INTRODUCTION

In the genome of hepatitis B virus (HBV) the coding region for the core-protein (HBc) is preceded by an in-phase open reading frame (ORF), known as pre-C (Chang et al., 1987). From this region, the two transcripts for HBc and pre-C are initiated (Buscher et al., 1985; Netter et al., 2008). When protein synthesis starts at the core-ATG, the resulting HBc is localized in the cytoplasm of liver cells with a strong tendency for assembly into viral core particles (Cohen & Richmond, 1982), while the pre-C protein, initiated upstream of the core ATG, is targeted to the endoplasmic reticulum for further proteolytic processing, resulting in a protein termed e antigen (eAg) or HBe, which is transported to the outer cell membrane (Schlicht & Schaller, 1989), from where it is secreted (Ou et al., 1986). HBe can be detected in sera of infected persons (Tiollais et al., 1985) and complex formation of a proportion of HBe with immunoglobulins has been observed (Takahashi et al., 1978). Seroconversion from HBe to anti-HBe antibodies serves as a clinically relevant marker for resolution of active HBV infection and for a long-term positive response to treatment.

As in HBe, C-terminally truncated polypeptides of 27 kDa, derived from the expression of the pre-C region of duck HBV (DHBV), which are analogically termed DHBe, can be detected in the sera of infected ducks. In contrast to HBe, DHBe harbours two glycosylation sites. In sera of infected ducks, the bulk of DHBe is found in its glycosylated form with molecular masses of 30 (GP30c) and 33 kDa (GP33c) (Schlicht, 1991; Schlicht et al., 1987).

The expression of a secretory protein derived from the pre-C ORF is a conserved feature among the family Hepadnaviridae (Enders et al., 1985; Moroy et al., 1985; Ou et al., 1986) and has only one parallel, in a gag-related protein of Moloney murine leukemia virus (Edwards & Fan, 1980; Schwartzberg et al., 1983). To investigate the functional relevance of DHBe, Chang and co-workers introduced a frameshift mutation into the pre-C region of the DHBV genome. Analysis of this DHBe mutant demonstrated that the expression of the pre-C region was not essential for virus infection and replication in vivo as well as in vitro; this was a surprise in relation to the small size and the extremely compact coding organization of the hepadnaviral genome (Chang et al., 1987). Furthermore, among human HBV carriers, a mixed population of HBV wild-type viruses and HBe-negative mutants is relatively widespread. Due to a higher prevalence of advanced fibrosis and a lower likelihood of spontaneous remission, infection with HBe-negative viral strains correlates with a poorer prognosis compared with wild-type infection. Although interesting experimental data obtained with transgenic mice suggest that HBe may act as an immune regulator in the infected host (Milich & Liang, 2003), little is still known about the role of eAg during natural infections with HBVs.

In this study, we concentrated DHBe from the supernatant of persistently infected primary duck-liver cells (PDLCs) and studied its influence on de novo infections of fetal PDLC cultures.
**METHODS**

**Cells and infection.** PDLCs were prepared from fetal duck livers as described previously (Bruns et al., 1998; Schlicht et al., 1993). Unless otherwise stated, PDLCs were infected with DHBV at an m.o.i. of 1 or 10 on day 1 after plating in a volume of 0.5 ml per well in six-well plates. Non-adsorbed virus particles were removed by washing with Williams’ medium E (Gibco). PDLCs were then cultured in 2 ml medium per well for 7 days. DHBBe [250 ng total protein collected from Sephadex G-75 fractions or 100 µl concanavalin A (ConA) eluate] was added to mock-infected or infected PDLCs with or without addition of 0.05 M α-methyl-D-mannopyranoside (mannoside) (Carl Roth) during virus adsorption for 4 or 24 h. In one experiment, mannoside was reduced or replaced with the equivalent amount of mannos or sucrose.

**Purification of DHBBe.** Supernatants (days 2–7) of DHBV carrier PDLCs served as the source of DHBBe. After removal of cell debris from the supernatants by low-speed centrifugation, virus particles were pelleted via ultracentrifugation at 100 000 g for 4 h. The supernatant was then dialysed against ConA buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 10 mM CaCl2, 10 mM MgCl2, 6H2O, 10 mM MnCl2, 4H2O). After dialysis, glycosylated and non-glycosylated proteins from the supernatant were separated via adsorption chromatography using the ConA coupled to CNBr-activated Sepharose-4B (Amersham Biosciences). Unspecifically adsorbed proteins were removed from the column with 1 M NaCl in ConA buffer and combined with the unbound material (termed ConA flow-through), whereas the bound glycosylated proteins were specifically eluted with 0.2 M mannoside in ConA buffer (termed ConA eluate). Eluted proteins were precipitated by addition of 50% (w/v) ammonium sulphate and centrifuged at 10 000 r.p.m. for 1 h with the GSA rotor in a Sorval centrifuge (Du Pont Nemours). The precipitated material was solubilized and thoroughly dialysed against PBS for direct application in cell culture. Most studies were performed using the ConA eluates enriched for DHBBe; the ConA flow-through, as well as eluates and flow-throughs of uninfected PDLC supernatants, were used as controls. For further purification, precipitates were dialysed against a salt-free isoelectric focusing (IEF) buffer (1% glycerol, 25 kDa), ovalbumin (45 kDa), which appeared approximately at the position of the DHBBe peak, and BSA (67 kDa), which was eluted in the void volume. From the IEF-peak, the sample was further purified using the ConA eluate. Eluted proteins were precipitated by addition of 50% (w/v) ammonium sulphate and centrifuged at 10 000 r.p.m. for 1 h with the GSA rotor in a Sorval centrifuge (Du Pont Nemours). The precipitated material was solubilized and thoroughly dialysed against PBS for direct application in cell culture. Most studies were performed using the ConA eluates enriched for DHBBe; the ConA flow-through, as well as eluates and flow-throughs of uninfected PDLC supernatants, were used as controls. For further purification, precipitates were dialysed against a salt-free isoelectric focusing (IEF) buffer (1% glycerol, 1% glycine and 0.1% Triton X-100). Insoluble proteins were removed by low-speed centrifugation and the DHBBe-containing supernatant was loaded on a preparative IEF column (LKB-Pharmacia,) and electrofocused for 72 h with a constant power of 2 W in a 0–40% sucrose gradient containing 1% ampholines, pH 3.5–10 (Amersham), and 0.1% Triton X-100, as described previously (Bruns & Frenzel, 1979). Fractions positive for DHBBe were collected, mixed with saturated ammonium sulphate solution to a final concentration of 50% and centrifuged for 10 min at 10 000 g. Precipitated material, concentrated at the top of the centrifuge tube, was subjected to molecular sieve chromatography in a Sephadex G-75 column (Amersham) (60 × 1 cm), using STE buffer (0.15 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.5) with 1 M KCl and 2 mM phenylmethane-sulfonyl fluoride (Sigma); void volume (Vv) and total volume (Vt) of the column were identified using blue dextran 2000 (Amersham) and phenol red (Sigma), respectively. Before the addition of DHBBe preparations to PDLCs, the samples were dialysed against PBS. For a rough estimation of the molecular mass of the proteins in the collected fractions, a Sephadex G-75 column was loaded with proteins of known sizes: chymotrypsinogen (25 kDa), ovalbumin (45 kDa), which appeared approximately at the position of the DHBBe peak, and BSA (67 kDa), which was eluted in the void volume.

**Isolation and analysis of DHBBe DNA.** DNA replicative intermediates of DHBV were purified from infected cells as described by Fernholz et al. (1993). The DNA was subjected to 1% agarose gel electrophoresis, followed by transfer onto Hybond-N membranes (Amersham) and UV cross-linking. For hybridization, DHBV-3 plasmid DNA was labelled with [32P]dCTP (Amersham). The DNA concentration of DHBBe in duck sera and in supernatants of infected PDLCs was measured by dot blot using a serial dilution of the DHBV-3 plasmid DNA as a standard (Maenz et al., 2007).

**Protein measurement and DHBBe ELISA.** The total protein content of each purification step was calculated using the Bio-Rad protein assay with the Bradford reagent (Bradford, 1976). For the determination of the relative amounts of DHBBe in the processed PDLC supernatants, an ELISA was carried out. Aliquots of the samples were adsorbed onto MaxiSorp Immunoplates (Nunc) for 2 h at room temperature, followed by incubation in 5% TWEEN 20 in PBS (T-PBS) at 4°C overnight. Thereafter, the protein-coated plates were incubated first with a polyclonal antisera against duck HBc (DHBc), which recognized DHBBe due to the sequence homology of large parts of both proteins, and then with horseradish peroxidase-labelled anti-rabbit immunoglobulins (Medac). Positive reactions were visualized by addition of O-phenylenediamine dihydrochloride in 0.2 M Na2HPO4 and 0.1 M citric acid (pH 5) containing an aliquot of H2O2 according to the manufacturer’s protocol (Sigma). After incubation for 5 min at room temperature, the reaction was stopped by addition of 2 M HCl, whereupon the colour change was measured at 490 nm.

**Detection of proteins in infected cells.** Cellular proteins were lysed in 20 µl sample buffer (4% SDS, 10% β-mercaptoethanol, 0.05 M Tris/HCl pH 7), boiled for 3 min and separated on 5–20% polyacrylamide gradient gels (Laemmli, 1970). For immunoblotting, the proteins were transferred to PVDF membranes (Bio-Rad), which were incubated with polyclonal rabbit antiserum raised against the small or large viral surface proteins (DHBs and DHBpreS, respectively) or DHBc (Fernholz et al., 1993; Schneider et al., 1991). Visualization was carried out by addition of peroxidase-labelled anti-rabbit immunoglobulins (Medac) and reaction with an enhanced chemiluminescent substrate (Pierce) followed by exposure to X-ray films.

**Analysis of PDLC subpopulations.** In order to demonstrate the presence of liver sinusoidal endothelial cells (LSECs), fluorescein-conjugated acetylated low-density lipoprotein (Bodipy FL–acLDL; Molecular Probes) was added to PDLCs, one day after plating, for 2 h; following this, cells were either examined directly by fluorescence microscopy or fixed and subjected to immunocytochemistry with anti-pig collagen antibody.

**Immunocytochemistry.** Cell layers grown on coverslips were washed with cold PBS, fixed for 5 min in methanol and 4 s in acetone at −20°C and air-dried. Fixed cells were blocked with 1% BSA in 0.1% T-PBS, incubated for 1 h with rabbit anti-DHBc antisera, washed three times with T-PBS and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulins (Invitrogen). Cell nuclei were counterstained using Hoechst 33342 (Invitrogen). Cells were again washed three times with T-PBS and mounted with Kaiser’s gelatin (Merck).

**RESULTS**

**Production of DHBBe**

We have studied the DHBBe titre in a duck persistently infected with DHBV from 2 days after hatching over the course of 2 years and compared it with the virion production throughout this period (Supplementary Fig. S1, available in JGV Online). There were fluctuations in the levels of viral DNA and DHBBe in the serum that did not correlate with each other. Thus, DHBBe production or secretion must be regulated independently of the level of viraemia.
To elucidate the source of naturally processed DHBe, we tested cell culture supernatants of the chicken hepatoma cell line LMH (Kawaguchi et al., 1987) transfected with a DHBV expression construct and supernatants of PDLCs either freshly infected in cell culture or prepared from duck embryos congenitally infected with DHBV. The DHBe production from transfected cells was low compared with that from freshly infected PDLCs, which secreted more DHBe relatively late after infection. DHBe could be detected on the first day of infection, possibly a remnant of the high viral input, but not on days 2 and 3. The different species of DHBe only became detectable on day 4 (Supplementary Fig. S2, available in JGV Online). Following this, PDLCs from the livers of ducklings persistently infected with DHBV were cultured, and secretion of DHBe was investigated in two parallel experiments by collecting and replacing the culture media at intervals of 3 days. Subviral particles (SVPs) were continuously released into the supernatants of the carrier-PDLCs during the study period (Fig. 1, lanes 2–4 and 9–11). The detection of the small and large viral surface proteins DHBs and DHBpreS in the supernatants served as an indirect measure of virus replication, as viral capsids cannot usually be visualized by Western blot of supernatants of infected PDLCs due to their low abundance (Franke et al., 2007). DHBe was detected with the polyclonal anti-DHBc serum. Secretion of both forms of glycosylated DHBe, GP30^c and GP33^c, could be detected, as well as the unglycosylated form P27. A preservation of P27 through the process of preparations from supernatants of infected PDLCs and, at the same time, an increase in DHBe titres. The first concentration step, the ConA column, resulted in an enrichment of DHBe in the eluate by a factor of 800 in relation to total protein content. The final collection of DHBe-positive fractions from the Sephadex G-75 column was concentrated 60,000 and 200,000 times, respectively. The Western blot (Fig. 2d) demonstrated the integrity of DHBe after the numerous purification steps. Both glycosylated species, GP30^c and GP33^c, could be detected, as well as the unglycosylated form P27. A preservation of P27 through the process of preparations from supernatants of infected PDLCs, which were either uninfected or infected with the same virus dose (Fig. 3a, lanes 9 and 10). This revealed that a reduction of viral replication could be achieved with the ConA eluate alone, whereas viral replication was considerably impaired by the addition of ConA eluate plus mannoside during infection (Fig. 3a, lanes 3 and 4). The ConA flow-through, with or without addition of mannoside (Fig. 3a, lanes 1 and 2), showed no effect on virus replication. No reduction of virus replication could be observed when the corresponding preparations from control cells were used (Fig. 3a, lanes 5–8). On the contrary, an

Concentration of DHBe

The kinetic studies performed with DHBV carrier cells exposed them as reliable producers of DHBe for protein purification. A further advantage of the employment of PDLCs for DHBe production compared with cell lines was the possibility to forego serum in the culture medium, thus ruling out that factors from calf serum would be co-enriched. DHBe was concentrated from supernatants via ConA–Sepharose columns. For a direct comparison of the amount of DHBe in flow-through and eluate, both were precipitated with ammonium sulphate, picked up in the same volume of PBS and extensively dialysed. ELISAs of supernatants and resolubilized precipitates confirmed that the entire amount of DHBe was always retained in the supernatants (data not shown). The DHBe concentration in the flow-through was approximately 1000 times below the concentration in the eluate (Fig. 2a).

The dialysed material was subjected to preparative IEF in a column containing a sucrose gradient together with a non-ionic detergent and ampholytes, which were used to establish a pH gradient between 3.5 and 10 (Fig. 2b). The IEF revealed a relatively broad range of DHBe-positive fractions with distinct enrichments at pH 5.3 and 5.7. The fractions containing the highest serological activities (fractions 22–24 and 27–28) were collected and concentrated with ammonium sulphate. The concentrated material was picked up in high-salt STE buffer and separated via molecular sieve chromatography (Fig. 2c). Again, DHBe-positive fractions, appearing shortly after the void volume, were collected and dialysed against PBS. Finally, the different preparation steps were compared by measuring and comparing the total protein concentrations and DHBe ELISA titres to calculate the magnitude of enrichment (Table 1).

This analysis demonstrated an overall decrease of proteins and, at the same time, an increase in DHBe titres. The first concentration step, the ConA column, resulted in an enrichment of DHBe in the eluate by a factor of 800 in relation to the probe volume and by a factor of 100 in relation to total protein content. The final collection of DHBe-positive fractions from the Sephadex G-75 column was concentrated 60,000 and 200,000 times, respectively. The Western blot (Fig. 2d) demonstrated the integrity of DHBe after the numerous purification steps. Both glycosylated species, GP30^c and GP33^c, could be detected, as well as the unglycosylated form P27. A preservation of P27 through the process of preparations from supernatants of infected PDLCs, which were either uninfected or infected with the same virus dose (Fig. 3a, lanes 9 and 10). This revealed that a reduction of viral replication could be achieved with the ConA eluate alone, whereas viral replication was considerably impaired by the addition of ConA eluate plus mannoside during infection (Fig. 3a, lanes 3 and 4). The ConA flow-through, with or without addition of mannoside (Fig. 3a, lanes 1 and 2), showed no effect on virus replication. No reduction of virus replication could be observed when the corresponding preparations from control cells were used (Fig. 3a, lanes 5–8). On the contrary, an
increased viral replication or accumulation of DHBpreS within infected cells was seen when infection was accompanied by treatment with ConA eluate from control cells, irrespective of the addition of mannoside (Fig. 3a, lanes 7 and 8). We concluded that the crude enrichment of glycoproteins from cellular supernatants still contained secreted factors that may influence DHBV infection. No toxic effects of any of the samples on the cells were observed (Fig. 3b).
Increased inhibition by extended incubation of PDLCs with the ConA eluate/mannoside combination

Our next experiment was performed to increase the effect of inhibition with ConA eluate/mannoside. To achieve this, the incubation of PDLCs with DHBV at an m.o.i. of 10 and the ConA eluate or flow-through, with or without mannose, was extended to 24 h (Fig. 4a). Immunoblotting of cellular proteins 7 days after infection demonstrated that the longer incubation period with ConA eluate of carrier PDLCs, in combination with mannose, reduced the virus replication to an almost undetectable level (Fig. 4a, lanes 5 and 7). Again, addition of only one of the components did not show any significant change in DHBpreS synthesis (Fig. 4a, lanes 1 and 3 or 13 and 15) compared with untreated cells (Fig. 4a, lanes 9 and 11).

Fig. 2. Concentration and purification of DHBe. (a) Elution profile of a ConA–Sepharose column loaded with proteins from cell culture supernatants. DHBe titres in the collected flow through and eluate fractions were measured by ELISA. The results, given below the profile, correlate to the last dilution step, where DHBe was still detectable. (b) IEF of DHBe (bars, indicating reciprocal ELISA titres) within a pH gradient of 3.5–10 (A, pH values). Peak fractions were observed at pH 5.2–5.4 and 5.6–5.8. (c) Molecular sieve chromatography in Sephadex G-75 for the separation of proteins according to their molecular mass (●, OD515) and anti-DHBc ELISA of respective fractions to detect DHBe (bars). (d) Separation of proteins after each purification step by electrophoresis, followed by either staining of the gel with Coomassie blue (left panel) or Western blotting with anti-DHBc antiserum (right). Lanes: M, marker; CO, positive control (ammonium sulphate precipitation of DHBV carrier culture supernatant). The positions of DHBe variants are shown on the right side. pr and sn, Precipitates and supernatants, respectively, of ConA flow-through and ConA eluate after dialysis against IEF buffer.
**Table 1. Concentration of DHBe**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total protein (% of volume)</th>
<th>ELISA (reciprocal titre)</th>
<th>ELISA (relative reciprocal titre)</th>
<th>Enrichment factor (based on volume)</th>
<th>Enrichment factor (based on total protein)</th>
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<tbody>
<tr>
<td>Centrifugation at 100000 g</td>
<td>418</td>
<td>52.25</td>
<td>12.5</td>
<td>240</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ConA eluate</td>
<td>11</td>
<td>8.94</td>
<td>81.3</td>
<td>5120</td>
<td>21</td>
<td>800</td>
<td>100</td>
</tr>
<tr>
<td>Dialysis</td>
<td>15</td>
<td>0.1</td>
<td>0.7</td>
<td>6400</td>
<td>26</td>
<td>700</td>
<td>10000</td>
</tr>
<tr>
<td>IEF</td>
<td>8.7</td>
<td>0.05</td>
<td>0.6</td>
<td>5200</td>
<td>21</td>
<td>1000</td>
<td>20000</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>0.5</td>
<td>0.02</td>
<td>4.0</td>
<td>19200</td>
<td>80</td>
<td>60000</td>
<td>200000</td>
</tr>
</tbody>
</table>

**Inhibition of virus replication by further-purified DHBe**

The concentrate of glycoproteins from supernatants of PDLCs still contained a wide variety of proteins that could potentially influence infection when added together with DHBV, as seen with ConA eluates from control PDLCs (Fig. 3). Therefore, we sought to purify further the DHBe preparation as detailed above (Fig. 2 and Table 1). PDLCs were again infected at an m.o.i. of 10 and at the same time treated for 24 h with buffer only (Fig. 4b, lane 1), with mannoside alone (Fig. 4b, lane 2) or with mannoside and DHBe-positive fractions that had been collected from the Sephadex G-75 column (Fig. 4b, lane 3). The supernatants were harvested on days 4 and 7; the cells were also subjected to Southern blotting to visualize viral DNA. In cells treated with DHBe plus mannoside, viral DNA replicative intermediates were not detectable at day 7 after infection. Mannoside alone somewhat reduced the amount of DHBV single-stranded DNA in infected cells but relaxed-circular DHBV DNA was synthesized to the same extent as in infected PDLCs treated with buffer only. For unknown reasons, incubation with mannoside alone also reduced the amount of virions released into the supernatants harvested on days 4 and 7. Viral DNA was undetectable at day 7 after infection, when PDLCs were treated with the combination of DHBe and mannoside (Fig. 4b, lane 3). However, on day 4, a weak signal was seen in the supernatant of infected PDLCs treated with this combination (Fig. 4b, lane 3); this probably stems from the input virus (Fig. 4b, lane V). In an additional experiment, inhibition of virus replication was also observed, when, instead of mannoside, either mannose or sucrose was combined with purified DHBe (Supplementary Fig. S3, available in JGV Online). However, when we treated congenitally DHBV-infected PDLCs it was evident that in an already established infection, DHBe plus mannoside had no effect (data not shown).

**Association of DHBe with PDLC subpopulations**

All experiments were performed with whole liver preparations of fetal ducklings, which are, as in the human liver, composed of roughly 60 % parenchymal cells (hepatocytes) and 40 % non-parenchymal cells, where the latter comprises about 70 % LSECs, 20 % liver-specific macrophages (Kupffer-cells) and 10 % Ito cells (Götz et al., 1990). Previous in vitro studies demonstrated the phagocytosis of colloidal Indian ink by Kupffer cells and the specific uptake of acetylated low-density lipoprotein (acLDL) by LSECs (M. Bruns, unpublished data; Irving et al., 1984; Okaji et al., 2004). To date, the cellular receptor of DHBV remains elusive. Carboxypeptidase D (GP180) has been suggested to play a role in virus binding and internalization (Breiner et al., 1998; Kuroki et al., 1994; Urban et al., 2000) but its expression does not lead to productive infection in cell lines. Thus, another receptor or additional factors are required for DHBV infection. Also, the participation of another cell type, LSECs, which are in close contact with hepatocytes in vivo, has been discussed previously (Breiner et al., 2001). The observation that DHBe inhibited DHBV infection prompted us to find out which cells were able to bind or take up DHBe. Purified DHBe with or without mannoside was added to a PDLC culture overnight. The association of DHBe with the cells was then analysed by immunocytochemistry using anti-DHBe antiserum. The study displayed the co-localization of DHBe with distinct cells (Fig. 5a), which was not detected when additional mannoside was added to the culture (Fig. 5b). These cells were characterized by the incorporation of acLDL. Fluorescent labelling of DHBe in cells that were incubated with the Sephadex G-75 DHBe-positive fractions and acLDL revealed that some of the acLDL-positive cells contained DHBe (Fig. 5c). We therefore suggest that cells that bind or adsorb DHBe are most likely to be LSECs, although acLDL uptake has also been described for macrophages, though to a much lower efficacy. PDLCs that were incubated with DHBV SVPs did not show any co-localization of DHBpreS with acLDL (Fig. 5d).

**DISCUSSION**

So far, a vital function of the hepadnaviral eAg in the viral life cycle has not been described. It does not influence infection in vivo or in cell culture, and successful eAg mutants have been described for HBV and DHBV. On the other hand, the conservation of that protein within the hepadnavirus family, whose characteristic is an extremely compact genome, implies that it has a function in vivo.
We attempted to enrich DHBe from a natural source to investigate its influence during the course of acute infection of PDLCs in cell culture. By the use of ammonium sulphate precipitation, enrichment of glycoproteins via a ConA–Sepharose column, preparative IEF and molecular sieve chromatography, we obtained DHBe in the last fraction that was augmented by a factor of 200 000 based on the total protein content; both glycosylation variants of DHBe, GP30c and GP33c, were present. Nevertheless, DHBe was a minor component in the preparation, as the Coomassie blue staining of the gel revealed two bands of slightly lower molecular mass; although it is easily possible to underestimate the DHBe content, as glycosylated proteins are often poorly stained with Coomassie blue.

When we added concentrated DHBe to PDLCs during incubation with DHBV, there was only a minor effect on the establishment of a productive infection. However, simultaneous addition of DHBe and mannoside during incubation with DHBV led to such a powerful inhibition of

**Fig. 3.** Influence of ConA-concentrated glycoproteins on DHBV infection. ConA flow-throughs (lanes 1, 2, 5 and 6) and ConA eluates (lanes 3, 4, 7 and 8) of DHBV carrier supernatants and supernatants of control PDLCs were added to PDLCs during infection with DHBV, either with or without additional mannoside (indicated below the blots). The supernatants were harvested on day 7 post-infection. (a) Secretion of DHBpreS-containing particles (P36 and P28) into the supernatants served as a measure for virus production. Proteins were visualised by Western blot. DS, DHBV-positive duck serum. (b) Amido black stain of cellular proteins. M, Marker; SN, supernatant.
the infection that, 7 days after infection, we were not able to detect viral DNA replicative intermediates in the cells or viral DNA in the supernatants. Mannoside was introduced in our experiments to inhibit the binding of DHBe to its target as we predicted that as mannoside outcompetes DHBe binding to ConA, it might also do so with its natural receptor. Contrary to expectations, mannoside amplified the inhibitory effect of DHBe on the infection. We also found that the ConA eluate of the supernatants from uninfected PDLCs enhanced the infection considerably. Naturally, the ConA column concentrated all glycoproteins of the cell supernatants, probably including factors that influence virus infection. Therefore, the optimal control for these experiments would be supernatants of PDLCs from ducks congenitally infected with DHBe-mutant DHBV. Such a duck colony remains to be established.

**Fig. 4.** Inhibition of virus replication. (a) Extended incubation (24 h) of PDLCs with ConA eluate during infection. Intracellular DHBpreS expression (particles P36 and P28) was analysed by immunoblotting as a measure of infection. Treatment conditions are detailed below the blot. (b) Analysis of DHBV replication by Southern blot. Supernatants of DHBV-infected or mock-infected and DHBe/mannoside-treated PDLCs were removed after 4 days (SN/d4) and replaced with fresh supernatants that were harvested 3 days later (SN/d7) together with the PDLCs (Cells/d7). Virus DNA was analysed by Southern blot using a DHBV-specific radioactively labelled probe for hybridization. V, One tenth of the viral input; M, markers obtained by cutting DHBV-3 plasmid DNA with EcoRI (3 kb) or HindIII (1.7 and 1.3 kb); RC, DHBV relaxed circular DNA; SS, DHBV single-stranded DNA.
In search for a possible entry pathway of DHBV into hepatocytes, it was postulated that DHBV needs to be transported across LSECs to reach the hepatocyte compartment in the liver and that this pathway is conserved in vitro (Breiner et al., 2001). By co-staining with fluorescently labelled acLDL, we found DHBe but not DHBs from SVPs associated with LSECs. Co-localization was abolished by addition of mannose. LSECs present a high number of lectin receptors and are known for their capability to clear glycoproteins from the plasma (Knolle et al., 2000). DHBe, once attached to the surface of LSECs, could be internalized in a comparable fashion, as reported for the uptake of ceruloplasmin by LSECs. Furthermore, ceruloplasmin experiences desialation within LSECs as a prerequisite for its binding to hepatocytes (Irie & Tavassoli, 1986). Another related pathway was also discussed for the liver iron-transferrin receptors, which are limited to LSECs and are not present in hepatocytes and Kupffer cells (Tavassoli et al., 1986).

In order to understand the influence of DHBe plus mannose during infection of PDLCs with DHBV, one could hypothesize that at an early time point of infection, the glycosylated DHBe is altered either by desialation, modification of its sugar moieties or proteolytic cleavage. Following release of the modified DHBe from LSECs, it may act by supporting the binding of DHBV virions to hepatocytes by binding of a receptor in close proximity to the virion receptor. In this scenario, mannose would inhibit processing of DHBe in LSEC by competition for binding lectin, and the remaining sugar residues of DHBe might pose a sterical hindrance to virion binding when glycosylated DHBe locates to its hepatocellular receptor. DHBe-negative mutants would thus have no disadvantage during infection compared with wild-type virus, as experimental evidence suggests (Chang et al., 1987). Admittedly, artificially high doses of mannose were used in our experiments and the amount of DHBe that was secreted from infected cells in situ, which is not known. Yet, if one assumes that sometimes the local amount of DHBe outnumbers the accessibility of lectins on LSECs, then this would result in a surplus of unmodified DHBe with the ability to inhibit the infection of hepatocytes.

HBe was proposed to interact with T helper cells to promote HBV persistence by modulating the immune response in favour of a Th2-like profile (Milich et al., 1995, 1998). In contrast with DHBe, HBe is not glycosylated, and the HBV surface proteins carry glycosylation. This may imply that although the existence of the secreted viral eAg

Fig. 5. Localization of DHBe in PDLC cultures. PDLCs were incubated with concentrated DHBe alone (a) or with the addition of mannose (b) for 24 h and then washed and fixed for indirect immunofluorescence with anti-DHBe antiserum for the detection of DHBe. (c) PDLCs were incubated with concentrated DHBe (red) for 24 h, and acLDL was added for the last 2 h (green), which showed that 3–5% of cells that incorporated acLDL were positive for DHBe. (d) Control experiment, in which SVPs were used instead of DHBe. Magnification, ×200 for all images.
Inhibition of DHBV infection by DHBe

is conserved among hepadnaviruses, its function is not. However, these hypotheses do not have to be mutually exclusive, as our experimental setting would not allow monitoring of immunological events.

It might also be interesting to consider the participation of LSECs in the process of HBV infection, as shown in a further study (Bruns & Maenz, 2007), to determine whether the mannose-binding lectin, which is a central component of the innate immune response, is important for HBV persistence (Thiol et al., 2005). The variation in DHBe synthesis during a persistent infection does not parallel the production of DHBV virions and SVPs (Jilbert et al., 1992; Supplementary Fig. S1) and could therefore be a regulating factor for the maintenance of infection. Infection of Pekin ducks with a DHBe-negative mutant correlated with increased anti-core antibody production, suggesting an immunoregulatory role for DHBe (Zhang & Summers, 1999). Thus, it seems feasible that eAg plays a dual role in hepadnaviral infections.

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