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Expression of the Hepatitis Delta Virus Large and Small Antigens in Transgenic Mice†

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Simultaneous infection with hepatitis delta virus (HDV) and hepatitis B virus (HBV) in humans is often associated with severe viral liver disease including fulminant hepatitis. Since HBV is thought to be noncytopathic to the hepatocyte, the enhanced disease severity observed during dual infection has been attributed to either simultaneous immune responses against the two viruses or direct cytotoxic effects of HDV products on the hepatocyte or both. To examine these alternate possibilities, we produced transgenic mice that express the small and large delta antigens (HDAg) in hepatocyte nuclei at levels equal to those observed during natural HDV infection. No biological or histopathological evidence of liver disease was detectable during 18 months of observation, suggesting that neither the large nor small form of HDAg is directly cytopathic to the hepatocyte in vivo.

Hepatitis delta virus (HDV) is a small single-stranded, circular RNA virus whose genome structure resembles that of plant viroids (43). This virus codes for only one protein product, the delta antigen (HDAg), which serves as its nucleocapsid, and is highly pathogenic, frequently causing fulminant hepatitis. HDAg is encoded on antigenomic RNA and is expressed in the cell via a cytoplasmic polyadenylated RNA of about 0.8 kb in size (7, 18). HDAg consists of two protein species of 214 and 195 amino acids, termed large and small HDAg, respectively. During HDV replication, a specific transition occurs at the second position of the amber termination codon (UAG to UGG) of the small HDAg open reading frame (ORF), thereby increasing the length of the ORF and allowing synthesis of the large HDAg containing a 19-amino-acid C-terminal extension (4, 23, 45).

Despite their extensive primary structure similarity, the small and large HDAg play very different functional roles in the life cycle of the virus. The small HDAg is required for genome replication, while the large HDAg is required for genome packaging (7, 22, 34). In addition, the large HDAg inhibits HDV genome replication (6). Virus particle formation requires not only HDV RNA and HDAg, which together form the nucleocapsid, but also the surface antigen of HBV (HBsAg), which provides the viral envelope which is necessary for viral infectivity (39).

Because of this obligatory association with HBV, characterization of the nature and extent of the disease attributable to HDV alone has been difficult to assess. Experimental HDV infection of animals that support hepadnavirus replication has been successful (29, 32), and considerable information concerning the natural history of HDV infection has been gathered from these animal models. However, it remains unclear whether HDV causes liver disease by a direct cytotoxic effect or by indirect, immune system-mediated destruction of the infected hepatocytes.

To evaluate the potential hepatocytotoxic properties of HDAg in vivo, we have developed transgenic mice that express the small and large forms of HDAg in the liver, separately and in combination with the small and large envelope polypeptides of HBV.

MATERIALS AND METHODS

Plasmid construction. To ensure high-level expression in the liver, we placed the HDAg ORF under the transcriptional control of the mouse albumin promoter. We used the 2333-A1 expression vector generously provided by Richard Palmiter (University of Washington). The 2335-A1 vector is similar to pUC18-Alb(e/p) described previously (15), except that the albumin enhancer/promoter *Nhe*I-*Kpn*I DNA fragment was inserted into pBluescript SK+ (Stratagene, La Jolla, Calif.) instead of pUC18. Briefly, in the 2335-A1 vector, the albumin promoter is linked to the 2-kbp *Nhe*I-*Bam*HI enhancer fragment located 8 kbp upstream of the albumin promoter in the mouse genome that is known to confer high-level expression of the human growth hormone in the mouse liver (28). The large HDAg ORF was generated from plasmid pECE-HDV-L (5) by *Bam*HI-and-*Bgl*II double digestion. The small HDAg construct was generated by inserting at the 2335-A1 *Bam*HI site a PCR-generated fragment containing nucleotides 1663 to 944 (in antigenomic orientation) of the HDV sequence (25). A *Stu*I-*Kpn*I fragment was subsequently exchanged for a blunt-ended, *Stu*I-*Eco*RI fragment extracted from plasmid pECE-SM (44) to reconstitute a small HDAg ORF. The large and small HDAg ORFs were inserted into the 2335-A1 expression vector at the unique *Bam*HI site, downstream of the albumin promoter.

DNA injection. The small and large HDAg minigene fragments were prepared by digestion with *Sac*I-*Nco*I and *Sac*I-*Kpn*I, respectively. DNA fragments were separated by electrophoresis on an 1% agarose gel and recovered by binding onto glass beads with a GeneClean kit, (Bio 101, Inc., San Diego,

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Calif.). Residual agarose traces were removed by filtration through a 0.45-μm-pore-size filter, binding of the DNA to a DEAE-cellulose column (Elutip; Schleicher & Schuell, Inc., Keene, N.H.) and eluted according to the manufacturer's recommendations. DNA microinjection into fertilized egg pronuclei was performed by DNX (Princeton, N.J.) as previously described (16).

Identification of HDAg DNA-positive transgenic mice expressing. Genomic DNA and total cellular RNA was prepared from tissues by standard techniques as previously described (11). Transgenic mice were identified by subjecting 1-μg samples of DNA to amplification by the PCR with two pairs of oligonucleotides as primers. Oligonucleotides delta 1 (GAAG ACATCCTCGAGCAGTG) and delta 2 (CGTCTATCTCCA TCTGGTCC) corresponding to nucleotides 1355 to 1374 and 1543 to 1562, respectively, of the HDV genome (25) were used to amplify a 208-nucleotide delta DNA fragment. In the same reaction, oligonucleotides ras 1 (TTTGCAGGACTCCTAC CGG) and ras 2 (ATGTACTGGTCCCGCATGGC) corresponding to nucleotides 458 to 476 and 549 to 568, respectively, of the rat c-Ha-*ras* gene (33) were used to amplify an 111-nucleotide *ras* DNA fragment. The *ras* fragment was amplified both in transgene-positive or -negative mice and was used as an internal control for PCR, while delta-transgene-positive mice were identified by the presence of the additional 208-nucleotide delta fragment. PCR amplification was performed as previously described (35) with the following parameters: DNA denaturation at 92°C for 5 min followed by 30 cycles, with 1 cycle consisting of 15 s at 92°C, 15 s at 52°C, and 30 s at 72°C. To further characterize the founders, Southern and Northern (RNA) blotting were performed as previously described (13). Restriction enzymes were purchased from New England Biolabs or Boehringer Mannheim and used according to the manufacturer's recommendations.

Production of HDAg × HBV transgenic hybrids. HDAg transgenic mice were crossbred with HBV transgenic mice to produce hybrids expressing both HDV and HBV gene products. A HBV transgenic lineage (107-5) (8) was used in these experiments. Lineage 107-5 expresses the HBV large and small envelope proteins in the hepatocyte. Because the HBV large envelope protein is overproduced by this lineage, particulate HBsAg filaments form and are trapped within the hepatocyte endoplasmic reticulum (8), resulting in increased sensitivity to bacterial lipopolysaccharide (LPS)-induced hepatocellular injury (12).

Preparation of liver nuclei. Nuclei were prepared by the procedure of Schibler et al. (36). Briefly, each liver sample was homogenized by ca. 10 strokes in a Potter-Elvehjem tissue grinder in 5 ml of buffer A [60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.3 M sucrose, 15 mM Tris Cl (pH 7.5)] and centrifuged over a 5-ml cushion of buffer B (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 0.85 M sucrose, 15 mM Tris Cl [pH 7.5]) at 10,000 × *g* for 20 min at 4°C. The pellet was resuspended in 3 ml of buffer C (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.1 mM EGTA, 0.1 mM EDTA, 2 M sucrose, 15 mM Tris Cl [pH 7.5]) and centrifuged over an 8-ml cushion of buffer C in a Beckman SW40 rotor at 36,000 rpm for 1 h at 4°C. Nuclei were resuspended in 1 ml of storage buffer (20 mM Tris Cl [pH 8], 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride, 50% glycerol) and stored at −80°C.

Western immunoblot analysis. Purified nuclei in storage buffer were pelleted by centrifugation at 5,000 × *g* for 2 min, resuspended in 200 μl of loading buffer, and boiled for 5 min. Genomic DNA was sheared by forcing the sample through a 28.5-gauge needle before loading. Proteins were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes as described previously (13). Rabbit antidelta antibody (44) was used at a 1/500 dilution, and then a biotinylated anti-rabbit whole antibody and streptavidin-alkaline phosphatase conjugate (Amersham, Arlington Heights, Ill.) were used. The color reaction was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in diethanolamine buffer. To compare HDAg levels expressed in transgenic mouse livers and in HDV-infected chimpanzee and woodchuck livers, proteins from transgenic mouse livers were extracted by the guanidine-HCl procedure described previously (1). Guanidine-HCl-extracted liver samples from HDV-infected chimpanzee and woodchuck livers obtained at the peak of HDV infection were kindly provided by Katherine Bergmann and John Gerin (Georgetown University).

Immunostaining. Frozen liver sections (5 μm thick) were fixed in ice-cold acetone (5 min). Human polyclonal antidelta antibody (AMAC, Westbrook, Maine) was applied at a 1/10 dilution in phosphate-buffered saline (PBS) overnight at 4°C, and then biotinylated goat $F(ab')_2$ affinity-isolated anti-human immunoglobulin G antibody (gamma chain specific) (Sigma Chemical Co., St. Louis, Mo.) at a 1/100 dilution at 4°C for 6 to 12 h and Extravidin-peroxidase were applied. The color reaction was developed with aminoethylcarbazole, and the slides were counterstained with Meyer's hematoxylin as previously described (8).

Immunoelectron microscopy. Indirect immunostaining for nuclear localization of HDAg was performed with protein A-colloidal gold (8-nm particle size) as the electron-dense label. Preparation of the protein A-colloidal gold conjugate was performed as previously described (37, 38).

Isolated nuclei were incubated with rabbit antidelta antibody at a 1/100 dilution in storage buffer overnight at 4°C and washed four times with PBS. The nuclei were incubated with protein A-colloidal gold at a 1/100 dilution in PBS containing 0.02% polyethylene glycol (molecular weight, 20000) for 2 h at 4°C and then washed in PBS four times. Nuclei were fixed for 1 h at 4°C in PBS containing 1% glutaraldehyde and 4% paraformaldehyde. After osmification in 1% aqueous solution of osmium tetroxide for 1 h at 4°C, the nuclei were processed for Epon embedding. The ultrathin sections were examined with a Zeiss 109 electron microscope.

RESULTS

Description of the transgenic mouse lineages. (i) DNA analysis. Three transgenic mouse lineages containing the small HDAg construct (DS-186, DS-188, and DS-190) and one transgenic mouse lineage containing the large HDAg construct (DL-11) were derived. Southern blot analysis of *Sac*I-digested genomic DNA revealed a distinct integration pattern for each lineage (Fig. 1A). For each lineage, the observed pattern is compatible with the presence of at least one full-length integrated fragment.

(ii) RNA analysis. Total RNA derived from the liver, kidney, spleen, stomach, small intestine, large intestine, testis, heart, lung, thymus, salivary glands, and brain from an adult DL-11 transgenic animal was analyzed by Northern blotting and probed with HDV and glyceraldehyde-3-phosphate dehydro-

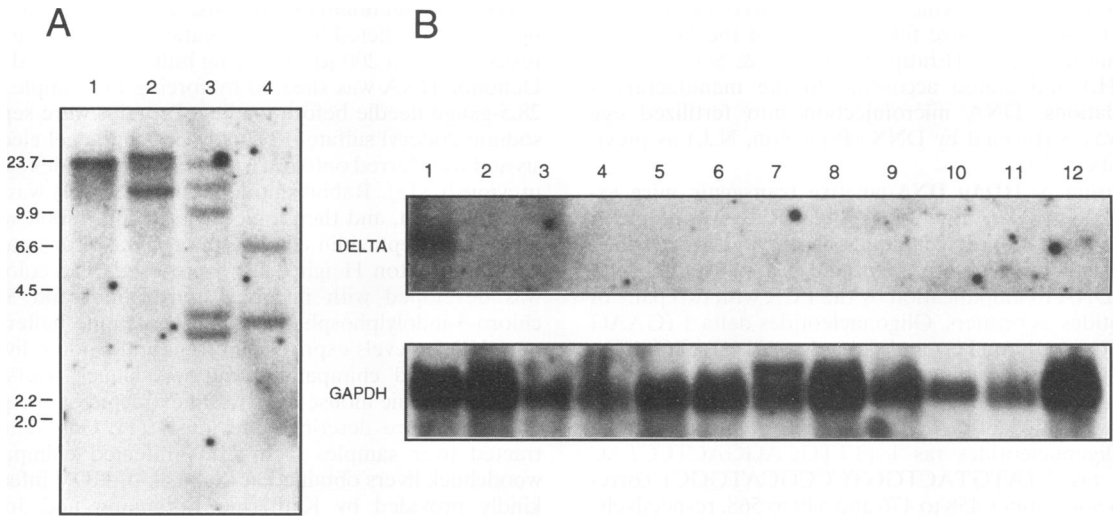

FIG. 1. Hepatitis delta large and small antigen transgenic mouse lineages. (A) Southern blot analysis of *Sac*I-digested genomic DNA prepared from transgenic lineages DS-186 (lane 1), DS-188 (lane 2), DS-190 (lane 3), and DL-11 (lane 4) and hybridized with an HDV probe. (B) Northern blot analysis of total RNA (10 μg) extracted from tissues of an DL-11 adult animal and hybridized with HDV and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences. Lanes: 1, liver; 2, kidney; 3, spleen; 4, stomach; 5, small intestine; 6, large intestine; 7, testis; 8, heart; 9, lung; 10, thymus; 11, salivary glands; 12, brain.

genase (GAPDH) (10) sequences (Fig. 1B). HDV-specific RNA was detected only in the liver.

(iii) Protein analysis. HDAg was detected in the livers of all four lineages as illustrated in Fig. 2 for lineages DS-186 and DS-188. HDAg-positive hepatocytes were located principally around the central vein of the liver acinus (zone 3). Approximately 25 to 30% of all hepatocytes stained positively for HDAg in each lineage. Within the hepatocyte, HDAg was located exclusively in the nucleus, especially in the nucleolus, with additional nucleoplasmic distribution in some lineages.

Western blot analysis performed on isolated transgenic mouse liver nuclei demonstrated HDAg-specific bands with mobilities corresponding to 27 and 24 kDa compatible with the sizes of the large and small HDAg, respectively (Fig. 3A).

To estimate the level of HDAg expression in the transgenic mice relative to HDV-infected livers, we performed Western blotting with liver protein extracts from transgenic mice and from HDV-infected chimpanzee and woodchuck livers obtained at the peak of infection. Chimpanzee and woodchuck protein extracts were kindly provided by Katherine Bergmann

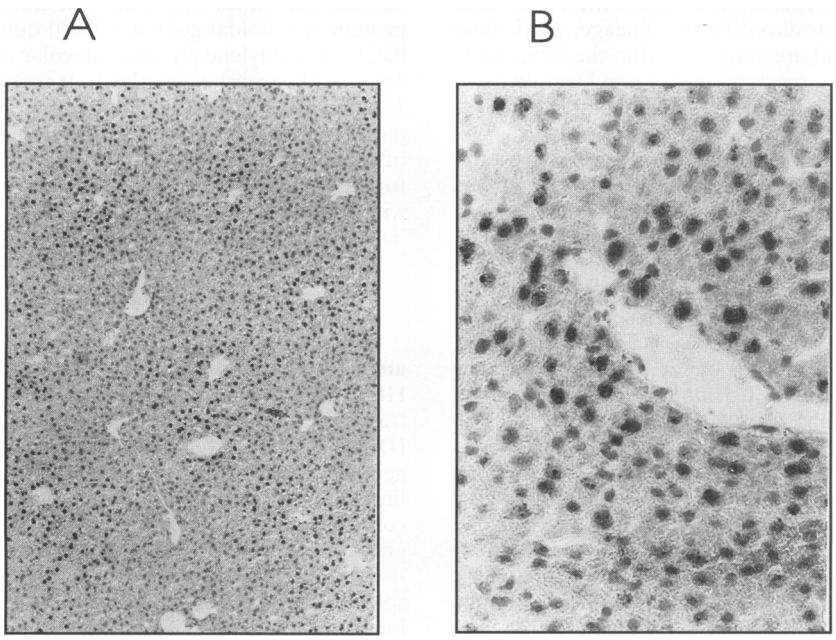

FIG. 2. Immunohistochemical localization of HDAg in transgenic mouse liver sections. Indirect immunoperoxidase staining of DS-186 (A) and DS-188 (B) liver sections incubated with human anti-HDAg antiserum is shown. Magnifications, ×75 for panel A and ×300 for panel B.

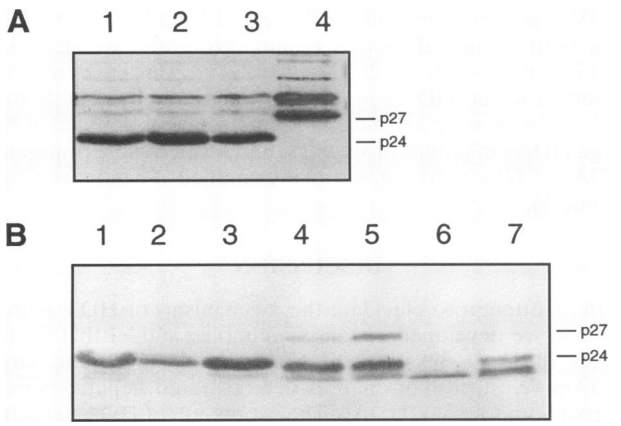

FIG. 3. Western blot analysis of HDAg. (A) Proteins extracted from 10^6 liver nuclei from DS-186 (lane 1), DS-188 (lane 2), DS-190 (lane 3), and DL-11 (lane 4) transgenic mouse lineages were separated in a sodium dodecyl sulfate–12% polyacrylamide gel and immunoblotted with rabbit anti-HDAg antiserum. (B) Comparison of HDAg expression levels in HDAg transgenic mice and HDV-infected chimpanzee and woodchuck livers at the peak of HDV infection. Transgenic mice DS-190 (lane 2) and DS-188 (lane 3), HDV-infected chimpanzees 56 and 1241 (lanes 4 and 5), and HDV-infected woodchucks 15 and 21 (lanes 6 and 7) were used. Total liver proteins (20 μg) extracted by guanidine-HCl treatment were used for lanes 2 to 7, and proteins from 10^6 DS-190 liver nuclei were used for lane 1. Chimpanzee and woodchuck protein extracts were kindly provided by Katherine Bergmann and John Gerin (Georgetown University) and described previously (1).

and John Gerin (Georgetown University) and previously described (1). Protein extracts (20 μg) from HDAg transgenic mouse livers (Fig. 3B, lanes 2 and 3), HDV-infected chimpanzee livers (lanes 4 and 5), and HDV-infected woodchuck livers (lanes 6 and 7) were used for detection of HDAg. The signal for p24 HDAg present in the transgenic mouse samples is as strong as the strongest signals for HDV-infected liver samples. Immunohistochemical analysis reveals HDAg in approximately 30% hepatocytes in the transgenic mice and 25 to 50% in chimpanzee 1241 (2), indicating similar levels of HDAg on a per-cell basis in transgenic hepatocytes and in infected hepatocytes. Obviously, the comparison of the overall HDAg load in the liver does not address the possibility that higher levels of HDAg expression may occur in a limited number of hepatocytes and be cytotoxic to these cells during natural infection.

Immunoelectron microscopy was performed with isolated DL-11 liver nuclei (Fig. 4). Positive immunolabeling was observed in about 25% of the transgenic mouse liver nuclei, while no labeling was noted in any of the nontransgenic littermate control liver nuclei. As shown in Fig. 4A, the labeling is more intense around the nucleolus, presumably associated with the perinucleolar chromatin, and to a lesser extent among the chromatin fibrils in the nucleoplasm. The observed intranuclear localization of HDAg in the transgenic mice is similar to the HDAg localization reported in HDV-infected livers (20).

(iv) Disease parameters. To determine whether HDAg expression was cytopathic to the hepatocyte, all four mouse lineages were examined for histological evidence of liver disease at monthly or bimonthly intervals for up to 18 months

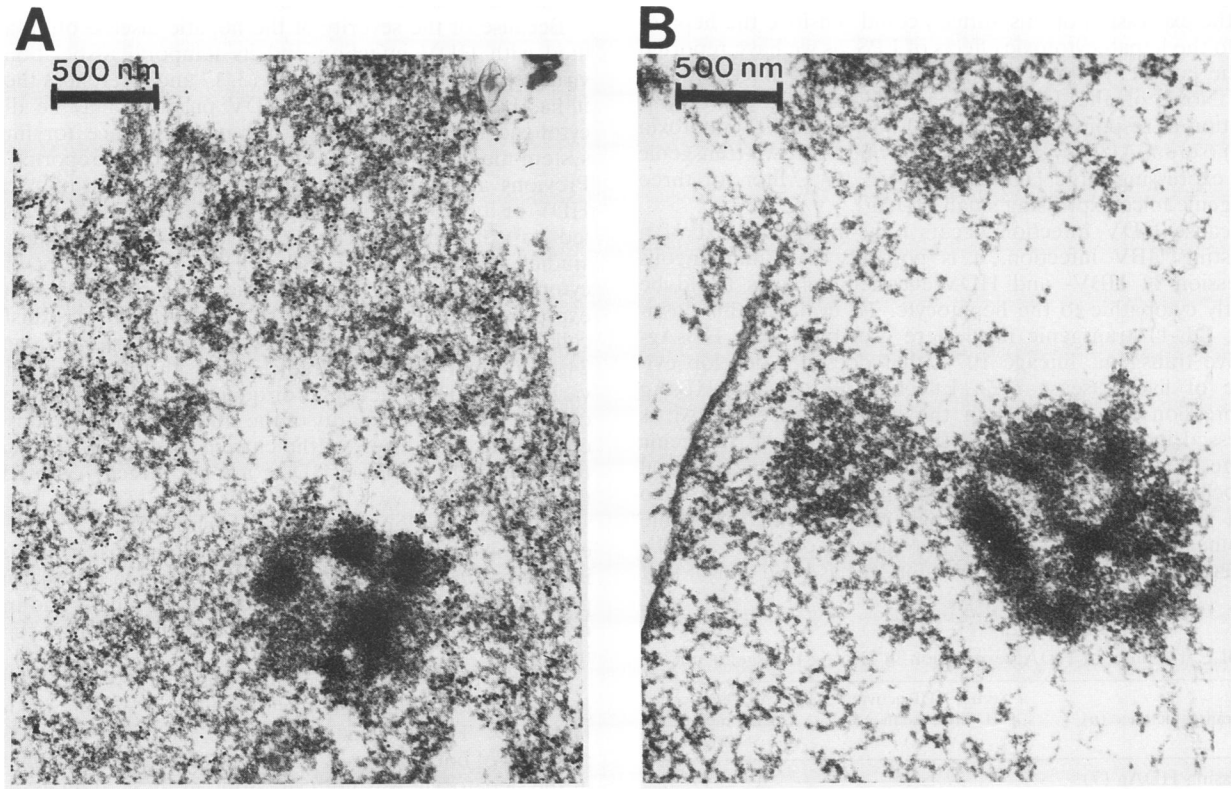

FIG. 4. Immunoelectron microscopic detection of HDAg in transgenic mouse lineage DL-11. Purified liver nuclei were reacted with rabbit anti-HDAg antiserum at a 1:100 dilution followed by protein A-gold (8-nm diameter). (A) transgenic mouse DL-11 liver nuclei; (B) nontransgenic littermate liver nuclei as control.

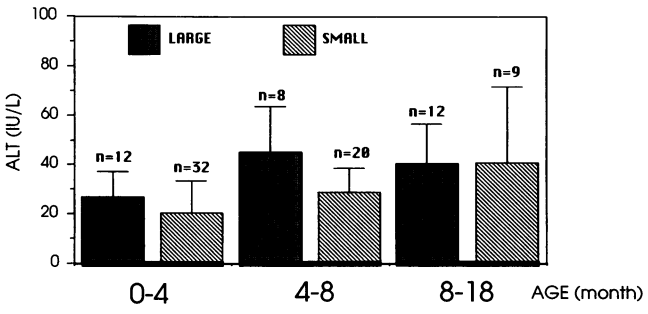

FIG. 5. Serum ALT levels in HDAg transgenic mouse lineages. Mice were bled by retro-orbital phlebotomy with heparinized capillary tubes, and serum ALT activity was determined with a Paramax autoanalyzer as previously described (7). Values are grouped for the three small HDAg transgenic lineages. The number of animals (*n*) in each category is shown. Results represent the mean ± standard error of the mean.

and for ultrastructural evidence of liver disease in lineage DL-11 at 2 months of age. No histopathological or ultrastructural (not shown) changes were associated with HDAg expression in any of the transgenic lineages. Serum alanine aminotransferase (ALT) activity as a biochemical parameter of liver disease was also monitored in the four transgenic mouse lineages. As shown in Fig. 5, serum ALT levels remained within the normal range (less than 100 U/liter) over an 18-month observation period for DL-11 lineage and a 10-month observation period for the three DS lineages.

While expression of HDAg is not directly cytotoxic to the transgenic mouse hepatocyte in vivo, we tested the possibility that the expression of this antigen could sensitize the hepatocyte to the hepatocytotoxic effects of LPS, as we have reported for the large envelope polypeptide of HBV in lineage 107-5 (12). None of the HDAg lineages displayed serum ALT elevation in response to 100 μg of LPS (1.6×10^6 endotoxin units) (serum ALT levels of 16 ± 11 U/liter for two transgenic mice expressing large HDAg and 44 ± 17 U/liter for three transgenic mice expressing small HDAg).

Because HDV infection occurs only in the context of a coexisting HBV infection, it is possible that simultaneous expression of HBV- and HDV-encoded antigens could be directly cytopathic to the hepatocyte. To evaluate this possibility, DL-11 transgenic mice were crossbred with HBsAg-positive transgenic lineage 107-5 that does not develop evidence of liver disease (12). Effective HDAg and HBsAg coexpression was achieved in the hybrids, since HBsAg is expressed in virtually 100% of the hepatocytes in transgenic lineage 107-5. In no instance did we observe histopathological or biochemical evidence of liver cell injury in groups of three to seven single and double transgenic mice from all possible combinations monitored for up to 6 months of age (Table 1).

Effect of HDAg on HBV expression. It has been shown that HDV superinfection leads to decreased hepadnavirus replication both in animal models (chimpanzees and woodchucks) and in humans (14, 21, 26, 27, 29, 32, 41). This effect was not observed in the HDAg × HBsAg transgenic hybrids. Serum HBsAg levels were identical in the presence or absence of the large HDAg in groups of six single and seven double transgenic HDAg × HBsAg transgenic mice examined at 2 months of age (Table 1).

TABLE 1. Effect of HDAg expression in HBsAg transgenic mice[a]

Transgenic mice (*n*)	Serum ALT activity (U/liter, mean ± SEM)	Serum HBsAg concn (ng/ml, mean ± SEM)
Expressing HDAg (7)	57 ± 35	19 ± 17
Not expressing HDAg (5)	40 ± 23	15 ± 8

[a] Serum ALT activity and HBsAg cell concentration were determined as described in Materials and Methods.

DISCUSSION

In an attempt to elucidate the mechanisms of HDV pathogenesis, we developed a transgenic mouse model that reproduces the liver-specific expression of the small and large HDAgs. Western blot analysis demonstrated hepatic expression of both forms of HDAg. The expression of HDAg-specific RNA was restricted to the liver. In the hepatocyte, HDAg expression was limited to the nucleus, similar to that reported for HDV infection of humans and animal models (29, 31, 32). The distribution of HDAg in transgenic mice was mainly nucleolar, with an additional prominent nucleoplasmic component in many cells. The same nuclear distribution observed in all four HDAg transgenic lineages suggests that the nuclear localization signal and conformation of the HDAg in transgenic mice faithfully reproduce the native structures of the large and small forms of HDAg. The intranuclear distribution of HDAg and its association with perinucleolar structures were further confirmed by immunoelectron microscopic observation. Thus, it appears that the expression of the two isoforms of HDAg in these transgenic mice reflects the characteristics of the HDAg found in infected livers. Therefore, any potential direct cytotoxic property associated with HDAg expression should be revealed in this model.

Because of the severity of the hepatic disease often associated with HDV infection and its temporal association with virus replication and expression (3, 17 and references therein), it has been postulated that HDV products may be directly cytotoxic to the hepatocyte, although evidence for immune system-mediated liver disease has also been reported (30). Previous attempts to demonstrate the direct cytotoxicity of HDV or its products by using in vitro expression systems have led to contradictory results. For example, although most studies suggested that HDV or HDAg alone is not directly cytopathic (7, 19, 22, 40, 42), one study based on inducible expression of small HDAg in clones derived from transfected cell lines suggested that small HDAg is cytopathic in vitro (9, 24). Whether these contradictory results reflect qualitative variations in HDV genomes or quantitative effects of the level of expression or peculiarity of the studied clones remains to be elucidated. The results of the current study, however, suggest that the small and large HDAg are not directly cytopathic to the hepatocyte in vivo.

To further explore the potential cytopathic effect of HDAg expression in the hepatocyte, we considered that in natural infection, the expression of HDAg occurs in a cell that is also perturbed by the presence of HBV gene products and inflammatory mediators released in the liver in response to HBV infection.

To determine whether coexpression of HDAg and HBV gene products is directly cytopathic, we produced hybrid HDAg × HBsAg transgenic mice that express the gene products of both viruses. Colocalization of HBsAg with HDAg in the hepatocyte was not cytopathic in these animals.

We have previously demonstrated that noncytopathic concentrations of the HBV large envelope protein in the hepatocyte can sensitize the hepatocyte to the cytopathic effect of LPS

(12). In contrast, HDAg expression does not sensitize the hepatocyte to the cytotoxic effects of LPS.

Thus, it would appear that expression of HDAg, even in the presence of HBsAg, is not directly cytotoxic to the hepatocyte nor does it potentiate the hepatocyte to injury by LPS. This model obviously does not address the potential cytotoxicity of other aspects of the HDV infection cycle, such as HDV genome replication and interactions with the cellular transcription machinery.

HDV superinfection of patients with chronic HBV infection is often associated with a transient but marked reduction of HBV DNA and HBV core antigen within the liver and a decrease in circulating HBV DNA and HBV e antigen (14, 21). The same is true for HDV superinfection of woodchuck hepatitis virus-infected woodchucks or HBV-infected chimpanzees (26, 27, 29, 32, 41). We did not observe any apparent down-regulation in the secreted HBsAg in our hybrid HDAg-HBsAg transgenic mice. These results suggest that down-regulation of HBV replication by HDV is not due to a direct interaction between HDAg and HBsAg. Since this transgenic mouse model does not include all the steps of complete HDV or HBV replication cycles, it is likely that other aspects of the HDV life cycle are responsible for this effect. Alternatively, the repression of HBV replication during HDV infection could be generated indirectly by the host response against HDV, by inducing the release of inflammatory cytokines such as tumor necrosis factor alpha and gamma interferon which we have shown inhibit HBV gene expression in vivo (11, 12).

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