In vivo electroporation improves therapeutic potency of a DNA vaccine targeting hepadnaviral proteins

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A B S T R A C T

This preclinical study investigated the therapeutic efficacy of electroporation (EP)-based delivery of plasmid DNA (pDNA) encoding viral proteins (envelope, core) and IFN-γ in the duck model of chronic hepadnavirus (DHBV) infection. Importantly, only DNA EP-therapy resulted in a significant decrease in mean viremia titer and in intrahepatic covalently closed circular DNA (cccDNA) levels in chronic DHBV-carrier animals, compared with standard needle pDNA injection (SI). In addition, DNA EP-therapy stimulated in all virus-carriers a humoral response to DHBV preS protein, recognizing a broader range of major antigenic regions, including neutralizing epitopes, compared with SI. DNA EP-therapy led also to significant higher intrahepatic IFN-γ RNA levels in DHBV-carriers compared to other groups, in the absence of adverse effects. We provide the first evidence on DNA EP-therapy benefit in terms of hepadnaviral infection clearance and break of immune tolerance in virus-carriers, supporting its clinical application for chronic hepatitis B.

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Introduction

Despite the existence of an effective prophylactic vaccine, chronic hepatitis B virus (HBV) infections remain a major public health problem (Zoulim, 2011). The failure of current antiviral therapies (interferon alpha, nucleoside analogs) is related to their limited ability to completely eliminate the intranuclear covalently closed circular DNA (cccDNA), viral minichromosome that represent the transcriptional template of HBV replication (Zoulim, 2011). Several studies reported that the clearance of acute HBV infection relies on the development of appropriate immune responses against HBV, including protective, virus neutralizing antibodies against HBV surface antigen (HBsAg), antigen-specific T-helper (Th) cells and cytolytic T lymphocytes (CTL) associated with an upregulated expression of type 1 inflammatory cytokines in the liver, especially gamma interferon (IFN-γ) (Bertoletti and Ferrari, 2003; Chisari and Ferrari, 1995; Ferrari et al., 1990; Guidotti and Chisari, 2001; Jung et al., 1995; Lohr et al., 1995; Marinos et al., 1995; Rehermann, 2000; Wang et al., 2003). Studies in HBV-transgenic mice demonstrated the crucial role of IFN-γ in the non-cytolytic mechanism of virus clearance (Guidotti and Chisari, 2001; Wieland and Chisari, 2005). In addition, such IFN-γ-mediated control of infection was demonstrated during recovery from acute HBV infection of chimpanzees and woodchuck HBV (WHV) infection of woodchucks (Guidotti et al., 1999; Hodgson and Michalak, 2001; Wieland et al., 2004). In this context, it has been shown that IFN-γ is highly effective in steering T-cell responses towards the Th1 type during resolution of infection. In contrast, the chronic HBV infection is accompanied by weak and narrowly focused HBV-specific humoral and cellular immune responses. Thus, therapeutic DNA vaccination, able to activate both defective humoral and Th1 cellular immune responses in HBV carriers, has been proposed as a particularly...
pertinent approach for chronic hepatitis B therapy (Cova, 2012; Michel et al., 2011; Michel and Loirat, 2001). However, the progress of DNA vaccines into the clinic has been hampered by the relatively low magnitude and inconsistent immune responses achieved by conventional DNA delivery methods in non-human primates and humans (Sardesai and Weiner, 2011). Thus, the results of the first clinical trials of HBV DNA vaccine in chronically infected patients were rather disappointing, indicating a need to increase the efficacy of this approach (Cavanaugh et al., 2011).

Electroporation (EP)-mediated delivery appears to be a promising novel approach to improve the immune responses to DNA vaccines. EP dramatically enhances the potency of DNA vaccines by increasing cell membrane permeability, thereby facilitating cellular uptake of DNA, and also by eliciting a low level inflammation, which enhances the influx of antigen-presenting cells to the injection site (van Drunen Littel-van den Hurk and Hannaman, 2010). Recent studies in several animal species (mice, rabbit, woodchuck, sheep, pig, cattle and rhesus macaques) (Babiuk et al., 2007; Chen et al., 2011; Donate et al., 2011; Kim et al., 2008; Liu et al., 2011; Luxembourg et al., 2006; Luxembourg et al., 2008; Nystrom et al., 2010; Peng et al., 2007; van Drunen Littel-van den Hurk et al., 2008; Yin et al., 2011; Zhao et al., 2006) convincingly demonstrated that EP-based delivery of DNA vaccines targeting HBV envelope and/or core antigens markedly enhanced humoral and cellular immune responses as compared to those induced by standard needle injection (SI) of the same DNA vaccine. However, evaluation of EP-based pDNA delivery in these studies was limited to analysis of immunogenicity or prophylactic efficacy of such vaccines in naïve animals. EP-based DNA delivery now has reached the clinical stage being actively investigated in several phase I clinical trials for therapeutic and prophylactic application against cancer (Lowers et al., 2009) and infectious diseases (Bodles-Brakhop et al., 2009; Sallberg et al., 2009; Sardesai and Weiner, 2011; van Drunen Littel-van den Hurk and Hannaman, 2010). Concerning HBV infection, a randomized controlled clinical trial of EP-mediated HBV DNA vaccine in chronic hepatitis B patients under lamivudine chemotherapy has been recently performed in China (Yang et al., 2012), although, its ability to clear intrahepatic viral cccDNA that cannot be eliminated by current antiviral therapies, remains unknown.

In this regard, the duck hepatitis B virus (DHBV) represents a pertinent model to evaluate the therapeutic efficacy of EP-based pDNA delivery. Thus, the DHBV is a related avian hepadavirus with replication strategies and genomic organization closely related to the human HBV. DHBV played a pivotal role in studying hepadavirus replication and testing the impact of novel anti-HBV therapeutic approaches on intrahepatic viral cccDNA elimination (Cova, 2012; Cova and Zoulim, 2004; Le Guerhier et al., 2000; Le Guerhier et al., 2003; Thermet et al., 2008). The experimental DHBV infection of neonatal ducklings leads to the establishment of a chronic DHBV-carrier similar to chronic HBV infection in humans (Cova and Zoulim, 2004; Rolllier et al., 1999). Moreover, as for the resolution of acute HBV infection, the resolution of DHBV infection in adult ducks is associated with a seroconversion to protective, virus-neutralizing antibodies, essentially directed against preS envelope protein and an increased expression of duck interferon-γ (DuIFN-γ) in the liver and peripheral blood mononuclear cells (PBMCs) (Narayan et al., 2006).

We have previously reported in this model that immunotherapy of chronically infected ducks with SI-delivered DNA vaccine encoding DHBV proteins (envelope, core), decreased viral replication and enhanced viral clearance in some animals (Rolllier et al., 1999; Thermet et al., 2008). In addition, we have shown that co-delivery of DuIFN-γ encoding plasmid with the DHBV DNA vaccine leads to the enhancement of the humoral anti-preS response in naïve animals (Saade et al., 2008) and enhanced therapeutic efficacy of DNA vaccine in DHBV-carriers (Saade et al., 2011).

In our recent study, we have shown that EP-based pDNA delivery in naïve ducks considerably enhanced the magnitude and breadth of humoral response to DHBV large envelope protein (preS/S), which was highly neutralizing as compared with that induced by the SI administration (Khawaja et al., 2012).

In our efforts to explore whether therapeutic efficacy of a generic vaccine targeting hepadnaviral proteins can be enhanced using EP-based immunization, we carried out a long-term (10 months) follow-up study in chronic DHBV duck model. We focused here on the ability of EP-based pDNA delivery to sustainably suppress viral infection, including clearance of the intrahepatic cccDNA viral form responsible for the persistence of infection, and to restore antiviral immune responses.

Results

EP-based pDNA delivery decreases viremia and enhances break of humoral immune tolerance in chronically infected ducks

To investigate the long-term therapeutic effect of EP-based DHBV pDNA vaccine delivery, chronic DHBV-carriers were randomly assigned into three groups. Two of the groups were immunized intramuscularly (i.m) with a total of 600 μg of the same DNA vaccine encoding DHBV envelope and core proteins as well as DuIFN-γ, but delivered either by SI or EP. A control group included untreated ducks and ducks receiving 600 μg of empty pC1 plasmid. The immunized animals received additional DNA vaccine administrations every 3–7 weeks during 10 months of follow-up. Analysis of serum DHBV DNA collected prior to immunization (weeks 1–3 of age) showed no significant difference between the mean viremia levels of the different duck groups. By contrast, starting from the third DNA administration (week 10), the EP-treated group showed a marked decrease in mean viremia levels, which reached a significant difference as compared to SI-treated and to the control group starting from week 31 to the end of the experiment at week 40 (p < 0.05) (Fig. 1C). This viremia decrease in the EP-treated group was largely due to 2 animals: duck no. 474 exhibited an early drop in viremia starting at week 10 that became undetectable from week 16 and duck no. 454 that cleared serum viral DNA after week 37, as illustrated on individual viremia titers follow-up (Fig. 1C). Importantly, viremia levels remained undetectable in these animals following immunotherapy cessation and up to the end of the follow-up, indicating a sustained effect (Fig. 1C). The remaining animals from the EP-treated group (nos. 440, 452, 475 and 459) responded also to the therapeutic vaccination by exhibiting 1.5 to 2.3 log10-fold decrease in viremia titers at week 40 as compared to pre-treatment levels (week 1). By contrast, none of the ducks from the SI-treated group or the control group cleared viral DNA levels in their sera (Fig. 1A and B). Although the SI-treated group showed higher mean viremia levels as compared to the control group, the difference was not significant and was essentially due to two animals (nos. 470 and 473) exhibiting viremia fluctuations or rebounds during the therapy that contributed to increase in mean titers in the group (Fig. 1B and G). Taken together, these results indicate a significant benefit of EP-based pDNA delivery by sustained decreasing serum viral load as compared with other groups.

To evaluate whether the anti-envelope antibody response, generally weak or undetectable in chronic-DHBV carriers, could be enhanced by pDNA EP therapy, we monitored the anti-preS antibody titers during the entire experimental period. As shown in Fig. 1H, the EP-treated group showed a strong and sustained
Fig. 1. Monitoring of viremia and anti-preS titers in chronic DHBV-carriers. Viremia and anti-preS titers were monitored throughout the entire experiment and titers are plotted on the graphs using a log scale. Individual viremia and anti-preS titers are represented for control ducks (panels A and D, respectively), SI-treated ducks (panels B and E, respectively) and EP-treated ducks (panels C and F, respectively). The mean viremia and anti-preS titers for each group are shown in panels G and H, respectively. Black arrows indicate DNA injections at weeks 3, 6, 9, 16, 21, 25, 29, 33 and 36. The dotted line indicates the cut-off detection level of the assays.
mean anti-preS response. By week 29 and until the end of the experiment, this response was significantly higher (4–34 fold) as compared to those of SI-treated and control groups ($p < 0.05$). The follow-up of individual anti preS titers within this group, indicated that two animals (no. 474 and 454) mounted a particularly high anti-preS response (by week 10 and 30, respectively), which was concomitant with serum viral DNA clearance (Fig. 1F and C). The remaining EP-treated ducks (4 out of 6) mounted a high anti-preS response (reaching titers of 1280–2560) at the end of the follow-up, even though they did not completely clear viral DNA (Fig. 1F). By contrast, two (no. 470 and 473) out of five SI-treated ducks and all control ducks showed very weak anti-preS responses not exceeding a titer of 160 (Fig. 1D and E). These data indicate that EP-based DNA delivery induced a break in humoral immune tolerance against the preS protein in DHBV carriers.

**Induction of a broader repertoire of anti-preS responses by EP-based DNA delivery**

To investigate the epitope pattern of antibodies generated in animals from different groups we compared, by peptide scanning analysis, the major antigenic pre-S regions (ARs) and pre-S neutralizing epitopes recognized by these antibodies. The scans in Fig. 2 illustrate the reactivity of sera (week 40) from the EP-treated, SI-treated, and control groups (panels A, B and C respectively) obtained with overlapping 9-mer peptides spanning the entire DHB pre-S region (peptides 1–77). All sera from EP-treated ducks recognized 14 major antigenic regions: AR1 (aa 1–17), AR2 (aa 15–31), AR3 (aa 27–47), AR4 (aa 41–53), AR5 (aa 53–65), AR6 (aa 65–81), AR7 (aa 79–95), AR8 (aa 93–107), AR9 (aa 101–117), AR10 (aa 115–127), AR11 (aa 121–133), AR12 (aa 135–151), AR13

**Fig. 2.** Screening of induced antibodies in ducks for pre-S B cell epitope recognition by pepscan analysis. Sera from immunized ducks with DHBV DNA vaccine delivered by (A) EP, (B) SI or (C) from control ducks, were taken at the end of the follow-up (week 40) and were screened for their reactivity with 77 overlapping 9-mer peptides covering the entire DHB pre-S region (1–161 aa). Note that the comparison of reacting spectrum of sera from animals exhibiting similar ELISA titers (640–1280) showed a broader anti-preS response for ducks from EP-immunized group as compared with SI-immunized group. Cumulative optical density values, as determined by the pepscan analysis, are shown. Background signal of control and nonreactive peptides was subtracted before graphic representation. AR1 to AR14 indicate the positions of recognized antigenic regions (AR).
eliminated all intrahepatic DHBV DNA intermediates, including very low levels of cccDNA (Fig. 3B). More importantly, duck no. 474 that showed an early drop in viremia levels (week 10), showed lower levels of viral DNA replicative forms compared to the control group. By contrast, all DNA EP-treated animals (nos. 468, 470, 100F-R107, 112T-P120, 123K-G133 and 58T-W65, respectively), that were previously identified by us and others (Chassot et al., 1993; Yuasa et al., 1991). By contrast, the reactivity spectrum of sera within the SI-immunized group appeared narrower, of lower magnitude and was restricted to fewer ARs (2, 3, 5, 7, 8, 10 and 13) as compared to that from EP-treated ducks (Fig. 2B). The sera of control ducks showed an extremely narrow B-cell epitope response pattern, which was limited to only one AR (8) (Fig. 2C).

Overall, these results indicate that unlike DNA SI therapy, the EP-based pDNA delivery was able to break humoral tolerance and generate in all chronic DHBV-carriers a broadly reactive humoral response to DHBV preS protein able to recognize several neutralizing epitopes.

**EP-based pDNA delivery enhances intrahepatic viral DNA clearance**

At the end of the 10-month follow-up period, we examined the impact of the immunotherapy on intrahepatic viral DNA in necropsy liver samples. Southern blot analysis of total intrahepatic DHBV DNA showed that control ducks (3 untreated and 2 empty vector-treated) had similarly high levels of replicative viral DNA intermediates (Fig. 3). In the duck group treated with SI-delivered DNA vaccine, none of the animals eliminated DHBV DNA. Within this group, one (no. 468) out of 5 animals showed low levels of DHBV DNA replicative forms, however 2 (nos. 470, 473) out of 5 animals had high DNA levels comparable to those of empty vector-treated ducks were highly reactive to the ARs (2, 4, 7, 8, 10 and 11) that represent the neutralization epitopes of R-P30, P52, 83L-P90, 100F-R107, 112T-P120, 123K-G133 and 58T-W65, respectively, that were previously identified by us and others (Chassot et al., 1993; Yuasa et al., 1991). By contrast, the reactivity spectrum of sera within the SI-immunized group appeared narrower, of lower magnitude and was restricted to fewer ARs (2, 3, 5, 7, 8, 10 and 13) as compared to that from EP-treated ducks (Fig. 2B). The sera of control ducks showed an extremely narrow B-cell epitope response pattern, which was limited to only one AR (8) (Fig. 2C).

Overall, these results indicate that unlike DNA SI therapy, the EP-based pDNA delivery was able to break humoral tolerance and generate in all chronic DHBV-carriers a broadly reactive humoral response to DHBV preS protein able to recognize several neutralizing epitopes.

**Effect of DNA immunization on intrahepatic DHBV DNA.** Total DNA was extracted at the end of the follow-up (week 40) from all necropsy liver samples and analyzed by Southern blotting. 10 μg of total DNA or cccDNA preparations were subjected to electrophoresis through 1.5% agarose gels, transferred to nylon membranes followed by specific hybridization with a full-length DHBV DNA probe. Control for equal cellular DNA loading in all lanes of the corresponding gel was established by spectrophotometric quantification and by ethidium bromide staining of the corresponding gel (data not shown). (A) Analysis of total DHBV DNA. Arrows show position of relaxed circular (RC-DNA), single-stranded DNA (ss-DNA) and covalently closed circular DHBV DNA (cccDNA). A 3 kb linearized DHBV DNA (‘’) was used as a control positive of hybridization. (B) Specific isolation of cccDNA. The black arrow indicates undetectable cccDNA.

To further examine the persistence of residual traces of intrahepatic viral DNA, we performed a real-time PCR assay for more precise quantification of cccDHBV DNA in these liver samples. The results indicated that duck no. 474, which had undetectable liver DHBV DNA replicative forms in Southern blotting, presented a cccDNA level below the cut-off of the real-time PCR (i.e. 0.0001 copies/cell), whereas duck no. 454 exhibited only traces of cccDNA (0.08 copies/cell) (Fig. 4). Moreover, a significant difference in terms of mean cccDNA levels was observed between EP-treated group and the other groups, i.e. cccDNA levels being more than 5 and 10-fold lower as compared to SI-treated group (p = 0.0441) and to control group (p = 0.0043), respectively suggesting a significant benefit of EP-based DNA delivery on cccDNA pool clearance.

**EP-based pDNA delivery increases the intrahepatic IFN-γ response**

To explore the impact of therapies on the intrahepatic expression of Th1 cytokines, we compared the levels of DulIFN-γ expression in the liver of all animals at the end of the follow-up period (week 40), using a quantitative RT-PCR. The analysis of uninfected duck livers showed very low DulIFN-γ mRNA amount (Fig. 5, horizontal bar), which was defined as the baseline level. Within the control and SI-treated groups, intrahepatic DulIFN-γ RNA levels were almost similar to the baseline level in some animals or slightly upregulated. By contrast, within the EP-treated group, an increase in DulIFN-γ expression was observed for all animals, particularly for duck no. 454, which peaked to a high

![Fig. 4. Intrahepatic viral cccDNA levels. Detection of DHBV cccDNA was performed by a specific real-time PCR in necropsy liver samples from all animals. The detection limit of this assay was 0.0001 cccDNA copies per cell. Horizontal bars represent mean cccDNA level per group. Statistically significant difference in mean cccDNA levels in the EP group (○) compared the SI group (Δ) (p < 0.05) and to the control group (■) (p < 0.01) are shown as assessed by two-tailed Mann–Whitney test.](image-url)
level that was correlated with the low level of intrahepatic cccDNA. Interestingly, the mean values of intrahepatic DuIFN-γ mRNA levels in EP-treated group were significantly 10 and 21-fold greater than those of the SI-treated group \(p = 0.0043\) and the control group \(p = 0.004\), respectively. Further analysis of the intrahepatic DuIFN-γ RNA in all DHBV-carriers revealed a significant inverse correlation \(r = -0.93, p < 0.0001\) with the intrahepatic cccDNA levels (supplementary Fig. 1). Thus, the diminished expression of DuIFN-γ in control and nonresponder ducks within the SI-treated group correlated with the chronic infection status of these animals that was characterized by a high level of intrahepatic viral cccDNA. In contrast, the increased expression of DuIFN-γ in all EP-treated ducks and slight increase in some partial responders to the SI-therapy correlated with lower levels of cccDNA.

These results suggest a significant benefit of EP-based pDNA delivery in its ability to enhance the Th1 cytokine response in the liver and the cccDNA clearance.

**Immunohistochemical analysis of liver samples**

Next, we examined the infiltration of CD3+ T cells and DHBV pre-S protein expression in necropsy liver samples of all animals and we correlated their immunohistological profiles with their serum and liver virus clearance. This comparison revealed different profiles of viral clearance as illustrated in Fig. 6 and summarized in Table 1. All five control ducks and two (nos. 470 and 473) out of 5 ducks from the SI-treated group, exhibiting a typical serological profile of chronic DHBV infection that is characterized by high viral load and very weak or narrow B-cell response, showed a massive expression of DHBV pre-S in virtually all hepatocytes and a mild portal lymphocyte infiltration (as illustrated in Fig. 6A for duck no. 470). By contrast, a different profile was observed for three responder animals (nos. 448, 458 and 468) from the SI-treated group and four ducks (nos. 440, 452, 459 and 475) from EP-treated group exhibiting lower viremia and higher anti-preS response than those of the control ducks at the end of the follow-up, suggesting a partial viral clearance (Table 1). Thus, immunoperoxidase staining of liver samples from these animals showed the presence of clusters of cells expressing DHBV preS antigen and a moderate to marked portal infiltration as illustrated for duck no. 440 from EP group (Fig. 6B, Table 1). A particularly interesting profile was observed for duck no. 454, from the EP-treated group, which cleared viremia relatively late (week 37) concomitantly with development of a high anti-preS response. This animal showed a residual DHBV preS antigen expression limited to only few hepatocytes and exhibited a marked portal lymphocyte infiltration that correlated with very high levels of DuIFN-γ expression in its liver, suggesting an advanced stage of clearance (Fig. 6C). An even more complete stage of clearance was observed for duck no. 474 from this group that completely cleared viremia at an early stage, by week 10, concomitantly with seroconversion to a sustained anti-preS response and undetectable levels of cccDNA by the end of the therapy. Interestingly, the
profile of this totally resolved animal was characterized by undetectable DHBV preS expression and a moderate portal lymphocyte infiltration, indicating an earlier clearance of infection (Fig. 6D). Such complete viral clearance was not observed in animals from SI-treated ducks and controls (Table 1). Collectively these results indicate that in spite of individual differences, more advanced degree of viral clearance was observed in the EP-treated group.

Adverse effects of therapy

Histological analysis of all duck liver samples did not show severe lesions, necrosis or fulminant hepatitis. Occasional presence of amyloidosis and frequent steatosis, known to occur in adult Pekin ducks (Cova et al., 1990), was observed regardless of treatment group. Even though several deaths have occurred during this long-term experiment (10 months), they were accidental and their incidence rate was similar between the SI, EP and control groups, indicating that these deaths were not related to the treatment (two deaths per group, excluded from the study). These data indicates the absence of side effects for the treatment we used at the described conditions.

Discussion

In this study, we report for the first time that EP-based delivery enhanced the therapeutic efficacy of DNA-based vaccination in terms of both viral clearance and breaking of immune tolerance in hepadnaviral-carriers, suggesting a benefit of this approach for chronic hepatitis B therapy. We used in this study co-immunization with DuIFN-γ encoding plasmid, since we have previously shown that co-delivery of DuIFN-γ gene with the DHBV DNA vaccine led to the enhancement of the humoral anti-preS response in naïve animals (Saade et al., 2008) and increased therapeutic efficacy of DNA vaccine in DHBV-carriers (Saade et al., 2011). The EP-delivered DHBV DNA vaccine was particularly effective, leading to a marked decrease in viremia in all animals. In contrast, delivery of the same vaccine by SI was less effective since 2 out of 5 ducks had similar viremia levels as those of the controls. The mean viremia levels in the EP-treated group was consistently lower and reached a significant difference starting at week 31 p.i. until the end of the follow-up, as compared to control ducks and SI-treated ducks. Importantly, EP-based pDNA therapy led to viremia clearance in 33% of animals (2 out of 6 ducks) whereas none of the SI-treated ducks or the control ducks cleared viral DNA levels in their sera. Moreover, the effects of the EP-based pDNA therapy on DHBV replication were sustained since no rebound in viremia was observed during 4 weeks after immunotherapy cessation in the 2 EP-treated ducks that cleared viral replication. Interestingly, within these two EP-treated animals, duck no. 474, which cleared viremia after only three DNA immunizations and duck no. 454, exhibiting a relatively late viremia clearance, completely eliminated or presented very low intrahepatic DHBV DNA intermediates, respectively.

Because studies in animal models and in chronically infected patients demonstrated the crucial role of the hepadnaviral cccDNA pool in the persistence and reactivation of virus replication (Le Mire et al., 2005; Mason et al., 1994; Werle-Lapostolle et al., 2004), it was of particular importance to precisely quantify the cccDNA levels in apparently resolved animals, using a highly sensitive quantitative PCR assay. We report here that an early drop in viremia was correlated with complete liver DHBV DNA clearance, including cccDNA, i.e. level below the cut-off of detection by the qPCR assay, as observed for duck no. 474. In contrast, a relatively late drop in viremia was associated with an advanced stage of intrahepatic viral DNA clearance represented by only traces of cccDNA (0.08 copies of cccDNA/cell for duck no. 454), which is consistent with our previous observations in another DNA vaccination-based therapeutic protocol in DHBV-carriers (Thermet et al., 2008). The low pre-treatment viral load observed for these two ducks may contribute to their advanced or complete stage of viral clearance as previously reported by us (Le Guerhier et al., 2003). Furthermore, intrahepatic viral cccDNA levels were found to be significantly reduced only in the EP-treated group as compared with control group, suggesting a marked benefit of EP based pDNA delivery in terms of decrease and even clearance of cccDNA pool in these DHBV-carriers.

The effect of EP-based pDNA delivery on restoring the anti-preS antibody response in DHBV-carriers was remarkable. In fact, this pDNA delivery method was associated with a strong and sustained seroconversion to anti-preS response in all animals that was significantly higher (p < 0.05) during the last 11 weeks as compared to other groups. We observed the presence of viral particles in some animal sera despite the presence of anti-preS antibodies, which may be likely related to the circulating immune complexes. Interestingly, an early seroconversion to high anti-preS titers was observed only in the 2 EP-treated ducks that cleared viral DNA from their sera and had either undetectable or traces of cccDNA in their liver, whereas a more delayed and lower anti-preS response was observed in animals that showed a decrease in viral load only at the end of the follow-up. Thus, these results suggest that viral clearance is tightly associated with an early induction of the humoral response.

Even though the mechanism of viral clearance is thought to be a Th1-cell dependent process, the antibody response to hepadnaviral infection is known to play a critical role in viral clearance by neutralizing free viral particles in the circulation and by preventing their attachment and uptake by hepatocytes (Chisari et al., 2001). In the case of DHBV, we and others have previously showed that anti-preS antibodies play an important role in

<table>
<thead>
<tr>
<th>Group</th>
<th>DHBV infection profile</th>
<th>Duck LD</th>
<th>% of DHBVpreS positive hepatocytes</th>
<th>Portal infiltration</th>
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<td>EP-treated</td>
<td>Partial clearance</td>
<td>440, 452, 475, 459</td>
<td>30–50 (clusters)</td>
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<td></td>
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<td>Complete clearance</td>
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<td>&lt; 0.001</td>
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<td></td>
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<td>SI-treated</td>
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<td></td>
<td>Chronic infection</td>
<td>470, 473</td>
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<td>Control</td>
<td>Chronic infection</td>
<td>431, 457, 441, 462, 467</td>
<td>&gt; 90</td>
<td>+</td>
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* Different status of chronic DHBV infection.

The DHBpreS-positive hepatocytes were determined by immunoperoxidase staining of liver tissue.

Portal infiltration of CD3+ T cells was determined by immunoperoxidase staining and graded semi-quantitatively as: +, mild; ++, moderate; +++ marked.
neutralization of DHBV infectivity and inhibition of virus interaction with host cell receptors (Sunyach et al., 1999; Urban et al., 2000). In addition, Zhang and Summers, 2004 have shown that neutralizing anti-preS antibodies prevented secondary cycles of infection, resulting in limited intrahepatic virus spread. Therefore, using the peptide scanning approach, we explored, in this study, the epitope pattern, particularly the neutralizing epitopes, of antibodies generated in all animals at the end of the follow-up. We report here that all sera from EP-treated ducks recognized a broad range of neutralizing epitopes. Indeed, these sera recognized 14 major ARs of the DHBV L protein, almost in the same manner as ducks that eliminated DHBV replication. Moreover, such multi-specific B-cell response is similar to that observed recently by us in naïve ducks immunized by EP with DNA vaccine targeting DHBV envelope (Khawaja et al., 2012). Importantly, the highly recognized ARs included several DHBV neutralization epitopes 12R-P30, 41P-T52, 58T-W65, 58T-P60, 100P-R107, 112T-P120 and 123K-G133 previously identified by us and others (Chassot et al., 1993; Chassot et al., 1994; Yuasa et al., 1991). By contrast, the reactivity spectrum of sera within the SI-treated group appeared narrower, less intense and was restricted to a few ARs, including only a subset of neutralizing epitopes as compared to sera from EP-treated ducks. These results suggest a significant benefit of EP-based pDNA delivery to stimulate, in all DHBV-sera from EP-treated ducks. These results suggest a significant decrease in viral load in these animals as compared to other groups.

At the end of follow-up, intrahepatic expression of IFN-γ, known to play a key role in the mechanism of HBV clearance through a non-cytolytic pathway (Hodgson and Michalak, 2001; Wieland et al., 2004) was investigated in all animals using a quantitative real-time RT-PCR assay. Using this approach, we have previously demonstrated that the resolution of transient DHBV infection is concomitant with an extremely rapid rise of intrahepatic DuIFN-γ RNA levels, which is thereafter followed by a decrease (Narayan et al., 2006). We demonstrate here that EP-treated group had significantly higher mean intrahepatic DuIFN-γ RNA levels than those of the SI-treated group and the control group (p < 0.001). Although all EP-treated ducks exhibited a break of immune tolerance, the fact that not all of them resolved the infection could be explained by the relatively late induction of anti-preS antibody response and the late expression of DuIFN-γ response. Interestingly, the highest DuIFN-γ RNA level observed for EP-treated duck (no. 454) was associated with the late drop in viremia, the strong seroconversion to anti-preS, the presence of traces of cccDNA and a marked portal infiltration, therefore confirming that this animal was in an advanced stage of infection clearance. Thus, our findings suggest a significant benefit of EP-based pDNA delivery for restoration of the intrahepatic IFN-γ response in all DHBV-carriers that may contribute to viral clearance from their livers. We were unable to monitor other features of the Th1 response in this study, since the tools for analysis of duck cellular response are still under development (\_ENREF\_34\_ENREF\_34).

The safety of an intensive therapeutic protocol including 12 injections of HB-100 DNA vaccine at 4 weeks interval has been previously reported in chronic hepatitis B carriers (Yang et al., 2012), indicating that further studies exploring such novel DNA vectors are still under development. In conclusion, this preclinical study provides the first report on the benefit of EP-based pDNA delivery, able to markedly restore multi-specific B-cell response and to sustainably decrease and even clear viral infection in chronic hepadnavirus-carriers. Additional improvements in the therapeutic protocol of EP-based pDNA delivery should be carried out such as optimization of the vector construct so as to minimize the amount of the pDNA that needs to be delivered, evaluation of the number of DNA injections/sites, and the immunization schedule. Since our results represent the net effect of DNA vaccine, the combination of EP-based pDNA delivery with approved antiviral treatments (Tenofovir, Entecavir) deserves to be further investigated. Thus a first clinical trial showed already encouraging results (Yang et al., 2012), indicating that further studies exploring such novel DNA vectors are still under development.

Materials and methods

Animals

Chronic DHBV-carrier ducks were obtained by intravenous inoculation of 3-day-old Pekin ducklings with a viremic serum pool (2 × 10^{10} viral genome equivalents (vge) per animal) as described previously (Cova and Zoulim, 2004; Rollier et al., 1999). Animal experimentation was performed in accordance with the guidelines for animal care of the ethical committee at the National Veterinary School of Lyon (Marcy l’Etoile, France).

Plasmid expression vectors

pCI-preS/S, pCI-C, pCI-INF\_\gamma plasmids were constructed and verified for the expression of DHBV preS/S, core, and DuIFN-γ respectively, as described previously (Rollier et al., 1999; Saade et al., 2008; Thermet et al., 2004). Plasmids were purified using Endotoxin-Free Giga Prep (Qiagen, Hilden, Germany).
Three weeks after hatching, the DHBV positivity of each duck was assessed by dot blot hybridization. DHBV-infected ducks were randomly assigned into 3 groups, which were intramuscularly (i.m) injected with a total of 600 µg of pDNA. Treated groups received i.m. injections of pCI-preS/S, pCI-C and pCI-FN-γ delivered either by a TriGrid electroporation (EP) device (Ichor Medical Systems, San Diego, CA) (group EP, n = 6) or by standard injection (group SI, n = 5). A control group included untreated ducks (n = 3) and pCI (empty plasmid, n = 2) injected ducks via EP was followed up in parallel. The DNA injections were performed in two sites; in breast and shoulder. EP was applied after general anesthesia with a four-needle electrode array using the TriGrid delivery device, with 6 mm intraelectrode spacing and electrical stimulation at amplitude of 250 V/cm. The total duration of electrical stimulation was 40 ms, applied over a 400 ms interval. Booster doses were administered at week 6, 9, 16, 21, 25, 29, 33 and 36 post-hatching. After the last immunization, animals were followed during additional 4 weeks and were thereafter sacrificed. Autopsy liver samples were snap-frozen in liquid nitrogen and stored at −80 °C before use for analysis described below.

Serological assays

Viremia was assessed by quantitative detection of DHBV DNA in duck serum using dot blot hybridization assay described elsewhere (Cova and Zoulim, 2004; Le Guerhier et al., 2000). The anti-preS antibody titers were determined on serum samples from each duck at each time point of follow-up using an end-point dilution ELISA titration assay described previously (Chassot et al., 1994; Rollier et al., 2000).

Screening of induced antibodies in ducks for pre-S B-cell epitopes recognition

The antibody response of individual duck sera to DHBV pre-S linear epitopes were analyzed using the PEPSAN method (Khawaja et al., 2012). Briefly, a pepset of 77 9-mers peptides with an overlap of 7 amino acids, spanning the entire DHBV pre-S protein (residues 1–166) was synthesized by Mimotopes peptide company (UK) in the format Biotin-SGSG-PEPTIDE-amide. Each duck serum taken at week 40 was tested for peptide binding as described previously in details (Khawaja et al., 2012).

Analysis of intrahepatic viral DNA replication

Frozen necropsy liver samples were submitted to two independent DNA extraction procedures for isolation of total viral DNA (protein bound) and for cccDNA (non-protein bound) as described previously (Le Guerhier et al., 2000) and then quantified by spectrophotometry. 10 µg of DNA obtained by each preparation were then subjected to Southern blot analysis as described previously (Cova and Zoulim, 2004).

For viral cccDNA quantification by real-time PCR, 500 ng of DNA were subjected to Plasmid-SafeTM ATP-dependent DNase (Epicentre, Madison, USA) digestion according to manufacturer’s recommendations before PCR amplification. For real-time PCR analysis, 4 µl of digestion product was amplified in 1 × SYBR green Supermix (Bio-Rad) with SYBR Green Supermix Kit (Bio-Rad) under the same conditions than those previously described by Narayan et al., 2006 with the selective primers for DuIFN-γ (forward, 5′−CAACATATCCGGATGTACG-3′ and reverse, 5′−GGGTTGATTTAATTAACG-3′). The reaction was standardized using 10-fold serial dilutions (1 × 108 copies to 1 copy) of pCI-DuIFN-γ plasmid samples as described previously in detail (Narayan et al., 2006). All samples from infected and uninfected ducks were tested in duplicate and run together. The detection limit of this assay was estimated to 1 copy of amplicon DNA per reaction. The assay was normalized against the housekeeping gene β-actin tested in the same samples using primers derived from the duck β-actin sequence (forward, 5′−TTGATGAGCTCAGAGC-3′; reverse, 5′−TCCTCAGGGCCCTCTC-3′).

Immunohistological analysis of liver samples

Five μm thickness formalin-fixed liver tissue sections were stained with hematoxylin–eosin–safran and examined under a light microscope. The level of hepatocyte necrosis (acidopholic bodies), steatosis, portal tract and intralobular inflammation were assessed semi-quantitatively under code (i.e. in a blinded manner). Liver sections were analyzed for DHBV pre-S antigen expression and for CD3+ T cells infiltration by immunohistochemistry. Tissue sections were stained with a previously described rabbit polyclonal antiserum raised against DHBV pre-S polypeptide (Lambert et al., 1991) or with an antibody to human CD3 (Clone F7.2.38, DakoCytomation). All slides were counterstained with haematoxylin.

Statistical analysis

The efficiency of therapies on viremia, anti-preS titers and liver viral cccDNA levels of experimental groups were compared using the nonparametric Mann–Whitney U test to determine the significance between groups. These statistical analyses were performed using GraphPad Prism 4.0 software (San Diego, CA). P values < 0.05 were considered statistically significant.

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