR4 and AR10, although the recognition of some ARs was less broad and less intense as compared to sera from EP group (Fig. 7B). Overall, immunization with high (150 μg) pDNA dose, delivered either by EP or SI, elicited antibodies that recognize a broad range of B-cell epitopes on the preS protein. Interestingly, antibodies induced in the two groups immunized with 150 μg of pCI-preS/S were highly reactive to AR5, AR6, AR7 and AR8 (Figs. 7A and B). These regions correspond to neutralization epitopes on the preS protein that have been previously identified by us and others. The AR5 almost coincide with a neutralization epitope ^{58}T-W^{65} described by Yuasa et al. (Yuasa et al., 1991). Similarly, AR6, AR7 and AR8 coincide respectively with the neutralization epitopes ^{83}I-P^{90}, ^{100}F-R^{107} and ^{112}T-P^{120} previously identified by us using mouse monoclonal antibody or polyclonal rabbit antibody that induces 100% protection *in vivo* (Chassot et al., 1993).

Concerning immunization with a low DNA dose (10 μg), delivery by EP induced antibodies that recognized all ARs (AR1, AR2, AR4 to AR10) except AR3, including high reactivity to neutralization epitopes AR5, AR7 and AR8 (Fig. 7C). Sera from this group (10 μg DNA/EP) showed less reactivity for some ARs compared to sera from the 150 μg DNA/EP group, as illustrated for AR4 and AR6 (Fig. 7C). However, the reactivity spectrum of antibodies induced by the same DNA dose (10 μg), administrated this time by SI, appeared extremely narrow and was restricted to only one of these ARs (AR7). In addition, recognition of the neutralizing epitope AR7 was restricted to 2 out of 4 ducks immunized with 10 μg of DNA by SI. These results clarified why pooled sera from group 10 μg DNA/SI were completely unable to neutralize the DHBV infectivity *in vitro* as compared with sera from group 10 μg DNA/EP or other groups (Fig. 7). Overall, even a very low DNA dose delivered with EP was able to maintain biological properties of generated antibodies. These antibodies recognized a broad range of neutralization epitopes as well as those elicited with a high DNA dose. In contrast, delivery of 10 μg of low dose DNA by the SI method did not generate broadly reactive antibodies.

Table 1
Amino acid sequences of the overlapping DHB preS synthetic peptides used for Pepscan analysis.

Peptide location aa	Peptide sequence	Peptide location	Peptide sequence
1–9	MGQHPAKSM	79–87	TPQEIPQPQ
3–11	QHPAKSMDV	81–89	QEIPQPQWT
5–13	PAKSMDVRR	83–91	IPQPQWTPE
7–15	KSMDVRRIE	85–93	QPQWTPEED
9–17	MDVRRIEGG	87–95	QWTPEEDQK
11–19	VRRIEGGEI	89–97	TPEEDQKAR
13–21	RIEGGEILL	91–99	EEDQKAREA
15–23	EGGEILLNQ	93–101	DQKAREAFR
17–25	GEILLNQLA	95–103	KAREAFRRY
19–27	ILLNQLAGR	97–105	REAFRRYQE
21–29	LNQLAGRMI	99–107	AFRRYQEER
23–31	QLAGRMIPK	101–109	RRYQEERPP
25–33	AGRMIPKGT	103–111	YQEERPPET
27–35	RMIPKGTLT	105–113	EERPPETTT
29–37	IPKGTLTWS	107–115	RPPETTTIP
31–39	KGTLTWSGK	109–117	PETTTIPPS
33–41	TLTWSGKFP	111–119	TTTIPPSSP
35–43	TWSGKFPTL	113–121	TIPPSSPPQ
37–45	SGKFPTLDH	115–123	PPSSPPQWK
39–47	KFPTLDHVL	117–125	SSPPQWKLQ
41–49	PTLDHVLDH	119–127	PPQWKLQPG
43–51	LDHVLDHVQ	121–129	QWKLQPGDD
45–53	HVLDHVQTM	123–131	KLQPGDDPL
47–55	LDHVQTMEE	125–133	QPGDDPLLG
49–57	HVQTMEEIN	127–135	GDDPLLGNQ
51–59	QTMEEINTL	129–137	DPLLGNQSL
53–61	MEEINTLQN	131–139	LLGNQSLLE
55–63	EINTLQNQG	133–141	GNQSLLETH
57–65	NTLQNQGAW	135–143	QSLLETHPL
59–67	LQNQGAWPA	137–145	LLETHPLYQ
61–69	NQGAWPAGA	139–147	ETHPLYQSE
63–71	GAWPAGAGR	141–149	HPLYQSEPA
65–73	WPAGAGRRV	143–151	LYQSEPAVP
67–75	AGAGRRVGL	145–153	QSEPAVPVI
69–77	AGRRVGLSN	147–155	EPAVPVIKT
71–79	RRVGLSNPT	149–157	AVPVIKTPP
73–81	VGLSNPTPQ	151–159	PVIKTPPLK
75–83	LSNPTPQEI	152–161	IKTPPLKKK
77–85	NPTPQEIPQ		

Discussion

In this study we demonstrated, using the DHBV model, that administration of pDNA by *in vivo* electroporation dramatically enhanced the magnitude, breadth and neutralizing potency of humoral responses against hepadnaviral large envelope protein. EP-based pDNA delivery induced a more rapid onset and higher magnitude of anti-preS antibody response. In addition, pDNA delivery with EP required fewer immunizations (2 DNA administrations) to reach significantly higher anti-preS antibody titers that were 16-fold greater than SI of the same pDNA dose. A similarly high-level antibody response to HBV envelope protein (HBsAg) following delivery by EP was observed in several animal models such as mice, rabbit, sheep and rhesus-macaques (Babiuk et al., 2007; Luxembourg et al., 2006; Zhao et al., 2006). Regarding the dose effect of pDNA administrated by EP, the anti-preS responses were dose dependent. In addition, EP improved considerably the dose efficiency of the pDNA vaccine, since this delivery method was able to induce an antibody response comparable in magnitude to SI even with a 15-fold lower dose of pDNA (150 *versus* 10 μg). Moreover, in comparison with SI, a stronger anti-preS antibody response was observed with a 3-fold lower dose of pDNA (150–50 μg) when delivered with EP. These findings are consistent with those of other studies where EP was shown to induce superior and long-lasting immunogenicity compared to SI even at relatively

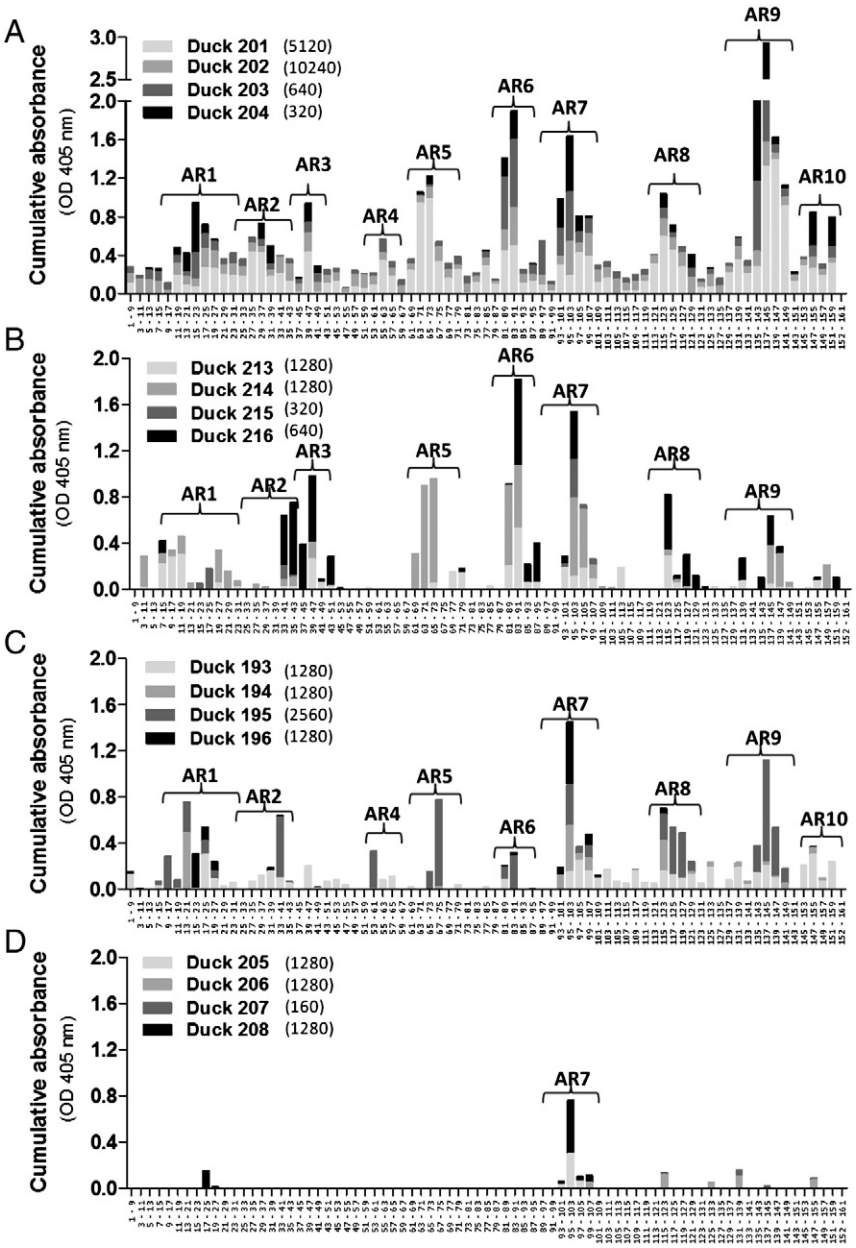

Fig. 7. Screening of duck antibodies for their reactivity to overlapping 9-mer peptides spanning the entire sequence of DHB preS envelope protein by pepscan analysis. Antisera from ducks immunized with A) 150 μg of pCI-preS/S by EP, B) 150 μg of pCI-preS/S by SI C) 10 μg of pCI-preS/S by EP or D) 10 μg of pCI-preS/S by SI, were taken at week 12 (Fig. 4) and were screened for their reactivity with overlapping 9-mer peptides covering the entire DHB preS region (1 to 161 aa). The anti-preS titer of each animal at week 12 is shown in brackets. Cumulative optical density values as determined by the pepscan analysis are shown. Background signal of control and nonreactive peptides has been subtracted before graphic representation. AR1 to AR10 indicate the positions of recognized antigenic regions (AR).

low DNA doses (Luckay et al., 2007; Luxembourg et al., 2008b; Rosati et al., 2008). We have not investigated the impact of this delivery method on duck cellular immune responses since tools for such studies are under development.

The mechanisms by which EP enhanced the breadth and magnitude of humoral response are likely multiple. We showed in this study that EP delivery of pDNA increases and prolongs antigen expression, induces local inflammation and infiltration of CD3+ T cells, and causes limited local tissue damage. Clearly, the increased and prolonged protein expression levels at the injection site are responsible for increasing the overall amount of antigen available for priming of immune responses. Given the absence of damage with EP or DNA alone, it is the combination of these two that leads to local tissue damage and the infiltration of APCs, as well as the recruitment of muscle-infiltrating $CD3^+$ cells, which play an important role in increasing the efficacy of pDNA EP. Indeed, these data suggest that the highly localized tissue damage and antigen expression results in a potent inflammatory response that most likely assists in the priming and recruitment of $CD3^+$ T cells. These observations are consistent with those of other reports where an inflammatory response at the antigen production site has been suggested to create an environment that is favorable to the establishment and/or maintenance of the immune response to the DNA vaccine (Babiuk et al., 2004; Widera et al., 2000).

A recent study in the woodchuck model has shown the ability of EP-based delivery to enhance both arms of immune responses to DNA vaccines for WHV, a hepadnavirus closely related to HBV. This study reported the induction of high antibody titers and strong T-cell responses with a shift toward a Th1 response following pDNA EP which could be very useful for the development of a therapeutic

HBV vaccine (Liu et al., 2011). However, the neutralizing efficacy of the humoral response to the WHV DNA vaccine was not investigated (Liu et al., 2011). Our study provided the opportunity to evaluate for the first time the DNA dose effect of EP-based delivery on the neutralizing antibody response against hepadnaviral proteins using PDH that are highly susceptible to DHBV infection. Importantly, EP dramatically enhanced neutralizing potency of humoral response since antibodies elicited with low pDNA dose (10 μg) were highly neutralizing, whereas SI of the identical pDNA dose was not able to generate neutralizing antibodies. Firstly, our results provide evidence that EP increases neutralizing antibody responses against hepadnaviral proteins. These findings are consistent with previous observations demonstrating that EP-based pDNA delivery facilitates the induction of neutralizing antibodies against different pathogens (Chen et al., 2008; Dupuy et al., 2011; Gardiner et al., 2009; Livingston et al., 2010; Luxembourg et al., 2008a; Mallilankaraman et al., 2011) and now extend them to hepadnavirus. Secondly, our data illustrate that more effective delivery of a low pDNA dose by EP induces a potent neutralizing response and further reinforces the finding that EP improves the dose efficiency of a DHBV DNA vaccine.

Another important aspect of this study concerned the identification, at the amino acid level, of major antigenic preS regions recognized by antibodies elicited either by pDNA EP or SI. The difference between these two immunization methods regarding the induction of neutralizing antibodies was clarified using pepscan analysis. We have previously used this approach for the determination of ARs of DHBV core protein and preS protein recognized by antibodies elicited in DHBV-carriers and during resolution of viral infection, respectively (Chassot et al., 1994; Thermet et al., 2004). In the present study, we demonstrate that antibodies generated by EP delivery of high dose pDNA were able to recognize 10 major preS ARs whereas the reactivity spectrum of antibodies induced by SI of the same dose was less broad and to a lesser extent. Interestingly, some of the common regions (AR5, AR6, AR7 and AR8) recognized by sera from groups immunized either by EP or SI coincide respectively with preS neutralizing epitopes (^{58}T-W^{66}, ^{83}I-P^{90}, ^{100}F-R^{107} and ^{112}T-P^{120}) that have been previously identified by us and others (Chassot et al., 1993; Yuasa et al., 1991). These findings are helpful to explain the ability of antibodies generated by EP or SI delivery of high dose pDNA to neutralize DHBV infectivity in PDH cultures. The higher neutralizing response induced by EP of high and low pDNA dose may be explained by the broader and better recognition of preS B-cell epitopes when compared to SI. Such antibody response induced by EP mimics that observed in ducks during the resolution of DHBV infection (Chassot et al., 1994). In addition, the better recognition of induced antibodies by EP to the minor and major forms of DHBV L preS/S envelope protein can be also explained by the recognition of a broader range of preS B-cell epitopes as compared to SI.

Interestingly, concerning the delivery of low dose (10 μg) of pDNA, EP was able to maintain the recognition of a broad range of B-cell epitopes, including neutralizing epitopes, comparable with that observed with the high DNA dose. However, immunization with such low pDNA dose by SI induced anti-preS antibodies with an epitope pattern restricted to only one AR (AR7) that was recognized by only 2 out of 4 animals of the group, which support their poor neutralizing ability *in vitro*. The difference between the reactivity spectrum of antibodies induced by EP or SI was not related to their anti-preS titers, since as shown in Fig. 7, these titers at week 12 differed only slightly between animals immunized with the same pDNA dose. Overall, these results provide a first evidence that EP is able to induce, even with low pDNA dose, an antibody response that recognize a broader repertoire of B-cell epitopes, including neutralizing epitopes, as compared to SI. Such an increase in potency of DNA vaccine, in a dose efficient manner, provides encouragement that EP may preserve the expression of plasmid-encoded antigen and immune responses to DNA vaccines across species including humans at clinically feasible DNA dose. In previous reports, induction of a broader repertoire of responses by pDNA EP has been demonstrated only for T-cell epitopes (Kim et al., 2008; Luckay et al., 2007; Luxembourg et al., 2006) and now our results allow to extend these findings to B-cell epitopes. These findings are relevant to therapeutic vaccination against chronic HBV infection, since multi-specific CTL responses are essential for intrahepatic viral clearance and highly neutralizing antibodies are particularly important for clearance of circulating viral particles and prevention of reinfection (Chisari et al., 2010). Based on these results, an evaluation of the therapeutic benefits of EP-based pDNA in the chronic DHBV infection duck model is warranted.

Materials and methods

Animals

DHBV-free Pekin ducklings (Anas domesticus) were purchased from a commercial supplier. Duck experiments were performed in accordance with the guidelines for animal care at the National Veterinary School of Lyon (Marcy l'Etoile, France).

DNA vaccine

The previously described pCI-preS/S plasmid, encoding the DHBV preS/S large envelope protein (Rollier et al., 1999) was used for genetic immunization of ducks. The empty plasmid pCI representing the plasmid backbone with no inserted gene was used as a negative control. Both pCI-preS/S and pCI plasmids were grown in E. coli, purified by an Endotoxin Free Giga Prep Kit (Qiagen, Hilden, Germany), resuspended in filtered water and quantified by spectrophotometry.

Duck DNA electroporation and immunization procedures

In the first study, five week-old naïve Pekin ducks were randomly divided into two vaccinated groups (5 ducks/group) and a control group (2 ducks/group). Ducks were anesthetized with a mixture of Dormitor/Zoletil and shaved over the two sides of the breast-bone to prepare the site of vaccination. Treated groups received intramuscularly a total of 300 μg pCI-preS/S plasmid in 600 μl of PBS per injection, delivered either by a TriGrid electroporation (EP) device (Ichor Medical Systems, San Diego, CA) (group 1) or by standard injection (SI) (group 2) (Fig. 1A). The control group received an i.m. injection of the same dose i.e. 300 μg of empty plasmid pCI by EP. For group 1, DNA injections were performed in two sites; in breast and shoulder. EP was applied with a four-needle electrode array (designed specifically for the duck model) using the TriGrid delivery device, with 6 mm intraelectrode spacing and electrical stimulation as previously described (Luxembourg et al., 2008b). For group 2, DNA immunization consisted of multiple injections (6 sites) in breast and shoulder as described previously (Rollier et al., 1999). Identical booster doses were administered at the same site 4 and 8 weeks later, followed by a delayed boost after 30 weeks (Fig. 1C).

To investigate the DNA dose effect, a second study was performed in which DHBV-free Pekin ducks divided randomly into six groups (4 ducks/group) received i.m. injections of different doses; 150, 50 or 10 μg of pCI-PreS/S were delivered either by EP or SI (Fig. 1B). Immunizations were conducted three times at weeks 0, 4, 8 (Fig. 1C).

Immunohistological examination of breast muscle at injection sites

Three ducks received pCI-preS/S plasmid (300 μg pDNA/600 μl PBS) or PBS alone (no DNA/600 μl PBS) either by SI or *in vivo* EP as described above. Six days post immunization, animals were euthanized with an overdose of Zoletil and muscle samples were obtained from all injections sites on these animals. Breast and shoulder muscles

were analyzed for the infiltration of CD3+ T cells and DHBV preS envelop protein expression by immunohistochemistry. Tissues were fixed in 10% neutral buffer formalin, dehydrated, and embedded in paraffin. 5 mm sections were cut, mounted onto glass slides and stained with hematoxylin and eosin (H&E), an antibody to human CD3 (Clone F7.2.38, DakoCytomation) or with a previously described rabbit polyclonal antiserum raised against DHB preS polypeptide (Lambert et al., 1991).

Detection of specific anti-preS antibodies by ELISA

The humoral anti-preS response of ducks was detected by a direct ELISA using microtiter plates (Falcon, Probind) coated with the recombinant DHBV preS polypeptide as described previously (Rollier et al., 2000). The antibody titers were determined on the serum samples of each duck at each time of the follow-up using an end-point dilution ELISA titration assay described previously (Rollier et al., 2000).

Immunoblotting analysis of generated antibodies

DHBV particles or mock (negative control) were concentrated by sucrose cushion (10%) ultracentrifugation of LMH-D2 or LMH supernatants (mock), respectively. The equivalent of 5 μg of denaturized proteins were separated on a 12% SDS polyacrylamide gel and subsequently electrotransferred to nitrocellulose filters. Immunoblotting was performed using pooled sera from each immunized duck group, which were brought back to the same anti-preS titer and diluted to 1:30. After washes, filters were incubated with anti-duck immunoglobulin G peroxidase conjugates (1:3000), and visualized by an ECL chemo-luminescence detection kit (Amersham, Courtaboeuf, France).

In vitro neutralizing activity of sera from ducks immunized by EP or SI

The neutralizing activity of duck sera was tested in duplicate in primary duck hepatocyte (PDH) cultures, which were infected with DHBV-positive serum (2×10^9 viral genome equivalents/well) that had been preincubated overnight at room temperature with various dilutions (1/4, 1/8, 1/16, 1/32, 1/64) of pooled sera from each immunized duck group of the dose effect study, collected one week after the last immunization (week 9) or with cell culture medium (negative control) as described previously (Lambert et al., 1991). The release of virions was followed during 6 days by quantitative dot-blot hybridization of DHBV DNA. The area under the curve of released DHBV DNA levels in PDH supernatants at days 3–6 reflects total viral secretion and was used to calculate the percentage of neutralization as previously described (Lambert et al., 1991). Neutralization was considered effective when the reduction of total released virions was at least 50%.

Peptide scanning analysis of induced antibodies

Preparation of biotinylated peptides

The antibody responses of individual duck sera to DHBV preS linear epitopes were analyzed using the Pepscan method (Skinner et al., 1999). A pepset of 77 overlapping nonapeptides (9 residues), spanning the entire DHBV preS protein (residues 1 to 166), were synthesized by Mimotopes peptide company (UK) in the format Biotin-SGSG-PEPTIDE-amide. These 9-mers peptides were designed with an overlap of 7 amino acids of the preS protein sequence as shown in Table 1 to ensure that all potential epitopes would be identified. A nonoverlapping peptide was used as an unrelated control peptide. Initially, each biotinylated peptide was solubilized in 400 μl of dimethyl sulfoxide/water (80%/20%) and 10 μl was used for each assay to make a 1:12 stock peptide solution diluted in 0.01 M sodium phosphate-buffered saline (PBS) pH 7.2 containing 0.1% sodium azide and 0.1% Tween 20. The biotinylated peptides were tested for antibody binding by ELISA according to the manufacturer's instructions, as described subsequently.

Pepscan assay

Briefly, streptavidin (Sigma) was diluted to 10 μg/ml in 0.1 M sodium bicarbonate buffer pH 9.6 and dispensed at 100 μl/well into 96-well microtiter plates (Nunc-Immuno® MaxiSorb), which were incubated overnight at room temperature (RT). Plates were then rinsed three times in wash buffer consisting of PBS with 0.1% Tween 20. The plates were blocked with 200 μl/well of PBS containing 1% bovine serum albumin (BSA) for 2 h at 37 °C and the rinse was repeated. 100 μl of each of the diluted biotinylated peptide solutions (1:12) were transferred into the corresponding well positions of the plate and incubated 1 h at RT with shaking. After the plates were rinsed, 100 μl/well of serum from immunized ducks, diluted each to 1:40 in wash buffer containing 0.1% sodium azide was incubated for 2 h. Plates were rinsed and 100 μl/well of secondary antibody, (peroxidase-labeled affinity-purified goat anti-duck IgG (KPL, 0.1 mg/ml stock solution) diluted to 1:500 in wash buffer containing 1% sheep serum and 0.1% BSA was incubated for 1 h. After being rinsed, the plates were loaded for 45 min with 100 μl/well of 2,2′-azino-bis-[3-ethylbenzthiazolinsulfonate] (ABTS, Pierce) in substrate buffer according to the manufacturer's protocol. The plates were read at 405 nm and data were processed by subtraction of mean values plus two standard deviations obtained with background reactivity of duck serum with the unrelated control peptide. The signals were expressed as optical density units (O.D.) (Skinner et al., 1999).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.0 (San Diego, CA). The mean anti-preS titers of experimental groups were compared at each sampling using a nonparametric, two-tailed, Mann–Whitney *U* test. In addition, the Student *t* test was used to assess significance in the neutralization assay. P values ≤ 0.05 were considered statistically significant.

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References

Ahlen, G., Soderholm, J., Tjelle, T., Kjeken, R., Frelin, L., Hoglund, U., Blomberg, P., Fons, M., Mathiesen, I., Sallberg, M., 2007. In vivo electroporation enhances the immunogenicity of hepatitis C virus nonstructural 3/4A DNA by increased local DNA uptake, protein expression, inflammation, and infiltration of CD3+ T cells. J. Immunol. 179 (7), 4741–4753.

Babiuk, S., Baca-Estrada, M.E., Foldvari, M., Storms, M., Rabussay, D., Widera, G., Babiuk, L.A., 2002. Electroporation improves the efficacy of DNA vaccines in large animals. Vaccine 20 (27–28), 3399–3408.

Babiuk, S., Baca-Estrada, M.E., Foldvari, M., Middleton, D.M., Rabussay, D., Widera, G., Babiuk, L.A., 2004. Increased gene expression and inflammatory cell infiltration caused by electroporation are both important for improving the efficacy of DNA vaccines. J. Biotechnol. 110 (1), 1–10.

Babiuk, S., Tsang, C., van Drunen Littel-van den Hurk, S., Babiuk, L.A., Griebel, P.J., 2007. A single HBsAg DNA vaccination in combination with electroporation elicits long-term antibody responses in sheep. Bioelectrochemistry 70 (2), 269–274.

Capone, S., Zampaglione, I., Vitelli, A., Pezzanera, M., Kierstead, L., Burns, J., Ruggeri, L., Arcuri, M., Cappelletti, M., Meola, A., Ercole, B.B., Tafi, R., Santini, C., Luzzago, A., Fu, T.M., Colloca, S., Ciliberto, G., Cortese, R., Nicosia, A., Fattori, E., Folgori, A., 2006. Modulation of the immune response induced by gene electrotransfer of a hepatitis C virus DNA vaccine in nonhuman primates. J. Immunol. 177 (10), 7462–7471.

Chassot, S., Lambert, V., Kay, A., Godinot, C., Roux, B., Trepo, C., Cova, L., 1993. Fine mapping of neutralization epitopes on duck hepatitis B virus (DHBV) pre-S protein using monoclonal antibodies and overlapping peptides. Virology 192 (1), 217–223.

Chassot, S., Lambert, V., Kay, A., Godinot, C., Trepo, C., Cova, L., 1994. Identification of major antigenic domains of duck hepatitis B virus pre-S protein by peptide scanning. Virology 200 (1), 72–78.
Chen, M.W., Cheng, T.J., Huang, Y., Jan, J.T., Ma, S.H., Yu, A.L., Wong, C.H., Ho, D.D., 2008. A consensus-hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses. Proc. Natl. Acad. Sci. U. S. A. 105 (36), 13538–13543.
Chen, H., Wen, B., Deng, Y., Wang, W., Yin, X., Guan, J., Ruan, L., Tan, W., 2011. Enhanced effect of DNA immunization plus in vivo electroporation with a combination of hepatitis B virus core-PreS1 and S-PreS1 plasmids. Clin. Vaccine Immunol. 18 (11), 1789–1795.
Chisari, F.V., Isogawa, M., Wieland, S.F., 2010. Pathogenesis of hepatitis B virus infection. Pathol. Biol. (Paris) 58 (4), 258–266.
Cova, L., Zoulim, F., 2004. Duck hepatitis B virus model in the study of hepatitis B virus. Methods Mol. Med. 96, 261–268.
Dupuy, L.C., Richards, M.J., Ellefsen, B., Chau, L., Luxembourg, A., Hannaman, D., Livingston, B.D., Schmaljohn, C.S., 2011. A DNA vaccine for venezuelan equine encephalitis virus delivered by intramuscular electroporation elicits high levels of neutralizing antibodies in multiple animal models and provides protective immunity to mice and nonhuman primates. Clin. Vaccine Immunol. 18 (5), 707–716.
Gardiner, D.F., Rosenberg, T., Zaharatos, J., Franco, D., Ho, D.D., 2009. A DNA vaccine targeting the receptor-binding domain of Clostridium difficile toxin A. Vaccine 27 (27), 3598–3604.
Kim, C.Y., Kang, E.S., Kim, S.B., Kim, H.E., Choi, J.H., Lee, D.S., Im, S.J., Yang, S.H., Sung, Y.C., Kim, B.M., Kim, B.G., 2008. Increased in vivo immunological potency of HB-110, a novel therapeutic HBV DNA vaccine, by electroporation. Exp. Mol. Med. 40 (6), 669–676.
Lambert, V., Chassot, S., Kay, A., Trepo, C., Cova, L., 1991. In vivo neutralization of duck hepatitis B virus by antibodies specific to the N-terminal portion of pre-S protein. Virology 185 (1), 446–450.
Liu, K.H., Ascenzi, M.A., Bellezza, C.A., Bezuidenhout, A.J., Cote, P.J., Gonzalez-Aseguinolaza, G., Hannaman, D., Luxembourg, A., Evans, C.F., Tennant, B.C., Menne, S., 2011. Electroporation enhances immunogenicity of a DNA vaccine expressing woodchuck hepatitis virus surface antigen in woodchucks. J. Virol. 85 (10), 4853–4862.
Livingston, B.D., Little, S.F., Luxembourg, A., Ellefsen, B., Hannaman, D., 2010. Comparative performance of a licensed anthrax vaccine versus electroporation based delivery of a PA encoding DNA vaccine in rhesus macaques. Vaccine 28 (4), 1056–1061.
Luckay, A., Sidhu, M.K., Kjeken, R., Megati, S., Chong, S.Y., Roopchand, V., Garcia-Hand, D., Abdullah, R., Braun, R., Montefiori, D.C., Rosati, M., Felber, B.K., Pavlakis, G.N., Mathiesen, I., Israel, Z.R., Eldridge, J.H., Egan, M.A., 2007. Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. J. Virol. 81 (10), 5257–5269.
Luxembourg, A., Hannaman, D., Ellefsen, B., Nakamura, G., Bernard, R., 2006. Enhancement of immune responses to an HBV DNA vaccine by electroporation. Vaccine 24 (21), 4490–4493.
Luxembourg, A., Evans, C.F., Hannaman, D., 2007. Electroporation-based DNA immunisation: translation to the clinic. Expert Opin. Biol. Ther. 7 (11), 1647–1664.
Luxembourg, A., Hannaman, D., Nolan, E., Ellefsen, B., Nakamura, G., Chau, L., Tellez, O., Little, S., Bernard, R., 2008a. Potentiation of an anthrax DNA vaccine with electroporation. Vaccine 26 (40), 5216–5222.
Luxembourg, A., Hannaman, D., Wills, K., Bernard, R., Tennant, B.C., Menne, S., Cote, P.J., 2008b. Immunogenicity in mice and rabbits of DNA vaccines expressing woodchuck hepatitis virus antigens. Vaccine 26 (32), 4025–4033.
Mallilankaraman, K., Shedlock, D.J., Bao, H., Kawalekar, O.U., Fagone, P., Ramanathan, A.A., Ferraro, B., Stabenow, J., Vijayachari, P., Sundaram, S.G., Muruganandam, N., Sarangan, G., Srikanth, P., Khan, A.S., Lewis, M.G., Kim, J.J., Sardesai, N.Y., Muthumani, K., Weiner, D.B., 2011. A DNA vaccine against chikungunya virus is protective in mice and induces neutralizing antibodies in mice and nonhuman primates. PLoS Negl. Trop. Dis. 5 (1), e928.
Mancini, M., Hadchouel, M., Davis, H.L., Whalen, R.G., Tiollais, P., Michel, M.L., 1996. DNA-mediated immunization in a transgenic mouse model of the hepatitis B surface antigen chronic carrier state. Proc. Natl. Acad. Sci. U. S. A. 93 (22), 12496–12501.
Michel, M.L., Deng, Q., Mancini-Bourgine, M., 2011. Therapeutic vaccines and immune-based therapies for the treatment of chronic hepatitis B: perspectives and challenges. J. Hepatol. 54 (6), 1286–1296.
Otten, G., Schaefer, M., Doe, B., Liu, H., Srivastava, I., zur Megede, J., O'Hagan, D., Donnelly, J., Widera, G., Rabussay, D., Lewis, M.G., Barnett, S., Ulmer, J.B., 2004. Enhancement of DNA vaccine potency in rhesus macaques by electroporation. Vaccine 22 (19), 2489–2493.
Peng, J.L., Zhao, Y.G., Mai, J.H., Pang, W.K., Guo, W., Chen, G.M., Mo, G.Y., Rao, G.R., Xu, Y.H., 2007. Non-cytolytic antigen clearance in DNA-vaccinated mice with electroporation. Acta Pharmacol. Sin. 28 (7), 1024–1030.
Rehermann, B., Nascimbeni, M., 2005. Immunology of hepatitis B virus and hepatitis C virus infection. Nat. Rev. Immunol. 5 (3), 215–229.
Rollier, C., Sunyach, C., Barraud, L., Madani, N., Jamard, C., Trepo, C., Cova, L., 1999. Protective and therapeutic effect of DNA-based immunization against hepadnavirus large envelope protein. Gastroenterology 116 (3), 658–665.
Rollier, C., Charollois, C., Jamard, C., Trepo, C., Cova, L., 2000. Early life humoral response of ducks to DNA immunization against hepadnavirus large envelope protein. Vaccine 18 (27), 3091–3096.
Rosati, M., Valentin, A., Jalah, R., Patel, V., von Gegerfelt, A., Bergamaschi, C., Alicea, C., Weiss, D., Treece, J., Pal, R., Markham, P.D., Marques, E.T., August, J.T., Khan, A., Draghia-Akli, R., Felber, B.K., Pavlakis, G.N., 2008. Increased immune responses in rhesus macaques by DNA vaccination combined with electroporation. Vaccine 26 (40), 5223–5229.
Skinner, S.M., Schwoebel, E.S., Prasad, S.V., Oguna, M., Dunbar, B.S., 1999. Mapping of dominant B-cell epitopes of a human zona pellucida protein (ZP1). Biol. Reprod. 61 (6), 1373–1380.
Thermet, A., Robaczewska, M., Rollier, C., Hantz, O., Trepo, C., Deleage, G., Cova, L., 2004. Identification of antigenic regions of duck hepatitis B virus core protein with antibodies elicited by DNA immunization and chronic infection. J. Virol. 78 (4), 1945–1953.
van Drunen Littel-van den Hurk, S., Luxembourg, A., Ellefsen, B., Wilson, D., Ubach, A., Hannaman, D., van den Hurk, J.V., 2008. Electroporation-based DNA transfer enhances gene expression and immune responses to DNA vaccines in cattle. Vaccine 26 (43), 5503–5509.
van Drunen Littel-van den Hurk, S., Lawman, Z., Wilson, D., Luxembourg, A., Ellefsen, B., van den Hurk, J.V., Hannaman, D., 2010. Electroporation enhances immune responses and protection induced by a bovine viral diarrhea virus DNA vaccine in newborn calves with maternal antibodies. Vaccine 28 (39), 6445–6454.
Widera, G., Austin, M., Rabussay, D., Goldbeck, C., Barnett, S.W., Chen, M., Leung, L., Otten, G.R., Thudium, K., Selby, M.J., Ulmer, J.B., 2000. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. J. Immunol. 164 (9), 4635–4640.
Yuasa, S., Cheung, R.C., Pham, Q., Robinson, W.S., Marion, P.L., 1991. Peptide mapping of neutralizing and nonneutralizing epitopes of duck hepatitis B virus pre-S polypeptide. Virology 181 (1), 14–21.
Zhao, Y.G., Peng, B., Deng, H., Chen, G., Yang, F., Shao, M., Lu, H., Li, Y., Peng, J., Xu, L., Xu, Y., 2006. Anti-HBV immune responses in rhesus macaques elicited by electroporation mediated DNA vaccination. Vaccine 24 (7), 897–903.