Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice

(large envelope polypeptide/“ground-glass” cells/hepatocellular injury/nodular hyperplasia)

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ABSTRACT Overproduction of the hepatitis B virus (HBV) large envelope polypeptide by transgenic mice containing the entire HBV envelope coding region leads to the formation of extremely long (up to 800 nm), occasionally branching, filamentous 22-nm-diameter hepatitis B surface antigen particles that accumulate within the endoplasmic reticulum of the hepatocyte and are not efficiently secreted. As the endoplasmic reticulum expands to accommodate the increasing cellular filament stores, the hepatocytes become enlarged, hydropic, and eosinophilic and also display the characteristic features of “ground-glass” cells. As filament storage progresses, the ground-glass cells undergo coagulative necrosis and the mice develop an age-dependent lesion, whose severity is related to the intracellular concentration of envelope polypeptide, that is characterized by focal hepatocellular degeneration and necrosis, lobular macrophagic inflammation, and increased serum transaminase activity. Advanced lesions demonstrate hepatocellular hyperplasia evident as lobular architectural disarray and microscopic hepatocellular nodules, many of which no longer contain detectable HBV envelope antigens. These changes may become extreme, producing a massively enlarged liver due to multifocal nodular regenerative hyperplasia. Overproduction of the large HBV envelope polypeptide exerts major structural constraints on HBV particle formation, leading to reduced secretion and progressive intracellular accumulation of hepatitis B surface antigen, which can reach sufficiently high concentrations to be directly cytotoxic to hepatocytes in this transgenic mouse system.

The mechanisms responsible for hepatocellular injury in hepatitis B virus (HBV) infection are not clearly defined. Since HBV infection may occur without attendant liver disease, direct viral cytopathicity is unlikely. Although the involvement of an HBV-specific hepatocytotoxic cellular immune response as the major cause of liver cell injury in HBV infection is generally accepted, the characteristics of that mechanism are not yet defined because the requisite model systems have not existed.

To address these issues we recently produced transgenic mice by microinjection of a subgenomic HBV DNA fragment (subtype ayw) containing the coding region for the viral envelope polypeptides (1). An internal HBV promoter within this fragment leads to expression of the major envelope polypeptide containing the hepatitis B surface antigen (HBsAg). An exogenous promoter element inserted immediately upstream of the HBV envelope region leads to expression of the large envelope polypeptide, which includes the entire major polypeptide sequence plus 163 extra N-terminal amino acids containing the viral pre-S(1) and pre-S(2) antigens (1, 2).

Transgenic mice that produce primarily the major polypeptide assemble 22-nm HBsAg particles that are efficiently secreted and do not accumulate within the hepatocyte (1, 2). In contrast, transgenic mice that produce the large polypeptide accumulate HBsAg in an aqueous-insoluble pre-Golgi compartment within the hepatocyte (2).

We now present evidence that overproduction of the large polypeptide, relative to other envelope polypeptides, exerts significant structural constraints on HBsAg particle formation. This results in the production of extremely long, nonsecretable filamentous structures that become entrapped within the endoplasmic reticulum of the hepatocyte, where they progressively accumulate as synthesis exceeds secretion. The progressive increase in intracellular concentration of HBsAg filaments causes a time- and concentration-dependent spectrum of changes in the hepatocyte including “ground-glass” cell formation, cell death, and nodular regenerative hyperplasia.

MATERIALS AND METHODS

Plasmid Constructions. Plasmid pAlb-PSX has been described (2). This plasmid contains the Bgl II-A fragment of HBV, subtype ayw, downstream of mouse albumin promoter and enhancer sequences (3). Transcription start sites and mRNA polyadenylation recognition sequences within the HBV Bgl II-A fragment define the HBV transcripts that encode the major envelope polypeptide. The large envelope polypeptide can only be produced from a longer transcript emanating from transcription start sites defined by the exogenous albumin promoter.

Microinjection and Production of Transgenic Mice. Production of transgenic mice containing the HBV Bgl II-A fragment downstream from the albumin promoter has been described (2). The founding transgenic mice and their offspring were identified by “dot hybridization,” and integrated DNA was analyzed by restriction enzyme digestion and electrophoresis of genomic DNA followed by blot hybridization (1, 2).

Analysis of Gene Expression. Serum, derived from blood obtained by retro-orbital plexus phlebotomy, and organ homogenates were prepared as described (1, 2). Total soluble protein was determined by Coomassie blue G-250 binding (Bio-Rad). HBsAg pre-S(1) and pre-S(2) antigens and HBV envelope polypeptide composition were measured by solid-phase radioimmunoassay (AUSRIA-II; Abbott), by enzyme-linked immunosorbent assays (4), and by immunoblot as described (1, 2). The cellular localization of HBV envelope antigens was assessed by indirect immunofluorescence (1, 2).

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; ER, endoplasmic reticulum; SGPT, serum glutamic-pyruvic transaminase.

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using monoclonal antibodies to HBsAg, pre-S(1) antigen, and pre-S(2) antigen (2). In addition, HBsAg was localized in formalin-fixed, paraffin-embedded tissue sections by staining with orcein (5) and with Victoria blue (6). Specific HBV transcription was assessed by blot hybridization of glyoxylated total RNA prepared according to the method of Holmes and Bonner (7) and subjected to electrophoresis in 1.4% agarose gels. After transfer to nitrocellulose and prehybridization, the RNA was hybridized with a HBV-specific DNA probe (5'-labeled by nick-translation), washed, and subjected to autoradiography as described (1).

**Subcellular Fractionation of Transgenic Mouse Liver**. Livers obtained immediately after cervical dislocation were perfused, excised, finely minced, and homogenized in 0.25 M sucrose/10 mM Hepes/1 mM phenylmethylsulfonyl fluoride at pH 7.2 by 15 strokes in a Dounce homogenizer with a type B pestle at 0°C. After centrifugation at 800 x g for 10 min at 4°C, the pellet was resuspended in a Thomas Teflon tissue grinder (type B pestle; Thomas, Swedesboro, NJ) and homogenized (Fig. 1). After centrifugation at 250,000 x g for 120 min at 4°C, the pellet was resuspended in homogenization buffer. The resultant nuclear pellet was saved for analysis and the supernatants were pooled and centrifuged at 10,000 x g for 20 min at 4°C. The pellet (containing mitochondria, lysosomes, peroxisomes, etc.) was saved for analysis. The supernant (containing microsomes, Golgi complex, and soluble molecules) was adjusted to 15 mM cesium chloride and centrifuged at 250,000 x g for 120 min at 4°C onto a cushion containing 1.3 M sucrose, 10 mM Hepes, 15 mM cesium chloride, and 1 mM phenylmethylsulfonyl fluoride. One-milliliter fractions were collected. The heavy microsome-containing pellet was resuspended in homogenization buffer. The interface containing the light microsomes was resuspended in cold distilled water, pelleted at 250,000 x g for 120 min at 4°C, and resuspended in homogenization buffer. All fractions were analyzed for total protein and HBsAg concentration as described above.

**Morphologic Analysis**. Routine light-microscopic techniques included histochemical detection of HBsAg by the orcein reaction of Shikata et al. (5) and with Victoria blue (6). Standard electron-microscopic procedures were applied to liver tissue and to the subcellular fractions, which were also subjected to immunogold electron microscopy as previously reported (1). Briefly, subcellular fractions were applied to Parlodion (Mallinckrodt) carbon-coated grids and incubated with a rabbit antisemur to HBsAg (Behring Diagnostics) and then with a colloidal gold-labeled, affinity-purified antibody to rabbit immunoglobulin G. Samples were negatively stained with aqueous uranyl formate and examined.

**Biochemical Evidence of Hepatocellular Injury**. Sera were analyzed for glutamic-pyruvic transaminase (SGPT) activity (7) by a Hitachi 705 autoanalyzer.

**Immune Response to HBV Envelope Antigens**. Antibodies to HBsAg, pre-S(1), and pre-S(2) were measured by solid-phase radioimmunoassay (AUSAB, Abbott) and by ELISA using immobilized preparations containing the HBsAg, pre-S(1) antigen, and pre-S(2) antigen (3). Cellular immune sensitization to each of the envelope antigens was assessed in transgenic and normal mouse spleen cells by antigen-induced [3H]thymidine incorporation (4).

**Serological Evaluation for Mouse Pathogens**. Sera were analyzed for the presence of antibodies to several mouse pathogens known to be associated with liver disease, including mouse hepatitis virus, Sendai virus, ectromelia virus, reovirus 3, and lymphocytic choriomeningitis virus, by the Research Animal Diagnostic and Investigative Laboratory at the University of Missouri, Columbia, MO.

**RESULTS**

**Gene Expression**. Ten transgenic mouse lines containing the Alb-PSX construct were derived. Blot hybridization analysis of restriction enzyme-digested genomic DNA was consistent with integration of microinjected DNA concatemers at a single random site within the genome of each lineage (data not shown). HBV gene expression varied significantly among lineages. As determined by immunofluorescence analysis, hepatocytes displayed lineage-dependent differences in the intensity, frequency, and distribution of HBsAg expression within the hepatic lobule (Fig. 1). Three lineages (45-2, 45-3, 50-4) were analyzed further. At the RNA level, all lineages displayed two dominant transcripts measuring 2.1 and 2.6 kilobases, which correspond to the predicted sizes of transcripts initiating from the endogenous HBsAg and the exogenous albumin promoters, respectively. Lanes 1, 2, and 3 in Fig. 2A represent total RNA derived from 4-month-old mice from lineages illustrating moderate (45-3), high (45-2), and very high (50-4) levels of expression, respectively. Similar quantitative relationships were observed at the polypeptide level (Fig. 2B). The high molar ratio of the large polypeptide (p43, gp45) to the major polypeptide (p25, gp27) in all lineages is due to the albumin promoter that controls synthesis of the large envelope polypeptide in the mice. In all lineages, intracellular HBsAg increased progressively during the first 4 months of life, as shown for lineage 50-4 (Fig. 2, lanes 3–5). This corresponded, histochemically and immunohistochemically, with the accumulation of large amounts of HBsAg within the cytoplasm of the hepatocytes, which became strikingly enlarged (Fig. 3 A and B). Thereafter, for this lineage, polypeptide signal intensities decreased with age (Fig. 2, lanes 7 and 8), presumably as a consequence of severe hepatocellular injury and regeneration (see below) with the formation of nodules of HBsAg-negative hepatocytes (Fig. 3C).

**Ultrastructural Analysis and Subcellular Fractionation**. Thin sections of liver from representative transgenic lineages displayed nuclear and cytoplasmic enlargement of hepatocytes (relative to nontransgenic normal control mice) corresponding in frequency with the total hepatic HBsAg content (45-3, few; 45-2, moderate; 50-4, many; data not shown). The enlarged nuclei were otherwise normal ultrastructurally. The cytoplasm, however, often displayed a conspicuously increased volume of ribosome-poor endoplasmic reticulum (ER) (Fig. 4A). The lumen of the ER contained one or more characteristic, long filaments ~22 nm in diameter that branched occasionally and appeared as spheres in cross section (Fig. 4B). These long filaments were not observed elsewhere in the cell; in particular the rough ER and the Golgi...

**Fig. 1.** Hepatic expression of HBsAg, shown by immunofluorescence of quick-frozen liver tissue from 10 Alb-PSX transgenic mouse families (lineage designation shown in each section). Note variable distribution and staining intensity of HBsAg-positive hepatocytes. (x900.)
apparatus were free of such structures. Thus the intracellular trapping of HBsAg by the large envelope polyepptide is associated with the formation of very long, occasionally branching filaments, which accumulate within a greatly expanded ER compartment and apparently fail to be efficiently transported to the Golgi apparatus and subsequently secreted.

The intracellular localization of these filaments was confirmed by subcellular fractionation coupled with ultrastructural and immunocytochemical analysis. The microsomal fraction was enriched in HBsAg, containing nearly 3600 ng of soluble HBsAg per mg of total protein. This specific activity was at least 25 times greater than that of any other subcellular fraction and represented >90% of the total soluble HBsAg extractable from the liver. The microsomal fraction contained numerous smooth-walled vesicles containing characteristic intraluminal filaments (Fig. 4C), consistent with HBsAg localization within the endoplasmic reticulum. Negatively stained free filaments were 22 nm in diameter and ranged from 100 to 800 nm in length. Occasionally, branched filaments were observed (Fig. 4 D and Inset). The filaments were shown to consist of HBsAg by immunogold electron microscopy (Fig. 4E), and they contained roughly equimolar concentrations of the large and the major envelope polypeptides as shown by immunoblot analysis (data not shown).

**Hepatocellular Injury.** SGPT activity rose in some of the transgenic mouse lineages, corresponding with the higher intrahepatic concentrations of HBsAg (Fig. 5). SGPT activity was uniformly normal in all lineages during the first 6 weeks of life. At varying intervals thereafter, depending on lineage, SGPT became abnormal [greater than (twice the upper limit of normal (50 units/liter) for age-matched nontransgenic mice) in lineages 50-4, 45-2, and 45-3. In lineages 50-4, 100% of animals displayed elevated SGPT activity beginning at 2.9 ± 1.0 months of age (mean ± standard error), reaching a peak of 441 ± 40.8 units/liter. Corresponding values for lineage 45-2 were 60%, 6.4 ± 2.4 months, 143 ± 31.5 units/liter and for lineage 45-3 were 38.9%, 9.6 ± 0.8 months, 97 ± 9.9 units/liter. SGPT activity did not become significantly elevated in the remaining 7 lineages, which displayed lower total intrahepatic HBsAg concentrations than lineages 50-4, 45-3, and 45-2. All mice, regardless of SGPT levels, were immu-

![Fig. 2.](image2) **Fig. 2.** (A) Blot of total RNA (15 μg per lane) hybridized with a 32P-labeled, HBV-specific DNA probe as described in Materials and Methods. Lane 1: lineage 45-3, mouse aged 4 months. Lane 2: lineage 45-2, 5 months. Lanes 3-8: lineage 50-4, 4 months, 1 month, 2 months, 5 months, 6 months, and 14 months, respectively. Markers at left show length in kilobases. (B) Immunoblot of cellular protein (100 μg per lane) extracted from the aqueous-insoluble liver pellet from the same mice as in A. Blot was probed with a rabbit antiserum specific for denatured HBsAg. Markers at left show size in kDa.

![Fig. 3.](image3) **Fig. 3.** Age-dependent hepatocellular expression of HBsAg in lineages 50-4. (A) Transgenic mouse 50-4-105, age 1 month; HBsAg is present as finely granular cytoplasmic deposits, barely detectable at this magnification. (B) Transgenic mouse 50-4-5, age 4 months; note the cellular enlargement and large clumped cytoplasmic deposits of HBsAg. (C) Transgenic mouse 50-4-8, age 6 months; note the central HBsAg-negative regenerative nodule within a field of HBsAg-positive, pleomorphic hepatocytes. [Orcein stain (5) for HBsAg; ×60.]

![Fig. 4.](image4) **Fig. 4.** Electron-microscopic features. (A) Hepatocytes of transgenic mouse 50-4-5, age 4 months; note increased ER membranes. (x2400.) (B) Note multiple long filaments within lumen of ribosome-poor ER (arrows), which appear as spheres in cross section. (x30,000.) (C) Smooth-walled vesicles derived from microsomal fraction; note intraluminal filaments. (x30,000.) (D and Inset) Negatively stained free filaments 22 nm in diameter, branching in places (arrows). (x30,000.) (E) Immunogold electron microscopy demonstrating HBsAg on surface of filament. (x30,000.)

![Fig. 5.](image5) **Fig. 5.** Kinetics and lineage-dependence of hepatocellular injury. Mean (± standard deviation) SGPT activities displayed as a function of age for lineage 50-4 (n = 36). (Inset) Mean peak SGPT (± standard error) for three lineages. Percent of each lineage with elevated SGPT and mean age of onset of elevated SGPT were as follows. Lineage 45-3: 38.9%, 9.6 ± 0.8 months (n = 17). Lineage 45-2: 60%, 6.4 ± 2.4 months (n = 10). Lineage 50-4: 100%, 2.9 ± 1.0 months (n = 36).
nologically tolerant to the HBV envelope antigens as determined by the absence of serum antibodies or cellular sensitization to the HBsAg or pre-S(1) or pre-S(2) antigens (data not shown). In addition, there was no association between injury and serologic evidence of infection with mouse hepatitis virus, Sendai virus, ectromelia virus, reovirus 3, or lymphocytic choriomeningitis virus.

Several characteristic histologic changes were detectable in the livers of transgenic mice with elevated SGPT levels (Fig. 6), the severity and onset of which corresponded with intracellular envelope polypeptide concentration. The most severe changes occurred in lineage 50-4, which displayed the most hepatic HBsAg. In this lineage, no significant changes were detectable at 1 month of age (at which time the SGPT level was normal) except for rare cells with finely granular cytoplasmic vacuoles. HBsAg and pre-S antigens were detectable as finely granular deposits uniformly distributed throughout the cytoplasm.

At 2–3 months of age the antigen-laden hepatocytes (Fig. 3B) in lineage 50-4 were enlarged due to hydropic swelling and the accumulation of increased amphoterin or eosinophilic proteinaceous cytoplasm containing acidophilic inclusions thereby resembling typical (8–11) ground-glass hepatocytes, as illustrated (in an older mouse) in Fig. 6C. There were rare, widely scattered foci of hepatocellular necrosis occasionally associated with a mixed inflammatory cell infiltrate. Thus, ground-glass hepatocytes dominated the histologic picture at this point when hepatocellular injury was relatively minor in this lineage.

At 3–4 months of age the cytolytic changes in lineage 50-4 became more severe, displaying single-cell necrosis with acidophil body formation, focal necrosis of multiple hepatocytes, and an inflammatory infiltrate of polymorphonuclear leukocytes and macrophages (Fig. 6 B and C). Hepatocellular pleomorphism, feathery degeneration, eosinophilic clumping, microvesicular steatosis, and abnormal mitotic figures were observed. Many nuclei were severely altered by a peculiar granulation of chromosomes resembling early karyorrhexis and by inclusions, presumably cytoplasmic invaginations. At this point the normal lobular architecture was severely distorted by displacement of portal tracts with severe lobular disarray (Fig. 6C). Kupffer cells were prominent, and there was extensive interfascicular cell proliferation, predominantly of macrophages. The lobular distribution of the envelope antigens, which previously in lineage 50-4 had been uniform in 100% of hepatocytes, changed at this point and became irregular and spotty by immunofluorescence, orcein (5), and Victoria blue staining. In some fields one or more entirely negative nodules (Fig. 3C) alternated with entirely positive nodules observed against a background of negative hepatocytes. The remodeling of the lobular architecture, together with the nodule formation, was compatible with regenerative hyperplasia. Moreover, the large cytoplasmic masses of Victoria blue-positive material seen in the ground-glass-like cells frequently displayed a bizarre central coagulative necrosis. In all cases the portal tracts were uninvolved, bile ducts were normal, and cholestasis was not observed.

The oldest mouse in the 50-4 lineage developed a large abdominal mass at 12 months of age, associated with exceptionally high SGPT activity (1800 units/liter). The animal, sacrificed at 14 months of age, had a massively enlarged multinodular liver (Fig. 6D). The degenerative changes described above were extensive. In addition, the nodules were composed of disorganized sheets of either cytologically normal hepatocytes (Fig. 6F) or hepatocytes displaying either microvesicular (Fig. 6G) or macrovesicular (Fig. 6H) steatosis. Notably, many nodules failed to express detectable HBsAg or pre-S antigen by immunofluorescence or orcein staining (Figs. 3C and 6F). This corresponded with decreased total hepatic content of HBV envelope polypeptide, as illustrated earlier (Fig. 2). These types of histopathologic changes were less severe and were delayed in onset in lineages 45-2 and 45-3 in keeping with their lower intrahepatic HBsAg content. In the remaining seven lineages the only detectable histologic change was low-level ground-glass cell formation without evidence of injury or regeneration.

**DISCUSSION**

The envelope polypeptide composition of HBV particles secreted during natural HBV infection has been described (12, 13). Unlike the major and middle polypeptide, the large envelope polypeptide is unevenly distributed among the three forms of HBV particles. Specifically, the abundant, serum-derived 22-nm subviral spheres contain not more than 2% of the large envelope polypeptide, which contributes up to 20% of the polypeptides of the less abundant subviral filaments and complete virions present in the circulation.

We previously reported (2) that preferential synthesis of the large envelope polypeptide, relative to the major envelope polypeptide, in transgenic mice inhibits the secretion of HBsAg by the hepatocyte, which becomes increasingly laden with both polypeptides in an aqueous-insoluble pre-Golgi or early Golgi compartment. Similar results have been reported by other investigators using different systems (14–17).

The production of approximately equimolar quantities of the large and major envelope polypeptides leads to the formation of long (at least 800 nm) 22-nm diameter HBsAg filaments that occasionally show a branched structure. These filaments accumulate within a ribosome-poor region of the ER that increases as a function of age. Notably, no filaments (or spheres) were observed in any other cellular compartment; specifically, the rough ER and the Golgi apparatus were entirely devoid of detectable particles. This suggests that transport from the ER to the Golgi is severely constrained, possibly for structural reasons. Filaments up to 200 nm in length can be secreted into the blood during human HBV infection, although shorter filaments...
are more frequently found in the serum of patients with hepatitis B (18). Since the filaments found in the serum of the transgenic mice contain less large polypeptide than the long, branching, nonsecreted filaments observed in these mice, the lack of secretion is presumably influenced by the large envelope polypeptide content of the particles. It is possible that the very long filaments cannot efficiently progress through the cellular secretion pathways, and therefore they accumulate within the ER.

Similar ultrastructural features have been observed in human ground-glass hepatocytes (8–11). Such hepatocytes are found in chronic HBV infection and also in animal hepatitis B virus infections and are usually not associated with a necro-inflammatory reaction since they are mainly encountered in the “healthy” carrier state (19). Thus it is possible that the large envelope polypeptide may also be overproduced in human ground-glass hepatocytes, but since the envelope polypeptide composition of HBsAg-positive ground-glass hepatocytes has not been described in quantitative terms, confirmation of this possibility must await further studies.

A totally unexpected feature of this model was the development of injury, hypertrophy, and hyperplasia in hepatocytes containing high levels of HBsAg. Our results indicate that injury was not due to an immune response to HBV-encoded antigens in these immunologically tolerant mice; nor was it due to infection by unrelated murine pathogens. It appears that liver injury may be due to generalized ER dysfunction, induced by retention of the large polypeptide-rich HBsAg filaments. Since the frequency and severity of this lesion were directly related to the total liver concentration of HBsAg, quantitative factors are critically important in the pathogenesis of this lesion.

While this model illustrates that overproduction of the large envelope polypeptide leads to the intracellular storage of cytotoxic quantities of HBsAg within the hepatocyte, there is no evidence that a similar mechanism might be responsible for HBV-induced hepatocellular injury in human viral hepatitis, since the degree of HBsAg retention seen in the transgenic lineages with injury has not been reported in humans. Quantitative analysis of the relative envelope polypeptide composition and intrahepatic HBsAg concentrations in human HBV-infected liver samples is needed to investigate this issue further.

The hypertrophy and hyperplasia of the hepatocytes in this mouse model, however, have a counterpart in human chronic HBV infection, particularly in the ground-glass hepatocytes. In humans, these cells may form nodules in which large and binucleated cells are frequent, and the nucleocyttoplasmic ratio of HBsAg-rich hepatocytes is increased, even when the cells are small (20). The hypertrophy characteristic of human ground-glass hepatocytes is well illustrated in all transgenic lineages, including those without injury. Hepatocellular hypertrophy proceeded to hyperplasia, culminating in nodule formation, in the lineages containing the most ground-glass cells, the highest intrahepatic HBsAg concentration, and the greatest amount of prior injury.

We conclude that overproduction of the large envelope polypeptide by the hepatocyte causes the progressive accumulation of nonsecreted HBsAg filaments within the ER. Filament storage causes expansion of the ER leading to formation of ground-glass cells similar to those reported in the healthy carrier state. At very high concentrations HBsAg has the potential to directly mediate hepatocellular injury in this transgenic mouse system, and injury probably leads to regenerative hyperplasia. Since current information suggests that the large envelope polypeptide is not overproduced during the HBV life cycle, appropriate analysis of human samples will determine whether similar mechanisms may be operative in viral hepatitis in man.

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