Hepatitis B virus produced by transfected Hep G2 cells causes hepatitis in chimpanzees

(hepatitis B virus antigens and antibodies/liver damage/serum enzymes)

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ABSTRACT We have reported that clonal cells derived from Hep G2 cells transfected with a plasmid containing hepatitis B virus (HBV) DNA secrete spherical and filamentous forms of hepatitis B surface antigen (HBsAg), core particles, and virions into the culture medium. Here we describe the development of typical hepatitis in two chimpanzees following intravenous inoculation with the medium in which the transfected cells had grown. The liver biopsies from these animals showed characteristic lesions in parenchyma and portal tracts, more conspicuous at an earlier time in the chimpanzee that had received a greater number of virions. The amount of HBsAg in the serum of one infected chimpanzee increased with time after the initial inoculation and then decreased concomitantly with the appearance of antibodies against HBsAg and core antigens. HBsAg remained detectable in the other animal throughout the course of the experiment. The levels of hepatitis B “e” antigen in both animals peaked at week 5, signifying the acute phase of the infection. The activities of serum enzymes that are markers for necroinflammation also increased. The hepatitis HBsAg subtype of the virions isolated from the patient whose DNA was cloned and then used for transfection of the Hep G2 cells was the same as that found in the chimpanzees. Furthermore, the restriction enzyme analysis of the viral DNA isolated from the chimpanzees was identical to the cloned DNA. Thus, HBV DNA-transfected Hep G2 cells can support the replication of virions that, in turn, produce hepatitis in chimpanzees.

The lack of a tissue culture system in which hepatitis B virus (HBV) replicates has long impeded the study of the replicative cycle and the oncogenic potential of the virus. Recently, we (1) and others (2, 3) have demonstrated that HBV DNA-transfected cells of lines Hep G2 and Hu6-c15, which are both derived from human hepatic tumors, can produce replicative intermediates and mature virions. The definition of HBV particles in all three of these reports is based primarily on their physical properties, polymerase activity, antigenicity, and morphological description. However, these data do not prove that the transfected cells produce infectious virus. We report that viral particles produced by the transfected cells elicit acute hepatitis in chimpanzees, as shown by the histologic findings on liver biopsy, and in serum, as shown by the appearance of HBV antigens and antibodies as well as by elevated activities of enzymes reflecting hepatic necroinflammation.

MATERIALS AND METHODS

Cell Cultures and Inoculum Preparations. The establishment of the clonal line of cells, 2.2.15, by HBV DNA transfection of Hep G2 cells and the analysis and mainte-

nance of these cells has been reported (1). One culture of cells that had been sustained for 25 days after passage, with medium changes at 5-day intervals, was used as the source of inoculum for chimpanzee 1303. Ten milliliters of medium that had been exposed to the cells in this culture from day 20 to day 25 after subculture was aspirated, centrifuged at 3000 × g for 10 min to remove cell debris, and intravenously injected. The medium (10 ml) injected into chimpanzee 1178 had been withdrawn from another culture, exposed to the cells for 12 days after passage, and centrifuged as described above.

Chimpanzees. The chimpanzees used in this study weighed 21–22 kg and were born and raised in captivity. They were fed and housed by methods recommended by the National Research Council (4) in individual isolators, maintained under reduced pressure. Chimpanzee 1178 had been experimentally infected with hepatitis A virus previously. Neither chimpanzee had been inoculated with blood-borne non-A/non-B hepatitis virus.

Serological Assays. Serum samples were obtained from both animals at the day of inoculation and at weekly intervals thereafter for 14 weeks. HBV “e” antigen (HBeAg), surface antigen (HBsAg), and antibodies to HBsAg (anti-HBsAg) and to HBV core antigen (anti-HBcAg) were assayed, respectively, by the Abbott-HBe, Ausria II, Ausab, and Corab RIAs (Abbott). Sample-to-negative-control ratios (S/N) of >2 were considered positive. The HBsAg subtype of the input particles and of the virus isolate from the chimpanzees were determined as described by Hoofnagle et al. (5).

Assessment of Liver Damage. Hepatitis was assessed in chimpanzees by assay of serum alanine aminotransferase, γ-glutamyl transpeptidase, and isocitrate dehydrogenase by standard methodologies. Liver biopsies were obtained by Menghini needle the day of inoculation and at weekly intervals thereafter and were examined by light microscopy.

Isolation and Analysis of Viral DNA from the Culture Medium and Chimpanzee Serum. Concurrently with the collection of growth medium for inoculations, 1.5-ml aliquots of the medium were removed and utilized for isolation of viral DNA. Polyethylene glycol (M, ~ 8000) was added to each sample of medium to 10% (wt/vol), and the samples were incubated at 4°C for 1 hr. The virus particles were pelleted by centrifugation at 8000 × g for 10 min at 4°C. The resulting pellet was resuspended in 10 mM Tris-HCl, pH 7.5/10 mM EDTA/1% NaDodSO₄. The DNA was purified by treatment with 400 μg of proteinase K per ml for 2 hr at 37°C and was deproteinized by two extractions with equal volumes of phenol/chloroform. The DNA isolated from each sample was electrophoresed on a 1.5% agarose gel, transferred to nylon (6), and hybridized to HBV DNA that was 32P-labeled by nick-translation. The number of virus particles in these

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B “e” antigen; HBcAg, hepatitis B core antigen.
samples was estimated by a densitometric comparison with 20 pg of EcoRI- and 100 pg of EcoRI/BamHI-digested, cloned, full-length HBV, both of which were Southern-blotted along with the culture medium.

Plasma (1.5 ml) was combined from three samples taken at weekly intervals either from 6 to 8 weeks (chimpanzee 1303) or from 10 to 12 weeks (chimpanzee 1178) after inoculation. Virus particles were precipitated by the addition of equal volumes of 20% PEG in 10 mM Tris-HCl, pH 7.5/1 mM EDTA/0.15 M NaCl (TEN buffer) and incubation at 4°C for 16 hr. The virus was pelleted at 8000 × g for 10 min, resuspended in TEN buffer containing 10% PEG, and centrifuged again. The pellet was resuspended in 2 ml of 10 mM Tris-HCl, pH 7.5/0.1 M KCl/0.01% 2-mercaptoethanol. The DNA was 32P-labeled by an endogenous polymerase reaction in the presence of 0.5% Nonidet P-40/0.4 M KCl/50 mM Tris-HCl, pH 7.5/20 mM dithiothreitol/40 mM MgCl2/0.5 mM dATP, dGTP, and TTP/0.3 μM dCTP/300 μCi of [α-32P]dCTP (300 Ci/mM; 1 Ci = 37 GBq) as described (7). The 5 ml of final solution was incubated for 5.5 hr at 37°C. An equal volume of 25% PEG was then added, and the sample was incubated for 16 hr at 4°C. The precipitate was pelleted, resuspended, and centrifuged, and the DNA was isolated as described above. The DNA was dissolved in 50 μl of H2O, and 8 μl was digested with Sp1, Bgl II, and Hpa II. The restriction endonucleases were purchased from New England Biolabs and were used according to the recommendations of the suppliers. The digests were electrophoresed on 10% polyacrylamide gels, which were then dried and autoradiographed for 6 hr at −70°C with intensifying screens.

**RESULTS**

**Viral DNA in the Growth Medium.** Chimpanzee 1303 (Fig. 1, lane A) and chimpanzee 1178 (Fig. 1, lane B) received approximately 9.0 × 10⁶ and 3.6 × 10⁸ virus particles, respectively, as indicated by Southern blot analysis of the DNA isolated from the growth-medium samples. Although a greater number of particles were present in the growth medium utilized to infect chimpanzee 1303, this medium had been exposed to the transfected Hep G2 cells for a shorter period of time (from day 20 to day 25 after subculture) than that which was injected into chimpanzee 1178 (from day 0 to 12 after subculture), indicating that the virus production was more efficient 20 days after subculture of the transfected Hep G2 cells than at earlier times.

![Fig. 1. Southern-blot analysis of extracellular DNA from cultures of 2.2.15 cells. The autoradiograph shows the DNA isolated from 1.5 ml of medium exposed to cells either from days 20–25 (lane A) or from days 0–12 (lane B) after subculture. The markers (lane C) consist of a mixture of 20 pg of EcoRI- and 100 pg of EcoRI/BamHI-digested pHBV-1. Molecular mass is shown in base pairs.](image)

**Production of Viral-Specific Markers.** The serological profile of chimpanzees 1178 and 1303 at inoculation and for 14 weeks thereafter is shown in Fig. 2. At inoculation all markers were absent. HBsAg was the first marker to appear at 1 and 2 weeks after inoculation in chimpanzees 1303 and 1178, respectively. In both animals, the levels of HBsAg peaked at around week 8, and in chimpanzee 1178, the levels dramatically decreased 1 week later coincident with the detection of anti-HBsAg. In chimpanzee 1303, the HBsAg levels remained high for 14 weeks. In both animals, HBeAg was detected by week 3 and peaked at week 5. The levels decreased to background by weeks 9 and 12 in chimpanzees 1303 and 1178, respectively. Anti-HBcAg was observed at week 7 in both animals. Alanine aminotransferase was increased in chimpanzees 1303 and 1178 at weeks 5 and 7, respectively, and continued to be high throughout the course of the experiment (Fig. 2). The activities of isocitrate dehydrogenase and γ-glutamyl transpeptidase paralleled those of alanine aminotransferase (data not shown).

**Histological Findings.** Both chimpanzees initially showed essentially normal livers (Fig. 3A). Chimpanzee 1303 first had insignificant abnormalities on day 21 after inoculation—namely,
conspicuous activation of sinusoidal lining cells in the perivenous zone. On day 42 the first hepatic lesion appeared, reflected in acidophilic bodies in the tissue spaces, in some multiple nuclei, in irregular clumping of the cytoplasm, and in a slight inflammatory reaction in the portal tract with increased numbers of bile ductules. These lesions were markedly accentuated on day 49, when cytoplasmic alterations were far more severe, acidophilic bodies more numerous, and focal necroses appeared (Fig. 3B). The inflammatory reaction in small and now also in large portal tracts was conspicuous and associated with focal necroses in the periportal parenchyma and irregularities of bile duct epithelium. On day 56 the parenchymal lesion had significantly regressed, barely any acidophilic bodies were seen, and the small portal tracts showed considerably less inflammation (Fig. 3C). However, on day 77 it was again more conspicuous and then gradually decreased, exhibiting a low degree of inflammation in the parenchyma and activity in portal tracts on day 98.

In chimpanzee 1178, on day 56, a mild hepatitis was present, mainly reflected in portal and periportal inflammation and some borderline alterations of the cytoplasm of the hepatocytes. It became more conspicuous on day 77 and florid with many lymphocytes on day 84. On day 91 (Fig. 3D) it was most severe, as reflected in variation of the cytoplasm and nuclei of lymphocytes and activation of sinusoidal lining cells. The portal and periportal inflammation was impressive, and on day 98 it was somewhat decreased.

**Viral Subtypes and Restriction Analysis of the DNA in Plasma from Chimpanzees.** The virions from which the DNA was cloned, those recovered from the transfected Hep G2 cells and used for inoculation, and the virions recovered from the chimpanzees were all subtype ayw. Restriction analysis of the viral DNA from all of the above sources yielded similar HBV DNA fragments (Fig. 4). The fragments obtained are those predicted if the majority of the 32P-labeling commences around the unique EcoRI site and proceeds through to the 3' of the short strand. Digestion with Bgl II generated two fragments [2.0 and 2.3 kilobases (kb)], the most pronounced of which was 2.3 kb (Fig. 4, lane b). The species of DNA obtained by Scp I digestion (Fig. 4, lane d) were of an apparent size of 3.2 kb and N4.5 kb, corresponding to the positions of linear and relaxed circular HBV DNA, and were identical to those in the untreated sample (Fig. 4, lane a), indicating the absence of any Scp I restriction recognition sites. Fragments of 2.0 kb were most prominent after digestion with Hpa II. A minor fraction of the DNA was approximately 2.4 kb (Fig. 4, lane c).

**DISCUSSION**

The chimpanzees inoculated with tissue culture medium containing HBV developed a hepatitis both in the acinar parenchyma and in the portal tracts. It appeared first in chimpanzee 1303 and was distinct, although of varying intensity for 8 weeks: as usual, portal alterations outlasted parenchymal ones. The hepatitis in chimpanzee 1178 set in later but was more severe, particularly portal and periportal alterations. After 7 weeks, it had not yet declined. The hepatitis observed in both chimpanzees is compatible with,
but not fully diagnostic for, hepatitis B although the amount of lymphocytes in the parenchyma speaks for this etiology. That the infectious virions responsible for causing hepatitis in the two chimpanzees are produced in vitro is substantiated both by the subtype of the viral envelope protein and by the identical restriction enzyme pattern of the viral DNA isolated from the serum of chimpanzees and the cloned DNA utilized for transfection. The restriction patterns obtained after digestion of the viral DNA with SpI and Hpa II as well as of our cloned HBV DNA differs from that cloned from other virions of the ayw subtype (8). The latter contain an SpI site that results in the linearization of the DNA to fragments of approximately 3.2 kb and an additional Hpa II site that results in the cleavage of the 2.0-kb DNA into 1.4-kb and 0.6-kb fragments. The time course of appearance/disappearance and relative levels of the markers of HBV infections in both animals are similar to those reported by others both in spontaneous and experimentally induced infections (9, 10).

The inoculum utilized to infect chimpanzee 1303 (which developed markers of hepatitis first) was taken from cell cultures 25 days after subculturing and contained more particles than that which was removed from cells in culture for 12 days and injected into chimpanzee 1178. These observations parallel those of Sureau et al. (2), who detected increased levels of episomal HBV DNA replicative intermediates during the stationary phase of cell growth (16–33 days after subculture).

The incubation period to the first appearance of nascent HBsAg and to the first increase of alanine aminotransferase in the two chimpanzees was short. In previous studies we have shown that there is an inverse relationship between the infectivity titer of the inoculum and the incubation period but no relationship between the inoculum and the severity of the hepatitis (11). In that study, $10^{7.5}$–$10^{8.0}$ chimpanzee infectious doses of three strains of HBV produced incubation periods to first antigenemia of 2–3 weeks and to first increase in alanine aminotransferase of 4.5–10.5 weeks. Berninger et al. (12) demonstrated that the number of HBV genomes present in these and other inocula, as measured by DNA hybridization, approximately equaled the number of chimpanzee infectious doses present. Based upon those studies, there is good agreement between the estimated number of HBV genomes present in the cell culture-derived inocula used in the present study ($10^{8.3}$–$10^{9.0}$) and the incubation periods to first appearance of nascent HBsAg (1–2 weeks) and first increase in alanine aminotransferase (5–7 weeks) in the chimpanzees, suggesting that virtually all of the HBV genomes present in the cell culture-derived inocula were capsidated in infectious virions.

Will and coworkers (13, 14) found one of two cloned HBV DNAs to be infectious in chimpanzees if exposed directly to hepatocytes, either by in vitro transfection or by intraperitoneal inoculation irrespective of whether it was either covalently closed circular DNA or part of a plasmid. However, when the infectious DNA was administered intravenously it was noninfectious even in high doses (13, 14). Thus, we have provided convincing evidence that the response of chimpanzees to intravenous inoculation with cell culture-derived hepatitis B virions was a true HBV infection and not a transfection or immune response to noninfectious viral antigens.

As with virus isolated from infected patients, the virus produced by the transfected Hep G2 cells has thus far not been capable of directly infecting Hep G2 cells. Consequently, it is assumed that the Hep G2 cells may not have receptor(s) for viral attachment and penetration and that episomal HBV DNA and virus production is maintained for longer than 9 months by these cells (which divide approximately every 30 hr) by a constant replenishment of circular episomal DNA from the chromosomally integrated HBV DNA sequences.

The presented observations complete the attempt to construct the long-sought-after in vitro system that can produce infectious HBV. By producing the characteristic disease and thereby fulfilling the Koch postulate, the system is ready for the exploitation that has been successfully carried out in other infections when such a strategy became available.

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4. Committee on Care and Use of Laboratory Animals, National Research Council (1978) Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington, DC), pp. 7–9, 33–34.