Rise in gamma interferon expression during resolution of duck hepatitis B virus infection

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Gamma interferon (IFN-γ) expression plays a crucial role in the control of mammalian hepatitis B virus (HBV) infection. However, the role of duck INF-γ (DuIFN-γ) in the outcome of duck HBV (DHBV) infection, a reference model for hepadnavirus replication studies, has not yet been investigated. This work explored the dynamics of DuIFN-γ expression in liver and peripheral blood mononuclear cells (PBMCs) during resolution of DHBV infection in adolescent ducks in relation to serum and liver markers of virus replication, histological changes and humoral response induction. DHBV infection of 3-week-old ducks resulted in transient expression of intrahepatic preS protein (days 3–14) and mild histological changes. Low-level viraemia was detected only during the first 10 days of infection and was accompanied by early anti-preS antibody response induction. Importantly, a strong increase in intrahepatic DuIFN-γ RNA was detected by real-time RT-PCR at days 6–14, which coincided with a sharp decrease in both viral DNA and preS protein in the liver. Interestingly, liver DuIFN-γ expression remained augmented to the end of the follow-up period (day 66) and correlated with portal lymphocyte infiltration and persistence of trace quantities of intrahepatic DHBV DNA in animals that had apparently completely resolved the infection. Moreover, in infected ducks, a moderate increase was detected in the levels of DuIFN-γ in PBMCs (days 12–14), which coincided with the peak in liver DuIFN-γ RNA levels. These data reveal that increased DuIFN-γ expression in liver and PBMCs is concomitant with viral clearance, characterizing the resolution of infection, and provide new insights into the host–virus interactions that control DHBV infection.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection remains a major public health problem worldwide. Investigation of the immune mechanisms underlying the resolution of HBV infection is therefore essential to design new therapies to fight chronic hepatitis B. Data accumulated in patients indicate that vigorous Th1 cell responses to HBV antigens, associated with upregulated expression of cytokines and especially the production of gamma interferon (IFN-γ) in the liver, lead to the resolution of acute hepatitis, whereas weak cellular responses result in its progression to chronicity (Guidotti & Chisari, 2001; Rehermann & Nascimbeni, 2005; Wieland & Chisari, 2005). Studies in HBV-transgenic mice have shown a crucial role for IFN-γ in the non-cytolytic mechanism of virus clearance (Cavanaugh et al., 1998; Guidotti & Chisari, 2001; Wieland & Chisari, 2005). In addition, such IFN-γ-mediated control of HBV infection was demonstrated during recovery from acute HBV infection of chimpanzees and from woodchuck hepatitis virus (WHV) infection of woodchucks (Guidotti et al., 1999; Hodgson & Michalak, 2001; Wieland et al., 2004). Both chimpanzee and woodchuck are extremely useful models, although they are not readily available for investigation of virus clearance during antiviral or immune therapy.

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Another pertinent model that plays a pivotal role in studies of hepadnavirus replication and the testing of novel therapeutic approaches is infection of ducks with duck HBV (DHBV) (Rollier et al., 1999; Le Guerhier et al., 2003; Cova & Zoulim, 2004). It is therefore essential to understand the immune-mediated mechanisms controlling DHBV infection. However, data on the duck cellular immune response are scarce and the involvement of host cytokines in the outcome of DHBV infection has not yet been investigated in vivo.

In this regard, in vitro studies in primary duck hepatocyte cultures have demonstrated the ability of recombinant duck IFN-γ (DuIFN-γ) to inhibit DHBV replication in a dose-dependent fashion and have suggested that this cytokine could modulate the DHBV life cycle (Schultz & Chisari, 1999). In addition, endotoxin-stimulated duck liver macrophages have been shown to release DuIFN-γ and DuIFN-α, which inhibit DHBV replication in vitro (Klöcker et al., 2000). Transient and chronic outcomes of DHBV infection have been investigated previously in several studies, which focused on neutralizing antibody response induction (Jilbert et al., 1992, 1998; Zhang & Summers, 2004; Le Mire et al., 2005). However, the role of DuIFN-γ expression during resolution of avian hepadnavirus infection in vivo has not yet been addressed.

Analysis of DuIFN-γ expression has been hampered by a lack of available tools. In the present study, we first developed a real-time quantitative RT-PCR assay for DuIFN-γ RNA detection. Using this approach, we explored the dynamics of DuIFN-γ expression in liver and peripheral blood mononuclear cells (PBMCs) during resolution of transient DHBV infection in adolescent ducks in relation to serum and liver markers of virus replication, histological changes and anti-preS humoral response induction.

We report here, for the first time, that resolution of DHBV infection in adolescent ducks is characterized not only by an early anti-preS antibody response but also by an increase in DuIFN-γ expression in liver and PBMCs. In addition, we showed that the kinetics of DuIFN-γ expression during recovery from infection was very rapid for DHBV (2–3 weeks) compared with mammalian hepadnaviruses (2–3 months). Moreover, we observed an augmented hepatic DuIFN-γ expression up to the end of the follow-up period, which correlated with portal lymphocyte infiltration and persistence of trace quantities of viral DNA in the livers of ducks that had apparently completely resolved DHBV infection.

**METHODS**

**Animals and experimental design.** Pekin ducklings, purchased from a commercial supplier, were housed at the facilities of the National Veterinary School of Lyon (ENVL, Marcy l’Étoile, France). Animal experimentation was performed in accordance with the guidelines for animal care of the ethics committee of ENVL. All animals were bled at 1 day post-hatch, serum samples were tested for the presence of DHBV DNA and ducklings that were congenitally infected with DHBV were excluded from the experiment. A total of 24, 3-week-old, DHBV-free Pekin ducks were infected intravenously with a DHBV-positive serum pool [5 × 10^6 virus genome equivalents (vge) per duck] as described previously (Cova & Zoulim, 2004) and an uninfected group of 24 ducks was followed in parallel. Three infected and three uninfected ducks were euthanized by lethal injection of pentobarbitral (Dolethal; Vetoquinol) on days 0, 3, 4, 6, 14, 28, 40 and 66 post-infection (p.i.) and liver samples were stored at −80 °C until analysis. In addition, two groups of three DHBV-infected and three uninfected ducks were monitored in parallel for 66 days for viraemia and anti-preS response follow-up as detailed below. This infection assay was reproduced twice using two series of infected and uninfected ducks and two series of inocula prepared independently.

**Analysis of viraemia.** Viraemia was assessed throughout the 66 day follow-up period by detection of DHBV DNA in serum samples obtained from three DHBV-infected and three uninfected ducks using a previously described dot-blot hybridization assay (Rollier et al., 1999). Duck sera were spotted in duplicate. Filters were exposed to autoradiographs, scanned using a PhosphorImager (Amersham Biosciences) and quantified using ImageQuant software (Cova & Zoulim, 2004).

**ELISA analysis of the anti-preS response.** Anti-preS antibodies were detected using a previously described direct ELISA test (Chassot et al., 1994; Rollier et al., 1999, 2000). Briefly, microtitre plates (Falcon Probind), coated overnight with recombinant DHB preS polypeptide, were washed, blocked with 3% casein in PBS, incubated with individual duck sera and detected with alkaline phosphatase-conjugated anti-duck IgG goat antibody (KLP). The cut-off for positivity was set as the mean λ_{0.05} + 3SD of age-matched control duck sera at a dilution of 1:20.

**Nucleic acids analysis.** DNA was extracted from frozen autopsy liver samples, as described previously (Le Guerhier et al., 2003). Intrahepatic DHBV DNA analysis was performed by Southern blot hybridization as described previously by loading 10 μg DNA per lane (Cova & Zoulim, 2004). In addition, total liver DHBV DNA was quantified by dot-blot hybridization (20 μg per dot), followed by hybridization with 32P-labelled full-length genomic DHBV DNA and viral DNA was quantified using a PhosphorImager, as described previously (Rollier et al., 1999).

DNA was isolated from liver tissue, pulverized under liquid nitrogen, using Extract-All reagent (Eurobio) according to the manufacturer’s instructions. For RNA extraction from PBMCs, equal volumes of heparinized blood and PBS were mixed. One volume of the mixture was layered over an equal volume of Ficoll and centrifuged at 1200 g for 20 min and PBMCs were collected from the interface as described previously (Vickery et al., 1997). The PBMCs were washed twice in PBS and RNA was extracted using Extract-All. Nucleic acids were quantified by spectrophotometry and stored at −80 °C prior to use in real-time PCR analysis.

**In vitro transient expression of DuIFN-γ.** The entire ORF of DuIFN-γ was excised from the previously described pcDNA3DuIFN-γ plasmid construct (Schultz & Chisari, 1999) and inserted into a pCI plasmid (Promega), resulting in a plasmid designated pCIDuIFN-γ. Avian hepatoma LMH cells were transiently transfected with pCIDuIFN-γ using Fugene-6 reagent (Roche Diagnostics). At 24 and 48 h post-transfection, the LMH cells were lysed and RNA was extracted as described below, treated with RQ1 DNase (Promega) for 1 h at 37 °C, re-extracted using phenol/chloroform and ethanol precipitated. The expression of DuIFN-γ RNA was confirmed by Northern blot analysis using a radiolabelled, cloned DuIFN-γ probe.
Detection of DuIFN-γ RNA and DHBV DNA by real-time PCR.
A real-time RT-PCR for DuIFN-γ RNA detection was set up using primers (forward, 5'-CAAGTATCCCGATGTAGC-3'; reverse, 5'-GCGTGGATTTTCAGGCC-3') designed using Oligo 5 software (MedProbe) based on DuIFN-γ sequence GenBank accession no. AF087134 (Schultz & Chisari, 1999). RNA was extracted from liver samples as described above and treated with RNase Q1 DNA prior to reverse transcription to ensure the absence of cellular DNA. The real-time RT-PCR was carried out by reverse transcription (30°C, 30 min) in a LightCycler (Roche), followed by denaturation (95°C for 10 min) and 40 amplification cycles as follows: denaturation at 95°C for 15 s; annealing at 55°C for 10 s and extension at 72°C for 15 s using a SYBR Green I RNA amplification kit (Roche) according to the manufacturer's instructions. The reaction was first standardized using 10-fold serial dilutions (1×10⁶ copies to 1 copy) of pCLDuIFN-γ plasmid samples. Melting-curve analysis of the DNA standards for DuIFN-γ showed a single peak at a melting temperature (Tm) of 82-91°C, confirming a single PCR product identity and specificity (data not shown). A standard curve was prepared using 10-fold serial dilutions (1×10⁴–1×10¹⁰ pg) of RNA extracted and purified from pCLDuIFN-γ-transfected LMH cells as described above, which were run in a one-step RT-PCR and used as quantification standards showing similar Tm values (82-91°C) to that obtained for cloned DuIFN-γ DNA. The relative concentration of target DuIFN-γ RNA was calculated automatically by reference to this curve by the LightCycler software (Roche). All samples from infected and uninfected ducks were tested in duplicate and run together. The detection limit of this assay was one copy of ampiclon DNA per reaction. The assay was normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA, tested in the same samples using primers derived from the chicken GAPDH sequence (forward, 5'-AAGGTGTCGCTTAGCGTG-3'; reverse, 5'-GCCAGGCAGTTGGTGTCG-3').

Quantification of total DHBV DNA was performed using a real-time PCR designed to amplify both relaxed-circular and viral covalently closed circular (ccc) DNA forms, as described previously (Seigneres et al., 2003; with minor modifications). Briefly, primers were used that specifically amplified a 160 bp DHBV DNA fragment (forward, 5'-CTGACGGACAAAGGGTCCTAG-3'; reverse, 5'-GCGTGGGCAGAGAGGAGGT-3'). Real-time PCR was performed according to the manufacturer's instructions (Roche) in a reaction containing 0.5 mM of each primer and 0.2 and 0.4 µM 3'-labelled fluorescein probe (5'-CCCTCCATCTCCTCCTAGGCTCCCTG-3') and 5.5'-labelled Red 640, 3'-phosphate probe (5'-TCCGAAATCTCTCGTGCTGTTTAACGCG-3'; respectively (TIB MOLBIOL). The real-time PCR comprised a denaturation step at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 10 s, 61°C for 10 s and 72°C for 15 s, with a single fluorescent reading taken at the end of each amplification step. GAPDH amplification was used for normalization of liver samples, as described previously (Seigneres et al., 2003).

Histology and immunochemistry of liver sections. Three micrometre thick, formalin-fixed duck liver tissue sections were stained with haematoxylin/eosin/safran and examined under code with a light microscope. Portal infiltrates were graded in three semiquantitative stages: +, mild; ++, moderate; ++++, marked (Barraud et al., 1999). DHBV preS proteins were detected by immunoperoxidase staining of liver sections using a previously characterized anti-preS murine monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse IgG (Vector Laboratories), as described previously (Barraud et al., 1999; Sunyach et al., 1999). All slides were counterstained with haematoxylin.

Statistical analysis. A Mann–Whitney test was used to compare the difference in IFN-γ expression between infected and uninfected animals. A value of P<0.05 was considered to be statistically significant.

RESULTS

Transient DHBV viraemia correlates with the early appearance of an anti-preS antibody response in duck sera

Viraemia was assessed by dot blots for the entire 9 week follow-up period by detection of DHBV DNA in sera collected from a group of three ducks daily during the first week after inoculation and thereafter once a week for the following 8 weeks. All inoculated ducks became infected and viraemia peaked during the first week on day 4 p.i. at a low level of approximately 4×10⁸–5×10⁹ vge ml⁻¹ (Fig. 1). Thereafter, the level of viraemia dropped to undetectable levels from day 7 p.i. to the end of the follow-up period. As illustrated in Fig. 1, all infected ducks mounted a rapid anti-preS response, which was detectable as early as day 3 p.i., reaching a plateau at days 5–17, followed by a decrease and fluctuations. Therefore, the resolution of transient DHBV infection in adolescent ducks was characterized by low-level viraemia and early anti-preS antibody response induction. None of the ducks from the uninfected group followed in parallel mounted a detectable anti-preS response.

Increase in IFN-γ expression during resolution of DHBV infection

Next, we investigated the evolution of intrahepatic DuIFN-γ expression during the resolution of viral infection in relation to DHBV DNA levels. The mean DHBV DNA content detected in liver samples obtained from three ducks sacrificed at each time point (days 0, 3, 4, 6, 14, 28, 40 and 66 p.i) was analysed. The presence of DHBV DNA was detectable in duck livers as early as day 3 p.i., peaking at days 4–6 to approximately 3.5×10⁵ vge (µg liver DNA)⁻¹ (Fig. 2a). Southern blot analysis of viral DNA confirmed the presence of all replicative DHBV DNA intermediates in these liver samples (data not shown). Thereafter, the level of intrahepatic viral DNA declined, sharply reaching baseline levels, and was undetectable by Southern blot or dot-blot analysis (Fig. 2a).

We then examined whether the resolution of transient DHBV infection was associated with DuIFN-γ expression using quantitative RT-PCR to monitor intrahepatic DuIFN-γ RNA expression at different time points. As a first step towards such a study, we developed a one-step real-time RT-PCR for DuIFN-γ RNA detection, using primers based on the cloned DuIFN-γ sequence (Schultz & Chisari, 1999). This assay was validated using a range of cloned DuIFN-γ plasmid DNA dilutions and cDNAs produced by reverse transcription of RNAs from pCL-DuIFN-γ-transfected LMH cells as standards. The constructed standard curves for DuIFN-γ RNA were reproducibly linear, with the same slope over different experiments, allowing a precise and reproducible quantification of DuIFN-γ expression in infected and uninfected duck liver samples (data not shown). Using this assay, the analysis of uninfected duck livers showed a very low basal content of DuIFN-γ RNA (Fig. 2a). By

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contrast, in the liver samples from infected ducks, DuIFN-γ RNA levels were upregulated, rising rapidly from day 3 p.i. and peaking at day 14 at levels 15–20-fold higher than those observed for uninfected animals, followed by a sharp decline to day 28 (Fig. 2a). From day 28 to 40, intrahepatic DuIFN-γ RNA levels remained slightly elevated, although to a lesser extent, in the group of DHBV-infected ducks compared with the uninfected controls (Fig. 2a). Interestingly, at the end of the follow-up period (day 66 p.i.), a significantly higher level \((P = 0.05)\) of DuIFN-γ expression was observed in infected duck livers compared with uninfected controls (Fig. 2a and data not shown).

To explore whether residual viral DNA persisted in these duck livers, we searched for the presence of DHBV DNA using a quantitative real-time PCR. Traces of DHBV DNA ranging from \(2 \times 10^5\) to \(1.8 \times 10^4\) vge (\(\mu g\) liver DNA)\(^{-1}\) (0.005–0.046 copies per cell) remained detectable by real-time PCR in the livers of all three ducks sacrificed on days 28 and 40 and two (nos 302 and 303) of the three animals sacrificed on day 66 p.i. Thus, the rapid fall in intrahepatic DHBV DNA content correlated with a strong increase in hepatic DuIFN-γ expression, which decreased thereafter but remained detectable for 9 weeks p.i. and correlated with traces of DHBV DNA in the livers of animals that had apparently resolved the infection. These results were reproductively observed in two independent experiments.

**Augmented DuIFN-γ expression in PBMCs**

Next, we asked whether DuIFN-γ RNA could be detected in PBMCs during transient DHBV infection. PBMCs were isolated from blood samples collected from a total of six DHBV-infected and six uninfected animals on days 3, 4, 5, 6, 12, 14, 19, 26 and 33, and the amount of DuIFN-γ RNA was quantified by real-time PCR. As illustrated in Fig. 2(b), DuIFN-γ RNA was only detectable in the PBMCs of DHBV-infected ducks, starting from day 5 p.i. and peaking at the same day (day 14 p.i.) as in the liver, although at lower levels, followed by a sharp decrease to baseline levels (Fig. 2b). Therefore, the peak in liver IFN-γ RNA levels coincided with...
an increase, albeit to a lesser extent, in PBMC DuIFN-γ RNA.

**Limited spread of virus infection in the liver during resolution of infection**

To analyse the spread of DHBV during resolution of infection, viral preS proteins were stained in liver sections from animals sacrificed at different time points, counted and expressed as a percentage of the total number of hepatocytes (Fig. 3). As illustrated in Fig. 3(c), cytoplasmic staining of viral preS proteins showed that about 15–25 % of hepatocytes were positively stained for this antigen at days 4–6 p.i., consistently with the peak of liver DHBV DNA at these time points. However, at day 14, only 1–4 % positive hepatocytes were detected (Fig. 3d and data not shown) and staining became undetectable from day 40 (Fig. 3e) to the end of the follow-up period (day 66). Thus, the increase in DuIFN-γ expression and the anti-preS antibody response during resolution of DHBV infection coincided with a very rapid fall not only in levels of intrahepatic viral DNA, but also of viral envelope proteins.

**Histological analysis**

Steatosis and amyloidosis, which are common features in ducks, were observed in a total of five animals, three of which were infected with DHBV and two of which were uninfected, age-matched controls. In addition, histological analysis revealed changes limited to portal lymphocyte infiltration that were observed starting at day 3 p.i. in all infected duck livers and persisted at days 14 and 28 and 40 in most of the animals. As summarized in Table 1, the degree of inflammation was variable, with individual bird-to-bird variation, ranking from mild to marked portal infiltration, the latter being observed at day 6 p.i., as illustrated in Fig. 3(a). Interestingly, at day 66 p.i., the presence of traces of intrahepatic DHBV DNA and augmented DuIFN-γ correlated for two ducks (nos 302 and 303), with persistence of moderate portal lymphocyte infiltration in their livers as shown in Fig. 3(b) for duck no. 302.

**DISCUSSION**

In this study, we examined the elimination kinetics of DHBV infection in adolescent ducks and revealed for the first time that resolution of infection is associated not only with an early humoral response induction, but also with increased hepatic DuIFN-γ expression. This is of particular interest, since studies of mechanisms involved in DHBV clearance have been hampered by poor knowledge of duck cytokines and their role in the outcome of DHBV infection. In our study, despite the relatively large amount of inoculum

![Fig. 3. Histological analysis and immunochemical detection of viral preS protein in duck livers. (a, b) Histological examination of liver sections from a duck sacrificed at day 6 p.i. showing marked portal lymphoid infiltration (arrows) (a) and persistence of portal infiltration at day 66 in the liver of duck no. 302, which correlated with increased expression of DuIFN-γ and traces of intrahepatic DHBV DNA (b). Magnification, × 190. (c)–(e) Expression of viral preS protein visualized by immunostaining using monoclonal antibody mAb900 and hydrogen peroxidase-labelled secondary antiserum in the liver section of a duck sacrificed at day 3 p.i. showing cytoplasmic distribution of L protein (arrows) (c), a duck sacrificed at day 14 p.i. (d) and a duck sacrificed at day 40 p.i. (undetectable staining) (e). Nuclei were visualized by counterstaining with haematoxylin, allowing counting of antigen-staining cells as a percentage of the total number of hepatocytes. A representative section for each time point is shown. Magnification, × 400.](http://vir.sgmjournals.org)
(5 × 10^{19} vge per duck), infection of adolescent Pekin ducks with DHBV resulted reproductively in a transient outcome. This is similar to the observations of Zhang & Summers (2004), but contrasts with the data of Jilbert et al. (1998), who reported that infection of adolescent ducks could lead to either transient or chronic infection. The differences observed in the outcome of infection in these studies may be related to several factors such as the origin of the ducks and their susceptibility to DHBV infection.

We found that resolution of DHBV infection in 3-week-old ducks was characterized by low-level viraemia not exceeding 4 × 10^{3}–5 × 10^{7} vge ml^{-1} and transient intrahepatic preS protein expression that was restricted to 15–25% of hepatocytes and correlated with early anti-preS antibody induction. Our previous studies showed that infection of neonatal ducklings with a similar DHBV inoculum pool resulted in a chronic outcome characterized by viraemia levels that were at least 2 logs higher and by massive expression of viral proteins in almost 100% of hepatocytes and correlated with early anti-preS antibody induction. Our data are consistent with and extend the findings of Zhang & Summers (2004), who analysed virus spread in the liver in detail during DHBV infection and showed that neutralizing anti-preS antibodies, arising in adolescent ducks but not in neonates, prevented secondary cycles of infection, resulting in limited intrahepatic spread of virus and a transient outcome of infection. Anti-preS antibodies play an important role in neutralization of DHBV infectivity and inhibition of virus interaction with the host cell receptor, as documented by us and others (Sunyach et al., 1999; Urban et al., 2000). However, these studies did not investigate the contribution of IFN-γ, known to play a key role in the outcome of WHV and HBV infections (Hodgson & Michalak, 2001; Wieland et al., 2004), in the resolution of DHBV infection.

To address this issue, we took advantage of previous DuIFN-γ cDNA cloning and sequencing (Schultz & Chisari, 1999) to develop a quantitative real-time RT-PCR assay for DuIFN-γ RNA monitoring, similar to the assay recently described for woodchuck IFN-γ RNA (Menne et al., 2002). Importantly, using this approach, we have provided the first evidence that DuIFN-γ is expressed strongly during viral clearance in transiently infected ducks. Thus, the sharp decrease in serum and liver DHBV DNA levels during resolution of DHBV infection was associated with a rise in intrahepatic DuIFN-γ RNA to levels that were approximately 15- to 20-fold greater than in uninfected ducks. This is similar to the resolution of WHV infection of woodchucks, although the increase in IFN-γ RNA in acutely infected woodchuck livers was lower, reaching levels about 4-fold higher than those detected in control animals (Hodgson & Michalak, 2001).

Notably, our results suggest a considerable difference in the kinetics of resolution of infection between avian and mammalian hepadnaviruses. We observed an extremely rapid rise in DuIFN-γ RNA levels and a sharp fall in intrahepatic viral DNA levels and antigen clearance during the first 2 weeks p.i. By contrast, the increase in IFN-γ expression starts only 2–3 months p.i. during resolution of WHV and HBV infection in woodchuck and chimpanzee models and correlates with a delayed and slower clearance of infection (Hodgson & Michalak, 2001; Wieland et al., 2004). The difference in the dynamics of IFN-γ induction in duck compared with chimpanzee or woodchuck may play a role in the differences in kinetics of IFN-γ expression during resolution of viral infection in these distant species. In addition, several factors such as virus replication rate, rapidity and magnitude of the neutralizing antibody response and turnover of hepatocytes may contribute to the observed differences in the kinetics of resolution of hepadnaviral infection in these models.

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### Table 1. Summary of intrahepatic expression levels of DHBV preS protein and inflammation during resolution of transient infection

The number of animals presenting different degrees of portal infiltration is indicated in parentheses. ND, Not detected.

<table>
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<tr>
<th>Time p.i.</th>
<th>Cells positive for DHBV preS protein (%)</th>
<th>Portal infiltration*</th>
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<tr>
<td>Days 3–6</td>
<td>15–25</td>
<td>+ (3)</td>
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<td></td>
<td></td>
<td>+ + (3)</td>
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<td>+ + + (2)†</td>
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<tr>
<td>Day 14</td>
<td>1–4</td>
<td>+ (1)</td>
</tr>
<tr>
<td>Day 40</td>
<td>ND</td>
<td>+ (2)</td>
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<tr>
<td>Day 66</td>
<td>ND</td>
<td>+ (1)</td>
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*Portal infiltration was graded semi-quantitatively as: +, mild; + +, moderate; + + +, marked. †Marked portal infiltration was observed only on day 6 p.i.
Histological analysis of duck liver samples showed changes limited to different degrees of portal lymphocyte infiltrations that were observed starting at the peak of infection and continuing to the end of the follow-up period. This is consistent with previous studies showing that resolution of DHBV infection in ducks is associated with only a very mild liver disease with no evidence of significant cell death contributing to viral clearance (Jilbert et al., 1992). Interestingly, we observed that DuIFN-\(\gamma\) remained upregulated from day 28 to 40 in the liver of ducks that had apparently resolved the infection and was associated with persistence of portal inflammation. At the end of the follow-up period (day 66 p.i.), although viral preS proteins were undetectable by immunostaining, traces of DHBV DNA were detected by real-time PCR in two duck liver samples (nos 302 and 303) and correlated with increased expression of DuIFN-\(\gamma\) and portal lymphocyte infiltrations. These findings suggest that residual viral DNA persists in the livers of ducks that have apparently recovered from viral infection and have seroconverted to anti-preS and is associated with an augmented DuIFN-\(\gamma\) expression. Our data extend previous observations in woodchucks that apparently completely recovered from acute WHV infection but showed an increased hepatic IFN-\(\gamma\) expression for years, which correlated with recurring mild inflammatory changes and trace quantities of virus (Hodgson & Michalak, 2001). Le Mire et al. (2005) analysed the residual DHBV DNA in duck livers in detail following recovery from transient infection and showed that cccDNA is a predominant form of viral DNA, although it appears to be inactive and non-replicative. Whether trace amounts of DHBV DNA are essential for the maintenance of augmented hepatic DuIFN-\(\gamma\) expression, which may persist lifelong and play a role in antiviral immunity as suggested for HBV-specific immunity in recovered individuals (Rehermann et al., 1996; Rehermann & Nascimbeni, 2005), is unknown and requires further studies on the cellular response during virus clearance and host cytokine expression monitoring during a long-term follow-up of ducks that have apparently resolved infection.

Using real-time RT-PCR, increased PBMC IFN RNA expression was recently reported in macaques during acute simian immunodeficiency virus infection (Abel et al., 2001, 2004). In the present study, using a similar quantitative RT-PCR we showed that PBMC DuIFN-\(\gamma\) RNA levels increased to moderate levels, which correlated with the peak of intrahepatic DuIFN-\(\gamma\) RNA (days 12–14). These findings suggest that follow-up of IFN-\(\gamma\) RNA in duck PBMCs may represent an informative and less invasive marker than liver biopsy for monitoring of IFN-\(\gamma\) expression during DHBV infection. The follow up of DuIFN-\(\gamma\) expression in liver and PBMCs will be of particular value for investigation of the mechanisms involved in virus clearance during DNA vaccine-based immunotherapy, the ability of which to decrease and even eliminate intrahepatic virus replication in chronic DHBV carrier ducks has been demonstrated recently by us (Rollier et al., 1999; Le Guerher et al., 2003; Thermet et al., 2003).

Taken together, our findings revealed that, in addition to humoral anti-preS responses, augmented levels of DuIFN-\(\gamma\) RNA in liver and PBMCs are concomitant with viral clearance and characterize the recovery from transient DHBV infection. In addition, our data suggest a role for DuIFN-\(\gamma\) expression in the resolution of DHBV infection, as well as in the control of persistent low levels of viral genomes in the liver. Moreover, we showed that the kinetics of IFN-\(\gamma\) expression is extremely rapid during the resolution of DHBV infection compared with mammalian hepadnaviruses. As DHBV-infected duck is a pivotal model for hepadnavirus replication studies and antiviral evaluation, these results provide important new insights into the host–virus interactions that control DHBV infection and warrant further studies on the cellular response during virus clearance in this model.

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