Experimental Transmission of Human Hepatitis Delta Virus to the Laboratory Mouse

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Human hepatitis delta virus (HDV), obtained from the serum of an experimentally infected woodchuck, was injected into either the peritoneal cavity or the tail vein of both adult CB17 mice and mice with a severe combined immunodeficiency (CB17-scid mice). Three lines of evidence indicated that the virus was able to reach the liver and infect hepatocytes: (i) the amount of HDV genomic RNA detected in the liver by Northern (RNA) analysis increased during the first 5 to 10 days postinoculation, reaching a peak that was about threefold the amount in the original inoculum; (ii) also detected in the liver was the viral antigenomic RNA, which is complementary to the genomic RNA found in virions, and is diagnostic for virus replication; and (iii) by immunoperoxidase staining of liver sections, the delta antigen was detected in the nuclei of scattered cells identifiable as hepatocytes. In all of the mice, clearance of the infection occurred between 10 and 20 days after inoculation. The half-life for clearance was about 3 days in CB17-scid mice, indicating that clearance of infection did not involve a T- and B-cell-dependent immune response. Cell-to-cell spread of the initial infection was not detected. One possible interpretation of our results is that HDV infection of hepatocytes is directly cytopathic. Also, the results imply that chronic infection of the liver in humans may require continuous spread of virus within the liver. Alternatively, HDV in the absence of helper virus may be unable to cause a chronic infection of hepatocytes in vivo.

Human hepatitis delta virus (HDV) is a naturally occurring subviral satellite of human hepatitis B virus (HBV). The assembly and release of HDV depends upon envelope proteins provided by HBV. The genome of HDV is a single-stranded RNA of about 1,700 bases in length. This RNA shares structural features with certain pathogenic subviral agents of plants known as viroids, in that it is a covalently closed circular molecule which is able to form an unbranched rod-like structure by intramolecular base pairing (11). The HDV genome replicates in the nucleus of infected cells by RNA-directed RNA synthesis, probably by using the polymerase II of the host (20). Genome replication requires the synthesis of a complementary RNA template, the antigenomic RNA (5, 16, 17a, 38, 40), which is not found in the virions. HDV encodes one protein, the delta antigen, which exists in two forms: a small 195-amino-acid polyepitope, δAg-S, and a large form, δAg-L, that is identical to δAg-S except for a 19-amino-acid carboxy-terminal extension. An RNA-editing process is needed to create the template for subsequent translation of the δAg-L mRNA (2, 42). δAg-S is required for genome replication, while δAg-L is an inhibitor of replication and required for assembly of HDV (3, 32, 40).

The histopathology and replication of HDV can be studied not only with liver biopsies taken from patients (15, 23, 30) but also by experimental transmission to chimpanzees (29, 31) and woodchucks (28, 29). For virus assembly, release, and transmission to occur, there has to be present in HDV-infected cells a helper virus, be it HBV for primates or woodchuck hepatitis virus (WHV) in woodchucks. It is also possible to study virus replication by infection of primary hepatocytes from humans, chimpanzees (36), and woodchucks (39). With the availability of cloned HDV sequences, it is even more convenient to study genome replication in permanent cell lines transfected with HDV cDNA and cRNA (9, 10, 19, 21, 42). By cotransfection of these cells with hepatitis B and hepatitis delta virus, the assembly of infectious virus can be achieved (33, 37).

To characterize the biology and pathogenesis of HDV infections in animals, it would be useful to have hosts smaller and more inbred than the chimpanzee and the woodchuck. Issues that remain to be addressed include the following. Does HDV cause chronic infections of hepatocytes? Does infection alter cell function and/or shorten the life span of normally long-lived hepatocytes? It has been reported that following the injection into the mouse peritoneal cavity of large doses of influenza virus, some virus was able to reach the liver, enter hepatocytes, and possibly induce virus replication (14, 22). Thus, the present study was undertaken to determine whether HDV could similarly infect a mouse. We were additionally encouraged to test this possibility because HDV is less host range restricted than its helper virus in that HDV can cross species (28, 31).

As reported here, we were successful in obtaining HDV infection of the mouse. Evidence is presented that the virus was able to reach the liver, infect cells identifiable as hepatocytes, and initiate replication of the HDV genome.

MATERIALS AND METHODS

Animals. Three woodchucks (Marmota monax) that were chronic carriers for WHV were infected with HDV-positive woodchuck serum (provided by J. Newbold, Chapel Hill, N.C.). When sacrificed at the peak of the acute HDV infection, 1 ml of the sera contained from 1 x 10^11 to 9 x 10^11 molecules of HDV RNA. These sera contained at least 500 times fewer molecules of WHV DNA. Adult mice (6 to 8 weeks old) were inoculated into either the peritoneal cavity (100 to 500 µl) or the tail vein (100 µl). Neonatal mice were injected with 100 µl into the peritoneal cavity. The mice used were either CB17 wild type or CB17-scid (1).

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RNA analysis. Total RNA was extracted from liver by using a modification of a method described previously (8). Briefly, tissue was homogenized in the presence of guanidinium isothiocyanate; then an equal volume of phenol equilibrated with sodium acetate (pH 4), was added, and the mixture was vigorously shaken. After the addition of 1/5 volume of chloroform-isoamyl alcohol (24:1), the sample was briefly centrifuged to separate phases. The aqueous phase was then transferred to another tube, and an equal volume of isopropanol was added. After incubation on ice for 5 min, the RNA precipitate was collected by centrifugation. The RNA pellet was resuspended in 1% sodium dodecyl sulfate, and a 0.5 volume of 3.5 M potassium acetate (pH 4) was added. After 5 min on ice, the sample was centrifuged, the resulting supernatant was extracted as described above with acid phenol and chloroform-isoamyl alcohol, and then the RNA was precipitated with ethanol. For Northern (RNA) analysis, RNA samples were glyoxalated and subjected to electrophoresis into horizontal gels of 1.5% agarose in phosphate buffer. The RNA was then transferred onto nylon membranes, and hybridization with HDV-specific probes was carried out as previously described (5). Quantitation was carried out by using either the direct scanning or the optical system of a radioanalytic imaging system (AMBIS, San Diego, Calif.).

Antisera. The HDV small delta antigen was expressed in Escherichia coli as a fusion protein with an amino-terminal tag of six histidines and purified by nickel chelate affinity chromatography as specified by the manufacturer (Qiagen, Chatsworth, Calif.) and used to immunize a rabbit.

Immunoperoxidase staining. Following euthanasia of the mice, pieces of liver were fixed with 10% formalin in phosphate-buffered saline, processed into paraffin wax, and sectioned at 4 μm onto glass slides. Delta antigen was detected by the standard peroxidase-antiperoxidase technique (13, 35). The incubation of the tissue sections with primary rabbit anti-delta antibody was followed by the sequential application of goat anti-rabbit immunoglobulin and peroxidase antiperoxidase (Organon Teknika, Durham, N.C.). Peroxidase activity was revealed by diaminobenzidine. Determination of the percentage of delta antigen-positive hepatocytes was performed by light microscopy on tissue sections. The number of positive hepatocytes was expressed as a percentage of the total hepatocytes examined (between 2,000 and 9,500 cells per section).

RESULTS
To determine whether HDV could infect a mouse, we used serum collected from woodchucks at the peak of an acute HDV infection and injected this serum directly into the peritoneal cavity of adult mice. We tested both normal CB17 strain mice and those with a severe combined immunodeficiency, CB17-scid mice (1). Mice were sacrificed at 5, 10, and 20 days postinoculation, and liver samples were assayed for evidence of HDV replication by Northern blot analysis. Figure 1A shows that HDV genomic RNA could be detected in the livers at 5 and 10 days after injection, and lower amounts were detectable at even 20 days. This HDV RNA was not just the inoculum virus because, as shown in Fig. 1B, we could also detect the HDV antigenomic RNA, which
is indicative of HDV genome replication (5). However, we could not detect any obvious difference between the results obtained for CB17 mice and to CB17-scid mice.

Given this result, we undertook a more thorough investigation of the time course of appearance and disappearance of HDV-related RNAs in the liver of injected mice. Twelve CB17-scid mice were injected with HDV, and pairs of mice were sacrificed at 1, 3, 5, 10, 16, and 20 days. Figure 2 shows the Northern analyses for both genomic, (Fig. 2A) and antigenomic (Fig. 2B) RNA. A quantitation of this more detailed set of data is presented in Table 1. We found that genomic RNA was detectable at days 1 and 3, reached a peak between days 5 and 10, and then progressively decreased by days 16 and 20. From an assay of the genomic RNA in the inoculum virus (Fig. 2A, lane 15), we deduced that the amount of genomic RNA in the liver at day 5 was about threefold more than the amount injected into the peritoneal cavity. This observation was consistent with the hypothesis that HDV was replicating in the liver. Additional and definitive evidence for HDV infection came from the detection of antigenomic HDV RNA (Fig. 2B). As expected, such antigenomic RNA was not detected in virions (e.g., Fig. 2B, lane 15), but its presence inside cells in amounts 5 to 20 times less than the amount of genomic RNA was indicative of HDV-specific RNA synthesis (5).

For the experiments presented above, the virus was injected directly into the peritoneal cavity. We also tested injection of virus into the tail vein. Using two groups of CB17-scid mice, we injected identical amounts of virus by either of these routes and then after 5 days isolated the liver RNA and examined it by Northern blot analysis for the amount of HDV genomic RNA. The results are shown in Fig. 3. From a quantitation of these data, we deduced that the two routes were of comparable efficiency, although the efficiency of the intravenous route was less reproducible, most probably for technical reasons, than that for the intraperitoneal route.

The various Northern assays described so far showed that HDV had replicated in the liver, but they did not show which cells were infected and to what extent. To answer this question, we used a second experimental approach, immunoperoxidase staining of fixed liver sections, so as to detect the HDV-specific protein, the delta antigen. An example of such staining is shown in Fig. 5A. This section was from a liver of a neonatal mouse at day 10 after injection. The delta antigen was detected in some cells. These cells were considered to be hepatocytes, both because of their appearance and by virtue of their occasional existence as binucleate tissues from two CB17-scid mice sacrificed at 5 days after injection. One mouse was injected into the peritoneal cavity, and the other was injected into the tail vein. For both animals, genomic RNA was detected in the liver (lanes 2 and 6) but not in the kidney (lanes 3 and 7), spleen (lanes 4 and 8), or lungs (lanes 5 and 9). From quantitation of results from a larger study in which we compared only kidney relative to liver, the amount of HDV genomic RNA was at least 50 times less in the kidney than in the liver (27). We conclude that the tissue specificity of HDV infections in mice is thus similar to that previously reported for HDV infections of woodchucks (12, 24).

FIG. 3. Northern analysis of HDV genomic RNA in the livers of infected CB17-scid mice by injection into either the peritoneal cavity or the tail vein. Lanes 2 to 8 represent mouse liver RNA samples (5 μg per lane). Lane 2 was from an uninfected animal; lanes 3 to 8 represent RNA from mice 5 days after being inoculated with $10^{10}$ particles of HDV into either the tail vein (lanes 3 to 5) or the peritoneal cavity (lanes 6 to 8). Lane 1 shows liver RNA (15 ng) from a woodchuck at the peak of an acute HDV infection, and lane 9 shows a standard of HDV RNA (1 ng). At the right side is indicated the mobility of 1.7-kb HDV RNA. The autoradiogram was obtained from an exposure of 5 h. i.v., intravenous; i.p., intraperitoneal.

FIG. 4. Northern analysis of HDV genomic RNA in the tissues of infected CB17-scid mouse. Membranes were hybridized with an RNA probe to detect genomic RNA. Lanes 2 to 9 represent RNA (8 μg per lane) from the tissues of two mice, isolated at 5 days after injection of $10^{10}$ particles of HDV either into the peritoneal cavity (lanes 2 to 5) or into the tail vein (lanes 6 to 9). The tissues examined were liver (lanes 2 and 6), kidney (lanes 3 and 7), spleen (lanes 4 and 8), and lung (lanes 5 and 9). Lane 1 shows liver RNA (15 ng) from a woodchuck at the peak of an acute HDV infection, and lane 10 shows a standard of HDV RNA (1 ng). At the right side is indicated the mobility of 1.7-kb HDV RNA. The autoradiogram was obtained from an exposure of 3 h. i.v., intravenous; i.p., intraperitoneal.

**TABLE 1.** Quantitation of HDV markers in livers of CB17-scid mice inoculated with HDV

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Time after infection (days)</th>
<th>HDV RNAa</th>
<th>Positive hepatocytes (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>51</td>
<td>&lt;8 0.03 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>&lt;14</td>
<td>&lt;8 0.04 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>58</td>
<td>20 0.05 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>410</td>
<td>116 0.60 ± 0.16</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>360</td>
<td>72 0.32 ± 0.09</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>400</td>
<td>42 0.20 ± 0.10</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>400</td>
<td>68 0.10 ± 0.03</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>90</td>
<td>&lt;8 0.01 ± 0.01</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>55</td>
<td>&lt;8 0.08 ± 0.06</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>14</td>
<td>&lt;8 0.04 ± 0.04</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>28</td>
<td>&lt;8 0.04 ± 0.04</td>
</tr>
</tbody>
</table>

a Liver RNA samples were assayed for HDV genomic and antigenomic RNA as described for Fig. 2A and B, respectively. Quantitation was obtained with a radioanalytic imaging system. Relative to HDV RNA standards, the results are expressed as nanograms of HDV RNA per milligram of liver RNA.

b Values were obtained from immunoperoxidase-stained sections as in Fig. 5. The indicated errors are the standard deviations based upon the square root of the number of cells counted.
cells. Also, the staining was largely restricted to the nuclei of these cells, just as others have found typical for HDV infections (26, 41). The staining was similar to that observed with the positive control (Fig. 5B), which was obtained with a liver section from a woodchuck taken at the peak of an acute HDV infection. In both the mouse and the woodchuck, we saw no evidence of a focal localization of positive cells.

We next quantitated the fraction of immunoperoxidase-positive cells in liver sections from the mice sacrificed at various time points after injection with HDV. These were the same animals as examined for HDV-specific RNA in Fig. 2 and Table 1. The immunoperoxidase data are also summarized in Table 1. We detected positive cells at day 3 but not at day 1 postinoculation. The percentage increased to a maximum of about 0.5% at day 5. This percentage dropped off somewhat by day 10, and HDV-positive hepatocytes were not detected at day 20. The time course data were therefore consistent with the Northern blot data for HDV genomic and antigenomic RNAs in the liver.

A comparable quantitation in the woodchuck at the acute phase of HDV infection (Fig. 5B) indicated around 25% positive hepatocytes. This value was about 50 times higher than that in adult mice at day 5 after injection. (Such a difference was not surprising because WHV present in the woodchuck would have facilitated the assembly and spread of progeny HDV.) Also, from these assays in combination with the Northern assays, we deduced that the extent of HDV genome replication per hepatocyte was about the same for both the mouse and the woodchuck. From the data summarized in Table 1, we deduced a half-life for the clearance of HDV infection from the mouse liver. From the change in the amount of genomic RNA between days 10 and 20, the half-life of clearance was estimated to be about 2.5 days. From the change in the number of positive-staining cells between days 10 and 20, a half-life of clearance of about 3 days was obtained. Because these experiments were performed in CB17-scid mice, the observed clearance of HDV-specific RNA sequences and positive-staining cells must be independent of both T and B cells.

**DISCUSSION**

We have obtained three lines of evidence that HDV injected into the mouse liver and infects hepatocytes: (i) after 5 to 10 days, the amount of genomic RNA in the liver was threefold more than the inoculum; (ii) antigenomic RNA, which is diagnostic of genome replication, was also detected in the liver; and (iii) using immunoperoxidase staining, we demonstrated delta antigen in the nuclei of cells identified as hepatocytes and distinct in appearance from Kupffer cells. The infection that we observed was only transient. Between days 10 and 20 postinoculation, HDV was rapidly and almost completely cleared from the liver. These findings have significant implications for several areas of HDV research.

First, consider how the virus reached the hepatocytes. For the experiments summarized in Table 1, the virus was injected into the peritoneal cavity. Similar results were obtained following injection into the tail vein (Fig. 3). Our interpretation is that for each of these different routes of administration, the virus was able to reach the liver via the bloodstream. The next question is how the virus was then able to enter hepatocytes. More experiments are needed to determine whether attachment and entry were by the same mechanism, and even by the same, as yet unidentified receptor, as is used by the WHV pseudotype of HDV to infect woodchucks. Maybe other important human viruses, such as hepatitis C virus, for which there are at this time only primate models, might be profitably studied by using such a mouse model.

From the levels of staining in immunoperoxidase-positive cells, it would seem that once HDV entered the mouse hepatocytes, the levels of HDV genome replication were comparable to those obtained in a natural infection of woodchuck hepatocytes (Fig. 5). This ability to achieve a comparable level of replication in the mouse hepatocyte was not surprising, since upon transfections of cultured cell lines with HDV cDNA, we have previously observed efficient induction of HDV genome replication in lines from different animal species and from tissues other than liver (4, 17). A pertinent example was transfection of mouse fibroblasts (4). However, in the present studies, the only tissue in which we detected HDV replication was the liver. Thus, in the mouse, as in the woodchuck and primate (12, 24), the limiting event for HDV infection and replication seems to be virus entry.

The level of HDV-specific markers did not increase beyond day 10. This finding was not unexpected because HDV is known to require for assembly and release the presence, in the same cell, of a hepadnavirus, such as HBV or WHV. The inoculum used in our studies contained less than 1 WHV DNA molecule per 500 molecules of HDV RNA. In other
words, the chance of a coinfection was minimal. Moreover, even if WHV could infect mouse hepatocytes as efficiently as could HDV, then in the experiments described above, that amount of WHV would have been undetectable. In future experiments, it will be necessary to determine whether WHV, in amounts comparable to those of HDV that we have used, can lead to a detectable infection of the mouse liver and, if so, whether a coinfection with comparable amounts of the two viruses would lead to the assembly of new HDV particles. An alternative approach to obtain HDV assembly might be to use HDV to infect mice transgenic for HBV (1a, 6, 7) or WHV. This could also lead to HDV release and possibly virus spread.

After day 10, we invariably saw a decrease in the level of HDV-infected hepatocytes. The rate corresponded to a loss of about 50% HDV-specific markers per 3 days. We can infer that T and B cells were not involved, in that the rate of this decrease was the same in both normal mice and those with a severe combined immunodeficiency, the CB17-scid mice (1). This being the case, three possibilities can be proposed as the basis for this decrease.

(i) The HDV infection could have directly caused cell death. Others have claimed from in vitro studies that the presence of the delta antigen, in the absence of HDV genome replication, shows some cytopathic effects; they reported pyknotic nuclei and karyolysis (9, 10). However, in contrast to this finding, Sureau et al. (36), in another in vitro study that monitored the infection of primary chimpanzee hepatocytes, observed that the infection was maintained for at least 42 days and without any obvious cytocidal effects. In addition, it should be noted that in vivo studies, based upon patient biopsies, are currently being assessed more cautiously in terms of ascribing direct cytopathic effects to HDV infections; more specifically, Negro et al. (25) reported that at most a minor fraction of infected hepatocytes showed any cytopathic effects; most cells seemed healthy. Our data for the infected mouse agree to the extent that we detected no obvious cytopathic effects via the light microscope. Our inability to observe such an effect does not exclude the existence of the effect. We say this because the observed rate of loss per unit time for positive-staining cells was relatively slow, and cells once killed could have been rapidly cleared.

(ii) A second possibility is that the infected hepatocytes disappeared as part of a natural level of cell death. This possibility must be considered unlikely in the face of knowledge obtained by other assays; specifically, hepatocytes are claimed to have life spans of 150 days (34) or more (18).

(iii) A third possibility is that the infected cells essentially cured themselves of the infection. Certainly the large form of the delta antigen is expected to accumulate during genome replication, and this protein is a dominant negative inhibitor of genome replication (4, 40). It has not been shown that this phenomena either controls the level of HDV RNA accumulated per infected cell or thereby leads to a net loss of HDV RNA per cell. Such a loss would be gradual and is thus inconsistent with our data (Table 1); that is, we observed that the rate of disappearance of HDV from the liver was essentially the same for total HDV RNA as for the loss of delta antigen-positive cells.

Of these three possibilities, we would favor the first; that is, HDV infections in an animal, and at least in the mouse, can lead directly to cell death without the involvement of a T- and B-cell-dependent immune response. This interpretation needs to be further qualified by pointing out that our observations refer to a situation in which virus assembly and release were not occurring. Nevertheless, it should be expected that additional studies of the variables involved in the clearance of infected hepatocytes will provide further information valuable for understanding HDV-induced liver disease. The convenience of this mouse model for HDV infection, combined with the powerful systems of mouse genetics, has considerable potential. We expect that this approach might have unique advantages in some areas of research on hepatotropic viruses, such as receptors, assembly, clearance, and the testing of antiviral agents and the interference between viruses.

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