An in vitro system for infection with hepatitis B virus that uses primary human fetal hepatocytes

(abstract)

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ABSTRACT  An in vitro culture of human fetal hepatocytes has been employed for infection by hepatitis B virus (HBV) virions that are produced by an established human hepatoma cell line, HB 611. HBV surface antigen and e antigen were released into the medium 3-4 days after infection, and production continued thereafter. RNA synthesis with similar kinetics was observed. Viral DNA replication started 2 days after infection, and replicative HBV DNA that included relaxed circles, single-stranded minus strands, and closed circles accumulated during 16 days of incubation. Immunofluorescent study using fluorescein isothiocyanate-labeled rabbit antisera directed against HBV core antigen revealed that this antigen is present in the nuclei in 12% of the infected cells. Particles containing HBV DNA were detected in the culture medium and were infectious. Thus, this in vitro infection system closely mimics infection in vivo and it allows detailed studies on early events associated with human HBV entry into cells and subsequent replication and integration.

Hepatitis B virus (HBV) is a world-wide pathogen that causes hepatitis. Infection with the virus may be chronic and is associated with subsequent development of hepatocellular carcinoma. Although chimpanzees can be infected in vivo with HBV (1) and woodchucks, ground squirrels, and Peking ducks can be infected with their respective HBV-like viruses (2-4), use of whole animals poses certain limitations for detailed molecular studies. The most vexing technical problem in the biology of HBV has been lack of a practical in vitro system that allows infection and replication of this virus under controlled experimental conditions. Several investigators have attempted to infect human hepatic cells by using human adult and fetal hepatocytes (5, 6), fetal hepatocyte organ culture (7), oval cells originating from human liver (8), and HeLa cells (6). However, no signs of viral penetration, replication, or particle production have been observed except for a transient expression of some viral markers.

We have established a cell line (HB 611) derived from a human hepatocellular carcinoma cell line (Huh6-cl5) by transfecting with a plasmid containing tandemly arranged HBV DNA. The HB 611 cells carry chromosomally integrated HBV genomes, and they allow expression of viral RNA, DNA, and proteins and produce Dane-particle-like virions (9). These particles have all the properties characteristic of the Dane particles from the patient's blood (10). However, it has not yet been proved that the virions are actually infectious.

Although the chromosomally integrated HBV genomes in the HB 611 cells allow viral replication, the replicative intermediates differ from those observed with the infected liver in that the closed circular form of HBV DNA was not detectable in HB 611 cells. Transfection of Hep G2 and Hu 7 derivatives, both from human hepatomas, showed the same effect (11, 12). In addition, none of these systems reproduce the in vivo process of viral infection, including virus penetration and the early events that ensue. Here we describe a system for experimental infection by the HBV virions that uses primary human fetal hepatocytes. Infection was carried out by cocultivation of human fetal hepatocytes with HB 611 cells continuously producing the infectious viruses. The fetal hepatocytes infected in vitro were found to initiate viral DNA replication and viral RNA and protein production, and they produced infectious viral particles.

MATERIALS AND METHODS

Virus Sources and Cell Culture. The cell line HB 611, which produces Dane-particle-like double-shelled particles from integrated HBV DNA, has been described (9). A monolayer cell culture of HB 611 in 10% fetal calf serum/Dulbecco's modified Eagle's medium (10% FCS/DMEM) kept at 37°C was used as the source of infection.

Hepatocytes were isolated from human fetal liver by a modification of the methods of Leffert and Paul (13). The liver tissue was placed immediately in cold 10% FCS/DMEM supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml), and then washed several times with the same medium. Other extraneous matter was trimmed away and the tissue was cut with scissors into 0.5- to 1.0-mm3 pieces. They were transferred to a new tube and gently stirred for 15 min at 37°C in 10% FCS/DMEM containing collagenase (Sigma type I) at 0.5 mg/ml and Dispase (Godoshusi) at 2000 units/ml. The larger fragments were allowed to settle and the supernatant was saved. These dissociation steps were repeated two or three times with fresh fluid until no large clumps were seen. The cell suspensions were pooled and passed through a Nitex Swiss nylon filter of 100-µm pore diameter. The filtrate was centrifuged at 40 x g for 60 sec, and the pelleted cells were washed with DMEM three times to remove collagenase, damaged cells, and nonparenchymal cells. The final pellet was resuspended in 10% FCS/DMEM supplemented with penicillin (100 units/ml). Such cells were used to seed six-well cluster dishes (Costar; diameter 60 mm) at 2 x 104 cells per well and incubated with 3 ml of 10% FCS/DMEM per well at 37°C under 5% CO2 in air. The medium was changed after the first 24 hr and then once every 3 days.

Infection of Cultured Hepatocytes with HBV. Under the conditions of plating, the culture of the human fetal hepatocytes became confluent after 7-10 days. After the cells reached confluence they were washed three times with

Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; HBCAg, HBV core antigen; HBeAg, HBV e antigen; CCC, covalently closed circular.

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DMEM and cocultivated without direct contact for 72 hr with monolayered HB 611 cells (1 × 10⁶ cells) that have been attached to Transwell dishes (Costar) and grown in 3 ml of 10% FCS/DMEM. The HB 611 cells were removed, and the fetal hepatocytes were carefully washed three times with DMEM and incubated with 3 ml of 10% FCS/DMEM. The start of this incubation was taken as time zero.

**Biochemical Characterization of the Hepatocytes.** Histochernical staining for enzymes, such as glycogen (14), glucose-6-phosphatase (15), and ϒ-glutamyl transpeptidase (16), were performed with unfixed cells. Albumin secretion into the medium was measured by using enzyme-linked immunosorbent assay (ELISA) (17).

**Detection of HBV Surface Antigen (HBsAg) and HBV e Antigen (HBeAg).** HBsAg and HBeAg were assayed in culture medium by using commercial enzyme immunoassay (EIA) kits (Abbott) (9). Titers were expressed as the ratios against cut-off values (A₄₅₀ of negative control + 0.06 for HBsAg and + 0.05 for HBeAg).

**Detection of HBV Core Antigen (HBCag) by Immunofluorescent Staining.** The cells were fixed in acetone at 4°C, treated with a proper dilution of polyclonal human antibody against HBCag (6), and stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human globulin.

**DNA and RNA Preparations from Hepatocytes.** Extrachromosomal DNA was prepared as described by Hirt (18). The cells were lysed in 0.5 M NaCl/10 mM Tris-HCl, pH 7.4/1 mM EDTA/1% sodium dodecyl sulfate and stored overnight at 4°C. Cellular DNA was pelleted at 16,000 × g for 60 min, and the extrachromosomal DNA recovered in the supernatant was incubated at 37°C for 1 hr with Pronase E (Wako Pure Chemical, Osaka, Japan, 1 mg/ml), treated with phenol/chloroform, and precipitated with ethanol. The DNA was dissolved in 10 mM Tris-HCl, pH 7.4/1 mM EDTA. Total RNA was extracted by homogenizing cells in guanidine thiocyanate and sedimenting them through a layer of 5.7 M CsCl as described elsewhere (9).

**Purification of Virus Particles.** Viral particles produced and released from the HBV-infected hepatocytes into culture medium at 4–12 days of infection were purified by CsCl density gradient centrifugation (9).

**Hybridization Studies.** DNAs were subjected to electrophoresis in 1.5% agarose, transferred to a nitrocellulose filter, and analyzed as described by Southern (19). RNAs were subjected to electrophoresis in a 1.0% agarose gel containing 6.6% formaldehyde, blotted onto nitrocellulose filters, and analyzed as described by Maniatis et al. (20). Hybridization was done under stringent conditions with random-primed ³²P-labeled whole HBV DNA probe prepared from pBRHB-adr4 (21) (specific activity, >2 × 10⁶ cpm/μg).

**RESULTS**

**Properties of the Hepatocytes Isolated from Human Fetal Liver.** Human hepatocytes were prepared from fetal liver at 20–24 weeks of gestation, and 2 × 10⁶ cells were plated on collagen-coated plastic plate (60-mm diameter) to make monolayers. Two hours after plating, the hepatocytes started to attach to the dish and began to flatten. Twenty-four hours after the plating, subconfluent monolayers were obtained. Most of the hepatocytes thus obtained were mononuclear, polyhedral, and arranged in trabeculae. At 7 days of culture greater than 95% of these plated cells were histologically positive for glycogen, glucose-6-phosphatase, and ϒ-glutamyl transpeptidase. These regular morphologies and biochemical characteristics were well retained for at least 2 weeks of subsequent culture, and an outgrowth of fibroblastic or oval cells was not detected. Albumin secretion in the medium of the cultivated hepatocytes was detected 2 days after the plating and continued for 16 days (Fig. 1). The secretion rate of albumin was maximal at 10–12 days after plating and leveled off thereafter.

**Production of Viral Antigens in Cells Infected with HBV.** Coculture infection was designed to provide independent access to two different cell layers: HB 611 cells, which produce HBV virions, were seeded in the upper layer, and the fetal hepatocytes were in the bottom layer. Thus, fresh virus particles were supplied as they were made. In the first experiment, primary fetal hepatocytes at 24 weeks of gestation (2 × 10⁶ cells in 3 ml of 10% FCS/DMEM) were cultured for 7 days on the plate and then cocultured for 3 days with monolayered HB 611 cells (1 × 10⁶ cells per well) that were producing approximately 1 × 10⁷ virions during the coculture. After the infection, the HB 611 cells were removed and the hepatocytes were washed three times with DMEM and further incubated in 3 ml of 10% FCS/DMEM (time zero). The culture medium was collected periodically during this incubation and the levels of HBsAg and HBeAg were assayed. As shown in Fig. 2, the HBV-specific proteins were first detected 4 days after the infection and continued to increase during the 2 weeks of incubation. They must have been synthesized de novo, because they appeared only after 4 days of incubation. Thus, the human fetal hepatocytes were likely to be infected by virions and were propagating HBV. We chose the time of HBV challenge (7 days after plating) because the cells were not susceptible to viruses either earlier (culture days 0–4) or later (culture days 12–16) (data not shown).

**Fig. 1.** Secretion of albumin by cultured human fetal hepatocytes. The amount of albumin secreted (μg·day⁻¹ per 10⁶ cells, mean ± SD) into the medium was assayed by ELISA.

**Fig. 2.** Production of HBsAg and HBeAg in cells infected with HBV. Human fetal hepatocytes were cultured for 7 days to make confluent monolayers. They were then cocultivated for 3 days with HB 611 cells for infection, washed, and incubated again (time zero) in the absence of HB 611 without change of medium. Aliquots of culture medium were taken at intervals and assayed for HBsAg and HBeAg.
Detection of Virus-Specific Transcripts. Total RNA was prepared from the cultured hepatocytes at day 12 after infection, when HBsAg and HBeAg were detected in the culture fluid. It was then subjected to Northern blot analyses using whole HBV DNA as a probe. Three major transcripts, approximately 3.6, 2.4, and 2.1 kilobases (kb) were detected (Fig. 3), in complete agreement with those detected in the liver of infected HBV (22) and in the cultured HBV-producing cells (9). The 3.6-kb mRNA represents the whole genome transcript, and is believed to serve as a pregenome for viral DNA replication (23). Other two mRNA species (2.4 and 2.1 kb) apparently correspond to the transcripts that are translated into large and small HBsAg (24). None of the bands were detected after RNase digestion or in noninfected cells. Thus, this system is actively producing HBV-encoded RNAs.

Replicative HBV DNA in Extrachromosomal Form. To see if HBV DNA was replicating in the human fetal hepatocytes, cells were harvested at various times after infection. Extrachromosomal DNA was then extracted according to Hirt and analyzed by Southern blotting using \( ^{32}P \)-labeled whole HBV DNA as a probe. If HBV DNA was replicating in our system, the replicative HBV DNAs would have to be recovered in this fraction (9). As shown in Fig. 4, virus-specific DNA replicative intermediates appeared 2 days after infection. These DNA molecules cannot be carryovers of the infecting virions, because no DNA was detectable in cells or on cell surfaces at 0 or 1 day after the infection. In the 6-day sample, three replicative DNA components (termed D, CC, and S) could be identified. The S component had the size of single-stranded viral (minus strand) DNA. It did not change upon heating, hybridized only to the plus strand probe, and was not cleaved by BamHI. Thus this component must be the single-stranded DNA, as expected. The D component had the mobility expected for the partially single-stranded, relaxed-circular viral DNA (9), and the CC component had the mobility expected for the covalently closed circular (CCC) form of HBV DNA. It shifted to the 3.2-kb position after digestion with BamHI (25), and it was maintained upon heating at 95°C for 5 min (Fig. 4, lane B). Thus, we concluded that the CC component must be the HBV DNA in CCC form.

With increasing time after infection, these replicative DNA molecules accumulated. Their accumulation, especially accumulation of the CCC form, has been reported in the active infected human liver (26), whereas the CCC component has never been detected in cultured human hepatoma cell lines that were transfected by HBV DNA and allowed its replication. Therefore, our experimental infection system not only allows infection by the HBV virion but also reproduces the full cycle of viral replication.

Viral DNA in the Culture Medium. Our preliminary surveys detected the HBV DNA in the culture medium. Because they were not detected at time zero, they must have been released from the infected hepatocytes. To characterize these DNA molecules, the culture media were collected from day 0 to day 3 and from day 4 to day 12, and particles were collected by using a CsCl density-gradient centrifugation. DNA was extracted from the purified particles and subjected to Southern blot analyses (Fig. 5). Particles prepared from the culture medium 4–12 days after infection were found to contain mostly the D form of HBV DNA (Fig. 5, lane b), whereas there was no HBV DNA in the culture medium at 0–3 days after infection (Fig. 5, lane a). Thus, this system is likely to release mature virus particles carrying DNA. Comparison with a standard amount of cloned viral DNA showed that in...
Table 1. Viral antigens produced by human fetal hepatocytes infected by extracellularly released virions from the first infection

<table>
<thead>
<tr>
<th>Time after infection, days</th>
<th>Titer</th>
<th>Viral antigen</th>
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<tbody>
<tr>
<td></td>
<td>HBsAg</td>
<td>HBeAg</td>
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<tr>
<td>0</td>
<td>0.08</td>
<td>0.04</td>
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<tr>
<td>4</td>
<td>40</td>
<td>15</td>
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<tr>
<td>8</td>
<td>70</td>
<td>23</td>
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Virus particles harvested and pooled from culture medium 4–16 days after the first infection were used for a second-infection experiment (see text).

the 6-day culture about 5 × 10⁶ particles per ml were contained in the medium.

Reinfection by the Extracellular Virus Particles. To learn whether the virus particles released from the infected cells were infectious, the culture media at 4–16 days after the infection were pooled and about 1–2 × 10⁷ particles in 1.0 ml of medium were added to another primary human fetal hepatocyte culture at the 7th day of plating (hepatocytes received 10–20 virions per cell). After 3 hr of incubation at 37°C the inoculum was removed and the cells were washed and cultured with 10% FCS/DMEM. The culture medium was harvested at 0, 4, and 8 days after the infection and tested for HBsAg and HBeAg. The results (Table 1) show that virus-specific antigens started to appear at 4 days after the second infection and increased thereafter. The mode of production of virus-specific antigens and relative infectivity were almost superimposable to those observed in the coculturing experiments using HB 611 cells. Because no viral antigens were detected in culture medium at time zero, these virus-specific antigens cannot be carryovers of the inocula. These results demonstrate that infectious viruses were in fact produced and released into the medium of the infected hepatocytes and that the infection can be achieved by virions in culture medium, without coculturing with HB 611 cells.

To characterize the infectious agent in the culture medium, we determined whether antibodies specific for viral proteins would block infectivity. The culture medium of infected cells was incubated with rabbit antibodies directed against HBsAg (anti-HBsAg) and against HBeAg (anti-HBeAg) and then the media were assayed for infectivity. The anti-HBsAg antibodies reduced the infectivity, whereas anti-HBeAg antibodies or normal rabbit serum did not (data not shown). Although we have not examined the infectious agents by electron microscopy, these results suggest that they are most likely to be Dane-particle-like bodies coated with surface antigen.

Detection of HBeAg in the Infected Hepatocytes by Immunofluorescent Staining. To estimate the population of infected cells, immunofluorescence assay was performed with polyclonal human antibody against HBeAg, taking advantage of the fact that the viral core proteins accumulate exclusively within the infected cells. Core antigens were detected in 10–12% of the cells 6 days after infection (Fig. 6), whereas no core antigens were detected in the uninfected cells (data not shown). Only nuclei were stained in the infected cells. The percentage of positive cells did not increase 12 days after infection (data not shown), even though the production of viral antigens and viral replication in the culture reached the maximum at this stage (Figs. 2 and 4), suggesting that spreading of virus to neighboring cells does not take place in this system.

DISCUSSION

Experimental transmission of HBV can be achieved in chimpanzees. However, many attempts to establish an in vitro system that allows infection by the virion have not been successful. Recently, replication of HBV in human hepatoma cell lines by using integrated or transfected HBV genomes as template has been achieved (9, 11, 12). In all these cases, the HBV DNA did express and replicate, and it produced some virion-like particles. However, such systems do not allow infection by virions, and furthermore, no extrachromosomal HBV DNAs in CCC form have been detected, in contrast to frequent observation of such molecules in infected livers in vivo. Our in vitro system using primary human fetal hepatocytes cultured for few days can mimic the in vivo process of infection with HBV in that (i) it is infected by virions, (ii) it produces all known viral materials that are observed in infected livers, (iii) it accumulates HBV DNA in CCC form, and (iv) it liberates infectious virions. Human livers obtained from 20-, 23-, and 24-week-old embryos were used in all the experiments, and the cells allowed reproducible infection by HBV virions and viral DNA replication. Although we did not show individual results, each sample demonstrated almost identical kinetics of infection and virus replication. The most important implication of this work is that we can now detect infectious virions without using live chimpanzees. Whether this system can be extended to other infectious agents, such as non-A non-B hepatitis virus, waits further studies. In addition, this study has confirmed that the HB 611 cells release infectious agents (9), which may be multiplied indefinitely.

Immunofluorescence study demonstrated that the infection took place in only about 12% of total hepatocytes. Recently, using primary duck hepatocytes, Tuttlemann et al. (27) have observed infection by duck hepatitis B virus,

Fig. 6. Immunofluorescent staining of infected cells by anti-HBeAg antibody. (A) Human hepatocytes 6 days after infection were stained with fluorescein-conjugated polyclonal human antibody against HBeAg. (B) Phase-contrast micrograph of the same field in A. (×220.)
initiating syntheses of viral DNA, virus-specific proteins, and virus particles. In their case again, only 10% of the primary duck hepatocytes displayed HBCAg. We do not know the reason for this limited percentage of infection, but it could mean that only \( \approx 10-20\% \) of the cells at this stage of growth were compatible for viral adsorption and internalization or for expression of viral genes. In fact, cells were no longer susceptible to HBV when challenged with virus particles at a late stage of incubation (day 10 through day 16). Therefore, at the time when infectious virions are released into the medium, cells are no longer susceptible to HBV and thus, spreading of virus to adjacent cells does not take place.

Last, the apparent discrepancy between this work and the previous failures in attempting infection of cultured human hepatocytes must be commented upon. As is well known, human hepatocytes are hard to maintain in cultures, particularly when one tries to maintain their typical functions, such as albumin production. Such functions are easily lost within 1 week in culture when the cells are inoculated in highly diluted condition in poor medium. Our primary human fetal hepatocytes were inoculated at high density so they formed a confluent monolayer within one cell doubling. Such cells can maintain relatively stable hepatocyte-specific phenotype during the subsequent incubation. For example, albumin synthesis was observed to start soon after the plating and continued for 10 days. For establishing the HBV infection some liver-specific functions may be needed. These hepatocytes are likely to have kept such functions active. Access to fresh HBV virions produced from HB 611 cells may also have helped the infection process.

Our in vitro experimental infection system will also be useful for detailed studies of HBV infection and replication, which have not been elucidated in molecular terms. For example, the process of viral attachment and entry into cells, the subsequent early reactions that occur in replication, and integration of virus DNA into host chromosomes may be the targets for next studies. In this connection, it may be of some interest to point out that HBV DNA in CCC form has not been found in other cultured cell systems, so far as we know.

After this paper was written a paper by Gripon et al. (28) appeared that reported infection and replication of HBV in some preparations of cultured adult human hepatocytes.

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