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Production of Infectious Hepatitis Delta Virus In Vitro and Neutralization with Antibodies Directed against Hepatitis B Virus Pre-S Antigens

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Hepatitis delta virus (HDV) particles were produced in Huh7 human hepatoma cells by transfection with cloned hepatitis B virus (HBV) DNA and HDV cDNA. The particles were characterized by their buoyant density, the presence of encapsidated viral RNA, and their ability to infect primary cultures of chimpanzee hepatocytes. Successful infection was evidenced by the appearance of increasing amounts of intracellular HDV RNA after exposure to particles. Infection was prevented when particles were incubated with antibodies directed against synthetic peptides specific for epitopes of the pre-S1 or pre-S2 domains of the HBV envelope proteins before exposure to hepatocytes. These data demonstrate that HDV particles produced in vitro are infectious and indicate (i) that infectious particles are coated with HBV envelope proteins that contain the pre-S1 and pre-S2 regions, (ii) that epitopes of the pre-S1 and pre-S2 domains of HBV envelope proteins are exposed at the surface of HDV particles, and (iii) that antibodies directed against those epitopes have neutralizing activity against HDV.

Hepatitis delta virus (HDV) is a unique viral agent in animals in that it contains a circular, single-stranded RNA genome with ribozyme activity (39, 46), it packages its genome with the envelope proteins of coinfecting hepatitis B virus (HBV) to form particles (2, 3, 35, 47), and it suppresses the replication of HBV (9, 19, 26). The HDV genome displays the structural characteristics of plant viroids and replicates its RNA by using a similar strategy (4, 11, 20, 21, 33, 39, 44). However, unlike viroids which do not encode any proteins, the HDV genome codes for at least one polypeptide that bears the hepatitis delta antigen (1, 34, 45). This protein has two important functions in the viral life cycle: it interacts with HDV RNA and the HBV envelope proteins in the assembly of viral particles (2, 3, 35), and it plays an indispensable role in the replication of viral RNA (20). The HDV envelope bears the hepatitis B pre-S1, pre-S2, and surface (HBsAg) antigens carried by the large, middle, and small envelope proteins of HBV but in a proportion that differs from that found on complete (Dane) HBV particles (2, 3). Coinfecting HBV is required for transmission of HDV but is dispensable for the replication of HDV RNA within an infected cell (20). Therefore, the role of HBV seems limited to the morphogenesis of HDV particles, thereby providing a mode of transmission. As in HBV virions, the large and middle envelope proteins of HBV origin may have a crucial role in the assembly of the viral envelope and in recognizing the viral receptor on the cell membrane. However, as suggested previously (26, 36), HBV and HDV may utilize different mechanisms of entry into cells despite the similarities in the nature of their envelopes (2).

Recently, we have developed an in vitro system in which primary cultures of chimpanzee hepatocytes are used to test the infectivity of HDV particles (36). Although the supply of chimpanzee hepatocytes is limited, the cells remain permissive for infection for more than 3 weeks in culture, allowing several successive assays to be performed after a single isolation. High levels of replicating HDV RNA were observed after exposure of hepatocytes to HDV particles in vitro. This criterion was used to assess successful infection. Furthermore, HBV and HDV particles were produced after infection of cells derived from an HBV-infected animal. In contrast to primary cultures of human hepatocytes (16), cultures of chimpanzee hepatocytes grown in serum-free medium were refractory to infection with HBV.

In a recent study, Wu and coworkers (47) produced HDV particles in Huh7 cells by transfection with cloned HDV cDNA and HBV DNA. In the present study, particles produced in the same fashion in Huh7 cells were shown to be infectious in an in vitro assay. In addition, antibodies directed against synthetic peptides specific for the pre-S1 and pre-S2 antigens of HBV envelope proteins were able to neutralize infection. We have therefore defined a system to analyze the morphogenesis and infectivity of HDV particles whereby mutants can be produced in Huh7 cells and tested for infectivity in primary hepatocytes and in which antibodies can be analyzed for neutralizing activity.

Human hepatoma cell lines such as HepG2 and Huh7 are amenable to the production of infectious HBV particles upon transfection with cloned HBV DNA (37) and to the production of HDV particles after cotransfection with HBV DNA and HDV cDNA (47). However, these cell lines are refractory to a quantifiable infection with HDV particles (unpublished observations). Infectivity assays for HDV therefore require the use of primary cultures of chimpanzee hepatocytes, which are thus far the only cells susceptible to infection with HDV in vitro (36), whereas the infectivity of the woodchuck pseudotype of HDV (32) can be assayed with primary cultures of woodchuck hepatocytes (30, 40).

For production of HDV particles, Huh7 human hepatoma cells were transfected with a mixture of cloned HDV cDNA and HBV DNA as described by Wu and coworkers (47). Cells were propagated in a mixture of Dulbecco modified

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Eagle medium and F12 medium supplemented with 10% fetal bovine serum. Cells were seeded at 4×10^5/60-mm-diameter petri dish and transfected with 10 μg of pCP10 (37) and 5 μg of pSVLD3 (HDV cDNA) (20) at 24 h postseeding by the procedure of Chen and Okayama (8). Cells were exposed to the DNA-phosphate precipitate for 5 h and washed with phosphate-buffered saline before incubation in culture medium. Cells were harvested on day 6 after transfection and analyzed for the presence of HDV RNA by hybridization to an HDV-specific probe. For analysis, total RNA was prepared by disruption of 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 previously (25, 37). RNA samples were subjected to electrophoresis through a 1.5% agarose–2.2 M formaldehyde gel and then transferred to nitrocellulose for hybridization to an HDV-specific DNA probe. Replication of viral RNA was evidenced by the appearance of high levels of HDV RNA molecules with an electrophoretic mobility corresponding to a length of approximately 1.7 kb (data not shown). In addition, a low level of HDV RNA sequences of larger molecular size, 3.5 kb in apparent length, which represented replicative dimeric forms of the genome was detected (data not shown) (10, 20, 21, 40, 47). Cellular RNA was also analyzed for the presence of HBV RNA by hybridization to an HBV-specific probe. HBV RNA species of approximately 3.5 and 2.3 kb were detected on days 3, 6, and 9 after transfection. These molecules are typical of HBV-specific transcripts present in HBV-infected cells (15, 37).

Characterization of HDV and HBV particles produced in Huh7 cells. Culture medium from transfected cells, harvested on days 6 and 9 after transfection, was clarified by centrifugation at 5,000 × *g* for 1 h at 4°C. The clarified medium was then layered onto a 5-ml 20% sucrose cushion in 150 mM NaCl–20 mM Tris HCl (pH 7.4) (TN buffer) and centrifuged at 25,000 rpm at 4°C in an SW28 rotor for 16 h. The pellet was resuspended in TN buffer, loaded on a 10 to 50% (wt/vol) CsCl gradient in TN buffer, and centrifuged 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. Each fraction was assayed for density and for the presence of HDV RNA and HBV DNA. HDV RNA was purified by incubation in 50 mM Tris-HCl (pH 7.8)–200 mM NaCl–20 mM Na_2EDTA–2% sodium dodecyl sulfate (SDS)–1 mg of proteinase K per ml–2 mM vanadyl ribonucleoside complex for 2 h and by phenol extraction as described previously (36). RNA samples were analyzed by hybridization to an HDV-specific DNA probe as described above. HBV DNA was isolated from culture medium by digestion with 500 μg of proteinase K per ml in 10 mM Tris-HCl (pH 7.4)–10 mM NaCl–10 mM Na_2EDTA–0.5% SDS for 2 h at 37°C and then extracted with phenol (36). DNA samples were subjected to electrophoresis in a 1.5% agarose gel and transferred to nitrocellulose membranes for hybridization to a ^{32}P-labeled HBV DNA probe. Gel-purified full-length HBV DNA and HDV cDNA derived from recombinant plasmids pCP10 (37) and pSVLD3 (20), respectively, were labeled with [^{32}P]dCTP as described elsewhere (37). HBV DNA was detected at a buoyant density of 1.24 g/cm^3 (Fig. 1), whereas HDV RNA was detected in the fraction with a density of 1.21 g/cm^3. Fractions 4, 5, and 6, corresponding to densities of 1.24, 1.21, and 1.17 g/cm^3, respectively, were also positive for the presence of HBsAg as measured with an enzyme-linked immunoassay (E.I.A. Auzyme II; Abbott Laboratories). These results indicated that HBV DNA and HDV RNA of genomic sizes were detected in particles with densities characteristic of mature HBV and HDV virions, respectively. Transfection therefore resulted in the synthesis and secretion of HBV- and HDV-like particles.

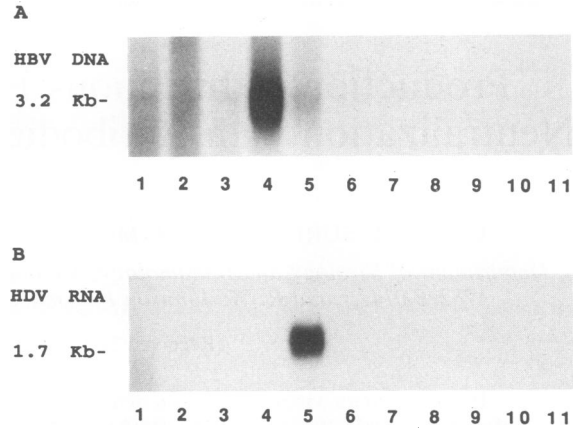

FIG. 1. Analysis of viral particles released into the culture medium of Huh7 cells transfected with HDV cDNA and HBV DNA. Viral particles obtained from 30 ml of culture medium harvested on days 6 and 9 posttransfection were prepared as described in the text (see "Characterization of HDV and HBV particles produced in Huh7 cells"). Fractions were collected from the bottom of the test tube; one-third of each sample was used for DNA extraction, and one-third was used for RNA extraction. Fractions 1 to 11 had densities of 1.38, 1.33, 1.30, 1.24, 1.21, 1.17, 1.14, 1.10, 1.06, 1.05, and 1.03 g/cm^3, respectively, as calculated by measurement of the refractive index. (A) 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 a ^{32}P-labeled HBV specific DNA probe. *Hin*dIII-digested bacteriophage lambda DNA fragments were used as DNA size markers. (B) RNA isolated from each fraction 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. Radiolabeled RNAs (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used as RNA size markers.

Infectivity of Huh7 cell-derived HDV-like particles in primary hepatocytes. The infectivity of HDV-like particles produced in Huh7 cells was determined with primary cultures of chimpanzee hepatocytes. Hepatocytes were isolated from a chimpanzee with no history of HDV infection whose serum was free of any HDV markers, including HDV RNA and anti-hepatitis delta antigen antibodies. The procedures used for the isolation and culture of primary hepatocytes in a serum-free medium formulation have been described previously (18, 24). Hepatocytes were exposed to HDV for at least 12 h on day 3 postseeding. As a positive control, 20 μl of infectious serum collected from chimpanzee X136 at the peak of infection (38) was added to a 22-mm-diameter well containing 10^6 cells in 1 ml of serum-free medium. Huh7 culture medium harvested on day 6 and 9 after transfection was used for purification of HDV particles. The culture medium was clarified by centrifugation at 5,000 × *g* for 1 h at 4°C and concentrated 20× in a Centricon 100 microconcentrator by centrifugation. The concentrate was resuspended in serum-free medium, resulting in a 5× concentration that served as inoculum. By measurement of HDV RNA extracted from the inoculum and comparison with a known amount of HDV cDNA, we estimated that cells from one well were exposed to an Huh7-derived inoculum containing

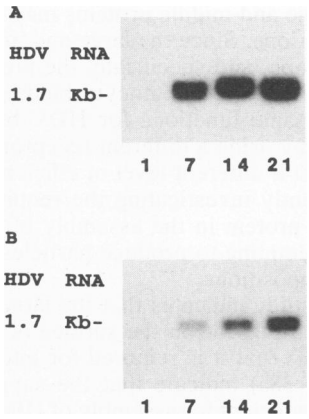

FIG. 2. Analysis of HDV RNA extracted from hepatocytes infected with HDV in vitro. Chimpanzee hepatocytes were infected in vitro with serum-derived particles (A) or with Huh7 cell-derived particles (B). Cells were harvested 1, 7, 14, and 21 days after infection. Total cellular RNA (5 μg) was separated 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. Radiolabeled RNAs (Bethesda Research Laboratories) were used as size markers.

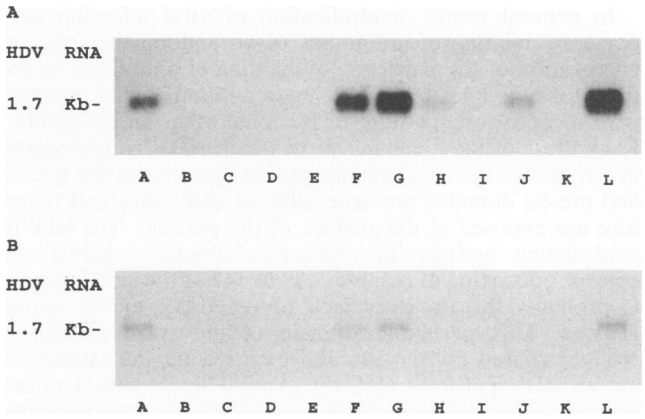

FIG. 3. Assay for neutralization of HDV infection of chimpanzee hepatocytes with antibodies directed to HBV surface proteins. Chimpanzee hepatocytes were infected in vitro with serum-derived particles (A) or with Huh7 cell-derived particles (B) that had been incubated with antibodies specific for HBV envelope proteins. Antisera were baboon (lanes B) or chimpanzee (lanes A and C through J) polyclonal antibodies directed to synthetic peptides specific for the HBV pre-S1, pre-S2, and S domains of the envelope proteins. Serum samples were from an unimmunized chimpanzee (lanes A); a baboon immunized with pre-S2-specific peptide 133–151 ay (lanes B); or chimpanzees immunized with pre-S1-specific peptide 12–47 ay (lanes C and D) or 12–47 ay–KLH (lanes E), S-specific peptide 139–147 ay (lanes F and G), pre-S2-specific peptide 120–145 ay (lanes H), or pre-S1-specific peptide 12–47 ay–KLH (lanes I) or 94–117 ay (lanes J). Uninfected cells (lanes K) were used as a negative control, and cells infected with 20 μl of chimpanzee infectious serum were used as a positive control (lanes L). Cells were harvested on day 9 after exposure to the inoculum. Total cellular RNA (5 μg) 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. Radiolabeled RNAs (Bethesda Research Laboratories) were used as size markers.

approximately 1×10^6 genomes and to a serum-derived inoculum containing approximately 2×10^7 genomes. After exposure, cells were washed and incubated in 1 ml of fresh serum-free medium. Cells were harvested every week thereafter for detection of intracellular HDV RNA. HDV RNA was not detected on day 1 after exposure but was present at increasing levels starting on day 7 either in cells exposed to HDV-infected chimpanzee serum (Fig. 2A) or Huh7-derived HDV (Fig. 2B). The most abundant species had an electrophoretic mobility of a 1.7-kb RNA similar to that present in the inoculum. Minor amounts of replicative intermediates not present in the inoculum (not shown), with a mobility of about 3.5 kb, were detected as well. Although not visible on the autoradiogram in Fig. 2, these molecules were clearly visible on an autoradiogram with a longer exposure (data not shown). These results demonstrate clearly that HDV particles produced in Huh7 cells following transfection are infectious.

Neutralization of HDV infection with antibodies to HBV surface proteins. For the neutralization assay, an inoculum containing approximately 1×10^6 genomes of Huh7-derived particles or 2×10^7 genomes of serum-derived particles was incubated overnight at 4°C in 500 μl of serum-free medium containing approximately 5 μg of purified immunoglobulin G antibody. This mixture was then added to a 22-mm-diameter well containing 10^6 cells and incubated at 37°C overnight. The inoculum was then removed, and fresh medium was added. Neutralizing activity was estimated by measurement of the level of intracellular HDV RNA 9 days after exposure. The antisera were baboon or chimpanzee polyclonal antibodies directed against synthetic peptides specific for the pre-S1, pre-S2, and S domains of the HBV envelope proteins. These antibodies were directed against synthetic peptides specific for the small envelope protein (S) (samples F and G), pre-S1 (samples C, D, E, I and J), or pre-S2 (samples B and H) domain of the HBV envelope proteins. Each antiserum was obtained from a different animal, either a chimpanzee (A, C, D, E, F, G, H, I, and J) or a baboon (B). As a control, a serum sample derived from a nonimmunized chimpanzee (animal A) was used. Immunizations were performed as described previously (5, 14, 17, 28, 41) by injection of the following synthetic peptides: a cyclized peptide from residues 139 to 147 of the S domain subtype ay (139–147 ay) (animals F and G), a pre-S2-specific linear peptide, 120–145 ay (animal H), a preS2-specific linear peptide, 133–151 ay (animal B), a pre-S1-specific linear peptide, 12–47 ay (animals C and D), a pre-S1-specific linear peptide, 12–47 ay coupled with keyhole limpet hemocyanin (12–47 ay–KLH) (animals E and I), and a pre-S1-specific linear peptide, 94–117 ay (animal J).

Samples B, C, D, E, H, and I, which contained antibodies directed against synthetic peptides specific for the pre-S1 or pre-S2 domain, had neutralizing activity on particles derived from chimpanzee serum as well as particles derived from transfected Huh7 cells, as shown by the absence or low level of HDV RNA detected in cells harvested 9 days after exposure (Fig. 3). Sample J, which contained antibodies against pre-S1 antigen residues 94 to 117, did not neutralize infection with the same efficiency. This peptide has been shown previously not to induce protective immunity against HBV (41). Serum derived from a nonimmunized animal (Fig. 3, lanes A) had no significant neutralizing activity, nor did antibodies directed against a peptide specific for S protein amino acids 139 to 147 of the ay subtype (Fig. 3, lanes F and G). Chimpanzees immunized with this peptide were not protected from a live HBV challenge (unpublished data).

In general terms, neutralization of viral infection may occur by binding of antibodies to an epitope that triggers aggregation of the particles, by binding of antibodies to the receptor-binding site, by binding of antibodies that interfere with receptor recognition, or by some other mechanism(s). Regardless of the mechanism by which HDV is neutralized in our assay, the results indicate that epitopes in the pre-S1 and pre-S2 domains are accessible to antibodies and therefore are exposed at the surface of the particle. The lack of neutralizing activity of antibodies directed against the epitope consisting of residues 139 to 147 of the small protein is probably due to their lack of reactivity to the native HBsAg. This particular domain of the small protein is N-glycosylated at Asn-146 and exposed on the outside of native HBV particles (15, 42). Antibodies F and G raised against a nonglycosylated synthetic peptide had no reactivity to native HBsAg particles and, as indicated above, failed to neutralize HBV in vivo. However, an HBsAg-specific monoclonal antibody raised against native HBsAg (Ortho Laboratories) was able to completely neutralize HDV in vitro (data not shown). The neutralizing activity of the anti-pre-S1 12–47, anti-pre-S2 120–145, and anti-pre-S2 133–151 antibodies indicates that, in addition to the small protein, the envelope of infectious HDV particles includes at least the large and possibly the middle HBV envelope proteins. More specifically, epitopes located between residues 12 and 47 of the pre-S1 polypeptide and between residues 120 and 151 of the pre-S2 domain are exposed at the surface of HDV particles as well as on HBV particles (17, 41). The antibodies to pre-S1 synthetic peptide 12–47 ay and pre-S2 synthetic peptide 133–151 ay used in this experiment were shown previously to neutralize HBV infection (17, 41) and to be directed against domains shown to contain epitopes involved in the binding of HBV to hepatocytes (27–29, 31). In addition, analysis of the topology of HBV envelope proteins (6, 7, 12, 13) on the membrane of the endoplasmic reticulum has demonstrated that the pre-S1 and pre-S2 domains of the large and middle proteins are exposed in the lumen of the reticulum. This is likely to represent their topology at the surface of the HBV virion (6, 7, 12, 13, 22, 23).

Taken together, these data strongly suggest that infectious HDV particles are coated with an envelope that contains at least the large HBV envelope proteins in addition to the small protein. It also suggests, but does not prove, that the pre-S1 or pre-S2 domain or both are involved in defining infectivity of HDV particles. This would indicate that the pre-S regions of HBV envelope proteins have similar functions on the envelopes of HBV and HDV. Despite this similarity, the infection step itself is likely to differ between the two viruses, as indicated by previous studies (26, 36). The HDV envelope has been described as containing low percentages of large and middle proteins (1 and 5%, respectively) in comparison with those of HBV (2). However, in contrast to HBV, which requires incorporation of large proteins for assembly of mature particles (6, 43), HDV may be able to assemble a particle including HDV RNA and hepatitis delta antigen proteins within an envelope made of the small HBV surface protein only. Those particles may be defective for infection, whereas only particles coated with an envelope made of the large, middle, and small proteins in proportions similar to those found in HBV particles may be infectious. Previously, the content of large and middle proteins of the envelope of infectious HDV may have been underestimated because of the presence in the pool of a substantial amount of particles coated with the small protein only. If this is true, the envelope of infectious HDV particles that include large and middle proteins may resemble that of mature HBV virions. Since the large and middle proteins of the HBV envelope, and specifically the pre-S domains, are likely to be involved in hepatocyte binding of HBV, they may fulfill the same functions for HDV but in a different manner: either by using a different receptor or by using the same receptor at a different level of efficiency.

We are currently investigating the requirement for each HBV envelope protein in the assembly of infectious HDV particles by attempting to produce particles with envelopes of different compositions.

The present study indicates that the large HBV envelope protein is indeed present on the surface of infectious particles and suggests that it is required for infectivity. Preliminary data (35a, 38a) indicate that the supply of the small protein only is sufficient for assembly of HDV RNA-containing particles. Whether particles devoid of pre-S1 and pre-S2 polypeptides are infectious is being tested.

The system described here will allow for the study of the HDV packaging mechanism, rapid analysis of the neutralizing ability of antibodies to the HBV envelope proteins, and mapping of the domain(s) of the HDV envelope involved in hepatocyte binding. Subsequently, experiments aimed at identification of the HDV receptor may be facilitated.

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