A Chronic Carrierlike State Is Established in Nude Mice Injected with Cloned Hepatitis B Virus DNA

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BALB/c nude mice were injected intrahepatically with hepatitis B virus (HBV) DNA prepared from recombinant plasmids. Hepatitis B surface antigen appeared in the circulation in 19 of 23 mice (82%) 3 to 20 weeks postinjection and persisted for more than 6 months in most animals. Hepatitis B e antigen appeared transiently in the circulation in 12 of the 23 mice (52%) within a few weeks after the appearance of hepatitis B surface antigen. Antibodies to the core, X, and/or polymerase gene products of HBV have also been observed in 14 (61%) of the mice. Histopathological examination of the livers at 7 months postinjection demonstrated that nearly half had characteristics consistent with chronic hepatitis. HBV DNA appeared to be integrated into host liver DNA. No evidence of viral replication was observed in sera or livers from these mice at 7 months postinjection. These results demonstrate that an HBV chronic carrierlike state can be established in mice and that such a model could be used to study host and virus factors important in the establishment and maintenance of HBV-associated chronic liver disease.

Hepatitis B virus (HBV) infection is a worldwide public health problem with more than 200 million estimated carriers (20). Until recently, an understanding of the biology of HBV infection has been hampered by the lack of a reproducible and long-term tissue culture system for viral propagation and by the very narrow host range of infection. The chronic carrier state has been successfully established by experimental infection of chimpanzees (2) with HBV or HBV DNA (22, 23), but the infection is self-limiting and chimpanzees are both expensive and endangered, thereby limiting the number and types of studies which could be done. The discovery and characterization of HBV-like viruses in other animals, such as ground squirrel hepatitis virus (12), woodchuck hepatitis virus (19), duck hepatitis B virus (14), and tree squirrel hepatitis B virus (8) has provided much information about their biology, including some information concerning the carrier state. The expense and difficulty in handling these animals, however, has limited their use. The recent construction of several transgenic mice expressing hepatitis B surface antigen (HBsAg) in serum and in some tissues (1, 3) will permit the detailed study of HBsAg biosynthesis *in vivo*. Although transgenic mice demonstrate evidence of chronic hepatitis (2a), the pathology described is due to overproduction of surface antigen and not likely to be immune mediated, as in natural infections. Therefore, transgenic mice will be of limited usefulness in studying the mechanism of liver disease associated with the chronic carrier state. In this study, inbred laboratory mice injected intrahepatically (i.h.) with HBV DNA develop circulating HBsAg, hepatitis B e antigen (HBeAg), and chronic hepatitis, providing a new system for studying the relationships between HBV gene expression, host immune response, and chronic liver disease.

MATERIALS AND METHODS

Mice. Female BALB/c nude mice (BALB/c *nu/nu*), 2 to 3 months of age, were obtained from matings of BALB/c (+/*nu*) heterozygote parents in the Laboratory Animal Facility of the Fox Chase Cancer Center. This nude mouse colony has been periodically screened for the presence of common mouse pathogens and evidence of disease in different organ systems. Mice used in these studies were derived from a colony free of any gross or microscopic liver abnormalities, including hepatitis. Mouse livers were also screened for the presence of mouse hepatitis virus (MHV), using a rabbit antiserum specific for common MHV nucleocapsid associated antigens, followed by a biotinylated goat anti-rabbit immunoglobulin, soluble avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, Calif.), and diaminobenzidine as substrate.

i.h. injections. For i.h. injection of DNA, mice were anesthetized intraperitoneally with 0.3 ml of 10% pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, Ill.). In each animal, the midline of the upper abdomen was opened to reveal the liver and a total of 100 µg of DNA was injected into several sites. The DNA species used for injection are as follows. (i) A total of 15 mice were injected with HBV DNA (adw clone [18]) derived from a full-length, single-copy, *EcoRI* insert of viral DNA in pBR322 (Fig. 1). Recombinant plasmids were grown in *Escherichia coli* and isolated exactly as previously described (16), cut with *EcoRI* to release the full-length HBV DNA insert, and finally ligated with T4 DNA ligase (New England BioLabs, Inc., Beverly, Mass.) under conditions favoring intramolecular ligation (11). The appearance of covalently closed circular monomers of HBV DNA (CCC HBV DNA) was monitored by agarose gel electrophoresis. For injection, the mixture containing religated monomers of HBV DNA was used without attempts at separation from pBR322 sequences. (ii) Another eight mice were injected with a head-to-tail *EcoRI* tandem HBV DNA dimer inserted into pBR322 sequences (pTKHH2, a gift from Hans Will; Fig. 1). This clone had been previously shown to be infectious in chimpanzees (22) without prior removal of virus sequences. In parallel experiments, (iii) five mice were injected with 100 µg each of

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FIG. 1. Outline of experimental design used for mouse injections. Mice were injected with 100 μ g of one of the DNA preparations as shown. Animals were bled every 2 weeks for 7 months and then sacrificed so that liver sections could be evaluated for pathology. The state of HBV DNA in liver cells was also determined by Southern blot analysis (see text).

pBR322 DNA and (iv) eight mice were injected with equivalent amounts of sheared calf thymus DNA.

Assays for HBsAg and HBeAg in sera. Serial sera were tested for the presence of HBsAg, using the following assays. (i) HBsAg particles were detected in a sandwich enzyme-linked immunosorbent assay in which a standard human antibody to HBsAg (anti-HBs) was used for coating microtiter wells (Immulon 2; Dynatech Laboratories, Inc., Alexandria, Va.) overnight at 4°C. Wells were washed eight times with phosphate-buffered saline (PBS), pH 7.2 (Fisher Scientific Co., King of Prussia, Pa.), and a 1:10 dilution of each mouse serum (in PBS containing 10% fetal calf serum [PBS-FCS]; GIBCO Laboratories, Grand Island, N.Y.) was added. Wells were incubated overnight at 4°C and washed as outlined above. Surface antigen was detected by addition of horseradish peroxidase (HRP)-conjugated anti-HBs (from the Auszyme kit, Abbott Laboratories) and *o*-phenylenediamine (OPD) substrate. Optical density values at 492 nm (OD_{492}) were determined. Any OD_{492} values due to nonspecific binding of prebleed sera (obtained before i.h. injections) for each animal were subtracted from the appropriate OD_{492} values of the serial sera. The resulting net values were then scored positive if they were greater than two standard deviations above the mean of the negative controls, which were serial sera from mice injected with control DNA and tested in the same assay. In these assays, the binding of the negative controls was not significantly different from the binding ($<0.05 OD_{492}$) of the HRP-conjugated antibody to wells previously incubated with PBS-FCS instead of normal mouse sera. The small serum volumes obtained from retro-orbital bleedings necessitated a modification of the standard solid-phase assay for HBsAg detection, using microtiter wells instead of beads so as to conserve serum and limit the dilutions used in such assays. (ii) For HBsAg quantitation, 5 μ l of sera found positive in the enzyme-linked immunosorbent assay described above were analyzed by agarose gel electrophoresis and then blotted to nitrocellulose (BA85, Schleicher & Schuell, Inc., Keene, N.H.) and detected by 125 I-anti-HBs (from the Ausria II kit, Abbott Laboratories) as previously described (15). Quantitation was achieved by densitometric comparison with HBsAg standards purified from human sera as previously described (6) and run on the same gels (Table 1). (iii) For electron microscopy, 10 μ l of selected sera found positive in the above assays was pelleted by ultracentrifugation overnight at 4°C in TE buffer (0.05 M

Tris hydrochloride [pH 7.5], 0.005 M EDTA) at 20,000 rpm, using a type 25 rotor (Beckman Instruments, Inc., San Ramon, Calif.). Pellets were resuspended in TE buffer, negatively stained by uranyl acetate, and viewed by electron microscopy (8). For immune electron microscopy, a human anti-HBs was added and the samples were evaluated after a 30-min incubation at room temperature. (iv) The appearance of the major HBsAg-associated polypeptides at 25,000 and 29,000 daltons in mouse sera was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (5, 8). In these experiments, 5- μ l samples of mouse sera per lane were reduced for 1 h at 37°C with 50 mM dithiothreitol (Sigma Chemical Co., St. Louis, Mo.) in TE buffer, pH 8. After SDS-PAGE and transfer to nitrocellulose, detection was carried out by 90-min incubation with an anti-HBs raised in rabbits against a synthetic peptide spanning residues 48 to 81 of the surface antigen gene (a gift from Richard Lerner). The second antibody used was affinity-purified alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, Md.), followed by addition of the substrates nitro blue tetrazolium and bromochloroindolyl phosphate (Sigma). (v) Several sera positive for HBsAg in the solid-phase assay described above were further characterized by density equilibrium gradient centrifugation in CsCl as previously described (6) except that 5-ml tubes were used in an SW65 rotor (60 h, 40,000 rpm, 10°C). Fractions were analyzed by Western blotting (immunoblotting) as described above to determine the density at which HBsAg-reactive polypeptides were present.

HBeAg in mouse serum samples was assayed by the HBeAg kit (Abbott Laboratories). HBeAg determinations were also carried out by an enzyme-linked immunosorbent assay in which microtiter wells (as used for HBsAg determinations) were coated with mouse serum samples (at a 1:5 dilution) to be tested. The plates were incubated overnight at 4°C, as outlined above, and washed, and a well-characterized human antibody to HBeAg (anti-HBe) from a chronic carrier was added. The plates were again incubated overnight at 4°C and washed, and a goat anti-human immunoglobulin conjugated to HRP (Kirkegaard and Perry Laboratories) was added. The plates were incubated for 1 h at 37°C, washed, and substrate was added. Calculations to determine HBeAg-positive samples were carried out as described for HBsAg determinations.

Detection of anti-HBc, anti-HBx, and anti-pol. Sera from nude mice were assayed for antibody to hepatitis B core antigen (anti-HBc) in the following manner. Hepatitis B core antigen particles were isolated from infected liver exactly as previously described (7) and used for coating microtiter wells. Approximately 1 μ g of core diluted to 50 μ l with PBS-FCS was added per well, and the mixture was incubated overnight at 4°C. After the wells were washed eight times with PBS, serial sera from HBV DNA-injected animals each diluted 1:10 in PBS-FCS were added and incubated overnight at 4°C. Wells were washed again with PBS, followed by the addition of HRP-conjugated goat anti-mouse immunoglobulin (Organon Teknika, Malvern, Pa.) and incubated for 1 h at 37°C. The wells were washed again, and OPD substrate was added. For antibodies to hepatitis B X (anti-HBx) and to hepatitis B polymerase (anti-pol), selected synthetic peptides deduced from the nucleic acid sequences (ayw clone; 9) encoding each of these HBV gene products were used for coating microtiter wells. Starting with the amino terminus of each gene product as position 1, the X peptides used spanned residues 10 to 29, 100 to 114, and 115

TABLE 1. Appearance of HBV-associated antigens, antibodies, and pathology^a

Plasmid injected and animal no.	HBsAg			HBeAg		Presence of antibody			Pathology	
	Presence	Time of appearance (weeks p.i.)	Amt (μg/ml)	Presence	Time of appearance after HBsAg (weeks p.i.)	Anti-HBc	Anti-HBx	Anti-pol	Presence	Type
CCC HBV DNA										
1	+	6	0.67	—		—	+	+	+	CAH
2	+	6	5.1	—		—	+	+	+	CPH
3	+	7	10	—		—	+	+	—	
4	+	13	2.5	+	2	—	—	—	+	CPH
5	—					—	—	—	—	
6	+	3	2.4	+	0	—	—	—	*	
7	+	3	5.1	+	0	—	—	—	*	
8	+	7	8.6	+	0	+	—	—	—	
9	+	7	11.2	+	8	+	+	+	—	
10	+	7	6.1	—		—	—	—	—	
11	+	7	1.3	+	2	—	+	+	+	CAH
12	+	7	4.1	+	6	+	+	+	—	
13	—					—	—	—	—	
14	+	7	0.74	+	0	+	—	—	—	
15	+	7	ND	—		+	+	—	+	CPH
pTKHH2										
16	+	4	3.4	+	6	—	—	—	+	CPH
17	+	4	12.2	—		—	—	—	+	CAH
18	+	12	7.7	+	6	+	+	+	+	CPH
19	—					+	+	—	+	CAH
20	—					—	—	—	—	
21	+	4	ND	+	8	+	—	—	+	CPH
22	+	6	ND	—		—	—	—	—	
23	+	4	ND	—		+	—	—	—	

^a Abbreviations and symbols: ND, not determined; p.i., postinjection; CAH, chronic active hepatitis; CPH, chronic persistent hepatitis; *, animals died before evaluation.

to 131, whereas the polymerase peptides used spanned residues 29 to 38, 781 to 795, and 822 to 838. In all assays, the OD₄₉₂ value of the first serum collected from a given mouse (before HBV DNA injection) was subtracted from the successive serial sera in the same animal. The net OD₄₉₂ values were scored as positive if they repeatedly yielded values greater than two standard deviations above the mean of the negative controls, as defined in the HBsAg assay described above.

Evaluation of liver tissue for HBV DNA and pathology. At 7 months postinjection, mice were sacrificed and the livers were removed for further analysis. Tissue sections were prepared from several regions of each liver, stained with hematoxylin and eosin, and evaluated for pathology by light microscopy. Most of each liver was frozen in dry ice and stored at -70°C . Total cellular DNA was extracted from approximately 0.3 g of tissue from each liver (13) and used for Southern analysis. Electrophoresis was carried out by using gels of 1% agarose loaded with 5 μg of DNA per lane as previously described (14). For each liver, DNA samples were examined before and after digestion with *Hind*III or with *Eco*RI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). After electrophoresis, the digestion products and size markers (*Hind*III fragments of phage λ DNA; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were visualized after staining with ethidium bromide, partially deproteinized with sodium acetate, and then blotted to nitrocellulose (BA85; Schleicher & Schuell). Hybridizations were carried out at stringent conditions (10), using full-length HBV DNA cut out from pBR322 by *Eco*RI digestion, isolated from pBR322 by agarose gel electrophoresis, electroluted, and radiolabeled by primer extension (4). Separate gel lanes were hybridized with pBR322 (Boehringer Mann-

heim) to test for the presence of vector sequences in the liver DNAs from HBV DNA-injected mice.

RESULTS

Detection of HBsAg and HBeAg in nude mice. The experimental design outlined in Fig. 1 was chosen to test for the possible appearance of HBV, HBsAg, or both in HBV DNA-injected mice from serial sera collected over a period of several months. All sera were negative for the endogenous DNA polymerase activity associated with HBV, consistent with the absence of complete virus (data not shown). However, testing of serial sera by solid-phase immunoassays demonstrated HBsAg appearing in the sera of about 80% of the mice from 3 to 13 weeks after i.h. injection of HBV DNA (Table 1). In addition, nearly 60% of the HBsAg-positive animals also became transiently seropositive for HBeAg within 2 months after the appearance of HBsAg (Table 1). From the time of onset, HBeAg remained detectable in different mice from 2 to 10 weeks in duration. Mice injected with either the HBV DNA-recircularized and -ligated monomer (CCC HBV DNA) or the HBV DNA tandem dimer in a recombinant plasmid (pTKHH2) produced both HBsAg and HBeAg. When the levels of surface antigen particles for each mouse were quantitated by Western blotting by using a ¹²⁵I-anti-HBs, the HBsAg concentration varied for different mice (Table 1) but remained relatively constant for a given mouse (data not shown). Mice which became HBsAg positive within a few weeks of injection remained positive for the remaining 6 months of the experiment, demonstrating that a chronic HBsAg carrierlike state could be established in mice by i.h. injection of HBV DNA.

Partial characterization of HBsAg particles. Characterization of the HBsAg-reactive species was carried out by using

FIG. 2. Western blot analysis of sera from HBV DNA-injected nude mice. Sera from different mice were analyzed by SDS-PAGE, and polypeptides were transferred to nitrocellulose. Surface antigen-related polypeptides were detected by addition of rabbit sera containing surface antigen peptide antibodies, followed by addition of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and finally substrate. Lanes: A, purified HBsAg from a chronic human carrier of HBV; B, sera from two mice injected with CCC HBV DNA; C, sera from two mice injected with pTKHH2; D, sera from two nude mice before injection with HBV DNA; E, sera from two nude mice injected with pBR322; F, sera from two nude mice injected with calf thymus DNA. Markers are in kilodaltons.

SDS-PAGE and Western blotting to detect surface antigen-associated polypeptides (Fig. 2). In lane A, HBsAg particles isolated from the serum of a human carrier demonstrate the major HBsAg-associated polypeptides at 25,000 and 29,000 daltons. Lane B shows the serum from two mice injected with CCC HBV DNA. Lane C shows the serum from two mice injected with pTKHH2. In lanes B and C, clearly reactive bands at approximately 25,000 and 29,000 daltons were present. In addition, there were a variable number of slower-migrating bands which might be HBsAg related. Lane D shows the Western blot of sera from two mice collected before injection of HBV DNA. The 25,000- and 29,000-dalton bands and some of the minor, larger bands were completely undetectable. Similarly, mice injected with control DNA (pBR322 DNA in lane E and calf thymus DNA in lane F) were also negative for these immunoreactive bands, demonstrating that the presence of these bands is associated only with prior injection of HBV DNA.

The density of the surface antigen particles in the sera of mice was determined by density equilibrium centrifugation in CsCl (Fig. 3). Fractions were analyzed for the presence of the major HBsAg components at 25,000 and 29,000 daltons by SDS-PAGE and Western blotting. Positive fractions had densities near 1.2 g/ml in CsCl, which is also the density of HBsAg particles derived from human sera.

Six of these sera were pelleted overnight by centrifugation and then suspended for examination by electron microscopy (Fig. 4). Small, spherical particles approximately 20 nm in diameter were observed in each of the sera (panel A). When anti-HBs from an HBV-infected human carrier was added, these particles aggregated in a time-dependent manner, consistent with the presence of HBsAg determinants (panel B). In the absence of anti-HBs or in the presence of normal human serum, the particles remained unaggregated. Particles were not observed in sera from mice either before DNA injection or after injection with control DNA (panel C).

Presence of antibodies to HBV gene products in nude mouse

FIG. 3. CsCl density equilibrium centrifugation of an HBsAg-positive serum from a nude mouse injected with HBV DNA. Fractions 14 to 17, indicated in the graph (+) and labeled above the appropriate gel lanes in the adjacent Western blot, were positive for the major pair of surface antigen polypeptides at a gradient density of 1.2. Markers on the Western blot are in kilodaltons.

sera. Serial sera from nude mice were assayed for anti-HBc, anti-HBx, and anti-pol in solid-phase assays (Table 1). Among the 23 nude mice injected with HBV DNA, 9 (39%) had evidence of anti-HBc, 9 (39%) had evidence of anti-HBx, and 7 (30%) had evidence of anti-pol in one or more serial sera after the appearance of HBsAg. Three animals had all of these markers, another four had both anti-HBx and anti-pol, and four more had anti-HBc alone. In addition, 2 animals had both anti-HBc and anti-HBx and the remaining 10 mice were negative for each of these antibody markers. The results are consistent with the conclusion that the core, X, and polymerase gene products may be made, at least transiently, in these hosts and give rise to antibody responses which are also observed in HBV-infected patients.

State of HBV DNA in nude mouse livers. Mice injected with HBV or control DNA were kept for 7 months and then sacrificed to examine the livers in more detail. The state of HBV DNA in these livers was determined by extracting whole-cell DNA and performing Southern blot hybridization with an HBV DNA probe (Fig. 5). For panel A, whole-cell DNA extracts from four HBV DNA-injected mice were analyzed before digestion with restriction endonucleases. The hybridization signals were confined to the high-molecular-weight region of the gel, consistent with integration of HBV DNA into the host genome. For panel B, the same

FIG. 4. Electron micrographs of an HBsAg-positive serum from a nude mouse injected with HBV DNA. Coded serum samples were pelleted overnight and resuspended for negative staining and observation. Panels: A, particles approximately 20 nm in diameter in serum from an HBV DNA-injected animal; B, same as panel A with the addition of anti-HBs; C, serum from a nude mouse before the injection of HBV DNA. Bar, 100 nm.

FIG. 5. Southern blot analysis of whole-cell DNA from different mice injected with control or HBV DNA. Panels: A, uncut DNA from four HBV DNA-injected mice; B, uncut DNA from two mice injected with control (calf thymus) DNA; C, DNA from the same four mice used for panel A, digested with *Hind*III before electrophoresis; D, DNA from the same mice used for panel A, digested with *Eco*RI before electrophoresis. Markers are in kilodaltons.

experiment was repeated, using whole-cell DNA from two animals injected with control DNA. The absence of signal demonstrates that under the conditions of hybridization, only HBV DNA sequences, and not host sequences, were detected. pBR322 sequences, however, were found associated with high-molecular-weight DNA in some of the HBV DNA-injected animals as well as pBR322-injected control animals (data not shown). For panel C, the liver DNAs from the four animals tested as shown in panel A were digested with *Hind*III before gel electrophoresis. Each of the animals demonstrated a smear of hybridizable DNA, consistent with random integration of HBV into the host genome. For panel D, the DNAs from the four animals tested as shown in panel A were digested with *Eco*RI before gel electrophoresis. Each animal demonstrated a smear of hybridizable DNA and a band approximately 3.0 to 3.2 kilobases. The appearance of a 3-kilobase band after *Eco*RI digestion is consistent with it being full-length HBV DNA, perhaps tandemly integrated into the host genome. The absence of replicating forms of HBV DNA in these experiments shows that HBV was not actively replicating in these livers at 7 months after injection of HBV DNA.

Liver pathology in HBV DNA-injected nude mice. Slices of tissue from different regions of the livers were fixed, hematoxylin- and eosin-stained, and examined for evidence of pathology. Several examples of these results are presented in Fig. 6, and all of the results are summarized in Table 1. In the HBV DNA-injected group, nearly half of the mice had one or more characteristics of hepatitis. Among the animals acquiring hepatitis, all demonstrated periportal inflammation involving many portal tracts in different parts of each liver (Fig. 6, panel A). These lesions were consistent with the diagnosis of chronic persistent hepatitis in 6 of 21 animals (29%) injected with viral DNA. In addition to periportal inflammation, another four animals (19%) had evidence of mild intralobular hepatitis, bridging inflammation, and fibrosis among neighboring portal tracts (Fig. 6, panels B and C, respectively); this was consistent with the diagnosis of chronic active hepatitis (Table 1). Among the 13 age-matched animals injected with control DNA, 12 had livers which had normal architecture and one (injected with calf

FIG. 6. Pathology associated with nude mouse livers approximately 7 months after injection with HBV DNA. Panels: A, periportal inflammation from a mouse injected with CCC HBV DNA; B, intralobular hepatitis from a mouse injected with pTKHH2; C, sample from a mouse injected as in panel B, showing bridging fibrosis and inflammation. All sections are hematoxylin and eosin stained. Magnification, $\times 300$ for panel A and $\times 400$ for panels B and C.

thymus DNA) had minimal periportal inflammation in one portal tract. The absence of MHV nucleocapsid expression, of syncytium formation, and of easily detectable regions of necrosis in the liver parenchyma from control and HBV DNA-injected animals argues against MHV infection. Clinical signs associated with MHV-mediated encephalomyelitis (17), chronic wasting disease (21), and high mortality were also absent from nude mice. Further, normal BALB/c mice sharing cages with these nude mice for 2 months showed no evidence of seroconversion to antibody to MHV (data not shown). In the same experiment, these normal mice also remained seronegative for ectromelia virus and lymphocytic choriomeningitis virus, which are known to be liver pathogens in mice (data not shown). Together, these results are inconsistent with MHV, ectromelia, and/or lymphocytic choriomeningitis virus infection(s) as a cause of chronic hepatitis in these nude mice.

DISCUSSION

These studies demonstrate that i.h. injection of HBV DNA into nude mice results in the establishment of an animal model sharing many characteristics with human chronic carriers of HBV. Approximately 80% of the mice injected with HBV DNA became seropositive for HBsAg within weeks of injection and remained HBsAg positive for 6 or more months. A similar phenomenon occurs in HBV-infected patients who become chronic carriers. More than half of the mice with detectable HBsAg also became positive for HBeAg for a duration of up to 2 months. A peak of HBeAg of variable duration is also characteristic of many patients with chronic HBV infection. The nude mice also maintain HBV DNA in the absence of viral replication. Long-term HBV-infected human carriers often have similar characteristics. In mice, the maintenance mechanism may be integration of viral DNA into host genomic DNA sequences, the presence of concatenated circular viral genomes, or both. In either host, the HBV DNA likely serves as the template for the sustained production of HBsAg. The nude mice also develop lesions in the liver consistent with the presence of chronic hepatitis, as seen in long-term HBV infected patients. Since HBV is not directly cytopathic, it is likely that the liver pathology observed in HBV DNA-injected mice, as in naturally infected patients, is immune mediated. If this is true, then partial or total reconstitution of the immune system in nude mice should significantly alter the frequency of chronic liver disease in these animals and permit identification of immune elements which contribute to the establishment or maintenance of chronic hepatitis.

The presence of HBeAg in the sera of several HBsAg-positive mice is consistent with a peak of virus replication, as it is in many chronically infected patients. Antibodies to the core, X, and polymerase gene products of HBV have also been detected in the sera of these mice. Together, these results are consistent with the expression of all of the known HBV gene products and with virus replication. The absence of HBV-associated DNA polymerase activity from HBeAg-positive sera may indicate low levels or lack of virus in the blood. Sera from transgenic mice carrying a plasmid containing a tandem head-to-tail dimer of HBV DNA were also negative for DNA polymerase activity, although low levels of replicative forms were observed in the liver (D. Paul, H. Farza, M. Hühne, and C. Pourcel, *Proc. Int. Symp. Viral Hepatitis Liver Dis.*, abstr. no. 154, p. 54A, 1987). Viral antigens and replicative forms of viral DNA may be present in the liver of HBV DNA-injected nude mice at times (1 to 4

months postinjection) when the sera are HBeAg positive, and experiments are now under way to test this hypothesis. Finally, if one or more of the viral gene products are targets for a cellular inflammatory response, which results in immune-mediated hepatitis, then the use of viral DNA mutants may identify which (if any) viral gene products alter the establishment, severity, and/or persistence of chronic liver disease.

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LITERATURE CITED

1. Babinet, C., H. Farza, D. Morello, M. Hadchouel, and C. Pourcel. 1985. Specific expression of hepatitis B surface antigen (HBsAg) in transgenic mice. *Science* 230:1160-1163.
2. Barker, L. F., F. Chisari, P. P. McGrath, D. W. Dalgard, R. L. Kirschstein, J. D. Almedia, T. S. Edgington, D. G. Sharp, and M. R. Peterson. 1973. Transmission of type B viral hepatitis to chimpanzees. *J. Infect. Dis.* 127:648-662.
- 2a. Buras, J., P. Filippi, A. McLachlan, C. A. Pinkert, R. D. Palmiter, R. L. Brinster, and F. V. Chisari. 1987. Suppression of HBsAg secretion and induction of an HBsAg filament storage disease by overproduction of the hepatitis B virus large envelope polypeptide in transgenic mice, p. 387-395. *In* W. Robinson, K. Koike, and H. Will (ed.), *Hepadna viruses*. Alan R. Liss, Inc., New York.
3. Chisari, F. V., C. A. Pinkert, D. R. Milich, P. Filippi, A. McLachlan, R. D. Palmiter, and R. L. Brinster. 1985. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. *Science* 230:1157-1160.
4. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
5. Feitelson, M. A. 1986. Products of the "X" gene in hepatitis B and related viruses. *Hepatology* 6:191-198.
6. Feitelson, M. A., P. L. Marion, and W. S. Robinson. 1981. Antigenic and structural relationships of the surface antigens of hepatitis B virus, ground squirrel hepatitis virus, and woodchuck hepatitis virus. *J. Virol.* 39:447-454.
7. Feitelson, M. A., P. L. Marion, and W. S. Robinson. 1982. Core particles of hepatitis B virus and ground squirrel hepatitis virus. I. Relationship between hepatitis B core antigen- and ground squirrel hepatitis core antigen-associated polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and tryptic peptide mapping. *J. Virol.* 43:687-696.
8. Feitelson, M. A., I. Millman, T. Halbherr, H. Simmons, and B. S. Blumberg. 1986. A newly identified hepatitis B type virus in tree squirrels. *Proc. Natl. Acad. Sci. USA* 83:2233-2237.
9. Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charney. 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature (London)* 281:646-650.
10. Ganem, D., L. Greenbaum, and H. E. Varmus. 1982. Virion DNA in ground squirrel hepatitis virus: structural analysis and molecular cloning. *J. Virol.* 44:374-383.
11. Hirschman, S. Z., P. Price, E. Garfinkel, J. Christman, and G. Acs. 1980. Expression of cloned hepatitis B virus DNA in human cell cultures. *Proc. Natl. Acad. Sci. USA* 77:5507-5511.
12. Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullard, and W. S. Robinson. 1980. A virus in Beechey ground squirrels that

- is related to hepatitis B virus of humans. *Proc. Natl. Acad. Sci. USA* **77**:2941–2945.
13. **Mason, W. S., C. Aldrich, J. Summers, and J. M. Taylor.** 1982. Asymmetric replication of duck hepatitis B virus DNA in liver cells (free minus strand DNA). *Proc. Natl. Acad. Sci. USA* **79**: 3997–4001.
 14. **Mason, W. S., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J. Virol.* **36**:829–836.
 15. **McMichael, J. C., L. M. Greisiger, and I. Millman.** 1981. The use of nitrocellulose blotting for the study of hepatitis B surface antigen electrophoresed in agarose gels. *J. Immunol. Methods* **45**:79–94.
 16. **Mukhopadhyay, M., and N. C. Mandal.** 1983. A simple procedure for large-scale preparation of pure plasmid DNA free from chromosomal DNA from bacteria. *Anal. Biochem.* **133**: 265–270.
 17. **Robb, J. A., and C. W. Bond.** 1979. Coronaviridae, p. 193–247. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.). *Comprehensive virology*, vol. 14. Plenum Publishing Corp., New York.
 18. **Sninsky, J. J., A. Siddiqui, W. S. Robinson, and S. N. Cohen.** 1979. Cloning and endonuclease mapping of the hepatitis B viral genome. *Nature (London)* **279**:346–348.
 19. **Summers, J., J. M. Smolec, and R. Snyder.** 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc. Natl. Acad. Sci. USA* **75**:4533–4537.
 20. **Szmunes, W.** 1978. Hepatocellular carcinoma and hepatitis B virus: evidence for a causal association. *Prog. Med. Virol.* **24**: 40–69.
 21. **Ward, J. M., M. J. Collins, Jr., and J. C. Parker.** 1977. Naturally occurring mouse hepatitis virus infection in the nude mouse. *Lab. Anim. Sci.* **27**:372–376.
 22. **Will, H., R. Cattaneo, G. Darai, F. Deinhardt, H. Schellekens, and H. Schaller.** 1985. Infectious hepatitis B virus from cloned DNA of known nucleotide sequence. *Proc. Natl. Acad. Sci. USA* **82**:891–895.
 23. **Will, H., R. Cattaneo, H.-G. Koch, G. Darai, H. Schaller, H. Schellekens, P. M. C. A. van Eerd, and F. Deinhardt.** 1982. Cloned HBV DNA causes hepatitis in chimpanzees. *Nature (London)* **299**:740–742.