DNA vaccination in combination or not with lamivudine treatment breaks humoral immune tolerance and enhances cccDNA clearance in the duck model of chronic hepatitis B virus infection

Alexandre Thermet, Thierry Buronfosse, Bettina Werle-Lapostolle, Michele Chevallier, Pierre Pradat, Christian Trepo, Fabien Zoulim and Lucyna Cova

1INSERM, Unit 871, F-69424 Lyon, France
2Université Lyon 1, IFR62, F-69008 Lyon, France
3Ecole Nationale Vétérinaire de Lyon, F-69280 Marcy l’Etoile, France
4Pathology Department, Marcel Mérieux Laboratory, F-69007 Lyon, France
5Hospices Civils de Lyon, Department of Hepatology, Hotel-Dieu, F-69002 Lyon, France

This study used a duck hepatitis B virus (DHBV) model to evaluate whether a novel DNA vaccination protocol alone or associated with antiviral (lamivudine) treatment was able to clear the intrahepatic covalently closed, circular viral DNA (cccDNA) pool responsible for persistence of infection. DHBV carriers received DNA vaccine (on weeks 6, 10, 13, 14, 28 and 35) targeting the large envelope and/or core proteins alone or combined with lamivudine treatment (on weeks 1–8) or lamivudine monotherapy. After 10 months of follow-up, a dramatic decrease in viraemia and liver DHBV cccDNA (below 0.08 cccDNA copies per cell) was observed in 9/30 ducks (30 %) receiving DNA mono- or combination therapy, compared with 0/12 (0 %) from lamivudine monotherapy or the control groups, suggesting a significant antiviral effect of DNA immunization. However, association with the drug did not significantly improve DHBV DNA vaccine efficacy (33 % cccDNA clearance for the combination vs 27 % for DNA monotherapy), probably due to the low antiviral potency of lamivudine in the duck model. Seroconversion to anti-preS was observed in 6/9 (67 %) ducks showing cccDNA clearance, compared with 1/28 (3.6 %) without clearance, suggesting a significant correlation (P<0.001) between humoral response restoration and cccDNA elimination. Importantly, an early (weeks 10–12) drop in viraemia was observed in seroconverted animals, and virus replication did not rebound following the cessation of immunotherapy, indicating a sustained effect. This study provides the first evidence that therapeutic DNA vaccination is able to enhance hepadnaviral cccDNA clearance, which is tightly associated with a break in humoral immune tolerance. These results also highlight the importance of antiviral drug potency and an effective DNA immunization protocol for the design of therapeutic vaccines against chronic hepatitis B.

INTRODUCTION

Despite the existence of an effective vaccine against hepatitis B virus (HBV), chronic hepatitis B remains a major health problem worldwide. The efficacy of current chronic hepatitis B treatments, relying on interferon-α and nucleoside analogues, is limited by the emergence of drug-resistant mutants and the persistence of intranuclear covalently closed, circular viral DNA (cccDNA), responsible for viral relapse after treatment withdrawal (Nafa et al., 2000; Lai et al., 2003; Villeneuve et al., 2003; Werle-Lapostolle et al., 2004; Zoulim, 2004). Recently, the concept of combination therapy has been proposed from initial observations in patients indicating that antiviral drug treatment, aimed at lowering viral load, can transiently restore antiviral immune responses, which can be stimulated in a sustained manner by an effective immunotherapy (Boni et al., 2001; Horiike et al., 2005).

Within different immunotherapeutic strategies aimed at restoring or reactivating HBV-specific immune responses, DNA-based vaccination appears to be a particularly pertinent approach, as, due to the endogenous synthesis of antigens, it is able to activate both humoral and cellular arms
of the antiviral response (Michel & Loirat, 2001; Cova, 2007). Moreover, the effectiveness of DNA vaccination can be considerably enhanced by adding immunostimulatory sequences, co-delivery of cytokine genes, modulation of DNA delivery and optimization of the DNA immunization schedule (Leitner et al., 1999; Cova, 2007). Several studies have demonstrated the ability of DNA vaccines to induce potent and specific immune responses against human HBV, woodchuck HBV (WHV) and duck HBV (DHBV) proteins in naïve mouse, chimpanzee, woodchuck and duck models (Michel et al., 1995; Davis et al., 1996; Triyatni et al., 1998; Lu et al., 1999; Rollier et al., 1999, 2000a, b; Michel & Loirat, 2001; Thermet et al., 2004; Wang et al., 2007). However, limited data are available on the immunotherapeutic efficacy alone or in combination with antiviral treatment (Rollier et al., 1999; Michel & Loirat, 2001; Foster et al., 2003; Le Guerhier et al., 2003). The combination of a DNA vaccine with antiviral drugs has, to date, been addressed only in a chronic DHBV infection model showing an absent or modest cccDNA clearance in protocols associating DNA vaccine therapy with entecavir (Foster et al., 2003) or adefovir (Le Guerhier et al., 2003).

The development of a therapeutic DNA vaccine has accelerated recently, with first clinical trials showing its safety and ability to activate T-cell responses in some HBV patients with lamivudine resistance, although no sustained serum HBV DNA clearance was achieved (Mancini-Bourgine et al., 2004). In addition, a proof-of-concept study in HBV carriers showed that association of lamivudine with a DNA vaccine comprising the HBV genes plus interleukin-12 DNA resulted in a detectable HBV-specific Th1-cell response, which was associated with a marked decrease in viraemia (Yang et al., 2006). However, the ability of such DNA vaccine-based immunotherapy to clear intrahepatic HBV cccDNA is largely unknown. Recent studies have pointed out a high stability of the cccDNA pool, a viral mini-chromosome responsible for persistence of infection, suggesting an urgent need for novel therapeutics that could enhance cccDNA elimination (Addison et al., 2002; Werle-Lapostolle et al., 2004).

The DHBV-infected duck is a reference model for evaluation of novel anti-HBV approaches and testing of their ability to clear viral cccDNA (Mason et al., 1994; Le Guerhier et al., 2000, 2003; Seigneres et al., 2003; Cova & Zoulim, 2004). Short-term antiviral drug treatment of DHBV carriers is usually associated with a rapid rebound in viraemia after therapy has been discontinued, indicating that cccDNA turnover is low. When treatment is continued for many months, a rebound in viraemia correlates with cccDNA persistence occurring after the cessation of drug treatment, as documented in various studies, even following administration of a combination of potent antiviral drugs (Mason et al., 1994; Le Guerhier et al., 2000; Addison et al., 2002; Foster et al., 2003; Seigneres et al., 2003).

There are relevant differences in the outcome of DHBV infection in ducks compared with HBV. Thus, in contrast to HBV infection of humans, which may have a transient or chronic outcome, DHBV infection of adult Pekin ducks is frequently self-limiting, indicating that immune control of viral infection works differently in humans and ducks. DHBV infection of newly hatched ducklings results in 100% chronic infection, similar to neonatal HBV infection of humans. This approach was used in the present study to establish a large flock of chronic DHBV carriers of the same age.

In this study, we carried out a long-term follow-up of chronic DHBV carrier ducks to study the antiviral efficacy of a novel DNA vaccine protocol targeting the envelope and/or core proteins, alone or in association with lamivudine treatment, focusing on its ability to enhance cccDNA clearance and to restore antiviral humoral responses. We report, for the first time, that DNA immunization was able to drastically suppress intrahepatic viral cccDNA in about one-third of DHBV carriers, and was tightly associated with an early drop in viraemia and a break in humoral immune tolerance. In addition, there was no rebound of virus replication after the cessation of immunotherapy. These findings are of particular interest, because, despite numerous studies investigating the efficacy of antiviral treatments in the chronic DHBV infection model, clearance of the cccDNA pool has not yet been reported.

**METHODS**

**Drug.** Lamivudine [β-D-3′,2′,3′-dideoxy-3′-thiacytidine], generously provided by J. Rhodes (GlaxoSmithKline), was dissolved in PBS (pH 7.2).

**Plasmid expression vectors.** DHBV large envelope (preS/S) and core (C) genes were cloned previously in pC1 vector and are referred to pC1-preS/S (Rollier et al., 1999) and pC1-C (Thermet et al., 2004), respectively. Plasmids were purified using an Endotoxin-free Giga Preparation kit (Qiagen).

**Animals.** Chronic DHBV infection was obtained by intravenous inoculation of 3-day-old Pekin ducklings with a viraemic serum pool [2 × 10^8 viral genome equivalents (vge) per animal] as described previously (Rollier et al., 1999; Cova & Zoulim, 2004). Animal experimentation was performed in accordance with the guidelines for animal care of the ethics committee of the National Veterinary School of Lyon.

**Therapeutic protocol.** DHBV-infected ducks were randomly assigned into eight groups (six to nine animals per group) with treatment protocols as follows: groups 1–3: combination of lamivudine with DNA vaccine against envelope, envelope and core, or core alone, respectively; group 4: lamivudine monotherapy; groups 5–7: DNA vaccine monotherapy against envelope, envelope and core, or core alone, respectively; group 8: untreated or empty vector-immunized controls (Table 1, Fig. 1). Lamivudine was administered to groups 1–4 at 25 mg kg^-1 day^-1 via the intraperitoneal route for 5 consecutive days of week 1 post-infection (p.i.) and three times a week for the following 7 weeks. DNA immunization consisted of multiple intramuscular injections (Rollier et al., 1999) of 300 μg endotoxin-free plasmid DNA per animal (on weeks 6, 10 and 14), followed by two delayed boosts at weeks 28 and 35 (Fig. 1).

**Duck liver biopsy and autopsy.** Liver biopsies were performed under general anaesthesia using 15 mg ketamine kg^-1 and 15 mg
Table 1. Summary of the therapeutic protocol for each group

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Group</th>
<th>Lamivudine</th>
<th>DNA vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combination therapy</td>
<td>1</td>
<td>+</td>
<td>PreS/S</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>PreS/S + core</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>Core</td>
<td></td>
</tr>
<tr>
<td>Lamivudine monotherapy</td>
<td>4</td>
<td>+</td>
<td>Vector or none</td>
</tr>
<tr>
<td>DNA monotherapy</td>
<td>5</td>
<td>–</td>
<td>PreS/S</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>PreS/S + core</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>Core</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>–</td>
<td>Vector or none</td>
</tr>
</tbody>
</table>

*Combination therapy groups 1–3 received lamivudine (weeks 1–8) plus DNA immunizations starting from week 6 p.i. with plasmids encoding either the viral envelope (group 1), the envelope and core (group 2), or the core alone (group 3), whereas DNA monotherapy groups 5–7 received an identical schedule of plasmid DNA immunizations but in the absence of lamivudine. Lamivudine monotherapy (group 4) received antiviral treatment alone or together with empty-vector immunizations. Untreated or empty vector-immunized controls (group 8) were followed in parallel. The weight of all animals was monitored regularly during the follow-up (10 months).

ELISA analysis of the anti-preS response. The humoral anti-preS response of ducks was determined in a direct ELISA using recombinant preS protein as described previously (Chassot et al., 1994; Rollier et al., 2000a). The end-point titres were taken as the reciprocal of the highest serum dilution that gave an A405 value above the mean signal of two replicates of control sera (Rollier et al., 2000a).

Analysis of viraemia. Viraemia was assessed by serum DHBV DNA detection using a previously described dot-blot hybridization assay (Le Guerhier et al., 2000; Cova & Zoulim, 2004). Filters were scanned using a PhosphorImager (Amersham Pharmacia Biotech) and counted using ImageQuant software as described previously (Cova & Zoulim, 2004). The limit of DHBV DNA detection was 0.5 pg.

Analysis of intrahepatic viral DNA. Total DNA was extracted from biopsy or autopsy liver samples according to procedures described previously (Le Guerhier et al., 2003; Cova & Zoulim, 2004). In addition, specific extraction of non-protein-bound cccDNA was performed as described previously (Le Guerhier et al., 2003; Cova & Zoulim, 2004). Total DNA or the cccDNA preparation (10 μg) was then subjected to Southern blot analysis as described by Cova & Zoulim (2004).

Viral cccDNA quantification using real-time PCR. The selective quantification of DHBV cccDNA was similar to that described for HBV cccDNA quantification (Werle-Lapostolle et al., 2004) except that primers were designed for amplification of a 272 bp DHBV cccDNA fragment. To enhance the specificity of cccDNA detection, 500 ng extracted liver DNA was digested for 1 h in a 20 μl final volume using 2 U Plasmid-safe DNase (Epicentre) to degrade the relaxed-circle and single-stranded forms of the viral DNA, followed by DNase inactivation at 70 °C for 30 min (Werle-Lapostolle et al., 2004). Four microlitres of this mixture was used for real-time PCR amplification in a LightCycler (Roche) as described previously in detail (Narayan et al., 2006), except that the primers used for amplification were 5’-GTCTGTTGCTCAGC-3’ (cccDS1 nt 2554–2571) for the forward primer and 5’-CCCTCTGTGAGTCTTCGAG-3’ (cccDS1 nt 2826–2843) for the reverse primer. All samples were analysed in duplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification was used for normalization of liver samples as described previously (Seignes et al., 2003; Narayan et al., 2006). The detection limit of this assay was estimated to be 0.04 copies of cccDNA per cell, after spiking of intrahepatic DNA from DHBV-negative duck livers with serial dilutions of plasmid containing DHBV monomer used as quantification standards. The cut-off was set as 0.08 cccDNA copies per cell (twofold detection limit).

Histological analysis of liver samples. Formalin-fixed liver tissue sections (3 μm) were stained with haematoxylin/eosin/safran, coded and examined blind under a light microscope.

Statistical analysis. A Mann–Whitney test was used to compare the efficiency of the therapies on viraemia. The association between DNA vaccination and cccDNA clearance, or between cccDNA clearance and seroconversion, was analysed using Fisher’s exact test. A level of P<0.05 was considered to be statistically significant.

RESULTS

Effect of therapy on DHBV viraemia

Lamivudine treatment led to a drop of about 70% in the mean values of the viraemia peak in weeks 1–3 (P<0.001) in all drug-treated (groups 1–4) compared with untreated (groups 5–8) ducks, although this effect was limited to the first 3 of 8 weeks of treatment (Fig. 2 and data not shown). During the following weeks, a decrease and fluctuations in viraemia were observed without significant differences between the mean viraemia values among duck groups (Fig. 2 and data not shown).

However, analysis of individual viraemia titres at the end of the follow-up period revealed an absence of detectable viraemia in the sera of a number of animals: nos 172, 173,
Fig. 2. Follow-up of individual viraemia levels in chronic DHBV carriers. Viraemia was monitored throughout the study period by DHBV DNA quantification using a dot-blot hybridization assay; a logarithmic scale of viraemia levels (vge ml$^{-1}$) for each duck is shown. DHBV carriers received combination therapy (a–c), lamivudine monotherapy (d) or DNA monotherapy (e–g). Untreated or empty vector-immunized controls are shown in (h). Individual viraemia levels are shown for ducks from groups 1–8 in (a)–(h), respectively. The bar represents the lamivudine (3-TC) treatment period. Arrows indicate DNA injections at weeks 6, 10, 14, 28 and 35.
174, 175 and 180 (group 1); nos 192, 197 and 199 (group 2); no. 211 (group 4); no. 218 (group 5); nos 239, 241 and 242 (group 6); nos 231, 232 and 233 (group 7); and nos 245 and 248 (group 8) (Fig. 2a–h). Interestingly, the fall in viraemia levels occurred relatively late, i.e. around weeks 20–28 for some DNA-vaccinated ducks and controls (nos 173, 174, 192, 197, 199, 211, 218, 239, 241, 242, 245 and 248), and was only rarely associated with intrahepatic viral cccDNA clearance (nos 192 and 242), and only two ducks (nos 218 and 242) mounted a detectable anti-preS response (Figs 2 and 3 and see below). In contrast, for DNA-vaccinated animals (nos 172, 175, 180, 231, 232 and 233) exhibiting an early drop in viraemia starting as early as weeks 10–12 p.i., there was a tight correlation with viral cccDNA elimination (Figs 2 and 3 and see below). In addition, this very early and drastic drop in viraemia levels was associated for all but one (no. 233) of these ducks with seroconversion to anti-preS (Fig. 2a, g; Fig. 3). This is further illustrated for duck no. 172 (group 1), in which viraemia levels had dropped by week 10 p.i., concomitantly with a rise in anti-preS response (Fig. 4). No rebound in viraemia was observed in these animals following DNA-based immunotherapy cessation up to the end of the follow-up period (week 40).

**DNA-based immunotherapy suppresses intrahepatic virus replication**

At week 8, i.e. at the end of lamivudine treatment, biopsies were taken from a few randomly selected animals (two ducks per group). Analysis revealed no differences in the intrahepatic DHBV DNA levels in ducks from different groups (groups 1–8), although these randomly chosen ducks did not correspond to animals that resolved infection at the end of the follow-up period (data not shown).

At the end of the 10 month follow-up period, we examined the impact of therapies on total DHBV DNA replicative intermediates in all autopsy liver samples by Southern blotting (Fig. 5, Table 2). All ducks on lamivudine monotherapy (group 4) had replicative DHBV DNA intermediates, except for duck no. 211, which showed very low but detectable levels of cccDNA (Fig. 5, Table 2). The presence of viral DNA intermediates was also observed for all but one control duck (no. 245), which received empty-vector immunization (group 8). By contrast, DHBV DNA, including cccDNA, was undetectable in the livers of several animals that received the specific DNA vaccine in association with lamivudine treatment (4/8 ducks in group 1; 2/3 ducks in group 2) or as DNA monotherapy (2/4 ducks in group 6; 3/7 ducks in group 7) (Fig. 5, Table 2).

To determine whether residual cccDNA might persist in these 12 liver samples showing undetectable DHBV DNA by Southern blotting, we searched for cccDNA clearance defined as cccDNA levels below the cut-off of real-time PCR, i.e. 0.08 cccDNA copies per cell. Thus, a control animal (no. 245, group 8), receiving empty-vector immunization, which had undetectable cccDNA levels by Southern blotting, showed cccDNA levels of 0.34 copies per cell, which were clearly detectable by real-time PCR (Fig. 3). Review of individual treatment groups (Figs 3 and 5, Table 2) showed no significant difference in terms of cccDNA clearance between animals receiving combination therapy and those receiving DNA monotherapy (3/8 vs 0/4 for DNA vaccine targeting the envelope, \( P=0.49 \); 2/3 vs 1/4 for DNA vaccine targeting the S protein, \( P=0.72 \)).
Further comparison indicated that a total of 5/15 (33 %; nos 172, 175, 180, 192 and 197) of ducks on combination therapy (groups 1–3) compared with 4/15 (27 %; nos 242, 231, 232 and 233) on DNA monotherapy (groups 5–7) cleared the cccDNA, indicating that association with the drug did not significantly \( (P = 1) \) improve DHBV DNA vaccine efficacy (Fig. 5, Table 2). Importantly, the decrease in cccDNA levels to below 0.08 cccDNA copies per cell was observed in 9/30 ducks (30 %) receiving DNA monotherapy or combination therapy, compared with 0/12 (0 %) from the lamivudine monotherapy or control groups, suggesting a significant \( (P = 0.041) \) antiviral effect of DNA immunization (Fig. 3, Table 2).

**Correlation between virus clearance and a break in humoral immune tolerance**

The ability of the DNA vaccine to induce an anti-preS and anti-C humoral response was first assessed in naïve ducks, showing a significant humoral response that reached a plateau level after the second DNA boost and was maintained by successive boosts (Fig. 6). No differences were found in the magnitude and kinetics of the anti-preS response following pCI-preS/S immunization alone or in association with the pCI-C plasmid (Fig. 6).

Next, we asked whether the humoral immune tolerance could be broken in DHBV carriers by a DNA vaccine and whether it could be correlated with viral cccDNA clearance. Among the DHBV carriers that did not clear cccDNA, only one duck (no. 218, group 5) mounted an anti-preS
No restoration of the anti-preS response was observed in the control and lamivudine monotherapy groups, including ducks nos 245 and 211, which showed a late drop in viraemia (at weeks 28 and 37, respectively), that correlated with an undetectable anti-preS response, and their residual cccDNA presence was clearly detectable by PCR (Figs 2, 3 and 5; Table 2), suggesting that these animals had not completely resolved the DHBV infection. Interestingly, seroconversion to anti-preS was observed in 6/9 animals that cleared cccDNA; 3/5 (nos 172, 175 and 180) belonged to the combination therapy group and 3/4 (nos 231, 232 and 242) belonged to the DNA monotherapy group (Fig. 3, Table 2). This seroconversion could be observed after the second or third DNA injection and was maintained by additional boosts as illustrated in Fig. 4 for a representative duck (no. 172) from combination therapy group 1. Therefore, seroconversion to anti-preS was observed in 6/9 ducks (67 %) with cccDNA clearance, compared with only 1/28 ducks (3.6 %) without clearance, whatever the treatment group (P<0.001), suggesting a significant correlation between viral cccDNA clearance and restoration of the humoral response in these animals. In addition, seroconversion to anti-preS was tightly correlated with a very early drop in viraemia (weeks 10–12), which was observed for five of these six ducks, and there was no rebound in virus replication in these animals following the cessation of immunotherapy, indicating a long-lasting effect.

Absence of adverse effects of therapy

No significant variations in animal weight were observed, indicating that the treatments were well tolerated (data not shown). During this long-term (10 month) study, a similar number of coincidental deaths occurred within the groups, whatever the protocol used. Liver histology showed the occasional presence of steatosis, amyloidosis and mild-to-moderate inflammatory infiltrates in the duck livers without marked differences among the different treatment groups (data not shown).

**Table 2. Effect of therapy on intrahepatic virus clearance and restoration of anti-preS humoral responses**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>DNA vaccine</th>
<th>Undetectable DHBV DNA in Southern blotting*</th>
<th>cccDHBV DNA clearance assessed by real-time PCR†</th>
<th>Anti-preS response‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combination therapy</td>
<td>1</td>
<td>PreS/S</td>
<td>4/8</td>
<td>3/8</td>
<td>3/8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>PreS/S + core</td>
<td>2/3</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Core</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>DNA monotherapy</td>
<td>5</td>
<td>PreS/S</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>PreS/S + core</td>
<td>2/4</td>
<td>1/4</td>
<td>1/4</td>
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<tr>
<td></td>
<td>7</td>
<td>Core</td>
<td>3/7</td>
<td>3/7</td>
<td>2/7</td>
</tr>
<tr>
<td>Lamivudine monotherapy</td>
<td>4</td>
<td>Vector or none</td>
<td>0/7</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>Vector or none</td>
<td>1/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

*Number of ducks presenting undetectable liver DHBV DNA/total number of treated ducks, as assessed by Southern blot analysis of autopsy liver samples.
†Intrahepatic viral cccDNA clearance was defined as cccDNA levels below 0.08 cccDNA copies per cell and was expressed as the number of ducks with cccDNA clearance/total number of treated ducks. Note that the number of ducks that cleared cccDNA was not significantly different (P=1) between combination therapy (5/15 for groups 1–3) and DNA monotherapy (4/15 for groups 5–7). However, a significant difference in cccDNA clearance (P=0.041) was found between DNA mono- and combination therapy-treated ducks (9/30, 30 %) and lamivudine therapy and untreated controls (0/12, 0 %).
‡Number of animals that seroconverted to anti-preS/total number of treated ducks.
DISCUSSION

We explored here whether a novel DNA vaccine-based protocol in combination or not with an antiviral drug (lamivudine) treatment was able to clear virus infection and break the humoral immune tolerance in chronic DHBV carriers. Analysis of viraemia and autopsy liver samples after 10 months of follow-up revealed that lamivudine monotherapy was ineffective in elimination of virus, whereas DNA vaccine mono- or combination therapy led to the resolution of infection in several animals, which had undetectable viraemia and replicative DHBV DNA intermediates as assessed by Southern blotting. As studies in animal models and in chronically infected patients have clearly demonstrated the crucial role of the hepadnaviral cccDNA pool in the persistence and reactivation of virus replication (Mason et al., 1994; Moraleda et al., 1997; Addison et al., 2002; Werle-Lapostolle et al., 2004; Le Mire et al., 2005), it was of particular importance to search for residual cccDNA in ducks that had apparently resolved infection, using a highly sensitive quantitative PCR, similar to the assay recently developed for HBV cccDNA detection (Werle-Lapostolle et al., 2004). We report here a dramatic decrease in the levels of liver DHBV cccDNA (below 0.08 cccDNA copies per cell) in 9/30 (30 %) ducks receiving DNA mono- or combination therapy, compared with 0/12 (0 %) receiving lamivudine monotherapy or control groups, suggesting a significant (P<0.05) antiviral effect of the DNA vaccine-based immunotherapy. No significant differences in cccDNA clearance were noted among individual treatment groups immunized with DNA vaccines targeting the viral envelope, core, or envelope and core. The efficacy of the preS/S vaccine appeared to be superior when combined with lamivudine (clearance in 3/8 ducks) compared with preS/S vaccine monotherapy (clearance in 0/4 ducks), whereas DNA vaccine targeting the core tended to be more effective in DNA monotherapy (clearance in 3/7 ducks) compared with combination therapy (clearance in 0/4 ducks) (Table 2). However, statistical analysis of these data did not show significant differences among groups, probably due to the relatively small number of animals per group as a result of coincidental mortality of ducks during this 10 month follow-up.

Whether trace amounts of cccDNA, undetectable by the real-time PCR in our study, are inert and non-replicative, as suggested by Le Mire et al. (2005), or whether such residual DNA persists, leading to replication-competent virion production as recently demonstrated in a WHV infection model (Menne et al., 2007), is unknown and deserves further investigation.

It was of interest to note that one empty-plasmid-immunized control duck (no. 245) had undetectable levels of cccDNA by Southern blotting. This is not surprising, as the plasmid backbone contains CpG sequences, which are known to have potent immunostimulatory properties (Leitner et al., 1999); thus, the non-specific stimulation of immune responses may have enhanced spontaneous virus elimination in this animal. However, its cccDNA level was 0.34 copies per cell, i.e. at least fourfold higher than the cut-off for the real-time PCR, and correlated with a late drop in viraemia and an absence of seroconversion to anti-preS, indicating that this carrier had not eliminated DHBV infection.

The concept of combination therapy relies on a synergistic association of a potent antiviral treatment, leading to a marked reduction in viral load and transient restoration of HBV-specific immune responses, with an effective immunotherapy able to stimulate these responses in a sustained manner (Boni et al., 2001). We have previously reported that the combination of adefovir with a DNA vaccine against DHBV led to an additive effect in terms of overall decrease in viraemia and liver DHBV DNA (Le Guerhier et al., 2003). In that study, a potent and sustained antiviral effect of adefovir was observed, leading to a drastic drop in viraemia to undetectable levels during the 4 weeks of drug administration (Le Guerhier et al., 2003). By contrast, in the present study, the decrease in mean viraemia titres did not exceed 70 % and was limited to only the first 3 of 8 weeks of lamivudine treatment, indicating a low antiviral potency of lamivudine in the DHBV infection model. This is consistent with previous observations showing that lamivudine exhibits only moderate anti-DHBV activity, which was 30 times less potent compared with B-L-FD4C (β-L-2',3'-dideoxy-2',3'-didehydro-5-fluorocytidine) (Le Guerhier et al., 2000; Tomita et al., 2000) or adefovir at a similar dose, i.e. 25 mg kg⁻¹ day⁻¹ (Le Guerhier et al., 2003). The dose of 25 mg kg⁻¹ day⁻¹ was previously found to induce the best antiviral effect in lamivudine-treated ducks without liver cell damage, as toxicity was observed following administration of a higher lamivudine dose (Le Guerhier et al., 2000; unpublished observations). Such a low antiviral potency of lamivudine in the duck model may explain why association with the drug did not significantly (P>0.05) improve DHBV DNA vaccine efficacy (33 % cccDNA clearance for combination therapy vs 27 % for DNA monotherapy). Interestingly, in the adefovir–DNA study, only a modest effect on viral cccDNA clearance and seroconversion was observed, in spite of the higher antiviral potency of the drug (Le Guerhier et al., 2003), whereas we demonstrated here a potent effect of DNA vaccine-based immunotherapy that was able to dramatically suppress, by at least 1000-fold, the cccDNA levels in DHBV carriers. As the present study differs from the adefovir–DNA combination study not only by choice of antiviral drug, but, importantly, also by the design of the DNA immunization protocol, other factors may play a key role in the potent antiviral efficacy of the DNA vaccine-based protocol presented herein, such as: (i) the larger amounts of plasmid DNA (300 vs 150 μg DNA per animal per boost), (ii) the larger number of DNA boosts (seven compared with five), including two delayed boosts, and (iii) the longer DNA immunization schedule (35 vs 22 weeks).

Seroconversion to anti-preS was observed in 6/9 ducks (67 %) that cleared cccDNA, compared with 1/28 (3.6 %) without clearance, suggesting a significant correlation...
(P<0.001) between viral cccDNA clearance and humoral response restoration. Interestingly, seroconversion was also tightly associated with an early drop in viraemia (weeks 10–11), which was observed for 5/6 of these ducks. By contrast, a large majority of animals exhibiting a late drop in viraemia only rarely mounted a detectable anti-preS response or cleared cccDNA. However, not all carriers that completely cleared virus replication mounted a detectable anti-preS response. This could be related to the presence of immune complexes in their serum, which hamper antibody detection. It was highly interesting that, out of six ducks that mounted an anti-preS response, four received pCI-preS/S plasmid immunization, whereas two were immunized with the pCI-C plasmid. Thus, a potent stimulation of the immune response with a DNA vaccine targeting the hepadnavirus core can break immune tolerance status in chronic carriers and induce a humoral response targeting the envelope. In the woodchuck model, a DNA vaccine targeting the WHV core induced a strong Th2 response that also provided help for the development of anti-WhSAg antibodies (Lu et al., 2005). Whether DHBcAg-specific helper Th cells induced by DNA vaccination of DHBV carriers can help B cells to produce antibody against viral envelope is not known and deserves further study. In this regard, we were unable to investigate the role of the T-cell response in virus elimination, as the tools for duck cellular response analysis are still under development (Narayan et al., 2006); this should provide a better understanding of the basic mechanism of immunity to DHBV infection and its modulation during viral clearance.

An analysis of the safety of the different therapeutic agents showed an absence of adverse effects of DNA-based vaccination against the viral core and/or envelope in combination or not with lamivudine. Several deaths occurred during this 10 month follow-up; however, they were coincidental, and liver histology revealed the occasional presence of changes that are known to occur in DHBV-infected adult Pekin ducks (Rollier et al., 1999; Le Guerhier et al., 2003), regardless of treatment group.

In conclusion, our report provides the first evidence of the ability of therapeutic DNA immunization to clear viraemia and intrahepatic hepadnaviral cccDNA in chronic virus carriers. This is particularly important in view of novel anti-HBV immunotherapeutic strategy development, as antiviral drugs tested to date in animal models of hepadnaviral infection are unable to cure viral infection, because rebound in viraemia, which correlates with cccDNA detection, has constantly been observed after the cessation of therapy. Although no significant benefit of lamivudine association with the DHBV DNA vaccine was found in this study, we demonstrated that an effective DNA immunization protocol led to cccDNA clearance in about one-third of carriers and was tightly associated with an early drop in viraemia and a break in humoral immune tolerance. Importantly, the antiviral effects of the DNA vaccine-based immunotherapy were long-lasting, as there was no rebound of virus replication several weeks after immunotherapy withdrawal. A number of factors determining the effectiveness of genetic vaccination such as the amount of plasmid DNA, number of DNA injections and immunization schedule may play a crucial role in the higher therapeutic potency of this novel DNA immunization protocol compared with previous studies in the chronic DHBV-carriage model. Additional improvements in DNA-based vaccines should be carried out in animal models for future clinical developments in patients chronically infected with HBV.

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