Vaccination of ducks with a whole-cell vaccine expressing duck hepatitis B virus core antigen elicits antiviral immune responses that enable rapid resolution of de novo infection

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Abstract

As a first step in developing immuno-therapeutic vaccines for patients with chronic hepatitis B virus infection, we examined the ability of a whole-cell vaccine, expressing the duck hepatitis B virus (DHBV) core antigen (DHBcAg), to target infected cells leading to the resolution of de novo DHBV infections. Three separate experiments were performed. In each experiment, ducks were vaccinated at 7 and 14 days of age with primary duck embryonic fibroblasts (PDEF) that had been transfected 48 h earlier with plasmid DNA expressing DHBcAg with and without the addition of anti-DHBcAg (anti-DHBc) antibodies. Control ducks were injected with either 0.7% NaCl or non-transfected PDEF. The ducks were then challenged at 18 days of age by intravenous inoculation with DHBV (5 × 10^8 viral genome equivalents). Liver biopsies obtained on day 4 post-challenge demonstrated that vaccination did not prevent infection of the liver as similar numbers of infected hepatocytes were detected in all vaccinated and control ducks. However, analysis of liver tissue obtained 9 or more days post-challenge revealed that 9 out of 11 of the PDEF-DHBcAg vaccinated ducks and 8 out of 11 ducks vaccinated with PDEF-DHBcAg plus anti-DHBc antibodies had rapidly resolved the DHBV infection with clearance of infected cells. In contrast, 10 out of 11 of the control unvaccinated ducks developed chronic DHBV infection. In conclusion, vaccination of ducks with a whole-cell PDEF vaccine expressing DHBcAg elicited immune responses that induced a rapid resolution of DHBV infection. The results establish that chronic infection can be prevented via the vaccine-mediated induction of a core-antigen-specific immune response.

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Keywords: Duck hepatitis B virus; Duck hepatitis B virus core antigen; Whole-cell vaccine; Cell-mediated immunity; Resolution of infection; Chronic virus infection

Introduction

Infection of immuno-competent adults with the human hepatitis B virus (HBV) results in transient infection in 90–95% of cases. However, the remaining 5–10% of adults, as well as 90–95% of neonates, develop chronic infection (Bertoletti and Ferrari, 2003). Worldwide, 350 million people have chronic HBV infection which often leads to liver disease, cirrhosis and hepatocellular carcinoma (Custer et al., 2004; Lavanchy, 2004). These individuals generally have levels of HBV-specific cytotoxic T lymphocytes (CTL) that are low or undetectable by in vitro assays (Bertoletti and Ferrari, 2003; Bertoletti and Naoumov, 2003; Nayersina et al., 1993; Penna et al., 1991; Rehermann et al., 1995), though in vivo, enough reactivity is retained to cause progressive liver damage. Current therapies for chronic HBV infections employ nucleoside analogues or interferon-alpha (IFN-α). However, the efficacy of these treatments is low, with sustained responses and seroconversion from HBV e antigen to anti-HBV e antibodies in only 10–30% of patients (van Zonneveld et al., 2004; Wright, 2004). Thus, the
development of new and improved treatments for chronic infection remains a major goal of HBV research.

Ideally, such new treatments would activate or boost both humoral and cell mediated immunity (CMI) to control and eliminate the infection, as occurs during resolution of a transient HBV infection. Indeed, chronically infected patients who respond to nucleoside analogue and IFN-α therapy appear to do so because their immune response to the virus is more active than in non-responders as they have more active liver disease at the start of therapy (van Zonneveld et al., 2004; Wright, 2004). Better approaches are therefore needed to activate humoral and CMI in a larger proportion of chronically HBV-infected individuals.

A recent attempt at therapeutic vaccination for chronic HBV infection involved a phase I clinical trial in which HBV carriers were vaccinated with DNA vaccines expressing HBV surface antigens (Mancini-Bourgine et al., 2004). Vaccination was correlated with decreases in the level of HBV DNA and transient boosting of HBV-specific interferon-gamma (IFN-γ) producing T cells, but no vaccinated patients resolved their HBV infection. It was therefore unclear if the DNA vaccines induced useful CMI that might have been therapeutic if the magnitude of the responses had been increased.

The efficacy of hepadnavirus vaccines can also be studied in animal models, which allow determination of humoral and CMI, as well as the outcome of viral challenge. A straightforward approach to addressing vaccine efficacy is to determine if the vaccine protects against de novo infection and, if so, to then commence analysis of the underlying immunological mechanisms. Ideally, protection with potential therapeutic value would prime CMI against infected hepatocytes.

Early vaccine studies in hepadnavirus animal models were directed towards generating neutralizing antibodies that protected against primary infection rather than clearance of an established infection. Thus, vaccines containing or expressing hepadnavirus surface antigens administered prior to viral infection were shown to protect animals against the development of chronic infection (Prince et al., 1997; Rollier et al., 1999; Triyatni et al., 1998). A disadvantage of assessing surface antigen vaccines in a virus challenge protocol is that the humoral response to surface, leading to a faster shut down of cell-to-cell spread of virus than in unvaccinated controls. However, in these previous studies, liver tissue was not examined to determine if vaccination: (i) reduced the amount of virus that reached the liver; (ii) slowed virus spread from initially infected hepatocytes or; (iii) enhanced the rate of elimination of infected hepatocytes. Rapid elimination of infected cells is essential for any vaccine strategy that might be used, not just for prevention, but also for treatment of chronic HBV infections.

Thus, the aim of the current study was to investigate the ability of a whole-cell vaccine comprised of primary duck embryonic fibroblasts (PDEF) expressing duck hepatitis B virus (DHBV) core antigen (DHBcAg) to induce an immune response that caused rapid resolution of de novo infections with clearance of infected hepatocytes. A whole-cell PDEF vaccine was used in an attempt to optimize and maximize delivery of viral core antigen to the cell type(s) most likely to potentiate CMI via a process designated cross-priming or cross-presentation (Heath and Carbone, 2001). It has been shown that major histocompatibility complex (MHC) compatibility between the cell donor and vaccinees is not required for this process to occur and that the cells used to present antigen do not have to be viable (Harshyne et al., 2001; Schulz and Reis e Sousa, 2002). The effect of adding anti-DHBcAg antibodies (anti-DHBc) to the PDEF vaccine was also assessed since antigen–immunoglobulin complexes are efficiently taken up and presented to dendritic cells in vivo (den Haan and Bevan, 2002). Four days following the second dose of vaccine, the ducks were challenged with 5 × 10^8 DHBV viral genome equivalents (vge) a dose of DHBV known from previous studies (Foster et al., 2005; Jilbert et al., 1998) to result in the rapid spread of the virus throughout the liver and chronic DHBV infection. Vaccine efficacy was therefore assessed as the ability to prevent the development of chronic DHBV infection.

We show that vaccination with the whole-cell vaccine did not block de novo infection by DHBV, but instead primed an immune response that rapidly cleared infected hepatocytes from the liver, while unvaccinated control ducks developed chronic DHBV infection. The results establish that chronic infection can be prevented via the vaccine-mediated induction of a core-antigen-specific immune response.

**Results**

Three experiments were performed to test the ability of a whole-cell PDEF vaccine expressing DHBcAg to protect 18-day-old ducks against the development of chronic DHBV infection. PDEF-DHBcAg vaccines were prepared as described in Materials and methods. In brief, PDEF were isolated from embryonated (non-homozygous) duck eggs then cultured in vitro for 24 h, transfected by electroporation with plasmid DNA expressing DHBcAg and cultured in vitro for a further 48 h to allow expression of DHBcAg. Ducks were vaccinated by subcutaneous injection with the whole-cell PDEF-DHBcAg vaccine with and without the addition of
anti-DHBc antibodies at 7 and 14 days of age and were challenged at 18 days of age by intravenous inoculation with a dose of DHBV (5 × 10⁸ vge) known to lead to chronic infection in ducks of this age. Control groups were injected prior to virus challenge with either 0.7% NaCl or non-transfected PDEF.

Control and vaccinated ducks were assessed for markers of DHBV infection in the liver at day 4 or 9 post-challenge (p.c.) and again at autopsy. Serum was tested throughout each experiment for the presence of DHBV surface antigen (DHBsAg) and for the development of anti-DHBc and anti-DHBs antibodies. Ducks that were protected from chronic infection did not develop detectable levels of serum DHBsAg but did develop anti-DHBsAg antibodies. Anti-DHBc (anti-DHBs) antibodies were detected in all vaccinated and control ducks with a more rapid onset in the vaccinated groups. At autopsy, carried out at 24–32 days p.c., none of the protected ducks had detectable levels of DHBV DNA in the liver, as assayed by PCR, or DHBsAg-positive hepatocytes, as assayed by immunoperoxidase staining of liver tissue sections. Each experiment is presented in detail below.

Experiment 1

In the first experiment, prior to virus challenge, three groups of five ducks were injected with 0.7% NaCl or with the whole-cell PDEF vaccine expressing DHBcAg alone or in combination with duck anti-DHBc antibodies. Analysis of antibody responses by ELISA showed that ducks vaccinated with the whole-cell PDEF-DHBcAg rapidly developed high titers of anti-DHBc antibodies following vaccination and virus challenge (Figs. 1B, C) compared to the 0.7% NaCl control ducks (Fig. 1A). There were no clear differences in the anti-DHBc antibody responses between ducks injected with the whole-cell

Fig. 1. Experiment 1. Levels of anti-DHBc antibodies (A–C), DHBsAg (D–F) and total anti-DHBs antibodies (G–I) in the serum following vaccination and DHBV challenge. Ducks were injected at 7 and 14 days of age with 0.7% NaCl (A, D, G), PDEF-DHBcAg (B, E, H) or PDEF-DHBcAg + anti-DHBc antibodies (C, F, I) and challenged with 5 × 10⁸ DHBV vge at 18 days of age. Liver tissue was collected by biopsy at 22 days of age (day 4 p.c.) and by autopsy at 47 days of age (day 29 p.c.). Titers of antibodies in each of the vaccinated and control ducks are shown and are expressed as the log of the reciprocal serum dilutions required to achieve an OD of 0.4 at 490 nm. The cut-off for DHBsAg, anti-DHBc and anti-DHBs-positive samples was set at two standard deviations above the average background, obtained by assaying normal duck serum. † = vaccination, ‡ = DHBV challenge, § = autopsy.
PDEF-DHBcAg vaccine alone and the ducks that received PDEF-DHBcAg vaccine plus anti-DHBc antibodies. Following challenge, the whole-cell PDEF-DHBcAg vaccinated ducks also produced anti-DHBs antibodies, a marker of resolution of infection (Figs. 1H, I), whereas most of the 0.7% NaCl-injected controls did not (Fig. 1G).

Analysis of DHBsAg levels in the blood following DHBV challenge provided initial evidence of the effectiveness of the PDEF-DHBcAg vaccination regime. None of the whole-cell PDEF-DHBcAg vaccinated ducks had detectable DHBsAg in the serum at any time following virus challenge (Figs. 1E, F). In contrast, all of the 0.7% NaCl-injected control ducks had detectable serum DHBsAg during the post-challenge follow-up (Fig. 1D). One of the vaccine control ducks (#34:35) had transient viremia detected as serum DHBsAg at 26 days of age (day 8 p.c.) (Fig. 1D). In this duck, the appearance of DHBsAg coincided with production of anti-DHBs antibodies, which persisted from 26 to 47 days of age (day 8–29 p.c.). We assume that these antibodies form complexes with DHBsAg, thereby removing it from the bloodstream. Duck #34:35 went on to have a fully infected liver at autopsy as determined by immunoperoxidase staining of DHBsAg and PCR detection of DHBV DNA in serum (Fig. 2A) and liver (Fig. 2B).

We have previously described the simultaneous detection of circulating anti-DHBs antibodies and DHBsAg during persistent DHBV infection in ducks (Foster et al., 2005). Detection of DHBsAg under these conditions can be difficult as the antibody masks the surface protein antigenic sites. A similar phenomenon is observed during HBV infection where immune complexes formed between circulating HBsAg and anti-HBs antibodies are readily detected in humans with acute and persistent HBV infection (Surelia and Boxall, 1990; Tsai et al., 1995, 1998).

In concordance with the DHBsAg data, PCR analysis of autopsy samples failed to detect DHBV DNA in the serum of PDEF-DHBcAg vaccinated ducks, while the serum of all 0.7% NaCl-injected control ducks was DHBV DNA-positive at week 9 p.c. (Fig. 2A).

Immunoperoxidase staining of liver tissue collected at biopsy on day 4 p.c. revealed similar percentages of DHBsAg-positive hepatocytes in both the PDEF-DHBcAg vaccinated ducks (1.4–3.0% DHBsAg-positive) and 0.7% NaCl-injected controls (0.6–2.7% DHBsAg-positive; Table 1). The level of infiltrating inflammatory cells was moderately increased in the animals that had received the PDEF-DHBcAg vaccines. This inflammation had disappeared by the end of the study at 29 days p.c. At autopsy, none of the PDEF-DHBcAg vaccinated ducks had any detectable DHBsAg-positive cells in the liver, while >95% of hepatocytes were DHBV-infected and stained positive for DHBsAg in the 0.7% NaCl injected control ducks (Table 1). To confirm that the liver of the PDEF-DHBcAg vaccinated ducks was DHBV DNA-negative, total DNA samples were extracted from autopsy liver and subjected to PCR analysis for DHBV DNA. Again, all PDEF-DHBcAg-vaccinated ducks (with and without the addition of anti-DHBc antibodies) had undetectable levels of DHBV DNA (less than 10 copies per 100 ng of extracted liver cell DNA), while the liver of all 5, 0.7% NaCl-injected control ducks, had $6.0 \times 10^5$–$1.7 \times 10^7$ DHBV DNA genomes per 100 ng (Fig. 2B).

**Experiment 2**

The next experiment was performed to determine if protection was due to expression of DHBcAg by the transfected PDEF, or to the antigenicity of the PDEF, as they were not derived from histocompatible or inbred ducks. Three groups of three ducks were vaccinated with non-transfected PDEF or PDEF-DHBcAg with or without the addition of duck anti-DHBc antibodies. All ducks were biopsied on day 4 p.c. and autopsied on day 32 p.c. Again, the humoral immune response in the PDEF-DHBcAg vaccinated ducks was rapid, with many of the ducks producing anti-DHBc antibodies shortly after the first dose of vaccine (Figs. 3B, C). Following DHBV challenge, the ducks vaccinated with the whole-cell PDEF-DHBcAg (Figs. 3B, C) developed high titers of anti-DHBc antibodies more rapidly than the PDEF control ducks where anti-DHBc antibodies were first detected at 28 days of age, on day 14 p.c. (Fig. 3A). Levels of anti-DHBs antibodies fluctuated throughout this experiment in most PDEF-DHBcAg vaccinated animals (Figs. 3H, I). Two of the PDEF vaccine control ducks (#54 and #56; Fig. 3G) also developed detectable anti-DHBs antibodies.

From the biopsy at day 4 p.c., it was clear that all ducks had been successfully infected with DHBV as liver tissue from all PDEF-DHBcAg vaccinated and PDEF control ducks had between 1.0 and 3.3% of DHBsAg-positive hepatocytes.
Unexpectedly, one of the ducks (56) that received the PDEF alone had <0.001% of hepatocytes DHBsAg-positive at day 4 p.c. and at autopsy at day 32 p.c. (Table 1). This duck was also serum DHBsAg-negative (Fig. 3D) but had developed serum anti-DHBc (Fig. 3A) and anti-DHBs antibodies (Fig. 3G), indicating that this duck had been infected but had cleared its DHBV infection.

The levels of serum DHBsAg revealed that 3 out of 3 ducks vaccinated with PDEF-DHBcAg (#57, #58 and #59) and 1 out of 3 ducks vaccinated with PDEF-DHBcAg with added anti-DHBc antibodies (#60) had no detectable DHBsAg present during the study (Figs. 3E, F) and were protected from the development of chronic DHBV infection. Testing of liver at autopsy also showed an absence of DHBV-infected DHBsAg-positive hepatocytes (Table 1). However, 2 ducks that received the PDEF-DHBcAg vaccine in combination with duck anti-DHBc antibodies (#61, #62) that had DHBV-infected cells present on day 4 p.c. were not protected from the development of chronic DHBV infection, and, by day 32 p.c., infection had spread throughout the liver to infect >95% of hepatocytes. The failure of vaccination to prevent the development of chronic DHBV infection demonstrates that the protection afforded by the PDEF-DHBcAg vaccine is not 100% effective. Although duck #62 developed anti-DHBc antibodies slightly later than the other 2 vaccinated ducks in this group (Fig. 3C), anti-DHBc antibodies were still detected earlier than in the PDEF control ducks (Fig. 3A), suggesting that vaccination had induced humoral immunity but the CMI was not sufficient to prevent the development of chronic DHBV infection.

Experiment 3

A third experiment was performed with 2 significant changes, firstly in order to determine if the number of cells in the vaccine had an effect, the number of PDEF in each dose was reduced to ~1/3 of that used in the first 2 experiments (that is, a

### Table 1

<table>
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<th>Vaccine</th>
<th>Duck #</th>
<th>% DHBsAg + ve hepatocytes Day 4 p.c.</th>
<th>% DHBsAg + ve hepatocytes Day 9 p.c.</th>
<th>% DHBsAg + ve hepatocytes autopsy</th>
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<td>36:37</td>
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<td>87:88</td>
<td>nt</td>
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* Liver biopsy tissue was collected in Experiments 1 and 2 at day 4 and in Experiment 3 at day 9 p.c. Autopsy tissue was collected in Experiments 1, 2 and 3 at day 29, 32 and 24 p.c.

b The percentage of DHBsAg-positive hepatocytes was determined by immunoperoxidase staining of ethanol acetic acid fixed liver tissue with anti-preS monoclonal antibodies and counting with an eyepiece graticule as described in the text. The minimum sensitivity of detection of DHBsAg-positive hepatocytes was 0.001% for biopsy and 0.0001% for autopsy liver tissue.
A dose of $1.5 \times 10^7$ PDEF was used compared to $5 \times 10^7$ in Experiments 1 and 2). Secondly, biopsy liver samples were collected at day 9 p.c. to determine if infected cells, detected on day 4 p.c. in Experiments 1 and 2, had been cleared from the liver by day 9 p.c., giving us an indication of the timing of the immune response and cleared of the DHBV-infected hepatocytes in Experiments 1 and 2.

Three groups of three ducks were vaccinated with non-transfected PDEF, or with PDEF-DHBcAg with or without the addition of duck anti-DHBc antibodies. Analysis of serum revealed that no PDEF-DHBcAg vaccinated ducks developed detectable anti-DHBc antibodies prior to challenge. However, following challenge, the levels of anti-DHBc antibodies developed more rapidly in the ducks that received the PDEF-DHBcAg vaccines (Figs. 4B, C) compared to the PDEF controls (Fig. 4A), suggesting that vaccination had primed the production of anti-DHBc antibodies that were boosted following virus challenge. One of the PDEF control ducks (#93:94) did not generate detectable anti-DHBc antibodies even after virus challenge (Fig. 4A), although this animal became chronically DHBV-infected (Table 1). Analyses of the raw data show that duck #93:94 had low levels of anti-DHBc antibodies, but these were below the cut-off for the assay which is set at two standard deviations above the average background, obtained by assaying normal duck serum. The low levels of anti-DHBc antibodies in duck #93:94 could also be explained in
part due to the fact the ducks are outbred, and each duck is likely to respond differently to the virus.

In this experiment, vaccination with the whole-cell PDEF-DHBcAg vaccine again resulted in protection against the development of chronic DHBV infection in 1 out of 3 ducks (#76:77) that received the vaccine alone and 2 out of 3 that received the vaccine plus anti-DHBc antibodies (#82:84, #87:88). In general, the protection afforded in this Experiment was lower than Experiments 1 and 2. This may have resulted from the lower dose of vaccine as each dose of PDEF-DHBcAg was reduced to ∼1/3 of that used in Experiments 1 and 2. Protection against chronic infection was demonstrated by an absence of DHBsAg in the serum (Figs. 4E, F). In concordance with the serum DHBsAg data, these protected ducks had no detectable DHBsAg-positive hepatocytes present in the liver on day 9 p.c. or at autopsy on day 24 p.c. (Table 1). In contrast, all three of the PDEF control ducks and the 3 other whole-cell PDEF-DHBcAg vaccinated ducks were not protected and had

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Fig. 4. Experiment 3. Levels of anti-DHBc antibodies (A–C), DHBsAg (D–F) and total anti-DHBs antibodies (G–I) in the serum following vaccination and DHBV challenge. Three groups of three ducks were injected at 7 and 14 of age with either PDEF (A, D, G), PDEF-DHBcAg (B, E, H) or PDEF-DHBcAg + anti-DHBc antibodies (C, F, I). Liver tissue was collected by biopsy at 27 days of age (day 9 p.c.) and by autopsy at 42 days of age (day 24 p.c.). Titers of antibodies and DHBsAg were determined as described in the legend to Fig. 1. § = vaccination, ¶ = DHBV challenge, ¶¶ = autopsy.
>95% of hepatocytes staining DHBsAg-positive at day 24 p.c. (Table 1).

Anti-DHBs antibodies were detected sooner in the ducks that received the PDEF-DHBcAg vaccine with highest levels in the ducks that were protected from chronic infection (Figs. 4H, I). In some cases, anti-DHBs antibodies were present in samples that also contained DHBsAg, presumably circulating as immune complexes (Foster et al., 2005; Surelia and Boxall, 1990; Tsai et al., 1995, 1998) as also described for HBV.

Statistical analysis of the outcome of infection

Although the numbers of ducks in each vaccine group were small, statistical analysis of the combined data from all 3 experiments was performed using log-binomial regression. Resolution of DHBV infection occurred in 9 of 11 ducks vaccinated with PDEF-DHBcAg compared to 1 of 11 control ducks ($P < 0.05$). Similarly, resolution of infection also occurred in 8 of 11 PDEF-DHBcAg vaccinated ducks that also received anti-DHBc antibodies, compared to 1 of 11 control ducks ($P < 0.05$). No statistical difference was observed between the 2 PDEF-DHBcAg vaccine groups with and without the addition of anti-DHBc antibodies ($P < 0.81$; Table 2).

Discussion

Effective IFN-α therapy for chronic HBV infection appears to induce an acute hepatitis that eliminates the infection in a similar way that the host is thought to clear a transient HBV infection, that is, through the production of inflammatory cytokines to block virus replication, antigen-specific CTL to kill infected hepatocytes and neutralizing antibodies to prevent reinfection of cells and virus rebound (Bertoletti and Ferrari, 2003). Thus, the immediate goal in the development of therapeutic vaccines for chronic HBV infection is to induce one or more of these steps in virus clearance. We report here on the development of a protective vaccine that appears to induce one or more of these steps in virus clearance.

Our vaccine approach involved the use of whole cells (PDEF) expressing DHBcAg. As the PDEF vaccine was derived from non-homologous duck embryos the vast majority of the vaccinated ducks would have received allogeneic fibroblasts. This same strategy could be used for vaccination of humans with allogeneic cells, thereby allowing non-customized vaccination with, for example, a common human fibroblast line. T-cell priming in the context of allogeneic vaccination is believed to occur via an in vivo cross priming or cross-presentation mechanism in which an early obligatory step is the capture by host dendritic cells of the antigen expressed by the vaccine cells. This mechanism would not be restricted to allogeneic transfer of the fibroblasts but would also operate in MHC-matched transfer during vaccination. This latter consideration means that customized vaccination using the patient’s own fibroblasts would also be a viable vaccine strategy although one that is a priori clearly less attractive, simply on a cost basis, than the use of allogeneic vaccination.

The current study used the DHBV model, in which the dose-and age-related outcomes of infection have been clearly defined. Our results showed that vaccination with PDEF-DHBcAg does not prevent, or reduce, the number of hepatocytes that are initially infected with DHBV. However, while unvaccinated control ducks rapidly developed chronic DHBV infection with widespread infection of hepatocytes, vaccinated ducks rapidly cleared their infected cells and resolved the infection (Table 1). Analysis of the combined data from all 3 experiments (Table 2) demonstrated that the differences in outcome of DHBV infection between vaccinated and control groups were statistically significant. Although the duration of protection was not tested later than 4 days after vaccination, it is likely that T cell responses persist and may still be increasing at the time of virus challenge.

The ability of hepadnavirus core antigens to protect against HBV and WHV infections has been assessed in previous studies in chimpanzees (Murray et al., 1987) and woodchucks (Schodel et al., 1993). In the study by Murray et al. (1987), two chimpanzees were vaccinated using SDS-treated and denatured HBV core particles combined with alum adjuvant. In the second study, recombinant WHV core antigen expressed in E. coli was combined with Freund’s adjuvant and used to vaccinate 6 woodchucks (Schodel et al., 1993). In both of these studies, levels of viremia were reduced following challenge, but liver tissue was not examined for viral replication early after virus inoculation. It was therefore unclear in these previous studies if vaccination reduced the percentage of initially infected cells,
enhanced the CMI response or facilitated development of an anti-surface response. In both the HBV and WHV models, challenge of unvaccinated adult animals results in transient infection, making it difficult to assess the effects of vaccination on outcome. In contrast in the current study performed in 18-day-old ducks, 10 out of 11 control unvaccinated animals developed chronic DHBV infection.

In other studies, DNA vaccines expressing WHV core antigen have been used to vaccinate woodchucks prior to challenge with WHV. The reproducibility of the vaccine response was not clear, and, because the studies were performed in adult woodchucks, prevention of chronic WHV infection was not used to assess the efficacy of vaccination. In an initial DNA vaccine trial (Lu et al., 1999), complete protection against WHV challenge was reported, based on measurements of viremia, whereas in a second DNA vaccine trial (Siegel et al., 2001) with the same challenge dose of WHV, vaccination did not protect. In fact, similar levels of viremia were observed in the WHV core vaccinated and control animals. Even in the unvaccinated control animals, the duration of viremia appeared short lived, and the appearance of anti-surface antibodies was rapid, compared to our own and other published data with the WHV model. This suggested that the inoculum itself might have induced an anti-surface response that shut down viremia and spread of virus, as apparently also happened in a study of transient WHV infection (Guo et al., 2000). Finally, no liver biopsies were taken, so it is unclear how much if at all spread of infection was limited prior to the early appearance of anti-surface antibodies. In brief, these initial studies appeared promising, but a need for a more detailed analysis examining the effects of vaccination on virus spread through the liver was clearly needed.

Our approach could in theory provide both the stimulus obtained with the DNA vaccination protocol in addition to the vaccine effect of the whole-cell preparation. That is, in addition to the likelihood that dendritic cells cross-presented cell-associated antigen, it is possible that some of the cell-associated plasmid DNA encoding DHBcAg was phagocytosed and associated antigen, it is possible that some of the cell-associated plasmid DNA encoding DHBcAg was phagocytosed and ultimately expressed within these cells. Finally, an important consideration that justifies a focus on cell-based vaccines is the present general low efficacy of naked DNA vaccines in humans, despite the success achieved in other mammalian systems.

Because it has been previously shown that antibodies bound to antigen facilitate Fc-receptor-specific uptake of the antigen (Amigorena, 2002), they can be considered as antigen receptors for phagocytes/dendritic cells. Theoretically, in the presence of antibodies to DHBcAg, core antigen released from dead or dying PDEF could form antigen/antibody complexes with enhanced uptake and cross-presentation to both the humoral and cell-mediated arms of the immune system, i.e., in both a class I and class II context. However, in our experiments, we found little evidence for an effect of anti-DHBc antibodies on the efficacy of vaccination.

With regard to the protection afforded by our vaccine, we know that it cannot be explained by opsonization of input virus by vaccine-induced anti-DHBc antibodies. Aside from the fact that core antigen has never been reported on the virus surface, this can be inferred from the results of immunoperoxidase staining of the day 4 p.c. biopsy tissues in Experiments 1 and 2. There was no significant difference in the number of hepatocytes that stained positive for DHBsAg at day 4 p.c. in the PDEF-DHBcAg vaccinated and control ducks. In addition, more direct assays have shown that anti-core antibodies are unable to opsonize either DHBV or HBV (D.S. Miller, in preparation) (Panda et al., 1988). The data instead are consistent with the possibility that CMI directed against DHBcAg is responsible for control of the infection. Given the difficulty in assaying for CMI activity of outbred avian species, this issue may be better addressed in a mammalian model of hepadnavirus infection. If CMI involvement proves key optimizing vaccine, protocols to induce CMI activity may offer the possibility of post-exposure treatments and therapy for those individuals who are chronically HBV-infected, especially in conjunction with nucleoside analogue treatment to reduce viral replication and antigen load.

Our studies demonstrate that hepadnaviral core antigen elicits protective immune responses that are able to stimulate clearance of virus-infected cells. The current HBV vaccine is composed of HBsAg. It is likely that addition of HBeAg to the vaccine may, firstly, provide additional coverage to immunized individuals and, secondly, may elicit immune responses in those individuals who do not respond to the current HBsAg vaccine. To develop an immuno-therapeutic vaccine is not an easy task and it is likely that it would need to contain several different vaccine antigens. Therefore, it is a requirement to validate the effectiveness of each individual antigen, albeit, in this case, in a protective setting. Based on the results presented in this study, we are investigating the effectiveness of combination antiviral and vaccine therapies to treat chronic DHBV infection.

**Materials and methods**

**Animals**

DHBV-negative, 1-day-old Pekin Aylesbury ducks (*Anas platyrhynchos domesticus*) and day-12 embryonated duck eggs were purchased from a commercial duck hatchery. Congenitally DHBV-infected ducks were purchased from a second commercial duck hatchery. Both hatcheries breed the animals as closed colonies. All animal handling protocols were assessed, approved and carried out in accordance with the guidelines of the University of Adelaide and Institute of Medical and Veterinary Science animal ethics committees and the National Health and Medical Research Council (NHMRC) of Australia.

**Isolation and in vitro culture of PDEF**

PDEF were isolated from DHBV-negative day-12 embryonated (non-homozygous) duck eggs, essentially as described (Vogt, 1969). Embryos were decapitated and eviscerated, loosely dissociated by mechanical disruption and then digested with 0.1% (w/v) trypsin (Gibco–BRL # 27250-18) in PBS for 30 min at 37 °C. The cells were then suspended in PDEF medium (RPMI 1640 containing 5% normal duck serum, 12 μg/
ml penicillin and 16 μg/ml gentamycin), dispensed into 150 cm² tissue culture flasks and allowed to attach and grow overnight in a 5% CO₂ incubator at 37 °C.

Transfection of PDEF with plasmid DNA expressing DHBcAg and addition of anti-DHBc antibodies

Plasmid DNA expressing DHBcAg, pTC-Dcore (von Weizsacker et al., 1995), was kindly provided by Dr. Fritz von Weizsacker from the Department of Medicine II, University Hospital, Freiburg, Germany. The plasmid DNA was grown in E. coli purified using the Plasmid Maxi kit (Qiagen #12162) and redissolved in distilled water at a concentration of 1 mg/ml. Following culture for 24 h, the monolayers of PDEF were washed, trypsinized and resuspended in Hanks Balanced Salt Solution at a concentration of 1 × 10⁷ cells/ml. 500 μl of the PDEF suspension and 50 μg of the plasmid DNA expressing DHBcAg were then placed into a 0.4 cm electroporation cuvette (Gene Pulser, Bio-Rad), chilled for 10 min and electroporated using a Bio-Rad Gene Pulser with capacitance extender at a voltage 0.35 kV. The time constant under these conditions was 31 ms. Following electroporation, the contents of three cuvettes (1.5 × 10⁷ cells) were pooled and cultured for a further 48 h in PDEF medium in a 10 cm diameter petri dish to allow antigen expression from the transfected DNA. The electroporation efficiency was assessed for each batch of cells by direct immunofluorescence using rabbit anti-DHBc antibodies as described below. The transfection efficiency was 20–40%. Under these electroporation conditions, approximately 50% of the PDEF were killed. This resulted in the formation of large clumps of transfected PDEF. For this reason, the wet weight of the transfected PDEF was determined by weighing on a microbalance rather than by counting individual cells.

On the day of vaccination (48 h after transfection), the transfected PDEF or non-transfected control PDEF were harvested from ten petri dishes by vigorous pipetting and scraping with a rubber policeman. Cells were washed in PBS, pelleted and the wet weight of the cells was measured. The PDEF were then resuspended in 3 ml of 0.7% NaCl. Where duck anti-DHBc antibodies were included, 200 μl of duck anti-DHBc antibodies (prepared as described below) was added to the transfected PDEF, and the mixture was incubated at 37 °C for 45 min. 0.7% NaCl was then added to increase the volume to ensure that each duck received 1 ml of the PDEF and antibody mixture. Animals were injected subcutaneously with 5 × 10⁷ transfected or non-transfected control PDEF in 1 ml of 0.7% NaCl or PBS.

Preparation of duck anti-DHBc antibodies

Recombinant DHBV core protein (rDHBcAg) was prepared as previously described (Jilbert et al., 1992) with some modifications to the procedure. Briefly, freeze/thaw lysates of E. coli expressing rDHBcAg were loaded onto a 10–30% linear sucrose gradient and centrifuged at 33,000 rpm for 3 h. Fractions were collected from the bottom of the gradient and subjected to PAGE analysis to assess the quantity and purity of the rDHBcAg. Three 50 μg doses of purified rDHBcAg in Freund’s incomplete adjuvant were administered by subcutaneous injection to three sites across the back of an adult duck at 2-week intervals. The levels of duck anti-DHBc antibodies were determined by ELISA, and serum was collected on days 7 and 14 following the final injection.

Detection of DHBcAg expression in transfected PDEF

PDEF transfected with plasmid DNA expressing DHBcAg, prepared as described above, were cultured in 24-well trays and assayed to determine the efficiency of transfection. Expression of DHBcAg was detected 48 h after transfection by fixation of the PDEF with 95% methanol for 10 min. The methanol was then removed, and the cells were washed two times with PBS then blocked with PBS + 10% fetal calf serum (FCS) for 1 h at 37 °C followed by immunofluorescence with rabbit anti-rDHBc antibodies (Jilbert et al., 1992) and goat anti-rabbit FITC (Kirkegaard and Perry Laboratories, Inc., Gaithsburg, MD).

Preparation of DHBV stocks and inoculum

The DHBV used for inoculation was derived from a pool (Pool 7) of serum from 34-day-old ducks congenitally infected with the Australian strain of DHBV (Triyatni et al., 2001). The serum was filtered through a 0.2 μM filter, aliquoted and stored at −80 °C. The serum pool contained 5 × 10⁷ DHBV vge/ml and 50 μg/ml of DHBSAg. Previous studies have shown it to be highly infectious in neonatal ducks (Jilbert et al., 1996). Ducks were inoculated intravenously at 18 days of age with 100 μl of pooled serum containing DHBV (5 × 10⁸ vge).

Vaccination and DHBV challenge protocol

Three experiments were performed using DHBV-negative ducks injected with 1 ml of PDEF vaccine, either with or without the addition of duck anti-DHBc antibodies, by the subcutaneous route on days 7 and 14 of age. In Experiment 1, ducks were vaccinated with 5 × 10⁷ PDEF-DHBcAg. A control group was injected with 0.7% NaCl only. In Experiment 2, ducks were vaccinated with 5 × 10⁷ PDEF-DHBcAg, while the control group received 5 × 10⁷ non-transfected PDEF. In Experiment 3, ducks were vaccinated with 1.5 × 10⁷ PDEF-DHBcAg, while the control group received 1.5 × 10⁷ non-transfected PDEF. All ducks were then challenged intravenously with DHBV (5 × 10⁸ vge) at 18 days of age. Liver biopsies were collected on day 4 (Experiments 1 and 2) or day 9 (Experiment 3) p.c., and autopsy tissue was collected on day 29, 32 and day 24 p.c. in Experiments 1, 2 and 3 respectively.

Analysis of serum and liver tissue

Serum samples were collected weekly and assayed for levels of total anti-DHBs, anti-DHBc antibodies and DHBSAg by ELISA as previously described (Foster et al., 2003; Jilbert et al., 1996; Miller et al., 2004; Pugh et al., 1995). To assess the extent...
of DHBV infection in the liver, a wedge biopsy was collected from each duck on day 4 p.c. (Experiments 1 and 2) or day 9 p.c. (Experiment 3). Liver, spleen, pancreas and kidney tissue were also taken from all ducks at autopsy during week 4 p.c. (day 24–32 p.i.). Biopsy samples of liver and autopsy samples of liver, kidney, pancreas and spleen were fixed in ethanol:acetic acid, wax-embedded, sectioned and examined for DHBsAg by immunoperoxidase staining as a marker of infected cells (Foster et al., 2003, 2005; Miller et al., 2004; Triyatni et al., 1998). The nuclei of hepatocytes staining positive for DHBsAg were counted in liver biopsy tissue with the aid of an eyepiece graticule in 200, 250 × 250 mm grid fields and expressed as a percentage of the average total hepatocyte nuclei. The minimum sensitivity of detection in liver biopsy tissue was 0.001%. In autopsy liver samples 2000, 250 × 250 mm grid fields were counted for each duck resulting in minimum sensitivity of detection of 0.0001% (Foster et al., 2005; Meier et al., 2003; Le Mire et al., 2005).

**Extraction and PCR amplification of liver and serum DNA**

A volume of 200 µl of each serum sample was treated with protease and extracted using a High Pure Viral Nucleic Acid kit (Roche 1 858 874). The final volume eluted from the column was 50 µl. To isolate total liver DNA, 25 µg of each liver sample was extracted using a DNeasy Tissue kit (Qiagen 69504). The volume eluted from the column was 100 µl. The efficiency of the extraction procedure was determined by measuring the optical density of each sample at 260 nm. DNA content ranged from 8 to 34 µg/ml. Samples were stored at −20 °C until required. PCR primers were designed to amplify part of the DHBV DHBcAg gene. The primers were located at nt 2554 (5′-TTCGGAGCTGCCTGCAGG-3′) for the forward primer and nt 2696 (5′-CTAGGTTCGAGTCCACGAGG-3′) for the primer that amplified the complementary strand (Triyatni et al., 2001). The predicted size of this PCR product was 743 bp. A standard 50 µl PCR reaction mix was used containing 20 µM of each primer, 1× reaction buffer, 200 µM dNTPs, 1.64 mM MgCl₂ and 1 U of Taq polymerase for each reaction. The template for each PCR reaction was 5 µl containing DNA extracted from 6.25 µg of liver or 5 µl of DNA extracted from 20 µl of serum. The limit of detection for DHBV DNA in the serum and extracted liver tissue was ~500 genomes.

DHBV DNA levels in autopsy liver tissues collected in Experiment 1 were also determined by quantitative PCR using the ABI Prism 7000. A head-to-tail dimer of DHBV inserted into pBluescript II KS (+) (p4.8BLx2) (Triyatni et al., 2001) was used to generate a standard curve of 10⁸–10¹ copies of the dimer in 10 µl supplemented with 100 ng of DNA extracted from normal duck liver. The volume of each PCR reaction was 25 µl and consisted of 100 ng of extracted DNA (2.5 µl adjusted to 10 µl with water), 12.5 µl of QuantiTect Sybr Green Master Mix, 50 nM of primer nt 390 (nucleotide sequence 5′-CAGATCCTCCCTCGCTTAGGA-3′) and 300 nM of the primer nt 666c (nucleotide sequence 5′-ATTGCTCATGCTGATCAC-3′). The cycling parameters for the reaction were an initial 95 °C for 10 min then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The limit of sensitivity was 10 copies of DHBV DNA per 100 ng of extracted liver cell DNA (equivalent to 40,000 cells).

**Statistical analysis**

The binary outcome (resolution of infection) was analyzed using log-binomial regression with adjustment for each experiment. The normally distributed outcome of the number of hepatocytes positive was analyzed using a mixed model ANOVA to allow for repeated measures over time with adjustment for each experiment. Post hoc testing was used to look at pair-wise comparisons between the treatments with no adjustment made for multiple comparisons. Significance was assessed at the 5% level. All analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA).

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**References**


Pugh, J.C., Di, Q., Mason, W.S., Simmons, H., 1995. Susceptibility to duck hepatitis B virus infection is associated with the presence of cell surface receptor sites that efficiently bind viral particles. J. Virol. 69 (8), 4814–4822.


Schulz, O., Reis e Sousa, C., 2002. Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells. Immunity 107 (2), 183–189.

Siegel, F., Lu, M., Roggendorf, M., 2001. Coadministration of gamma interferon with DNA vaccine expressing woodchuck hepatitis virus (WHV) core antigen enhances the specific immune response and protects against WHV infection. J. Virol. 75 (11), 5036–5042.


