Antiviral therapy with entecavir combined with post-exposure “prime-boost” vaccination eliminates duck hepatitis B virus-infected hepatocytes and prevents the development of persistent infection

D.S. Miller a,b, D. Boyle c, F. Feng a,b, G.Y. Reaiche a, I. Kotlarski a,d, R. Colonno e, A.R. Jilbert a,b,*

a School of Molecular and Biomedical Science, University of Adelaide, SA 5005, Australia
b Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Adelaide, SA 5000, Australia
c CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Vic, 3220, Australia
d Faculty of Health Sciences, University of Adelaide, SA 5005, Australia
e Bristol-Myers Squibb Pharmaceutical Research, Wallingford, CT, USA

Received 12 September 2007; returned to author for revision 26 October 2007; accepted 27 November 2007

Abstract

Short-term antiviral therapy with the nucleoside analogue entecavir (ETV), given at an early stage of duck hepatitis B virus (DHBV) infection, restricts virus spread and leads to clearance of DHBV-infected hepatocytes in ~50% of ETV-treated ducks, whereas widespread and persistent DHBV infection develops in 100% of untreated ducks. To increase the treatment response rate, ETV treatment was combined in the current study with a post-exposure “prime-boost” vaccination protocol. Four groups of 14-day-old ducks were inoculated intravenously with a dose of DHBV previously shown to induce persistent DHBV infection. One hour post-infection (p.i.), ducks were primed with DNA vaccines that expressed DHBV core (DHBc) and surface (pre-S/S and S) antigens (Groups A, B) or the DNA vector alone (Groups C, D). ETV (Groups A, C) or water (Groups B, D) was simultaneously administered by gavage and continued for 14 days. Ducks were boosted 7 days p.i. with recombinant fowlpoxvirus (rFPV) strains also expressing DHBc and pre-S/S antigens (Groups A, B) or the FPV-M3 vector (Groups C, D). DHBV-infected hepatocytes were observed in the liver of all ducks at day 4 p.i. with reduced numbers in the ETV-treated ducks. Ducks treated with ETV plus the control vectors showed restricted spread of DHBV infection during ETV treatment, but in 60% of cases, infection became widespread after ETV was stopped. In contrast, at 14 and 67 days p.i., 100% of ducks treated with ETV and “prime-boost” vaccination had no detectable DHBV-infected hepatocytes and had cleared the DHBV infection. These findings suggest that ETV treatment combined with post-exposure “prime-boost” vaccination induced immune responses that eliminated DHBV-infected hepatocytes and prevented the development of persistent DHBV infection.

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Keywords: Hepatitis B virus; Antiviral therapy; Entecavir; Prime boost vaccination; DNA vaccination; Recombinant fowlpoxvirus vaccines; Cell-mediated immunity; Resolution of infection; Persistent virus infection

Introduction

Worldwide ~2 billion people alive today have been infected with the human hepatitis B virus (HBV). Most adults infected with HBV experience a transient infection that is cleared by both humoral and cell mediated immune responses (CMI). However, approximately 2–5% of adults, and 90–95% of children exposed to HBV before the age of 1 year, develop chronic HBV infection (Bertoletti and Ferrari, 2003; Bertoletti and Naoumov, 2003; Hoofnagle, 2006; Lavanchy, 2004). Chronic HBV infection results in increased risks of chronic liver disease, cirrhosis and progression to hepatocellular carcinoma (Lavanchy, 2004). Current therapies for chronic HBV infection involve treatment...
with either interferon-α (IFN-α), or monotherapy with the nucleoside analogues, lamivudine, adefovir dipivoxil or the Bristol-Myers Squibb drug, entecavir (ETV) (Boni et al., 2003; Lai et al., 2002, 2006; Lavanchy, 2004; van Zonneveld et al., 2004; Yin and Lok, 2006). With these approaches, the rate of seroconversion from HBeAg to anti-HBe antibodies which is accompanied by persistently lower levels of virus replication is approximately 20%. In the remaining 80% of patients, cessation of treatment usually results in rebound of HBV infection (Bertoletti and Naoumov, 2003; Hoofnagle, 2006). There is currently no effective therapeutic vaccination protocol for chronic HBV infection.

Duck hepatitis B virus (DHBV) is a member of the hepadnaviridae family that shares with HBV similarities in genome structure, virus replication strategy and outcomes of infection (Bertoletti and Ferrari, 2003; Foster et al., 2005; Gibert et al., 1998, 1996, 1992). For example, infection of adult ducks is usually transient, whereas infection of 14-day-old ducks, generally leads to widespread and persistent DHBV infection. DHBV-infected ducks provide a useful model for testing novel antiviral and vaccine approaches for human HBV infection.

In recent work using the DHBV model, we reported that treatment of 14-day-old ducks with ETV from the time of DHBV infection until 14 or 49 days p.i. did not prevent initial DHBV infection of the liver, but markedly reduced the spread of infection, and allowed ∼50% of ducks to successfully clear their DHBV infection (Foster et al., 2005). In contrast, all untreated ducks developed widespread and persistent DHBV infection. It was concluded from this study that short-term suppression of virus replication and spread by ETV provided an opportunity for the host to mount an effective antiviral immune response that was able to control the infection in at least some ducks.

In a related work, we demonstrated that DNA vaccines expressing the DHBV pre-S/S and S antigens protected against subsequent challenge with DHBV in a virus dose-dependent manner (Miller et al., 2006b), providing significant protective immunity due to virus neutralization by anti-DHBs antibodies (Miller et al., 2006a; Rollier et al., 2000, 1999; Triyatni et al., 1998). In an additional study, we showed that pre-exposure vaccination with whole cell vaccines expressing DHBV core antigen, prevented the development of persistent DHBV infection, presumably by stimulating antigen specific CMI that targeted DHBV-infected hepatocytes (Miller et al., 2006a,b). Interestingly, a combination of DNA vaccination and ETV treatment offered no additional therapeutic benefit over ETV alone in ducks with established and widespread DHBV infection of the liver (Foster et al., 2003), again suggesting that further immune stimulation is required to elicit therapeutic responses in persistently DHBV-infected ducks.

Fowlpox virus (FPV) is the type member of the Avipoxvirus genus that causes disease in chickens (Gallus domesticus). “Shuttle vectors” enabling insertion of foreign genes into the non-essential thymidine kinase gene of the vaccine strain of FPV (FPV-M3) have previously been constructed (Boyle et al., 2004; Boyle and Coupar, 1988; Coupar et al., 2006) and used to derive recombinant FPV-M3 (rFPV) vaccine strains that induce vaccine specific immune responses in mice (Ramsay et al., 1999), chickens (Boyle and Heine, 1993; Heine and Boyle, 1993; Heine et al., 1997), macaques (Dale et al., 2004, 2000; Kent et al., 2000) and humans (Coupar et al., 2006; Dale et al., 2004; De Rose et al., 2007, 2006; Kelleher et al., 2006). Furthermore, “priming” with a DNA vaccine followed shortly afterwards with a rFPV “boost” was shown to enhance both CD4+ and CD8+ immune responses when compared to a dual rFPV “prime” and “boost” approach (Dale et al., 2004, 2006, 2000; De Rose et al., 2007; Kent et al., 2005; Ramsay et al., 1999).

As the next step toward developing a potential therapeutic DHBV vaccine, in the current study, we defined and optimized short-term combination therapies given to the ducks immediately after DHBV infection. In these experiments, ETV treatment and a vaccination regime consisting of a “prime” with DNA vaccines expressing the DHBc and DHBpre-S/S antigens (Triyatni et al., 1998; von Weizsacker et al., 1995), followed by a “boost” with rFPV-M3 strains expressing DHBV core and surface antigens (rFPV-DHBc and rFPV-pre-S/S) (Boyle et al., 2004; Boyle and Coupar, 1988; Triyatni et al., 2001), were administered following DHBV infection. Combination treatment slowed initial spread of DHBV through the liver and also induced anti-DHBV immune responses that targeted DHBV-infected hepatocytes and ultimately, prevented development of persistent DHBV infection in 100% of ducks. The effectiveness of this combination strategy in DHBV-infected ducks suggests that similar strategies could be employed to target HBV-infected hepatocytes in humans.

Results

Construction of rFPV strains expressing DHBV antigens

Recombinant FPV-M3 strains expressing the DHBV core and surface antigens (rFPV-DHBc and rFPV-pre-S/S) were constructed by homologous recombination between the FPV-M3 vaccine strain and the “shuttle vector” pAF09 into which we had previously cloned the DHBc and pre-S/S genes, as described in Materials and Methods and as shown in Table 1 and Fig. 1. Stocks of both rFPV-DHBc and rFPV-pre-S/S were grown in primary chicken embryonic fibroblasts (PCEF) cell cultures and the titer of each virus stock was determined as plaque forming units (p.f.u.) per ml. Infection of PCEF with FPV-M3, rFPV-DHBc or rFPV-pre-S/S caused widespread cell lysis and partial destruction of the PCEF monolayers within 24 h of infection, determined by staining of the monolayer with crystal violet (data not shown). In contrast, infection of primary duck embryonic fibroblasts (PDEF) with FPV-M3, rFPV-DHBc or rFPV-pre-S/S caused no visible cell lysis or cytopathic effects.

Western blot analysis of PCEF and PDEF infected with the recombinant strains (Fig. 2) confirmed the expression of a DHBc protein band (33 kDa) with minor degradation product running at ∼26 kDa (Fig. 2B). Expression of the DHBV pre-S/S protein (36 kDa) was also confirmed. A smaller band migrating at ∼28 kDa has been previously described in DHBV-infected liver (Rollier et al., 1999) and is thought to result from internal initiation of translation or post-translational degradation (Triyatni et al., 2001). PDEF monolayers, infected with the FPV-M3, rFPV-
DHBc or rFPV-pre-S/S strains, were tested for DHBV antigen expression by immunofluorescence. DHBV antigen expression was not detected in PDEF monolayers infected with FPV-M3. In contrast, DHBc and pre-S/S were detected in ~80% of the PDEF in each monolayer infected with either the rFPV-DHBc or rFPV-pre-S/S 24 h after infection (data not shown).

Finally, the cloning vector pAF09 contains the β-galactosidase gene under the control of an FPV late promoter. FPV-infected PCEF and PDEF monolayers were stained for β-galactosidase as a measure of FPV late gene expression. As expected, β-galactosidase staining was not detected in monolayers infected with the FPV-M3 strain, which lacks pAF09. β-galactosidase staining was detected in ~30% of the surviving cells in the PCEF monolayer and ~50% of cells in the intact PDEF monolayers infected with rFPV-DHBc and rFPV-pre-S/S 24 h after infection (data not shown). Thus, though cytopathic effects were not seen, at least some FPV late gene expression occurred in the PDEF monolayers.

Post-exposure ETV treatment and “prime-boost” vaccination study

We next tested the ability of a 14-day course of ETV therapy together with a DNA vaccine “prime” followed by a rFPV “boost”

DHBc or rFPV-pre-S/S strains, were tested for DHBV antigen expression by immunofluorescence. DHBV antigen expression was not detected in PDEF monolayers infected with FPV-M3. In contrast, DHBc and pre-S/S were detected in ~80% of the PDEF in each monolayer infected with either the rFPV-DHBc or rFPV-pre-S/S 24 h after infection (data not shown).

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Post-exposure ETV treatment and “prime-boost” vaccination study

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Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide number</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHBc-UCP b</td>
<td>2563–2580</td>
<td>AGAACAGGATCCATGGATACATGCTTCTAGAGCCTTAG</td>
</tr>
<tr>
<td>DHBc-DCP k c</td>
<td>414–393</td>
<td>GGTAGAAGGCTTAAATAATTTTCTTAGGCAGGAGA</td>
</tr>
<tr>
<td>Pre-S/S-UCP b</td>
<td>801–829</td>
<td>AGAAGGGATCCATGGGAGACACACTTGCAATAATAGGA</td>
</tr>
<tr>
<td>Pre-S/S-DCP k c</td>
<td>1793–1767</td>
<td>GGTAGAAGGCTTAAATAATTTTCAGATGAGAGAGCAGAG</td>
</tr>
<tr>
<td>Pre-S/S-DMP1 d</td>
<td>1370–1325</td>
<td>TTCAGGAAGTTTCAAAACGCTACAGTAATGCTGTA</td>
</tr>
<tr>
<td>Pre-S/S-UMP1 d</td>
<td>1325–1366</td>
<td>TACCAGGAATTCTTGGTAGCTTCTTGGTATGAAAAAAAA</td>
</tr>
<tr>
<td>Pre-S/S-DMP2 d</td>
<td>1551–1505</td>
<td>GCAGATTAAGAGGAGGAGATGAGAAAAACGCTTAGAGAGGAG</td>
</tr>
<tr>
<td>Pre-S/S-UMP2 d</td>
<td>1509–1551</td>
<td>GGACCTATTCACGGTTTTCATCATTCCTCTTATA</td>
</tr>
<tr>
<td>qPCR-F e</td>
<td>390–410</td>
<td>CAGATCTCCCTCGGCAAGA</td>
</tr>
<tr>
<td>qPCR-R f</td>
<td>666–646</td>
<td>ATTCGCTCATGCTGATCA</td>
</tr>
<tr>
<td>Seq. 1 (pAF09) g</td>
<td>1331–1351</td>
<td>GCCCTTCTCAGTAATGCAAGCTAG</td>
</tr>
<tr>
<td>Seq. 2 (pAF09) g</td>
<td>1622–1603</td>
<td>ATCGAATCCATCCGTGTA</td>
</tr>
</tbody>
</table>

a The nt numbers shown for each primer can be referenced to the AusDHBV genome (Genbank sequence AJ0006350 which is numbered from the EcoRI site at nt 1). b The BamHI and HindIII sites introduced into the 5’ and 3’ ends of the cloning primers are shown in italics. c Underlined sequences represent the (T5)NT FPV transcription terminator added to the 3’ end of the cloned DHBc and pre-S/S genes. In each case, the complimentary sequence, (A5)NA, is shown. d FPV termination sequences, (T5)NT, present in the original pre-S/S gene that were targeted for PCR mutagenesis are shown in bold. The underlined and bolded nt is the position of the introduced mutation. e Forward primer used for qPCR detection of DHBV DNA. f Reverse primer used for qPCR detection of DHBV DNA. g The plasmids pAF09-DHBc and pAF09-pre-S/S were sequenced in both directions using primers Seq. 1 and Seq. 2 designed within pAF09.
to prevent the development of widespread and persistent DHBV infection. A small-scale pilot study was initially carried out in 14-day-old ducks infected with 5×10^8 DHBV genomes by intra-venous inoculation. 5/5 ETV-treated and "prime-boost" vaccinated ducks resolved their DHBV infection by day 14 p.i., while 5/5 non-treated control ducks developed widespread DHBV infection in >95% of hepatocytes by day 14 p.i. (data not shown). Based on these observations, a more detailed study was carried out as described below.

To start, 4 Groups (A–D) of 14-day-old ducks were inoculated intravenously with 5×10^8 DHBV genomes. At the same time, oral ETV treatment (1.0 mg/kg/day for 14 days) was commenced in Groups A and C while ducks in the control Groups B and D were treated with water. At the same time, a single "priming" dose of DNA vaccine expressing DHBc, pre-S/S and S antigens was administered to Groups A and B while the control ducks (Groups C and D) received the DNA vector (pcDNA1.1 Amp) alone. Seven days later the ducks in Groups A and B received a boost with rFPV-DHBc and rFPV-pre-S/S, while the control ducks (Groups C and D) received the parental strain, FPV-M3.

At day 4 p.i., ducks in all Groups A to D had detectable DHBsAg-positive hepatocytes in the liver (Fig. 3, Table 2A). On average, the percentage of DHBsAg-positive hepatocytes was about 10-fold lower in the ETV-treated ducks than in those not treated with ETV (Table 2A: compare Groups A and B and Groups C and D) and was determined to be statistically significant with p values < 0.0001 (Table 3).

Also at day 4 p.i., the average percentage of DHBsAg-positive hepatocytes was slightly lower in the vaccinated than in the corresponding unvaccinated ducks (Table 2: compare Group A with Group C, and Group B with Group D). This result indicated that unlike the ETV treatment, DNA vaccination given at the time of infection did not have a major effect on early DHBV infection of the liver assessed at day 4 p.i.

ETV treatment was then continued for another 10 days, until day 14 p.i. Again ETV treatment markedly reduced the percentage of DHBsAg-positive hepatocytes (Table 2: compare Group A with Group B and Group C with Group D) and the differences were highly statistically significant with p values ≤ 0.0001 (Table 3). Interestingly, the percentage of DHBsAg-positive hepatocytes in ducks receiving ETV (Group A and Group C) was lower at day 14 than at day 4 p.i. The percentage was also lower at day 14 p.i. in the Group A ducks, which received ETV plus the "prime-boost" vaccination, than in the Group C ducks, which received ETV plus the control vectors, suggesting that "prime-boost" vaccination had induced immune responses that were helping to reduce the percentage of DHBsAg-positive hepatocytes.

The liver was again analyzed at day 67 p.i., 53 days after withdrawal of ETV to allow assessment of the outcome of DHBV infection. At this time, DHBV infection had rebounded and was present in >95% of hepatocytes in the liver of 6 of the 10 Group C ducks that received the short course of ETV in conjunction with the control vectors (Fig. 3, Table 2A). As found in our previous studies (Foster et al., 2005), DHBV infection had cleared from the liver of the remaining 4 out of 10 ducks (Table 2A). In contrast, in 10/10 Group A ducks, which received the short course of ETV together with the "prime-boost" vaccination, DHBsAg-positive hepatocytes were no longer detected and the difference in outcome of DHBV between the Group A and Group C ducks was statistically significant with a p value ≤ 0.0036 (Table 3).

Results of the immunoperoxidase detection of DHBsAg-positive hepatocytes were confirmed by testing available liver tissue from 5 ducks in each of Groups A and C for levels of total...
DHBV DNA by quantitative PCR (qPCR) (Table 2B). As expected, low levels of DHBV DNA (0.002–0.047 copies per cell) were detected in all tested ducks from Group A and C on day 4 p.i. However, by day 14 p.i., only 1/5 of the Group A ducks had detectable levels of DHBV DNA (0.002 copies per cell) and in autopsy liver collected on day 67 p.i., 3/5 Group A ducks had undetectable levels of DHBV DNA, while 2/5 ducks had low levels of DHBV DNA (0.002 and 0.003 copies per cell). These low-levels of DHBV DNA are not unexpected as similar levels of “residual” DHBV DNA have been detected in the liver of ducks following recovery from acute DHBV infection (Le Mire et al., 2005; Foster et al., 2005; Reaiche et al., manuscript in preparation). The biological significance of residual DHBV DNA is under investigation within the laboratory.

Also as expected based on the percentage of DHBsAg-positive hepatocytes, higher levels of DHBV DNA were detected in liver tissue from the Group C ducks compared to the Group A ducks on day 14 and 67 p.i. At day 14 p.i., 5/5 Group C ducks had DHBV DNA at 0.001–0.045 copies per cell (Table 2B), reflecting the higher percentage of DHBsAg-positive hepatocytes found in the same ducks (Table 2A). By day 67 p.i., 2/5 Group C ducks had resolved their DHBV infection and had undetectable levels of DHBV DNA while the 3 remaining ducks had DHBV DNA present at 2.25–490 copies per cell (Table 2B), again reflecting the higher percentage of DHBsAg-positive hepatocytes (1.53–>95%) (Table 2A).

A different outcome of DHBV infection was observed in the ducks that either received the control vectors (Group D) or received the prime-boost vaccination but not ETV (Group B) (Table 2A). Although samples were not available to test by qPCR, immunoperoxidase staining showed that all 5 ducks in Group D and 4 of 5 in Group B had >95% of DHBsAg-positive hepatocytes by day 14 p.i., and remained fully infected until
Table 2
The outcome of DHBV infection, ETV treatment and “prime boost” vaccination

A. Percentage of DHBsAg-positive hepatocytes

<table>
<thead>
<tr>
<th>DHBV DNA vaccines + rFPV-DHBc + rFPV-pre-S/S</th>
<th>Control vector DNA + FPV-M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>% DHBsAg-positive hepatocytes</td>
<td>% DHBsAg-positive hepatocytes</td>
</tr>
<tr>
<td>+ETV A A1 0.52 &lt;0.001 b 0.003 c</td>
<td>+ETV C C1 1.23 0.16 &lt;0.001 b</td>
</tr>
<tr>
<td>A2 0.33 &lt;0.001 b &lt;0.001 b</td>
<td>C2 0.49 0.12 &gt;95</td>
</tr>
<tr>
<td>A3 0.14 &lt;0.001 b &lt;0.001 b</td>
<td>C3 1.07 0.48 &gt;95</td>
</tr>
<tr>
<td>A4 0.26 &lt;0.001 b &lt;0.001 b</td>
<td>C4 0.73 0.3 &gt;95</td>
</tr>
<tr>
<td>A5 0.38 &lt;0.001 b Died</td>
<td>C5 0.82 0.07 &lt;0.002 b</td>
</tr>
<tr>
<td>A6 0.09 &lt;0.004 b &lt;0.006 b</td>
<td>C6 0.21 0.05 &lt;0.008 b</td>
</tr>
<tr>
<td>A7 0.05 &lt;0.003 b &lt;0.003 b</td>
<td>C7 0.22 0.08 &gt;95</td>
</tr>
<tr>
<td>A8 0.1 &lt;0.003 b &lt;0.007 b</td>
<td>C8 0.5 0.31 &gt;95</td>
</tr>
<tr>
<td>A9 0.24 &lt;0.003 b &lt;0.007 b</td>
<td>C9 0.07 0.03 &lt;0.004 b</td>
</tr>
<tr>
<td>A10 0.15 &lt;0.002 b &lt;0.005 b</td>
<td>C10 0.14 0.06 1.53</td>
</tr>
</tbody>
</table>

B. Levels of total DHBV DNA detected in duck liver by qPCR

<table>
<thead>
<tr>
<th>DHBV DNA vaccines + rFPV-DHBc + rFPV-pre-S/S</th>
<th>Control vector DNA + FPV-M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHBV DNA copies/cell</td>
<td>DHBV DNA copies/cell</td>
</tr>
<tr>
<td>+ETV A A6 NT e &lt;0.0002 f &lt;0.0002 f</td>
<td>+ETV C C6 0.007 0.001 &lt;0.0002 f</td>
</tr>
<tr>
<td>A7 0.002 &lt;0.0002 f &lt;0.0002 f</td>
<td>C7 0.047 0.008 123.46</td>
</tr>
<tr>
<td>A8 0.005 0.002 &lt;0.0002 f</td>
<td>C8 0.008 0.045 490.38</td>
</tr>
<tr>
<td>A9 NT &lt;0.0002 f 0.002</td>
<td>C9 0.03 0.016 &lt;0.0002 f</td>
</tr>
<tr>
<td>A10 NT &lt;0.0002 f 0.003</td>
<td>C10 0.013 0.001 2.25</td>
</tr>
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</table>

Table 3
Statistical analysis of the differences of the percentage of DHBsAg-positive hepatocytes at all time points

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Day 4 p.i.</th>
<th>Day 14 p.i.</th>
<th>Day 67 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean differences (95% CI) p value</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Group A vs. Group B - ETV+“prime-boost” vs. water+“prime-boost”</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>−2.17 (−3.07, −1.28)</td>
<td>−78.34 (−94.41, −62.27)</td>
<td>−76.00 (−114.18, −37.83)</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.0001 b</td>
<td>p&lt;0.001 b</td>
<td>p&lt;0.002 b</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group C vs. Group D - ETV+ control vectors vs. water+ control vectors</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>−3.51 (−4.41, −2.62)</td>
<td>−94.83 (−110.90, −78.77)</td>
<td>−47.35 (−84.99, −9.70)</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.0001 c</td>
<td>p&lt;0.0001 c</td>
<td>p&lt;0.0147 c</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group A vs. Group C - ETV+“prime-boost” vs. ETV+ control vectors</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>−0.32 (−1.05, 0.41)</td>
<td>−0.17 (−1.39, 12.95)</td>
<td>−47.66 (−79.04, −16.27)</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.3799 d</td>
<td>p&lt;0.9798 d</td>
<td>p&lt;0.0006 d</td>
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</table>

<table>
<thead>
<tr>
<th>Group B vs. Group D - Water+“prime-boost” vs. Water+ control vectors</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>−1.66 (−2.69, −0.63)</td>
<td>−16.66 (−35.21, 1.89)</td>
<td>−19.00 (−62.47, 24.47)</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.0022</td>
<td>p&lt;0.0773</td>
<td>p&lt;0.3844</td>
<td></td>
</tr>
</tbody>
</table>

* Statistical analysis was performed as reported in Materials and methods.
* Differences in the average percentage of DHBsAg-positive hepatocytes between the ducks in Group A vs. Group B were highly statistically significant at all time points.
* Differences in the average percentage of DHBsAg-positive hepatocytes between the ducks in Group C vs. Group D were highly statistically significant at days 4 and 14 p.i. (p<0.0001) but were less significant at day 67 p.i. (p=0.0417) due to the rebound of DHBV infection of 40% of Group C ducks by day 67 p.i.
* The percentage of DHBsAg-positive hepatocytes was not significantly different in ducks in Group A vs. Group C at day 4 and 14 p.i. due to the effect of ETV treatment on reducing the spread of DHBV infection, but was statistically significant at day 67 p.i. (p=0.0036) reflecting the effect of prime-boost vaccination on altering the outcome of DHBV infection.
autopsy at day 67 p.i. (Fig. 3). One of 5 ducks in Group B showed a slightly higher level of infection at day 14 than day 4 p.i., but eventually recovered from the infection (Table 2A). It is possible that “prime-boost” vaccination resulted in recovery from DHBV infection in this duck.

In an attempt to detect markers of the immune response that might contribute to the protection observed in the Group A ducks, liver tissue was studied to assess levels of infiltrating mononuclear cells and quantitative reverse transcription PCR (qRT-PCR) assays were used to determine levels of duck interferon alpha (IFN-α), interferon gamma (IFN-γ) and the duck T-cell markers, CD4 and CD8, mRNA. However, no significant differences were found in levels mononuclear cell infiltration or mRNA (data not shown). It is hypothesized that the failure to detect a difference may in part be due to the timing of the collection of biopsy samples after rFPV boosting.

Analysis of serum DHBsAg levels confirmed the patterns of DHBV infection in the liver: Only 1 Group A duck had a low-level transient rise in DHBsAg on day 63 p.i, whereas the remaining 9/10 ducks remained DHBsAg-negative throughout the experiment (Fig. 4A) and liver tissue collected at autopsy showed that all 10 combination treated ducks had resolved their

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**Fig. 4.** Levels of DHBsAg (A–D), anti-DHBs antibodies (E–H) and anti-DHBc antibodies (I–L) in the serum following DHBV infection, ETV treatment and “prime-boost” vaccination. All ducks were infected at day 14 of age with $5 \times 10^8$ DHBV genomes then vaccinated in treatment Groups A to D as described in the text. Titers of antibodies in each group 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-DHBs and anti-DHBc-positive samples was set at two standard deviations above the average background, obtained by assaying normal duck serum. DHBV challenge, DHBV DNA vaccine, control vector DNA vaccine, rFPV-DHBc+rFPV, pre-S/S FPV-M3, entecavir, water. NB: A colour version of Fig. 4 has been supplied for use as a supplementary figure.
DHBV infection. In contrast, 4/5 Group B (Fig. 4B), 4/10 Group C (Fig. 4C) and 5/5 Group D (Fig. 4D) ducks had detectable levels of serum DHBsAg reflecting the extensive DHBV infection in the liver of the same ducks (Table 2).

Titters of anti-DHBs antibodies fluctuated throughout the experiment in most groups. The ducks in Groups A and B that received the “prime-boost” vaccine, either in the presence or absence of ETV developed anti-DHBs antibodies faster (Figs. 4E and F) than the vector-vaccinated ducks in Groups C and D (Figs. 4G and H). The ducks that received ETV treatment in conjunction with “prime-boost” (Group A) maintained low levels of anti-DHBs antibodies throughout the study (Fig. 4E). Many of the ducks in Groups B and C that developed widespread DHBV infection had detectable levels of both DHBsAg and anti-DHBs presumably circulating in the bloodstream as immune complexes, as previously described (Foster et al., 2003, 2005; Miller et al., 2006a,b; Surelia and Boxall, 1990; Tsai et al., 1998, 1995). This finding is suggestive of production of anti-DHBs antibodies albeit at levels insufficient to completely remove circulating DHBV from the bloodstream. The water treated Group D ducks had widespread DHBV infection in the liver and high levels of circulating DHBsAg (Fig. 4D) and the lowest levels of anti-DHBs antibodies (Fig. 4H).

Titters of anti-DHBC antibodies were also monitored by ELISA. The ducks in Groups A and B that received the “prime-boost” vaccine, either in the presence or absence of ETV, again developed anti-DHBC antibodies faster (Figs. 4I and J) than the vector-vaccinated ducks in Groups C and D (Figs. 4K and L). The ducks in Group A and C that received ETV had on average slightly lower titers of anti-DHBC antibodies, possibly the result of lower levels of DHBC replication and antigen expression due to the antiviral effects of ETV.

**Discussion**

Although post-exposure treatment with Hepatitis B immunoglobulin (HBIG) and HBV surface antigen (HBsAg) vaccination can prevent the development of chronic HBV infection, vaccination of HBV carriers with the HBV vaccine with or without supplementary HBIG treatment provides no therapeutic benefit or change in HBV carrier status (Eren et al., 2000; Galun et al., 2002; Reed et al., 1973) and there is no effective cure for chronic HBV infection. The development of new and improved treatments for chronic HBV infection thus remains a major goal of HBV research and approaches to activate humoral and CMI responses in patients with chronic HBV-infection are required. Ideally, such new treatments might be most effective if they were given in combination with antiviral drugs that reduce the virus load and decrease the percentage of HBV-infected hepatocytes.

Therefore the aim of the current study was to develop and test a post-exposure antiviral and “prime-boost” vaccination strategy designed to prevent the development of persistent DHBV infection by stimulating immune responses that target and eliminate DHBV-infected hepatocytes.

Using the DHBV model and our knowledge of the dose-related outcome of DHBV infection in 14-day-old ducks, we previously showed that post-exposure treatment with ETV from the time of DHBV infection for either 14 or 49 days restricted the spread of DHBV infection within the liver and prevented the development of persistent DHBV infection in approximately 50% of ducks infected with $1 \times 10^6$, $1 \times 10^8$ and $5 \times 10^8$ DHBV genomes (Foster et al., 2005). This finding suggested that short-term suppression with ETV provides opportunity for the immune response to successfully control DHBV infection. However, ETV therapy was only able to alter the outcome of infection in 50% of ducks with the remainder developing widespread and persistent DHBV infection. Similarly, in the current study, ETV treatment following infection of 14-day-old ducks with a dose of $5 \times 10^8$ DHBV genomes, prevented the development of persistent DHBV infection in 40% of ducks in Group C (Table 2) validating previous results (Foster et al., 2005), but again indicating that additional immune stimulation needs to be applied over and above antiviral treatment alone if therapeutic outcomes are to be achieved.

As the first step in the assessment of rFPV strains for use as vectors to deliver vaccine antigens in ducks, we tested the ability of rFPV strains to infect and replicate in PDEF. Infection of PDEFs with FPV-M3, rFPV-DHBc or rFPV-pre-S/S caused no visible cell lysis or cytopathic effects. Furthermore, in contrast to previous reports in chickens (Boyle et al., 1997), wing web inoculation of rFPV strains into newly hatched ducks showed no visible lesions. Thus, these in vitro and in vivo results suggested that rFPV is an efficient and safe vaccine vector for delivery of vaccine antigens in ducks.

It is currently not clear if the rFPV boost provided additional immune responses over those observed with DNA vaccination alone. In preliminary experiments with rFPV strains in our laboratory, vaccination of ducks with rFPV-DHBc induced low-level anti-DHBc antibodies while anti-DHBs antibody responses were not detected following rFPV-pre-S/S vaccination (Darren Miller and Stephen Blake, unpublished results). This result is not entirely surprising as titers of anti-DHBs antibodies are generally lower than anti-DHBc antibodies following recovery from acute DHBV infection (Foster et al., 2005; Jilbert et al., 1998; Miller et al., 2006a,b). Therefore, these data are suggestive that the rFPV boost is contributing to the protection observed in the current study. In addition, it has been demonstrated that DNA vaccination followed by a rFPV boost elicited enhanced CMI responses than either of the delivery vehicles alone (Coupar et al., 2006; Kent et al., 1998; Ramsay et al., 1999).

Ideally we would like to measure CMI responses in ducks following vaccination with rFPV constructs as it is hypothesized that intracellular expression of the rFPV delivered antigens should elicit potent CMI. Unfortunately, due to a lack of duck specific reagents, we are at present unable to detect CMI responses in ducks. It is hoped that the recent availability of monoclonal antibodies specific for duck CD4 and CD8 will enhance our understanding of immune mechanisms of DHBV clearance (Kothlow et al., 2005). Additional ongoing work in the laboratory is aimed at answering some of these questions and also simplifying the combination treatment regime. To this end we are testing the efficacy of ETV treatment combined with DNA vaccines and rFPV vaccines alone and also “prime-boost”
vaccination with DNA vaccines and rFPV strains expressing either DHBc or pre-S/S as a single vaccine antigen. Although multiple vaccine antigens may induce broad humoral and CMI responses, it is possible that the DHBc or pre-S/S antigens alone may be sufficient to provide protective or therapeutic immune responses.

Finally, the results of the study demonstrated that combined post-exposure treatment with ETV and “prime-boost” vaccination significantly enhanced the rate of elimination of DHBV-infected hepatocytes and prevented the development of persistent DHBV infection in 100% of ducks. The improved efficacy of the combination ETV and “prime-boost” protocol suggests it may provide the basis for future therapeutic DHBV and HBV vaccines.

Materials and methods

Animals

Pekin Aylesbury ducks (Anas domesticus platyrhynchos) were purchased at 1 day of age from commercial hatcheries. All animal handling procedures and protocols were assessed, approved and carried out in accordance with the guidelines of the University of Adelaide and Institute of Medical and Veterinary Science (IMVS) animal ethics committees and the National Health and Medical Research Council (NHMRC) of Australia.

Preparation of DHBV stocks

The DHBV inocula were derived from a pool of serum from 34-day-old congenitally DHBV-infected ducks infected with the Australian strain of DHBV (AusDHBV; Genbank AJ006350) (Triyatni et al., 2001). The serum was filtered through a 0.2 μm filter, aliquoted and stored at 80 °C. The pool of serum was then purified using a Jetstar Maxi kit (Promega) and redissolved in distilled water at a concentration of 1 mg/ml.

DNA vaccines

The AusDHBV pre-surface (pre-S/S) and surface (S) genes were cloned into pcDNA1.1Amp as previously described (Triyatni et al., 1998). Plasmid DNA expressing DHBcAg, pTC-Dcore (von Weizsacker et al., 1998). Plasmid DNA expressing DHBcAg, pTC-Dcore (von Weizsacker et al., 1998) was kindly provided by Dr. Fritz von Weizsacker from the Department of Medicine II, University Hospital, Freiburg, Germany. All plasmid DNA constructs were produced in E. coli and then purified using a Jetstar Maxi kit (Genomed-Astral Scientific) and resuspended in distilled water at a concentration of 1 mg/ml.

PCR amplification of the DHBc gene prior to cloning into pAF09

Analysis of the AusDHBV pre-S/S gene (Triyatni et al., 2001) revealed two poxvirus transcription termination sequences, (T5)NT, at nt 1344 and 1525 which were then mutated using PCR (Higuchi et al., 1988) to prevent termination of transcription of the cloned pre-S/S gene while maintaining the encoded amino acid sequence. To enable directional cloning into the shuttle vector pAF09, a BamHI restriction enzyme sequence was included in the upstream cloning primer, pre-S/S-UCP and a HindIII restriction enzyme sequence was included in the downstream primer, pre-S/S-DCP (Table 1). The poxvirus transcription terminator sequence, TTTTTAT, shown as its complimentary sequence, (A5)TA, in Table 1. All PCR reactions were performed in a volume of 50 μl and contained 20 μM of both primers and 1 × reaction buffer, 200 μM dNTPs, 1.64 mM MgCl2 and 1 U of Pfu polymerase. The cycling parameters were 94 °C for 10 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. 2.5 × 10^4 AusDHBV genomes were amplified using pfu-DHBc-UCP and DHBc-DCP to yield a PCR product of 818 bp that contained the entire DHBc gene.

A series of stepwise PCR mutagenesis reactions were performed to mutate the two (T5)NT terminators: (Reaction 1) 1.64 μM of each primer and 1 × reaction buffer, 200 μM dNTPs, 1.64 mM MgCl2 and 1 U of Pfu polymerase. The cycling parameters were 94 °C for 10 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. 2.5 × 10^4 AusDHBV genomes were used as a template for PCR amplification with pre-S/S-UCP and pre-S/S-DCP (Table 1) to yield a product of 585 bp; (Reaction 2) 2.5 × 10^4 AusDHBV genomes were used as a template for PCR amplification with the upstream cloning primer (pre-S/S-UMP1) and the downstream cloning primer (pre-S/S-DCP) (Table 1) to yield a product of 1026 bp, which was purified and cloned into pBluescript (pBs-pre-S/S) (S/S-DCP (Table 1)) to yield a product of 1026 bp, which was purified and cloned into pBluescript (pBs-pre-S/S) (S/S-DCP (Table 1)) to yield a product of 1026 bp, which was purified and cloned into pBluescript (pBs-pre-S/S) (S/S-DCP (Table 1)) to yield a product of 1026 bp.
Cloning of the DHBc and pre-S/S genes into pAF09

The vector pAF09 (Boyle et al., 2004) and the gel-purified 818 bp DHBc, or the 1026 bp pre-S/S PCR products described above were digested with BamHI and HindIII, ligated and then transformed into DH5-α to create plasmids pAF09-DHBc and pAF09-pre-S/S (Fig. 1). Colonies were isolated and the plasmid DNA was sequenced using pAF09 forward primer (Seq. 1), and reverse primer (Seq. 2) (Table 1) to confirm the correct identity of the plasmids and the nucleotide changes introduced into the pre-S/S gene by PCR mutagenesis (data not shown). Sequence ladders were generated using 15 ng of template DNA and 8 μl of Big Dye dideoxynucleotide mix, with PCR cycling at 96 °C for 10 min, then 30 cycles of 96 °C for 10 min 50 °C for 10 min and 60 °C for 4 min. Sequences were determined using an ABI Prism 377 DNA sequencer.

Preparation of primary chicken and duck embryo fibroblasts

Primary embryonic fibroblasts were isolated from 12-day-old chicken (PCEF) and duck (PDEF) embryos as previously described (Miller et al., 2006a,b; Vogt, 1969). Briefly, the embryos were aseptically eviscerated; the remaining tissue was loosely dissociated with a pipette then digested with 0.1% trypsin at 37 °C for 15 min and further dissociated to produce a cell suspension. The primary fibroblasts were then plated in RPMI 1640 containing 12 μg/ml penicillin, and 16 μg/ml gentamycin, plus 5% normal duck serum (PDEF) or 5% normal chicken serum (PCEF) and grown overnight in a 5% CO2 incubator at 37 °C. The resulting monolayers were trypsinized, harvested and stored in liquid nitrogen prior to use.

Derivation and confirmation of rFPV-M3 vaccine strains expressing DHBc and pre-S/S antigens

The rFPV strains, rFPV-DHBc and rFPV-pre-S/S, were created dominant selection protocols for the insertion of genetic material into the FPV-M3 genome (Boyle et al., 2004): creation of the strains involved homologous recombination between the FPV thymidine kinase gene present within FPV-M3 strain and pAF09-DHBc and pAF09-pre-S/S, followed by selection using mycophenolic acid. Stocks of both rFPV-DHBc and rFPVpre-S/S were grown in PCEF cell cultures and the titer of each virus stock was determined as p.f.u. per ml. The expression of specific DHBV proteins was confirmed by Western blotting using PCEF infected with nil, FPV-M3, rFPV-DHBc or rFPV-pre-S/S using a modified protocol. PCEF were grown in a 6 well plates until they reached 85~90% confluence. The culture media were then replaced with 1 ml of fresh media (per well) containing 2.5% fetal calf serum and ~4 × 10^8 p.f.u. of FPV-M3, rFPV-DHBc or rFPV-pre-S/S, respectively. At 2 h after inoculation, the media were changed and the infected PCEF were incubated for a further 48 h. The PCEF were then lysed with 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% NP40 and protease inhibitor cocktail (Roche, 1 tablet for 10 ml lysis buffer). Cell lysates were separated by 12% SDS-PAGE and subjected to Western blot analysis with either polyclonal rabbit anti-DHBc antibodies (Jilbert et al., 1992) or monoclonal anti-DHBV pre-S antibodies (Pugh et al., 1995). The protein content of each lysate was compared by stripping of the membranes followed by Western blotting with monoclonal anti-human actin antibodies (CHEMICON #MAB1501R). The secondary immunoreactions were performed with horseradish peroxidise-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G (Pierce) and detected using ECL™ Western blotting reagents (GE Healthcare).

Assessment of the FPV-M3, rFPV-DHBc and rFPV-pre-S/S strains in cell culture

Monolayers of PCEF and PDEF were used to assess the cytotoxicity, DHBV antigen production and FPV late gene expression of the rFPV strains. 6 × 10^5 PCEF or PDEF were plated into 48-well trays and allowed to attach overnight at 37 °C. The following day the media were removed and the monolayers were infected with 5 p.f.u./cell of FPV-M3, rFPV-DHBc or rFPV-pre-S/S. The virus was allowed to attach for 1 h at 37 °C, the monolayers were then washed twice with PBS before addition of the appropriate growth medium, and incubated at 37 °C until assayed. Infected monolayers were stained with 1% crystal violet after fixation in 100% methanol, to assess cytopathicity. Separate cultures were stained for the presence of B-galactosidase after fixation in 0.2% glutaraldehyde, by incubation in 1 mg/ml of X-gal in 5 mM potassium ferricyanide and 2 mM magnesium chloride at 37 °C overnight. For immunofluorescent detection of DHBc or pre-S/S expression, monolayers incubated for 24 h after infection were washed with PBS, fixed with 95% methanol and blocked with 5% skim milk/PBS for 1 h. The monolayers were then incubated with primary antibodies, polyclonal rabbit anti-DHBc (Jilbert et al., 1992) or monoclonal anti-DHBV pre-S antibodies (Pugh et al., 1995), for 1 h at 37 °C. Following two PBS washes, the monolayers were incubated with secondary antibodies, goat anti-rabbit Alexa 488 (A-11008; Invitrogen) or goat anti-mouse Alexa 488 (A-21121; Invitrogen), were washed with PBS and were mounted in PBS containing 25% glycerol. Fluorescence was detected with a UV microscope at 495 nm.
Assessment of the FPV-M3 strain in vivo

The ability of the FPV-M3 strain to cause lesions in vivo was assessed by inoculation of the wing web of ducks, the standard industry practice for vaccine delivery in poultry. Inoculation of the FPV-M3 vaccine strain into the wing web of chickens causes local poxvirus lesions at the wing stab site over a 10^{-3}–10^{9} range of vaccine dilutions (Boyle et al., 1997). Three, 7-day-old ducks were inoculated via the wing web with a 22 G needle dipped into a neat stock of FPV-M3 (5 × 10^{8} p.f.u./ml). Three control 7-day-old ducks were housed in the same pen to assess the possible transfer of FPV infection from duck-to-duck. All ducks were monitored daily for 2 weeks following inoculation. No signs of respiratory distress or lesions at the site of inoculation of the wing web were observed in ducks. We concluded that although the FPV-M3 strain replicates and causes clinical lesions in chickens (Boyle et al., 1997), evidence of FPV lesions or disease was not seen in the inoculated ducks or in their cage controls.

DHBV challenge, antiviral drug administration and vaccination regime

Four groups of 14-day-old ducks, Groups A to D, were inoculated intravenously with 5 × 10^{8} AusDHBV genomes as previously described (Foster et al., 2003, 2005; Jilbert et al., 1998, 1996, 1992; Meier et al., 2003; Miller et al., 2006a,b; Triyatni et al., 1998). At the same time, oral treatment with ETV (1.0 mg/kg/day for 14 days) was commenced in Groups A and C. Groups B and D were treated with water. A single “priming” dose of DHBV DNA vaccine expressing DHBc, pre-S/S and S antigens (500 μg of each construct) was administered intramuscularly (IM) to Group A and B at the time of infection, followed at day 7 p.i. by a second IM “boost” dose of 5 × 10^{7} p.f.u. of both rFPV-DHBc and rFPV-pre-S/S. Groups C and D received the control DNA vector (pcDNA1.1 Amp), followed on day 7 by IM inoculation of 5 × 10^{7} p.f.u. of rFPV-M3, the parent FPV vaccine strain.

Analysis of serum and liver tissue

Serum samples were collected weekly and assayed for levels of DHBsAg and anti-DHBs and anti-DHBc antibodies by ELISA (Miller et al., 2004; Triyatni et al., 1998). To assess the extent of DHBV infection in the liver, a wedge biopsy was taken from all ducks at autopsy at day 67 p.i. Liver biopsy samples were also taken from all ducks at autopsy at day 67 p.i. Tissue fixation, embedding, sectioning and immunoperoxidase staining of DHBsAg and staining with hematoxylin and eosin were performed as previously described (Foster et al., 2003, 2005; Miller et al., 2004). Nuclei of hepatocytes staining positive for DHBsAg were counted and expressed as the percentage of the average total hematoxylin stained hepatocyte nuclei in the same fields using an eyepiece with a graticule. For each duck, 200, 250 × 250 mm grid fields were counted resulting in minimum sensitivity of detection of 0.001% (Foster et al., 2005; Meier et al., 2003). To assess the degree of liver inflammation, 100, 250 mm × 250 mm grid fields were counted using the 10X objective lens. The percentage of each section that contained infiltrating mononuclear cells was then calculated.

DNA extraction from liver tissue and quantitative PCR (qPCR) for total DHBV DNA

Total cellular and viral DNA was extracted from 20 mg of duck liver tissue using the DNeasy Tissue extraction kit (Qiagen) as previously described (Foster et al., 2005; Le Mire et al., 2005). qPCR was performed on an ABI 7000 qPCR machine. The reaction mix contained 10 μl of SYBR green PCR master mix (Applied Biosystems), 5 μl of extracted DNA containing 150 ng (equivalent of 52,000 cells), 10 μM of the forward primer, qPCR-F, and reverse primer, qPCR-R (Table 1), and 3.8 μl of DW. Standard curves were constructed using plasmid pBl4.8 containing the complete AusDHBV genome (Triyatni et al., 2001) with 90 ng of plasmid DNA being equivalent to 10^{10} copies of DHBV DNA. Tenfold dilutions of the plasmid resulted in a linear amplification curve between 10^{8} and 10^{1} copies (data not shown). The PCR reaction included an initial 50 °C denaturation step of 2 min and then 95 °C for 10 min. The amplification program consisted of 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Analysis of the data was performed using ABI Prism 7000 SDS software. The minimum sensitivity of detection was 10 copies of DHBV DNA in 52,000 cells or 0.0002 copies of DHBV DNA/cell.

RNA extraction, quantitative reverse transcription PCR (qRT-PCR) and primer design

Twenty-five milligram liver samples collected from ducks in Groups A and B on days 4 and 14 p.i. were extracted using an RNAQeous 4-PCR RNA extraction kit (Ambion). The extracted RNA was collected into RNase-free centrifuge tubes and treated with DNase I following the manufacturer’s instructions (RNAQeous 4-PCR RNA; Ambion). The reverse transcription step from 1 μg of RNA into cDNA was performed using Reverse Transcription System (Promega). Primers were designed for the detection of mRNA expression of the cytokines duck IFN-α, IFN-γ and duck T-cell markers CD3, CD4 and CD8, using duck GAPDH as an internal control (data not shown, Reaiche et al., manuscript in preparation). qRT-PCR was performed using a SYBR green PCR master mix (Applied Biosystems) on an ABI prism 7000 real-time PCR machine. The conditions for the qRT-PCR were 50 °C for 2 min followed by 10 min at 95 °C with amplification of 40 cycles at 15 s at 95 °C and 1 min at 60 °C. The data were later analyzed using ABI Prism 7000 SDS software.

Statistical analysis

The chi-square test was used to determine if there was a statistically significant association between the percentages of DHBV-infected hepatocytes in each treatment Group A-D. The normally distributed outcome of the average number of DHBsAg-positive hepatocytes in each Group was analyzed using a mixed
model ANOVA to allow for repeated measures over time. 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).

Acknowledgments

We thank Dr. Miriam Triyatni for producing the DHBV pre-S/S and S expressing DNA vaccines, Dr. Fritz von Weizsäcker and Dr. Stefan Wieland for providing the DHBe expressing DNA vaccine, pTC-Dcore, Dr. Barbara Coupar for assistance in the supply of the vector pAF09 and derivation of the rFPV and Ms, Emmea Ramsay, Department of Public Health, Faculty of Sciences, University of Adelaide, for performing the statistical analysis. We also acknowledge Ms, Wendy Foster and Cathy Scougall for technical assistance and wish to thank Dr. William Mason and Professor Christopher Burrell for critical reading of the manuscript. This work was supported by a project grant from the NHMRC and a Royal Adelaide Hospital, Dawes post-graduate scholarship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.11.032.

References
