Tissue Culture System for Infection with Human Hepatitis Delta Virus

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An *in vitro* culture system was developed for assaying the infectivity of the human hepatitis delta virus (HDV). Hepatocytes were isolated from chimpanzee liver and grown in a serum-free medium. Cells were shown to be infectible by HDV and to remain susceptible to infection for at least 3 weeks in culture, as evidenced by the appearance of RNA species characteristic of HDV replication as early as 6 days postinfection. When repeated experiments were carried out on cells derived from an animal free of hepatitis B virus (HBV), HDV infection occurred in a consistent fashion but there was no indication of infection with the HBV that was present in the inoculum. Despite numerous attempts with different sources of HBV inocula free of HDV, there was no evidence that indicated susceptibility of these cells to HBV infection. This observation may indicate that HBV and HDV use different modes of entry into hepatocytes. When cells derived from an HBV-infected animal were exposed to HDV, synthesis and release of progeny HDV particles were obtained in addition to HBV replication and production of Dane particles. Although not infectible with HBV, primary cultures of chimpanzee hepatocytes are capable of supporting part of the life cycle of HBV and the entire life cycle of HDV.

Hepatitis delta virus (HDV) represents a novel class of pathogenic agents in humans (28, 31). HDV is a defective virus that cannot replicate autonomously; it requires the helper functions of hepatitis B virus (HBV). The helper hepadnavirus is acquired either simultaneously with HDV (coinfection) or before HDV infection (superinfection). Although the interaction between HBV and HDV is not fully understood, one of the helper functions provided by HBV is the outer lipoprotein coat bearing the HBV surface antigen (HBsAg) that surrounds the HDV particle (2, 3). Experimental transmission of HDV has been accomplished in chimpanzees coinfecting with HBV and in woodchucks coinfecting with the woodchuck hepatitis virus (WHV), a member of the hepadnavirus family (24, 25, 29). In the case of superinfection, major suppression of HBV replication occurs at the peak of HDV replication (6, 13, 15, 16), but the mechanism of this interaction is as yet undetermined. HDV replicates its RNA genome by a strategy employed by plant viroids (4, 5, 7, 10, 27, 35, 40), but unlike the viroids, the HDV genome codes for at least one polypeptide that bears the hepatitis delta antigen (HDAg) (18, 38, 39). This protein interacts with HDV RNA and HBsAg in the assembly of viral particles (2, 3, 30) and is necessary for the replication of the viral RNA to proceed (17).

Recent studies have demonstrated the requirement for coinfecting HBV for transmission of HDV, although HBV is entirely dispensable for the replication of the HDV genome. Therefore, the role of HBV may be limited to supplying the viral envelope, allowing the HDV RNA to be packaged and released as a viral particle, thereby providing a mode of transmission. The composition of the HDV envelope includes proteins encoded by the surface antigen genes of HBV (large, middle, and major surface proteins), but the relative amount of each protein differs from that of HBV particles (2, 3). As in the HBV virions, the large and middle proteins may have a crucial role in recognizing the virus

receptor on the cell membrane and assembly into particles. However, the following circumstantial evidence indicates that HBV and HDV may utilize different mechanisms of entry into cells: (i) contrary to hepadnaviruses, which demonstrate a restricted host range, HDV of human origin can be transmitted to woodchucks, and the woodchuck progeny virions surrounded by an envelope of WHV origin can reinfect chimpanzees (24, 25); (ii) studies performed on WHV and HDV have shown a different extrahepatic tropism between the two viruses (22); and (iii) the compositions of HBV and HDV envelopes, although similar, differ in the proportion of the different envelope proteins of HBV origin. Mature HBV (Dane) particles contain an equimolar ratio of large, middle, and major surface proteins, while the HDV envelope contains only 1% or less of the large protein (2, 3). In addition, the envelope of HBV interacts with the viral nucleocapsid that bears the HBV core antigen (HBcAg), whereas the envelope of HDV interacts with HDAg and/or HDV RNA, which are not preassembled into a detectable nucleocapsid structure (2, 3, 30). This interaction may expose different domains of the surface proteins of HDV particles and/or allow the uncoating of HDV to proceed in a different manner, thereby influencing the penetration of particles into the cell.

To study the infection step of HDV, we have undertaken experiments aimed at developing a reliable culture system for *in vitro* infection. We have used primary cultures of chimpanzee hepatocytes under conditions defined by previous studies (14, 20). These experiments had established the conditions for long-term maintenance of primary liver cells that retain the properties of highly differentiated hepatocytes, including the ability to synthesize most of the liver-specific plasma proteins and to support the production of HBV particles (14). Here we demonstrate that this culture system is quite adaptable for testing the infectivity of HDV particles *in vitro*. High levels of replicating HDV RNA were observed following exposure to HDV particles *in vitro*, and susceptibility to infection was shown to persist for several weeks in culture. Furthermore, by infecting cells derived

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from an HBV-infected animal, sustained production of both HBV and HDV particles was demonstrated. However, infection *in vitro* with HBV remained unsuccessful. This may be a further indication that HBV and HDV indeed utilize a different mechanism of entry into hepatocytes.

MATERIALS AND METHODS

Primary cultures of chimpanzee hepatocytes. Hepatocytes were isolated from noninfected and HBV-infected chimpanzees. These animals were shown previously to be free of any HDV markers, including HDV RNA and HDVAg antibodies in the serum, and to have no history of HDV infection. Viral particles used for *in vitro* infections were obtained from the serum of an HBV-infected chimpanzee (X136) that had been transfected with a cDNA clone of HDV and that developed a typical HDV infection (33). Inocula therefore contained both HDV and HBV particles. The procedures used for the isolation and culture of primary hepatocytes in a serum-free medium formulation have been described previously (14, 20).

***In vitro* infections.** Routinely, cells were exposed to HDV for at least 12 h 3 days after plating. Unless otherwise specified, 20 μ l of X136 serum collected at the peak of infection (33) was added to a 22-mm-diameter well containing 10^6 cells in 1 ml of serum-free medium. By comparing HDV RNA extracted from the inoculum with a known amount of HDV cDNA, the concentration of HDV genomes was estimated to be 10^9 /ml. Following exposure, cells were washed and incubated in 1 ml of fresh serum-free medium.

Detection of HDV RNA and HBV DNA. Total cellular RNA was prepared by disrupting the cells in 6 M guanidinium isothiocyanate–5 mM sodium citrate (pH 7.0)–0.1 mM β -mercaptoethanol–0.5% sarcosyl and by centrifugation through a CsCl cushion as described before (21). Purification of HDV RNA from culture medium was performed by incubation in 50 mM Tris-HCl (pH 7.8)–200 mM NaCl–20 mM Na₂EDTA–2% sodium dodecyl sulfate (SDS)–1 mg of proteinase K per ml–2 mM vanadyl ribonucleoside complex for 2 h and phenol extraction as described before (34). For analysis of mRNAs, total cellular RNA was further purified by separation with oligo(dT)–cellulose chromatography as described before (21). Poly(A)–containing and poly(A)–negative fractions were subjected to electrophoresis through a 1.5% agarose–2.2 M formaldehyde gel and then transferred to nitrocellulose for hybridization to ³²P-labeled HDV DNA- and HBV DNA-specific probes.

Total cellular DNA was isolated by digestion with 500 μ g of proteinase K per ml in 10 mM Tris-HCl (pH 7.4)–10 mM NaCl–10 mM Na₂EDTA–0.5% SDS for 2 h at 37°C, followed by extraction with phenol. HBV DNA was isolated from culture medium by the same procedure. DNA samples (5 μ g) were subjected to electrophoresis in a 1.5% agarose gel and transferred to nitrocellulose membranes for hybridization to an HBV DNA probe. Gel-purified full-length HBV DNA and HDV cDNA, derived from recombinant plasmids pCP10 (17) and pSVLD3 (34), respectively, were labeled with [α -³²P] dCTP as described before (34).

Detection and characterization of HDVAg polypeptides by immunoblotting. Cells derived from a 22-mm-diameter tissue culture well were disrupted in 200 μ l of sample buffer containing 2% SDS and 2% β -mercaptoethanol, sonicated and heated at 100°C for 5 min, and then stored at –70°C prior to analysis by the immunoblot procedure as described previously (19). Particles from culture medium were sedimented through a 20% sucrose cushion at $190,000 \times g$ for 5 h at 4°C.

Pellets were resuspended in sample buffer, heated at 100°C for 5 min, and stored frozen at –70°C prior to immunoblotting. A 1:1,000 dilution of a human anti-HDVAg antibody-positive serum was used for immunoblots, followed by incubation with ¹²⁵I-labeled protein A. Immunoblots were exposed to XAR-5 film at –70°C with an intensifying screen.

Purification of HDV and HBV particles. Culture medium was clarified by centrifugation at $5,000 \times g$ for 1 h at 4°C. The clarified medium was layered on a 1-ml 20% sucrose cushion in TNE (150 mM NaCl, 20 mM Tris-HCl [pH 7.4]), and sedimented at 38,000 rpm for 5 h at 4°C in an SW41 rotor. The pellet was resuspended in TNE buffer, and isopycnic centrifugation was performed in a 10 to 50% (wt/vol) CsCl step gradient in TNE buffer. The gradient was subjected to centrifugation at 38,000 rpm in an SW41 rotor for 18 h at 4°C. Fractions were collected from the bottom of the tube, and the density was determined by measurement of the refractive index. Aliquots of each fraction were used for DNA, RNA, and protein analysis.

Immunofluorescence staining of primary hepatocyte cultures. Cells were grown on collagen-treated glass coverslips in 22-mm-diameter tissue culture wells. Coverslips were washed three times in phosphate-buffered saline, air dried, fixed in acetone, and processed for indirect immunofluorescence staining with human anti-HDVAg or mouse anti-HBcAg antiserum as described before (19).

RESULTS

In this study, we have investigated whether primary cultures of chimpanzee hepatocytes could serve as target cells in an *in vitro* HDV infection system. For this purpose, hepatocytes were obtained from either uninfected or HBV-infected animals and cultured in a serum-free medium as described previously (14, 20). Three days after plating, cells were exposed to HDV-containing serum. This serum was recovered from an HBV-infected animal (chimpanzee X136) that had been transfected with a cDNA clone of HDV and thus contained both HDV and HBV particles at concentrations of approximately 10^9 and 10^6 genomes per ml, respectively. As a control, cells were exposed to serum containing HBV only at approximately 10^8 genomes per ml (33), that had been collected from the same animal prior to HDV infection. Cells were seeded in 22-mm-diameter wells, exposed to HDV on day 3 postseeding, and harvested every 3 days thereafter to monitor for signs of infection. To determine whether HDV RNA or HBV DNA was replicating in hepatocyte cultures, nucleic acids were extracted and examined after electrophoresis and transfer to nitrocellulose membrane by hybridization to the HDV and HBV probe, respectively. For protein analysis, cells from one well were harvested every 3 days after infection by disruption in SDS– β -mercaptoethanol buffer, and samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting for the presence of HDVAg. For immunofluorescence staining, cells were seeded on glass coverslips, harvested every 3 days after infection, and stained for the presence of HDVAg and HBcAg.

***In vitro* infection with HDV of hepatocytes derived from HBV-noninfected animals.** Following exposure to an HDV inoculum, cells were monitored for the presence of HDV RNA for up to 6 weeks. HDV RNA was detected starting 6 days following exposure (see Fig. 3C). Viral RNA was maintained throughout the culture period at levels equal to or greater than those detected in an HDV-infected chimpanzee liver. The most abundant HDV RNA had an electropho-

FIG. 1. Analysis of HDV RNA extracted from cells derived from an HBV-negative liver and infected *in vitro* with HDV. Cells were harvested 3, 6, 9, 12, 15, 18, 21, and 42 days after infection. Total cellular RNA (5 μ g) was separated on a 1.5% agarose-2.2 M formaldehyde gel, transferred to nitrocellulose, and hybridized to a 32 P-labeled HDV-specific DNA probe. Total liver RNA (5 μ g) was purified from an HDV-infected chimpanzee liver (L). Radiolabeled RNAs (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used as size markers.

retic mobility of a 1.7-kb RNA similar to that present in the inoculum. These molecules represent monomeric circular and linear forms of the HDV genome and antigenome (Fig. 1). Minor amounts of RNA with a mobility of about 3.5 kb were also detected, which have been suggested to represent replicative intermediates of the HDV genome (7).

To determine whether the two forms of HDAg polypeptides described previously (1, 32) were also present in hepatocytes infected *in vitro*, an immunoblot analysis was conducted on cell lysates harvested every 3 days after infection. Intracellular HDAg appeared simultaneously with HDV RNA in both HBV-infected and noninfected cells (data not shown). Two distinct polypeptides with apparent molecular masses of 22 and 24 kDa in proportions different from that of the inoculum were detected (Fig. 2). By densitometric analysis, the relative amounts of p22 and p24 in the inoculum were estimated at 60 and 40%, respectively, whereas a lysate of cells harvested 12 days after infection contained 30 and 70% of the 22-kDa and 24-kDa polypeptides, respectively.

Although the inocula contained both HDV and HBV particles, no evidence of HBV infection was observed. Furthermore, when cells were exposed to inocula containing only HBV, including (i) serum derived from chimpanzee X136 prior to HDV infection, (ii) serum derived from an-

FIG. 2. Immunoblot analysis of proteins extracted from cells derived from an HBV-noninfected liver and infected *in vitro* with HDV. Immunoblot analysis of HDAg-related proteins extracted from infectious serum X136 (lane 1) and cells 12 days after infection *in vitro* with HDV (lane 2). Samples were treated with 2% SDS-2% β -mercaptoethanol, sonicated, and heated at 100°C for 5 min prior to analysis by the immunoblot procedure. The size markers were prestained proteins (Bethesda Research Laboratories).

other HBV carrier chimpanzee, and (iii) sucrose gradient-purified Dane particles, HBV infection could not be demonstrated either. The criterion used for positive HBV infection was the appearance of intracellular HBV mRNAs. The number of HBV DNA molecules in the inocula was estimated at 10^9 /ml in the chimpanzee serum and 10^{11} /ml in the preparation of purified Dane particles. Therefore, because 10^6 cells were exposed to 20 μ l of each inoculum, the number of HBV DNA molecules per cell was estimated at 20 and 2,000 in the chimpanzee serum and Dane particle preparation, respectively.

Experiments were conducted to estimate the optimal multiplicity of infection. Intracellular HDV RNA was quantitated from cultures extracted 9 days after exposure of 10^6 cells to various amounts of HDV inoculum. The HDV RNA was quantitated by laser densitometric scanning of an autoradiogram of a Northern (RNA) blot hybridization analysis (Fig. 3A). A quantity of serum as low as 0.3 μ l (3×10^5 genomes) was shown to be infectious, whereas an inoculum of 300 μ l (3×10^8 genomes) had an inhibitory effect on infection with HDV.

To determine whether primary cultures of chimpanzee hepatocytes remained susceptible to HDV infection for an extended period after plating, 10^6 cells were exposed to HDV on day 3, 6, 9, 12, 15, or 21 postseeding. Cells were harvested 9 days after exposure, and cellular RNA was extracted and analyzed for the presence of HDV sequences. High levels of HDV RNA of genomic size (1.7 kb) and low levels of the 3.5-kb RNA species that was not present in the inoculum were detected at the same level in all samples (data not shown). Therefore, primary chimpanzee hepatocytes remain susceptible to HDV infection for at least 3 weeks in culture. Similar results were obtained when cells were maintained in serum containing medium supplemented with 10^{-6} M hydrocortisone, insulin (10 μ g/ml), and glucagon (4 μ g/ml), suggesting that the presence of fetal calf serum did not induce the loss of functions required for infection. Whether the presence of these hormones in the culture medium had an influence on infectibility was not addressed in this study. For routine infection assays, we used the serum-free medium formula, which allows better maintenance and growth of primary hepatocytes. Previous studies demonstrated that cells could be maintained in this medium for up to 90 days after plating without significant growth of nonhepatic cells while maintaining the characteristic morphology and functions of differentiated hepatocytes (14, 20).

To determine whether a higher proportion of cells could be infected in culture medium supplemented with dimethyl sulfoxide (DMSO), cells (10^6 /well) were plated in serum-free medium in the presence or absence of 1.5% DMSO and exposed to 20 μ l of HDV inoculum 3 days later. Cells were harvested every 3 days following infection, and the amount of replicative forms of HDV RNA was measured by Northern blot hybridization analysis. No significant difference was observed in cells grown in serum-free medium in the absence and presence of DMSO (data not shown). In addition, no indication of HBV infection was detected in the presence of DMSO-containing medium with HDV-free, HBV-containing serum at 10^8 genomes per ml or purified Dane particles at 10^{11} genomes per ml.

To investigate the time required for the uptake of HDV by primary chimpanzee hepatocytes, infections were performed by exposing the cells to the inoculum for 0.1, 0.5, 1, 2, 6, or 12 h. Following exposure, cells were washed twice in fresh medium and grown in serum-free medium for 9 days before being harvested for HDV RNA analysis. The maximum level

FIG. 3. Quantitation of intracellular HDV RNA in cells infected with HDV in vitro. Autoradiograms of Northern blot hybridizations were quantitated with an LKB Ultrosan XL densitometer. The relative amount of intracellular HDV RNA is indicated as a function of the volume of HDV inoculum (A), as a function of the exposure period to the inoculum (B), or as a function of time (3, 6, 9, 12, 15, 18, and 21 days) after infection (C). The quantitation in panel C was obtained from analysis of the autoradiogram shown in Fig. 1.

of infection was reached when cells were exposed to the inoculum for at least 12 h (Fig. 3B).

In vitro infection with HDV of cells derived from an HBV-infected animal. Cells derived from an HBV-infected animal were exposed to an HDV inoculum, and signs of infection were monitored as described above.

To determine the percentage of cells infected with HBV and HDV, coverslips of primary hepatocytes were harvested and stained for HDAg by an indirect immunofluorescence assay with an anti-HDAg antibody-positive human serum and an anti-HBcAg hyperimmune mouse serum. Cells not exposed to HDV were used as a control. HDAg was de-

FIG. 4. Indirect immunofluorescence staining of cells derived from an HBV-infected liver and infected in vitro with HDV. Hepatocytes grown on glass coverslips were fixed for 3 min in acetone and processed for immunofluorescence staining. (A) Hepatocytes harvested 21 days postinfection were stained for HDcAg with a human serum with anti-HDAg reactivity and fluorescein-conjugated rabbit anti-human IgG. (B) Hepatocytes harvested 2 days postinfection with HDV were stained for HBcAg with a mouse anti-HBcAg and fluorescein-conjugated rabbit anti-mouse IgG.

ected in the nucleus of approximately 10% of the exposed cells as intense granular staining often associated with nucleoli (Fig. 4A). When stained for HBcAg, up to 50% of the cells displayed nuclear staining of various intensities (Fig. 4B). There were neither morphological alterations nor foci of infection in cells producing either viral antigen.

To better monitor the replication of HBV in the HDV-infected cultures, total cellular DNA was extracted and analyzed for HBV DNA. Replication of HBV DNA was detected throughout the culture period, as evidenced by the presence of sustained amounts of relaxed circular, linear double-stranded, and single-stranded HBV DNA molecules,

FIG. 5. Kinetic analysis of HBV DNA and HDV RNA in culture medium and cells derived from an HBV-infected liver and infected in vitro with HDV. Cells and culture medium were harvested on days 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 after infection. (A) RNA and DNA were extracted from each sample. Total cellular RNA (5 μ g) from each sample was separated on a 1.5% agarose-2.2 M formaldehyde gel and analyzed for the presence of HDV RNA after transfer to nitrocellulose and hybridization to a 32 P-labeled HDV-specific DNA probe. (B) Total cellular DNA (5 μ g) from each sample was separated on a 1.5% agarose gel and analyzed for the presence of HBV DNA after transfer to nitrocellulose and hybridization to a 32 P-labeled HBV-specific DNA probe. (C) RNA isolated from 7 ml of culture medium harvested at the indicated time points was separated on a 1.5% agarose-2.2 M formaldehyde gel and analyzed for the presence of HDV RNA after transfer to nitrocellulose and hybridization to a 32 P-labeled HDV-specific DNA probe. (D) DNA isolated from 7 ml of culture medium harvested at the indicated time points was separated on a 1.5% agarose gel and analyzed for the presence of HBV DNA after transfer to nitrocellulose and hybridization to a 32 P-labeled HBV-specific DNA probe. *Hind*III-digested bacteriophage lambda DNA was used as DNA size markers. Radiolabeled RNAs (Bethesda Research Laboratories) were used as RNA size markers. RC, relaxed circular DNA; L, linear double-stranded DNA; SS, single-stranded DNA.

which represent replicative intermediates of the HBV genome (Fig. 5B). Analysis of total cellular RNA revealed that, following exposure to HDV, replication of HDV RNA appeared on day 6 postinfection, as in HBV-noninfected cells, and persisted throughout the culture period (Fig. 5A). HDV RNA species of 1.7 and 3.5 kb similar to those found in an infected liver were detected. As a control, a culture of HDV-noninfected cells derived from the same animal was monitored. Analysis of HBV DNA and RNA in both HDV-

infected and control cells did not reveal any significant differences (data not shown). These results contradict observations made in vivo. In both experimentally infected animals and naturally infected humans, a transient suppression of HBV replication usually results from superinfection.

To monitor the production of viral particles, culture medium was harvested every 3 days throughout the culture period and examined for the presence of either HBV DNA or HDV RNA-containing particles. Particles were sedimented and nucleic acids were purified before analysis for the presence of HBV or HDV sequences. HBV DNA was detected at constant levels throughout the culture period, indicating a sustained production of HBV particles (Fig. 5D). HDV RNA was also detected at each time point throughout the culture period, with a slight peak on day 15 postinfection (Fig. 5C). In this experiment, cells were exposed to the inoculum for 72 h; therefore, the high level of HDV RNA detected on day 3 postinfection represents HDV RNA contained in the inoculum. The intensity of the HDV RNA signal on day 6 may indicate that particles derived from the inoculum had been carried over. This signal decreased until day 9 before increasing between days 12 and 18 and was maintained at a constant level until day 30, indicating that progeny HDV RNA-containing particles were released into the culture medium.

To verify that HDV RNA was indeed contained in viral particles rather than released as naked molecules from cells replicating high levels of viral RNA, particles were purified from a pool of culture medium samples harvested from days 12 to 30 postinfection, therefore excluding the possibility that particles were carried over from the inoculum. Particles were sedimented from this pool and subjected to centrifugation on a CsCl gradient. RNA was extracted from each fraction before analysis for the presence of HDV RNA. HDV RNA was detected at a buoyant density of 1.24 g/ml (Fig. 6A), similar to that of serum-derived HDV particles, indicating that HDV RNA was indeed contained in typical HDV virions.

To ascertain that HBV DNA was contained in mature viral particles, the buoyant density of HBV DNA-containing particles was measured by centrifugation on a CsCl gradient. Following centrifugation, fractions were collected and DNA was extracted from each fraction. HBV DNA was detected at a density of 1.24 g/ml (Fig. 6B), similar to that of serum-derived HBV particles, indicating that a sustained production of Dane particles occurred as well.

When total RNA derived from HBV- and HDV-infected cells was further fractionated to separate poly(A)-containing RNA, we were able to identify an HDV-specific mRNA in the poly(A)-positive fraction of approximately 0.8 kb (Fig. 7). This molecule may represent the messenger for HDV proteins as described by Hsieh and coworkers (12). The HDV RNA molecules of genomic size as well as the 3.5-kb forms were detected in the poly(A)-minus fraction, confirming the absence of a poly(A) tail. When hybridized to an HBV-specific DNA probe, two species of HBV mRNAs (3.5 and 2.3 kb) were detected exclusively in the poly(A)-positive fraction.

DISCUSSION

The present study has described a tissue culture system suitable for in vitro infection of primary chimpanzee hepatocytes with HDV. As early as 6 days after infection, replicative forms of HDV RNA were detected and the amount of intracellular viral RNA was similar to that present

FIG. 6. Analysis of viral particles released into the culture medium of hepatocytes derived from an HBV-infected liver and infected with HDV *in vitro*. Culture medium (30 ml) harvested on days 12 to 30 postinfection was subjected to centrifugation at 5,000 rpm for 1 h at 4°C. The clarified medium was then layered on a 5-ml 20% sucrose cushion in TNE and subjected to centrifugation at 25,000 rpm in an SW28 rotor for 16 h at 4°C. The pellet was resuspended in TNE buffer, loaded on a 10 to 50% (wt/vol) CsCl gradient in TNE, and subjected to centrifugation for 18 h at 38,000 rpm in an SW41 rotor at 4°C. Fractions were collected from the bottom of the tube, and one-third of each fraction was used for DNA and RNA extraction. (A) RNA was isolated and separated on a 1.5% agarose–2.2 M formaldehyde gel and analyzed for the presence of HDV RNA after transfer to nitrocellulose and hybridization to an HDV-specific DNA probe. (B) DNA isolated from each fraction was separated on a 1.5% agarose gel and analyzed for the presence of HBV sequences after transfer to nitrocellulose and hybridization to an HBV-specific DNA probe. Fractions 1 to 12 had a density of 1.46, 1.43, 1.40, 1.37, 1.32, 1.28, 1.24, 1.21, 1.16, 1.13, 1.10, and 1.09 g/ml, respectively, as calculated by measurement of the refractive index. *Hind*III-digested bacteriophage lambda DNA was used as DNA size markers. Radiolabeled RNAs (Bethesda Research Laboratories) were used as RNA size markers.

in liver cells of an infected animal. The high level of RNA replication in infected cells therefore renders this system very sensitive as an *in vitro* infection assay.

According to immunofluorescence data, the proportion of cells infected by HDV (10%) is significantly greater than that reported for woodchuck hepatocytes (2%) (36). In addition, we have demonstrated that cells remained susceptible to

FIG. 7. Northern blot analysis of RNA extracted from cells derived from an HBV-infected liver and infected *in vitro* with HDV. Total cellular RNA was fractionated by chromatography on oligo(dT)-cellulose. Poly(A)-containing RNA (A+) and poly(A)-negative RNA (A-) were analyzed for the presence of HDV RNA or HBV RNA by electrophoresis on a 1.5% agarose–2.2 M formaldehyde gel, transfer to nitrocellulose, and hybridization to a ³²P-labeled HDV- or HBV-specific DNA probe, respectively. Radiolabeled RNAs (Bethesda Research Laboratories) were used as RNA size markers.

infection for at least 3 weeks in culture (infection experiments were not carried out to investigate whether infectibility was retained beyond this period). Considering that a large number of cells (up to 5×10^8 to 6×10^8 viable cells) can be recovered from a single biopsy sample and that cultures can be maintained for up to 90 days, our system represents a substantial improvement over those described to date. Conditions for passing cells and reviving frozen cells are being explored.

Maximum levels of HDV infection *in vitro* required prolonged exposure of the cells to the inoculum (at least 12 h), similar to infection of primary duck hepatocytes with duck hepatitis B virus (26, 37). Several possible explanations may account for this phenomenon: (i) a limited number of receptors may be present on the cell surface at any given time; (ii) binding and internalization of the viral particle may be a slow process; or (iii) subviral HBsAg particles also present in excess in the inoculum may compete for binding to the receptors. Cells could be infected once receptors occupied by subviral particles are recycled and become available to bind to HDV.

Although infection with HDV was reproducible from several different cell isolations, infection with HBV was not observed despite numerous attempts involving the use of different inocula, different culture conditions, and multiple sources of hepatocytes. To explain this phenomenon, we can make two opposing assumptions. (i) Cells are equally susceptible to both HBV and HDV infections, but only HDV infection is detectable by Northern blot analysis due to the very high copy number of HDV RNA molecules per infected cell. An HDV-infected cell may contain 100,000 molecules of viral RNA, versus 50 to 100 copies of HBV DNA in an HBV-infected cell. Combined with the fact that 10% of the cells are infected following exposure to an inoculum, an infected culture should contain 10,000 copies of HDV RNA molecules per average cell and 5 to 10 copies of HBV DNA. This latter number should permit the detection of HBV DNA sequences by Southern blot analysis (in our assays, the sensitivity for detection of HBV DNA molecules is greater than 1 copy per cell). Therefore, this explanation seems unlikely. (ii) Primary cultures of chimpanzee hepatocytes are not equally susceptible to HBV and HDV infection. Therefore, uptake of HDV and HBV is performed by different mechanisms that may, as suggested previously (22), involve distinct receptors. Alternatively, a unique receptor may be utilized by both viruses, but the uptake of HDV would proceed more efficiently due to the different characteristics of the viral particle, including its envelope. Our culture system, in contrast to the human system described previously (11), does not permit infection with HBV and yet allows quite efficient HDV infection.

The detection of *de novo* synthesis of HDV particles in cells derived from an HBV-infected animal indicates that this system can support all stages of the HDV life cycle and can sustain the synthesis of both viruses to levels suitable for practical experimental use. The amount of HDV RNA molecules in a 3-day-old culture medium harvested on day 15 postinfection was estimated at 10^6 /ml. No significant suppression of HBV replication was observed after infection with HDV *in vitro*. However, it is possible that cells supporting a dual infection did display an inhibitory effect on HBV replication, but this effect would remain undetected in our system due to the presence of a large excess of cells infected only by HBV.

From this study, it remains unclear whether reinfection occurred in cells producing HDV. Repeated cycles of virus

infection could not be demonstrated by analyzing the levels of intracellular viral RNA, and a kinetic analysis by immunofluorescence staining of HDAG did not reveal a dramatic increase in the number of positive cells. The possibility that HDV-infected cells were lost from the culture at the same rate that new cells became infected seems unlikely. No cytotoxic effect from HDV replication was observed in the HBV-noninfected cultures that do not produce particles, since the level of intracellular HDV RNA did not decline over a 42-day period after infection.

Nevertheless, this system represents a substantial improvement over the primary woodchuck hepatocyte system described previously (8, 9, 23, 36) in that (i) it allows for infectivity assay of human HDV versus the woodchuck pseudotype, (ii) the cells retain infectibility for an extended period of time (3 weeks or more), (iii) the high level of intracellular HDV RNA following infection provides a very sensitive assay, and (iv) it allows for de novo synthesis and release of progeny virions. Despite its limitations, reflected by the failure to support HBV infection, this tissue culture system represents a valuable model for analysis of the entire life cycle of HDV in vitro and, in particular, the identification of its receptor.

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