A newly identified hepatitis B type virus in tree squirrels
(chronic carrier/hepadnavirus)

MARK A. FEITELSON*, IRVING MILLMAN, THERESA HALBHERR, HEIDI SIMMONS, AND BARUCH S. BLUMBERG

Fox Chase Cancer Center, Philadelphia, PA 19111

Contributed by Baruch S. Blumberg, December 11, 1985

ABSTRACT Virus-associated particles have been isolated from the livers of three common gray tree squirrels (Sciurus carolinensis pennsylvanicus) that have histological evidence of hepatitis. Two of these livers were also positive by orcein staining, suggesting the presence of surface antigen in the cytoplasm of hepatocytes. Fractionation of these particles by CsCl density equilibrium gradient centrifugation and assay of the fractions for surface antigen, core antigen, and DNA polymerase activities demonstrate the presence of all three at an approximate density peak of 1.27. Electron microscopic examination of purified virus preparations showed spherical particles with a mean diameter of 25 nm. Initial characterization of the DNA polymerase product by gel electrophoresis showed a single DNase I sensitive band, migrating slightly faster than the woodchuck hepatitis virus DNA polymerase product. The presence of apparently cross-reacting antibodies was demonstrated by purified hepatitis B surface and/or core antigens binding to some squirrel sera in solid phase assays. Infected tree squirrels appear to lack detectable antigen in their sera. These results suggest that the tree squirrels studied are chronic carriers of a hepatitis B type virus. The host–virus interaction described herein may be useful in understanding the chronic carrier state associated with hepatitis B in man.

Elucidation of hepatitis B virus (HBV) molecular biology relevant to the asymptomatic carrier state and disease, including chronic hepatitis and hepatocellular carcinoma, has been hampered by its narrow host range of infection and its inability to replicate in a tissue culture system. The discovery of hepatitis B type viruses in woodchucks (1), ground squirrels (2), and ducks (3) has provided important systems to address questions concerning host–virus interactions (4), including hepatocellular carcinoma (1, 4), and has recently made significant contributions toward better understanding the viral replication scheme (5). The continued development of these systems and the search for new ones will be important in learning more about their biology and that of HBV. In this study virus-associated particles have been found in liver extracts obtained from locally captured tree squirrels. Characterization of such particles demonstrates that they are distinct from, but similar to, HBV and related viruses. It is proposed that this tree squirrel hepatitis virus (THBV) is another member of the group including HBV, woodchuck hepatitis virus, ground squirrel hepatitis virus, and duck hepatitis B virus.

MATERIALS AND METHODS

Animal Studies. Ninety-four tree squirrels (Sciurus carolinensis pennsylvanicus) were collected with humane traps in areas adjacent to the Fox Chase Cancer Center (Philadelphia and Montgomery Counties, PA), around Reading, PA, and in selected sites within several neighboring states.

Isolation of Tree Squirrel Antigen. The liver was obtained from each tree squirrel immediately after capture and frozen at –70°C until use. Approximately 200 mg of each liver was homogenized in 20 mM Tris-HCl, pH 7.5 (grinding buffer), at 0°C (6). The mixture was sonicated (twice for 30 sec) and centrifuged at 10,000 rpm at 4°C for 30 min. The pellet was resuspended in fresh grinding buffer, sonicated, and centrifuged again as described above. The supernatants were pooled, layered over 20% (wt/vol) sucrose in grinding buffer, and centrifuged at 20,000 rpm at 4°C for 18 hr in a Beckman Ti 60 rotor. Pellets were resuspended in grinding buffer and centrifuged once more at 50,000 rpm at 4°C for 4 hr in a Ti 60 rotor. These pellets were further analyzed by CsCl density equilibrium centrifugation by resuspension in phosphate-buffered saline (PBS, 0.015 M sodium phosphate, 0.15 M sodium chloride), pH 7.3, followed by addition of enough CsCl to make the density 1.2090 g/ml. Centrifugation was carried out at 40,000 rpm, 10°C, for 72 hr in a Beckman SW 55 rotor. Alternatively, pellets were taken up in 0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl (Tris/saline) and chromatographed over a Sepharose 4B column (3 × 60 cm, LKB) equilibrated with the same buffer.

Antigen and Antibody Assays. Crude liver extracts (antigen samples prior to CsCl density equilibrium centrifugation or Sepharose 4B chromatography), sera, column, or gradient fractions were tested for cross-reactive hepatitis B surface antigen (HBsAg) by the Ausria II or Auszyme assay kits (Abbott). For hepatitis B core antigen (HBcAg) assay, samples were spotted onto nitrocellulose (BA85; Schleicher & Schuell) and developed with 125I-labeled anti-HBe from the Corab assay kit (Abbott). Samples were also tested for core reactivity by direct assay in the Corab kit.

Tree squirrel sera were screened for cross-reacting anti-HBs or anti-HBc by using several different assays. For anti-HBs detection, the commercially available Ausab (Abbott) or passive hemagglutination assay kits (Hemasure-Ab, Electro-Nucleonics, Bethesda, MD) were used. Alternatively, squirrel sera were serially diluted in PBS, pH 7.3, containing 10% (vol/vol) fetal bovine serum (PBS/FBS) and used to coat Immulon 2 Removawell (Dynatech, Alexandria, VA). Following overnight adsorption, the wells were washed six times in PBS/FBS, and 1 μg of purified HBsAg (7,8) was added per well. Following overnight binding, the wells were washed and then incubated for 1 hr at 37°C with goat anti-HBs conjugated to horseradish peroxidase obtained directly from the Auszyme II kit (Abbott). After washing, binding was detected colorimetrically by using o-phenylenediamine. Anti-HBe screening was carried out using the Corab kit (Abbott) or by counterimmunoelectrophoresis. After electrophoresis, the slides were fixed and stained in

Abbreviations: HBV, hepatitis B virus; DNA, DNA polymerase; THBV, tree squirrel hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; anti-HBs, anti-hepatitis B surface antigen; anti-HBe, anti-hepatitis B core antigen.

*To whom reprint requests should be addressed.
Coomassie blue to detect antigen–antibody precipitation lines. Alternatively, purified HBcAg particles (9) were added to the Immulon wells in place of HBsAg as described above. Sheep anti-HBc (a kind gift from Wolfram Gerlich) was added, followed by incubation at 37°C for 1 hr. After washing, horseradish peroxidase-conjugated rabbit anti-sheep Ig (Cappel, West Chester, PA) diluted 1:100 in PBS/FBS was added, and binding was detected by using α-phenylenediamine.

DNA Polymerase Reaction and Analysis of Labeled Products. Tree squirrel antigen pelleted from a crude lysate, from Sepharose 4B peaks, or from CsCl gradient fractions was radiolabeled for 1 hr by the endogenous DNA polymerase (DNAP) reaction as described (10, 11) and then digested for 30 min at 37°C with pronase at a final concentration of 0.5 mg/ml in 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 0.1% NaDodSO4. The digest was electrophoresed in 1.5% agarose gels using buffer and electrophoretic conditions described (3). The gels then were dried under vacuum and exposed to X-ray film for screen-intensified autoradiography.

DNase I Digestion. Some agarose gels containing the tree squirrel antigen-associated DNAP reaction product were run in the presence of 0.1 μg of ethidium bromide/ml (Calbiochem) next to HindIII-digested phage λ DNA markers (Bethesda Research Laboratories). A trough was cut just in front of the 2-kilobase λ DNA fragment in the lane containing the DNAP product, and the radioactive band recovered by electroelution. The material was ethanol precipitated in the presence of 20 μg of carrier salmon sperm DNA and washed twice, and then half was treated with DNase I (Sigma; ribonuclease free) in 0.05 M Tris-HCl (pH 7.2), 0.01 M MgSO4, 0.1 mM dithiothreitol, and 50 μg of bovine serum albumin/ml (fraction V, Sigma). The product before and after DNase I treatment was then analyzed on agarose gels as described above.

Electron Microscopy. Semi-purified antigen resulting from CsCl density equilibrium centrifugation or Sepharose 4B column chromatography was layered on continuous CsCl gradients (density 1.15–1.30) and centrifuged at 10°C for 2.5 hr in an SW 40 rotor at 34,000 rpm. The gradients were then fractionated and assayed for crossreactive surface and core antigen containing fractions as well as for DNAP activity. One hundred microliter fractions positive by all three criteria were diluted 1:10 in 0.005 M Tris-HCl/0.0005 M EDTA (pH 7.5) (TE) and centrifuged overnight at 10°C in a type 25 rotor at 20,000 rpm. After carefully decanting the tubes, the pellets were resuspended in 10 μl of TE and transferred to nitrocellulose–carbon coated 300 mesh copper grids for electron microscopy. Grids were negatively stained with saturated, aqueous uranyl acetate and viewed on a Philips 420 electron microscope.

RESULTS

Assay of Tree Squirrel Sera and Livers for Markers of an HBV-Type Infection. Ninety-four tree squirrels (Sciurus carolinensis pennsylvanicus) were trapped, their livers were removed, and each was assayed for histological evidence of hepatitis and possible virus markers cross-reacting with HBsAg or HBcAg. Sera were collected from 53 of the 94 animals and assayed for cross-reacting HBsAg, anti-HBs, and anti-HBc using commercially available assays. None of the sera were positive for HBsAg by Ausria II, for anti-HBs by passive hemagglutination and by Ausab, or for anti-HBc by counterimmunoelectrophoresis. These results suggest that at least with reagents suitable for the detection of HBV and its corresponding antibodies, no serological evidence for infection was found. However, when these tests were reconstructed so that the binding of purified HBsAg or HBcAg to increasing dilutions of squirrel sera was determined, many

![Fig. 1](image-url) Liver sections from two infected tree squirrels, 308957 (A) and 308960 (B), showing representative areas of inflammation. (×80.) (C) Similar section from a squirrel with no signs of hepatitis. (×80.) Liver extracts from this animal were negative in the DNAP and surface antigen assays. The serum was also negative for the presence of apparently cross-reacting anti-HBs and anti-HBc. Sections were stained with hematoxylin/eosin. (D) Liver section from squirrel 308960 stained with orcein and counterstained with fast green. (×150.) (E) Same liver as in D at lower magnification. (×80.) (F) Orcein staining of a liver section from a tree squirrel negative for hepatitis and markers of viral infection. (×80.) The orcein staining is striking in the liver cell cytoplasm of the infected animals.
Tree squirrels demonstrated the presence of apparently cross-reacting anti-HBs and anti-HBC, suggesting a high frequency of exposure to an HBV-type agent. The nature of these responses will be considered in greater detail below.

Independent examination of hematoxylin/eosin stained liver sections from each of the 94 animals showed marked scattered focal necrosis, portal perinuclear proliferation, pericholangitis, and/or bile duct proliferation consistent with possible viral hepatitis in 14 animals, two of which are shown in Fig. 1. A small piece of liver tissue (0.1–0.2 g) from each of these animals as well as from each of the 80 normal appearing animals was homogenized and centrifuged at low speed to remove cell debris and at higher speed to pellet material for the DNAp assay. Three of the 14 livers that demonstrated pathology by hematoxylin/eosin staining were also positive in the DNAp assay and were used for further characterization (squirrels 308957, 308960, and 309017). Similarly prepared crude extracts from all livers were tested for HBsAg by the Austria II and Auszyme assays. Only the three livers that were DNAp positive were also positive for cross-reacting surface antigen. Material from liver 308960 had a positive (P/N) ratio of 2.42; material from 309017 had a ratio of 4.46; and material from 308957 had a ratio of 2.59 by Austria II. A result was scored as positive if the P/N ratio was greater than 2.1. Respective positive values (P/N) in the Auszyme assay were 15 for 308960, 25 for 309017, and 21 for 308957. The immunological specificity of these reactions was demonstrated by the ability of anti-HBs to block these surface antigen tests when mixed with the crude extracts prior to assay. Identical analyses of the 80 livers that showed no sign of pathology and of the other 11 livers with evidence of pathology were negative in these assays. These results suggest the presence of HBsAg cross-reactive and DNAp positive material in crude liver extracts from 3 out of the 14 different tree squirrels showing a histological picture consistent with viral hepatitis.

The presence of tree squirrel antigen in liver sections was also assayed using orcein, a histochemical stain that detects HBsAg in HBV-infected hepatocytes (12). In a group of 32 tree squirrels tested for orcein staining, two of the three livers containing virus were strongly positive (Fig. 1). As with HBsAg, hepatocyte staining was strictly cytoplasmic. Livers lacking detectable tree squirrel antigen by the criteria described above were uniformly negative. These results are consistent with the presence of material similar to HBsAg exclusively in the cytoplasm of infected hepatocytes in virus-containing animals.

**Purification and Partial Characterization of Tree Squirrel Antigen Particles.** CsCl density equilibrium centrifugation of crude extracts from squirrels 308957 or 309017 was carried out to isolate the reacting fractions and ascertain whether the markers expected for HBV and related viruses copurified. Gradient fractions from liver 308957 assayed for surface antigen by Austria II demonstrated a peak P/N ratio of 3.83 at density 1.271 g/ml (Fig. 2). Fraction samples spotted on nitrocellulose and blotted with radiolabeled anti-HBc from the Core kit demonstrated binding in the HBsAg positive fractions (Fig. 2). Fractions pelleted overnight and assayed for DNAp demonstrated such activity only within the HBsAg and HBCAg positive peak (Fig. 2). The same results were obtained from an independent analysis of tree squirrel antigen from liver 309017. Further characterization of the DNAp product by agarose gel electrophoresis consistently showed a small band equivalent to 2.0–2.4 kilobases of linear DNA marker (Fig. 3A). Since this band migrated faster than similarly labeled DNA of HBV and related viruses, a crude extract from another DNA positive liver was processed differently to see if the same DNA product was still present. Purification of crude extracts from squirrel 308960 by chromatography on Sepharose 4B yielded five discrete peaks by absorbancy (unpublished data). Assay of each pooled peak for DNAp activity showed that such activity resided exclusively in the second eluted peak and that further analysis of the product resulted in exactly the same result as that obtained from CsCl fractions (Fig. 3B). The DNase I sensitivity of the DNA product following pronase digestion also suggests that the radiolabeled band is due to the presence of DNA (Fig. 3C). These results are consistent with the presence of a nucleic acid species that becomes endogenously radiolabeled in the DNAp assay and copurifies with the crossreactive surface and core antigen containing fractions using two independent approaches.

Tree squirrel antigen partially purified by density equilibrium ultracentrifugation or column chromatography was further purified by rate zonal sedimentation in preformed CsCl gradients. Fractions positive for DNAp activity, and crossreactive surface and core reactivities were used to prepare samples for electron microscopy. Fraction aliquots were centrifuged overnight, and pellets were resuspended. Negatively stained samples were examined (Fig. 4). Particles having a mean diameter of 25 nm, with a size range between 21 nm and 35 nm, were observed. Such particles were not observed in DNAp negative fractions derived from isolates of infected livers, nor from parallel analyses of four uninfected livers. These results are consistent with the conclusion that these particles are virus associated, although it is not clear whether they represent virions or subviral particles. Their size and appearance, however, suggest that they are similar
The product, untreated.

The results of this study demonstrate the presence of surface antigen and endogenous DNAp activity in particles anti-HBc in each serum resulted in three different types of response to infection (Fig. 5). Twelve sera demonstrated a pattern of reactivity consistent with there being a strong anti-HBc response and little or no anti-HBAs, as exemplified in Fig. 5A. In 28 sera, neither anti-HBc nor anti-HBAs responses were detectable, as shown in Fig. 5B. In the remaining 13 sera, both activities were present, as presented in Fig. 5C. The titers in many squirrel sera were similar to positive controls using known anti-HBAs and anti-HBc containing sera (Fig. 5D). Further, the P/N ratios in Fig. 5B with tree squirrel sera were similar to those obtained using human sera with the Ausab and Corab kits known to be negative for the corresponding antibodies. The apparent presence of an anti-HBAs response without anti-HBc in tree squirrel sera was not detected.

Comparison of these serological responses to the appearance of hepatitis in the liver showed no correlation. Tree squirrel antigen carrying animals also showed different serological responses. Animals 308960 and 308957 did not demonstrate apparently cross-reacting anti-HBc or anti-HBAg, while animal 309017 assayed strongly positive for both binding activities. All three antigen positive animals, however, demonstrated one or more features characteristic of hepatitis upon examination of their liver sections.

**DISCUSSION**

The discovery of histological evidence of hepatitis in 14 of 94 tree squirrel livers examined prompted the search for a possible viral etiology. In 3 of the 14 diseased livers, crude extracts were positive for DNAp and crossreactive surface antigen activities. None of the 80 livers normal by histological criteria or of 11 others with histological signs of hepatitis were positive for these viral markers. The presence of crossreactive HBAs and HBCAg determinants copurifying with DNAp positive particles together suggest that tree squirrel antigen is a virus similar to, but distinct from, HBV. These data suggest the presence of a new HBV type of virus that we propose to name tree squirrel hepatitis B virus (THBV).

Fig. 3. Analysis of the DNAp reaction product from livers of different infected squirrels by using two independent methods of purification and sensitivity of the product to DNase I. (A) DNAp-positive fractions (lanes 4–6) from a CsCl density equilibrium gradient of tree squirrel antigen from squirrel 308957. Only fractions 4–6 from this gradient, as shown in Fig. 2, were positive in the DNAp reaction and were further analyzed by agarose gel electrophoresis. The numbers to the right are molecular size markers derived from HindIII-digested lambda DNA and are expressed as kilobasepairs. (B) Sepharose 4B absorbancy peaks (lanes 1–5) analyzed for the tree squirrel antigen 308960 DNAp product. Fractions in each absorbancy peak were pooled and reduced by a factor of approximately 10 in volume. Ten percent of the concentrated materials (by volume) was pelleted and finally assayed for DNAp activity. The polymerase product (lane WC) from woodchuck hepatitis virus liver-derived core particles used as a positive control and for comparison of mobilities to that of the tree squirrel antigen DNAp product. (C) DNase I sensitivity of the tree squirrel antigen DNAp reaction product. Lane 1, gel purified tree squirrel antigen DNAp product was treated with DNase I and analyzed by agarose gel electrophoresis. Lane 2, same product, untreated. Lane 3, HBV-DNAp product labeled under the same conditions as the tree squirrel antigen sample. Lane 4, HindIII-digested lambda DNA markers terminally labeled by polynucleotide kinase (a gift from A. O'Connell). The numbers in the right-hand column are in kilobase pairs.

Serological Profiles Associated with Tree Squirrel Antigen Infection. The binding of purified HBAs and/or HBCAg to different dilutions of tree squirrel sera in solid-phase assays from 26 of the 53 sera tested is consistent with the presence of crossreacting antibodies and suggests that exposure to the tree squirrel antigen is widespread. Analysis of anti-HBAs and anti-HBc in each serum resulted in three different types of response to infection (Fig. 5). Twelve sera demonstrated a pattern of reactivity consistent with there being a strong anti-HBc response and little or no anti-HBAs, as exemplified in Fig. 5A. In 28 sera, neither anti-HBc nor anti-HBAs responses were detectable, as shown in Fig. 5B. In the remaining 13 sera, both activities were present, as presented in Fig. 5C. The titers in many squirrel sera were similar to positive controls using known anti-HBAs and anti-HBc containing sera (Fig. 5D). Further, the P/N ratios in Fig. 5B with tree squirrel sera were similar to those obtained using human sera with the Ausab and Corab kits known to be negative for the corresponding antibodies. The apparent presence of an anti-HBAs response without anti-HBc in tree squirrel sera was not detected.

Comparison of these serological responses to the appearance of hepatitis in the liver showed no correlation. Tree squirrel antigen carrying animals also showed different serological responses. Animals 308960 and 308957 did not demonstrate apparently cross-reacting anti-HBc or anti-HBAg, while animal 309017 assayed strongly positive for both binding activities. All three antigen positive animals, however, demonstrated one or more features characteristic of hepatitis upon examination of their liver sections.

**DISCUSSION**

The discovery of histological evidence of hepatitis in 14 of 94 tree squirrel livers examined prompted the search for a possible viral etiology. In 3 of the 14 diseased livers, crude extracts were positive for DNAp and crossreactive surface antigen activities. None of the 80 livers normal by histological criteria or of 11 others with histological signs of hepatitis were positive for these viral markers. The presence of crossreactive HBAs and HBCAg determinants copurifying with DNAp positive particles together suggest that tree squirrel antigen is a virus similar to, but distinct from, HBV. These data suggest the presence of a new HBV type of virus that we propose to name tree squirrel hepatitis B virus (THBV).

The results of this study demonstrate the presence of
from THBV-infected tree squirrel livers. Particles having similar characteristics have also been isolated from HBV-infected human livers (14). In the case of infected human liver, the major particle-associated DNA forms are double-stranded relaxed circular and linear, both of which are analogous to the DNA forms predominant in serum-derived Dane particles. In this study, characterization of the DNAp reaction product of THBV resulted in a broad band 2–2.4 kilobases in size, which is smaller than the 3–3.3 kilobases full-length genomes of HBV and other related viruses. Similar results have been obtained when virus-derived ground squirrel hepatitis virus DNA was compared to HBV DNA by gel electrophoresis and Southern blotting under nonstringent conditions (15). Since these DNA molecules were detected by nonstringent hybridization prior to the DNAp reaction, a simple explanation for the relatively fast migration of THBV DNA in comparison to HBV DNA following the DNAp reaction may be incomplete filling in of the single-stranded region. The DNase I sensitivity of the DNAp reaction product from THBV is also consistent with it being DNA. Extending the endogenous polymerase reaction from 1 to 6 hr resulted in an increased incorporation of radioactivity with time (unpublished data) further suggesting that the reaction product has a partially single-stranded structure that is filled in over time, as observed in HBV and other related viruses.

The absence of detectable virus particles from the sera of infected animals could indicate either that viral replication is proceeding at a low rate, with release of a few virions into the blood, or that a vigorous antibody response is present. While the latter explanation may be operative in squirrel 309017, the lack of any detectable binding of HBsAg or HBCag to the sera of 308960 and 308957 suggests that perhaps low levels or complete lack of viral replication, which is characteristic of the HBsAg-negative chronic carrier state in HBV infection (16), may be present in these animals. Alternatively, noncross-reacting viral antibodies may exist in these sera. If antibodies cross-reacting with purified HBsAg and/or HBCag roughly reflect the frequency of virus exposure, then the THBV-negative carrier state may be the dominant one in infected adult tree squirrels. Among the hepatitis B type viruses, long-term duck hepatitis B virus infection is also often characterized by a lack of circulating antigen (4, 17). The inverse relationship between liver disease and serum DNA or endogenous polymerase activity observed with duck hepatitis B virus infection (17) also seems to be consistent with the observations herein. Since HBV replication generally decreases as liver disease progresses from chronic passive to chronic active hepatitis and is infrequent or absent in primary hepatocellular carcinoma (17–21), it may be possible to study this type of disease progression in a mammalian host. If further work more firmly establishes the presence of a chronic carrier state, comparison of the host–virus relationship in this system with that of infected woodchucks and ground squirrels may help elucidate some of the factors in related mammalian hosts that account for the differences in pathogenesis among them.

We would like to thank Drs. Richard Crowell and John Taylor for their critical reviews of the manuscript and helpful suggestions for improvement. We would like to acknowledge Drs. Crane and Custer for their help in screening hematoxylin/eosin-stained liver sections and Dr. M. E. Bayer and S. Shepardson for help in obtaining the electron micrographs of purified THBV. Dr. Howard Blatt arranged the trapping, transport, and care of the tree squirrels. This work was supported by Public Health Service Grants CA-06551, RR-05539, and CA-06977 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.