A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks

(DNA polymerase-containing particles/hybridization)

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ABSTRACT Particles with properties similar to those associated with human hepatitis B were found in serum from woodchucks with chronic hepatitis and hepatocellular carcinoma. It is suggested that woodchuck hepatitis virus is a second member of a novel class of viruses represented by the human hepatitis B virus.

Recently, considerable information concerning the morphology of the human hepatitis B virus (HBV) and its genome structure has accumulated. Thus, HBV is thought to be a 40- to 50-nm double-layered virus (1) containing a circular, partially double-stranded DNA genome (2, 3) and a DNA polymerase (4). In vitro, the DNA polymerase fills in a large heterogeneous single-stranded region in the genome, generating a fully double-stranded circular DNA (3).

Antigenic determinants on the surface of the HBV (HBsAg) are found also on spherical and tubular particles approximately 20 nm in diameter that occur in the blood in vast excess over the 40- to 50-nm DNA-containing particles (1, 5). The smaller particles are free of nucleic acid and apparently represent excess viral coat protein.

The properties of HBV distinguish it from any known class of DNA-containing viruses. In fact, evidence presented in this paper indicates that HBV belongs to a novel class of DNA viruses which may be causative agents of hepatitis in species other than human beings. In this report, we describe the probable occurrence of one such virus in a colony of woodchucks (Marmota monax) at the Penrose Research Laboratory at the Philadelphia Zoo.

MATERIALS AND METHODS

Animals. The animals used in this study were from a colony of woodchucks (M. monax) at the Penrose Research Laboratory, which has been studied for 18 years, from 1960 to 1978, with the objective of identifying diseases that may serve as animal models for human diseases (6). As of 1 May 1977, the records on this colony, which included postmortem studies of 102 animals, contained 23 cases of primary hepatocellular carcinoma (22.5%). These cancers occurred at a mean age of 59 months, with a range of 23–106 months. All of the animals with hepatomas had degenerative and regenerative changes in the uninvolved liver, suggesting preexisting chronic active hepatitis as reported (7). In addition, 3 of the 102 woodchucks had developed acute hepatitis judged to be the cause of death. Several animals without hepatocellular tumors had inflammatory and regenerative changes in the liver consistent with chronic active hepatitis. A more complete review of the postmortem studies will be published elsewhere (8).

Histology. Tissues fixed in 6.6% neutral formalin were embedded in tissue-Prep (Fisher Scientific), sectioned at 5 μm, and stained with hematoxylin and eosin, alchroime, Weigert–Van Gresen light green, Verhoeff, Prussian blue, and Gomori’s silver stain.

Materials. HBsAg-positive plasma was kindly supplied by B. S. Blumberg (Institute for Cancer Research, Philadelphia, PA). Avian myeloblastosis (AMV) DNA polymerase was a gift of L. Loeb (Institute for Cancer Research, Philadelphia, PA). S1 nuclease was purified according to Ando (9). Deoxyribonucleoside triphosphates were purchased from P-L Biochemicals and thymidine ([α-32P]triphosphate (>200 Ci/mmol) was from New England Nuclear Corporation (Boston, MA).

Assay of Serum for Endogenous DNA Polymerase Activity. One milliliter of serum was layered on a 3.0-ml gradient of 10–20% (wt/vol) sucrose containing 10 mM Tris-HCl, pH 7.4/1 M NaCl. After centrifugation for 3 hr at 55,000 rpm in a Spinco SW56 rotor at 20°C, the supernatant was thoroughly removed by aspiration, and the pellet was dissolved in 0.01 ml of a DNA polymerase reaction mixture (0.1 M Tris-HCl, pH 8.0, 20 mM MgCl2, 0.5 M NaCl, 200 μM each dATP, dGTP, and dCTP, 5 μM [α-32P]dCTP, 1 mM dithiothreitol, and 0.1% Triton X-100). The reactions were incubated at 37°C for 2 hr, and stopped by addition of 0.05 ml of a solution containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.2% sodium dodecyl sulfate (NaDodSO4), and 0.5 mg of Pronase per ml. After a further incubation for 2 hr, the total and acid-precipitable radioactivities were determined as described (3).

Electron Microscopy. Fractions of CsCl gradients were examined directly by negative staining with uranyl acetate on carbon films. Grids were examined in a Siemens 101 transmission electron microscope.

Agarose Gel Electrophoresis. The electrophoresis was performed in a vertical slab gel apparatus. Two percent agarose slab gels secured between glass plates by insertion of a sponge strip between the plates along the bottom of the slab gel were run in a continuous buffer system (40 mM Tris acetate, pH 6.8/20 mM sodium acetate/1 mM EDTA). Electrophoresis was at 0.5 V/cm.

Extraction of DNA from Tissues. Fresh frozen tissue (0.5–1.0 g) was minced with scissors, suspended in 10 ml of 10 mM Tris-HCl, pH 7.4/10 mM EDTA/0.15 M NaCl, and homogenized with a loose-fitting Dounce homogenizer. Nuclei and cell debris were pelleted by centrifugation at 20,000 × g for 10 min, resuspended in 10 ml of Tris-HCl, pH 7.4/10 mM

Abbreviations: HBV, hepatitis B virus; NaDodSO4, sodium dodecyl sulfate; HBsAg, hepatitis B surface antigen; AMV, avian myeloblastosis virus.
EDTA, and adjusted to 0.2% NaDodSO₄ and 500 μg of Pronase per ml. After 2 hr at 57°C, the DNA was deproteinized by three extractions with phenol, followed by two chloroform extractions. The aqueous phase was adjusted to 0.1 M with NaCl and the nucleic acids were precipitated with 2 vol of absolute ethanol. The DNA was dissolved in 3.0 ml of 10 mM Tris-HCl, pH 7.4/10 mM EDTA and stored at 4°C.

Hybridizations. DNA for hybridization was heated at 100°C in 0.3 M NaOH for 20 min, then thoroughly chilled at 0°C. One-tenth volume of 4 M HCl was added and the precipitated DNA was collected by centrifugation at 20,000 × g for 5 min. The pellets were immediately dissolved in 0.2–0.4 ml of 0.1 M Tris-HCl, pH 9.0. The denatured DNAs (270 μg) were precipitated with ethanol in siliconized glass tubes (6 × 60 mm), dried, and dissolved in 0.16 ml of annealing solution (10 mM Tris-HCl, pH 7.5/10 mM EDTA/1 M NaCl). [32P]DNA from an endogenous reaction with woodchuck-derived particles, prepared and purified through a CsCl gradient as described in the legend to Fig. 3, was used as the probe. The probe was denatured at 100°C in 0.3 M NaOH for 20 min, neutralized with HCl, and added to the annealing mixtures. After an initial heating for 5 min at 98°C to dissolve the DNA, the annealing mixtures were overlaid with 0.2 ml of mineral oil and incubated at 68°C. Aliquots containing about 300 cpm of probe (0.02 ml) were removed at various times during the annealing and assayed by digestion with S1 nuclease (9).

RESULTS

Serum samples from woodchucks at the Penrose Research Laboratory were tested for the presence of DNA polymerase-containing particles as described in Materials and Methods. Approximately 15% of the sera tested contained such particles in amounts comparable to those found in some HBsAg-positive sera.

Three animals (CW307, CW326, and CW316) died subsequently and were examined further to determine the possible relationship of these particles to lesions of the liver. At autopsy, both CW307 and CW326 were found to have prominent tumorous nodules in the liver. Uninvolved portions of the liver showed evidence of multifocal regeneration and bile retention. The liver of CW316 was free of tumors and regenerative changes. Death of CW316 was attributable to a myocardial infarction.

The distinctive nodules in the livers of CW307 and CW326 were multicentric, malignant tumors designated as primary hepatocellular carcinoma by microscopic study. Hepatocellular carcinomas in human beings are subdivided according to histologic appearance into several variants depending on the degree of anaplasia. All such tumors, however, are considered to arise from one cell type, the polygonal hepatocyte. The tumor in CW326 was characteristic of those commonly encountered in woodchucks (7), i.e., multicentric with cells arranged in a trabecular pattern (Fig. 1A). The tumor cells resembled hepatocytes, but possessed hyperchromatic nuclei and large, prominent nucleoli.

The tumor in CW307 represented an unusual form in woodchucks, being multicentric with both hepatocellular (Fig. 1B) and cholangiomaticus (Fig. 1C) elements in acinar or tubular patterns. In addition, anaplastic spindle-shaped cells resembling sarcoma cells were arranged in whorls around proliferations of blood vessels (Fig. 1D). A similar rare variant in human beings has been ascribed to progressive anaplasia in which the arrangement of the epithelial cells around blood vessels simulates perithelioma (10).

The histological features of the degenerative and regenerative changes in the nontumorous parts of the liver are summarized in Table 1. The following description of the liver of CW307 applies as well to CW326. The microscopic picture consists of inflammatory cells within the portal zones and sinusoids of the parenchyma, proliferation of bile ducts and ductules, and piecemeal necrosis and degeneration of hepatocytes (Fig. 2A and B). All forms of inflammatory cells were present, i.e., neutrophils, eosinophils, lymphocytes, plasma cells, and histiocytes. Many typical acidophilic nuclear inclusions and some variation in nuclear size were further prominent features.

The microscopic appearance of the liver of CW316 was not remarkable. A few inflammatory cells were found within the portal triads, there were apparently more Kupffer cells than normal, and the reticula of the portal triads were collagenized to some extent (Fig. 2C and D).

The differences in the histopathology of CW307 and CW326 on the one hand, and CW316 on the other, resemble differences observed in hepatitis in human beings that are important in prognosis. In chronic active hepatitis the inflammatory exudate spills over into the peripheral portion of the lobular parenchyma as in Fig. 2A and B, and is accompanied by continuing necrosis of hepatocytes. This form of chronic hepatitis with erosion of the limiting plate of the parenchyma tends to progress to cirrhosis regardless of the degree of lobular necrosis (11). The mild portal inflammation observed in the liver of CW316 (Fig. 2C and D), however, is common to many diseases, and rarely leads to cirrhosis.

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<th>Table 1. Microscopic appearance of liver tissue uninvolved by tumor</th>
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<tr>
<td>Nuclear inclusions</td>
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<td>Piecemeal necrosis</td>
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<td>Portal inflammation</td>
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<tr>
<td>Intrahepatic inflammation</td>
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<tr>
<td>Bile duct proliferation</td>
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<td>Kupffer cell proliferation</td>
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Fig. 2. Hematoxylin/eosin-stained sections of uninvolved liver: (A) portal area, CW307; (B) intralobular area, CW326; (C) portal area, CW316; and (D) intralobular area, CW316. (×272.)

Sera from the three animals were tested for the presence of DNA polymerase-containing particles. Serum from both animals with tumors and showing evidence of chronic active hepatitis (CW307 and CW326) contained detectable levels of endogenous DNA polymerase, while serum from the woodchuck with no apparent hepatic lesion (CW316) had no detectable DNA polymerase-containing particles (data not shown).

The DNA polymerase-containing particles in the serum from CW307 were concentrated and characterized. As shown in Fig. 3A, DNA polymerase-containing particles banded in CsCl at an average density of 1.215 g/cm³, while a prominent protein peak was found at a density of 1.196 g/cm³. A similar purification carried out on serum from a healthy HBsAg-positive blood donor (Fig. 3B) showed DNA polymerase-containing particles banding at a density of 1.238 g/cm³. No DNA polymerase-containing particles were detected after a similar purification performed on serum from CW316 (Fig. 3C).

The DNA polymerase- and protein-containing fractions from all three samples were examined by electron microscopy. Numerous spherical and tubular particles approximately 25 nm in diameter were seen in fractions of density 1.192–1.204 g/cm³ from CW307 (Fig. 4A). Similar particles measuring approximately 20 nm in diameter were seen in fractions of density 1.198–1.214 g/cm³ from the HBsAg-positive blood donor (Fig. 4B). The DNA polymerase-containing fractions from CW307 (density 1.205–1.234 g/cm³) contained lower amounts of the tubular and spherical forms but in addition contained many double-layered particles 55 nm in diameter (Fig. 4C). These particles were similar in size and appearance to 50-nm particles seen in the DNA polymerase-containing fractions from the HBsAg-positive donor. These particles, variously reported as 40–50 nm in diameter, contain the HBV DNA genome and DNA polymerase, and are thought to be the complete virus of hepatitis B.

The labeled DNA from endogenous DNA polymerase reactions of woodchuck and HBV DNA were extracted and compared by electrophoresis through a 2% agarose gel (Fig. 5). In addition, a sample of the labeled DNAs was incubated with DNA polymerase purified from AMV to fill in any remaining single-stranded regions, and compared with the untreated samples by electrophoresis. The results in Fig. 5 show that the labeled product of the woodchuck endogenous reaction (gel c) is heterogeneous in size, migrating slightly more slowly than the HBV DNA (gel b). Much of the heterogeneity of the woodchuck and HBV products is due to the continued presence of substantial single-stranded regions not filled in by the endogenous reaction. Treatment with AMV DNA polymerase converts both DNAs to two discrete bands (gels d and e) which probably correspond to linear and circular forms of the same length. The faster migrating linear forms were used to estimate the molecular weight of the DNAs relative to linear DNA standards (gel a). HBV DNA appeared to be 3300 nucleotides long, in agreement with values reported for the circular form (12), while woodchuck particles DNA was slightly larger at 3400 nucleotides. Similarly, the putative circular form of woodchuck particle DNA appears to be slightly larger than that of the HBV DNA (Fig. 5, gels d and e). Circular markers of known molecular weight were not available for estimating the length of the circular forms.

A number of tissues from CW307, CW326, and CW316 were examined for DNA complementary to the DNA of the woodchuck particles. The assay was performed by annealing, in vast excess, DNA extracted from each tissue to a small amount of [32P]DNA probe labeled in an endogenous reaction of the woodchuck particles. Annealing was assayed by resistance of
the double-stranded product to digestion with S1 nuclease. Data from these experiments are presented in Fig. 6.

Although no detectable DNA sequences complementary to the labeled woodchuck particle DNA were detected in tissues from CW316 (Fig. 6C), both animals showing the inflammatory and regenerative changes in the liver contained detectable amounts of such DNA, with the largest amounts found in the liver (Fig. 6 A and B).

**Fig. 4.** Electron micrographs of negatively stained particles from the CsCl gradients in Fig. 2: (A) fraction 11, Fig. 2A (CW307); (B) fraction 9, Fig. 2B (HBsAg-positive serum); (C) fraction 9, Figure 2A (CW307); and (D) fraction 5, Fig. 2B (HBsAg-positive serum). Bar represents 100 nm.

**Fig. 5.** Agarose gel electrophoresis of the [32P]DNA product from the woodchuck-derived particles and from HBV. Approximately 10,000 cpm of each product was added to 10 μl of a DNA polymerase reaction mixture containing 100 mM Tris-HCl, pH 8.0; 20 mM MgCl2; 100 μM each of dATP, dGTP, dCTP, and dTTP; 100 mM KCl; and 1 mM dithiothreitol. Each reaction was split. To one half was added 0.2 μg of AMV DNA polymerase; the other half received no DNA polymerase. After 1 hr at 37°C, 5 μl of 20 mM EDTA/0.1% sodium sarcosinate was added to each tube and each sample was layered on a 2% agarose gel. Electrophoresis was performed at 0.5 V/cm for 4 hr. The gel was dried and developed by autoradiography. (gel a) EcoRI fragments of λ DNA; (gel b) [32P]DNA from HBV; (gel c) [32P]DNA from woodchuck particles; (gel d) [32P]DNA from HBV, treated with AMV DNA polymerase; and (gel e) [32P]DNA from woodchuck particles, treated with AMV DNA polymerase. Length of the λEcoRI fragments is expressed in kilobases (kb).

**Fig. 6.** Annealing rate assay for woodchuck particle DNA in woodchuck tissues. The data are plotted as the reciprocal of the fraction of the [32P]DNA remaining single stranded against the Cot of annealing of the tissue DNA. (A) DNA from CW307; (B) DNA from CW326; (C) DNA from CW316. DNAs analyzed were extracted from liver (○), hepatoma (●), spleen (▲), lymph nodes (■), kidney (▲), lung (△), salivary glands (●), and thymus (⊙).

The numbers of complementary DNA molecules per diploid cell in the tissues were calculated by using a C0/1.2 (initial DNA concentration X time required for 50% reannealing) for the woodchuck particle DNA equal to 1.03 times that of HBV DNA, previously determined under these conditions to be 2.4 X 10^-1 mole-sec/liter (see Table 2). The large amounts of complementary DNA in the liver compared to other tissues strongly suggests a relation between these DNA-containing particles and the hepatitis changes observed in these animals.

**DISCUSSION**

On the basis of histopathology, Snyder suggested a viral etiology for hepatitis in woodchucks (7). In fact, a variety of particles with remarkable similarities to those associated with human hepatitis B were found in woodchucks with hepatitis. First, large numbers of spherical and tubular forms approximately 25 nm in diameter were found in the blood of two woodchucks showing histopathologic evidence of chronic active hepatitis. These forms strongly resemble the well-known hepatitis B surface antigen (HBsAg), but band at slightly lower density than HBsAg in CsCl gradients. A second class of particles banding at a density of 1.215 g/cm³ contain a DNA and DNA polymerase activity. The DNA found in this second class of particles is similar in size and structure to the HBV DNA, consisting of two electrophoretic species that are probably linear and circular forms of the same length. Each DNA molecule appears to contain a substantial single-stranded region, as does HBV DNA. The action of the internal DNA polymerase on the single-stranded region of the DNA accounts for the endogenous DNA polymerase activity observed in the serum of some woodchucks. Finally, fractions of the CsCl gradient that have endogenous DNA polymerase activity contain double-layered particles, 55

**Table 2.** Woodchuck particle DNA in various tissues*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CW307</th>
<th>CW326</th>
<th>CW316</th>
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<tbody>
<tr>
<td>Liver</td>
<td>623</td>
<td>496</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>338</td>
<td>12</td>
<td>1.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>8</td>
<td>58</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>9</td>
<td>2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>30</td>
<td>9</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>0.8</td>
<td>0.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>1.6</td>
<td>0.6</td>
<td>&lt;0.2</td>
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*Molecules per cell DNA equivalent.*
nm in diameter, which resemble so-called Dane particles, thought to be the complete virus of hepatitis B. It is likely, therefore, that these double-layered particles contain the DNA and DNA polymerase activity.

The virus-like particles found in the diseased woodchucks are similar but not identical to those associated with hepatitis B. Preliminary experiments indicate that the exposed antigenic sites on the small particles do not crossreact with the HBsAg. Moreover, nucleic acid homology is limited to 3–5% of the genome (data not presented).

Large amounts of DNA complementary to that of the woodchuck-derived particles were found specifically in the diseased livers of the two animals examined. This finding implicates the liver as the site of particle production, and together with the histopathologic data suggests that these particles represent various forms of a woodchuck hepatitis virus that is phylogenetically related to the HBV of human beings. Further studies will be required to determine if hepatitis is indeed transmitted among woodchucks by this agent.

Snyder has observed that hepatocellular carcinoma in the Penrose Research Laboratory woodchuck colony is always associated with chronic active hepatitis. Likewise, the relationship between chronic hepatitis B and hepatocellular carcinoma in human beings is a subject of intense current interest (13). Hepatitis in woodchucks may provide an experimental system for investigating the oncogenic potential of HBV-like viruses.

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7. Snyder, R. L. (1968) Am. J. Path. 52, 32a (abstr.).