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Apparent Helper-Independent Infection of Woodchucks by Hepatitis Delta Virus and Subsequent Rescue with Woodchuck Hepatitis Virus

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Hepatitis delta virus (HDV) is a subviral agent of humans which is dependent upon hepatitis B virus as a helper for transmission. HDV can be experimentally transmitted to woodchucks by using woodchuck hepatitis virus (WHV) as the helper. We used this model system to study two types of HDV infections: those of animals already chronically infected with WHV and those of animals without any evidence of prior exposure to WHV. At 5 to 10 days after infection with HDV, liver biopsies of these two groups of animals indicated that around 1% of the hepatocytes were infected (HDV antigen positive). Moreover, similar amounts of replicative forms of HDV RNA were detected. In contrast, by 20 days postinfection, the two groups of animals were quite different in the extent of the HDV infection. The animals chronically infected with WHV showed spread of the infection within the liver and the release of high titers of HDV into the serum. In contrast, the animals not previously exposed to WHV showed a progressive reduction in liver involvement, and at no time up to 165 days postinfection could we detect HDV particles in the serum. However, if these animals were inoculated with a relatively high titer of WHV at either 7 or even 33 days after the HDV infection, HDV viremia was observed. Our data support the interpretation that in these animals, hepatocytes were initially infected in the absence of helper virus, HDV genome replication took place, and ultimately these replicating genomes were rescued by the secondary WHV infection. The observation that HDV can survive in the liver for at least 33 days in the absence of coinfecting helper virus may be relevant to the reemergence of HDV infection following liver transplantation.

Human hepatitis delta virus (HDV) is a subviral agent with a small single-stranded RNA genome (18). Replication of this genome takes place by RNA-directed RNA synthesis using the host RNA polymerase II (7, 10) and is also dependent upon a single, virus-encoded protein, the delta antigen (9). Moreover, assembly of HDV genomes into progeny virions is dependent not only upon the delta antigen but also upon envelope proteins provided by a second, helper virus (18). The natural helper of HDV is human hepatitis B virus (HBV), but HDV can be experimentally transmitted to woodchucks, in which woodchuck hepatitis virus (WHV) can serve as a helper for subsequent intrahepatic spread (15). Because of the helper requirement, the serum of an infected host must contain not only HDV but also at least some hepadnavirus, be it HBV or WHV.

HDV infections of humans are typically described in terms of the nature of the associated helper virus infection. The most commonly recognized infection, probably because it is most damaging, is a superinfection in which an established chronic hepadnavirus infection is exacerbated by infection with HDV. A second type of infection, known as a coinfection, is one in which both viruses are received at the same time. With the extensive practice of liver transplantation, a third class has been recognized. This is an infection which occurs within 2 weeks of liver transplantation, apparently by HDV alone (5, 8, 19, 24). Such helper-independent infections are expected to be nonproductive, although they may become productive because of a subsequent infection with helper HBV. The present experiments were carried out to begin to determine how long a nonproductive infection will survive in the liver before it is no longer rescuable by superinfection with a helper virus. Our results revealed that HDV can survive for at least a month.

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MATERIALS AND METHODS

Infections and liver biopsies. For these studies, woodchucks were housed either at the Fox Chase Cancer Center or at Cornell University, Ithaca, N.Y. Prior to all procedures, woodchucks were anesthetized either by intramuscular injection of ketamine or by intraperitoneal injection of both ketamine and xylazine. For HDV infection, we used the sera from two woodchucks; these animals were chronic carriers of WHV that had been infected with HDV and subsequently sacrificed at the peak of the ensuing HDV viremia. A volume of 0.1 to 0.5 ml of such infectious serum, containing a total of around 2×10^{11} particles, was injected into the anticubital vein of the hind leg. For WHV superinfections, we pooled sera from three animals chronically infected with WHV. A total of 18 ml of this infectious serum was injected into the portal vein; this contained 7.5×10^{10} particles. Biopsies were performed by surgery through an epigastric paramedian incision and removal of liver tissue (0.5 to 1 g). Blood samples (1 ml) were taken from the femoral artery.

Serum assays. To assay HDV RNA, serum samples (20 μl) were extracted by a sodium dodecyl sulfate (SDS)-pronase procedure (3). RNA treated with glyoxal was then quantitated either by a slot blot assay or by Northern (RNA) analysis (3). Antidelta antibody was assayed with an enzyme-linked immu-

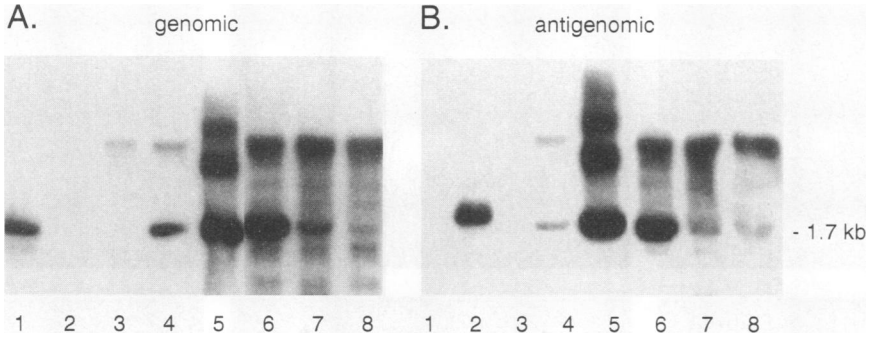

FIG. 1. Northern analysis of HDV RNA in liver biopsy samples. Extracted RNA was treated with glyoxal, and aliquots were subjected to electrophoresis on gels of 1.5% agarose followed by Northern analysis to detect HDV genomic RNA (A) and antigenomic RNA (B). Lanes 1 and 2, 1.8-kb HDV genomic and antigenomic RNA standards (1 ng), respectively, as synthesized in vitro. Lanes 3 to 5 are biopsy RNA samples (0.5 μg) taken at 90 days before and 10 and 21 days after HDV superinfection of a woodchuck chronically infected with WHV. Lanes 6 to 8 are similar RNA samples (5 μg) taken at 5, 25, and 62 days after HDV infection of a woodchuck with no prior exposure to WHV. At the right side is indicated the position of unit-length 1.7-kb HDV RNA. For this figure and Fig. 2, images were digitized and processed with Adobe Photoshop and Canvas software.

nosorbent assay (ELISA) kit, used according to the manufacturer's instructions (Abbott).

To assay WHV DNA, serum samples were extracted by an SDS-pronase procedure, treated with glyoxal, and then quantitated by Southern analysis. The tests for WHV surface antigen, antisurface antibody, and anticore antibody were performed in duplicate by using enzyme immunoassays as previously described (4).

Liver RNA extraction and analysis. Liver RNA was extracted with guanidine isothiocyanate as previously described (14). For Northern analysis, RNA samples were treated with glyoxal and subjected to electrophoresis on horizontal gels of 1.5% agarose in phosphate buffer. After transfer of the samples to a nylon membrane, HDV genomic RNA was detected as previously described (14).

Immunofluorescence assays of liver sections. Liver biopsy samples were prepared as described by Netter et al. (14). Paraffin-embedded sections, fixed with formalin, were triple stained essentially as previously described (1). Sections were blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 15 min at 37°C; blocking was followed by a series of incubations, each for 1 h at 37°C, with the following antibodies, each diluted in PBS. The first antibody, specific for the hepatitis delta virus antigen, was raised in a rabbit by inoculation of the delta antigen prepared in *Escherichia coli*. The second antibody, used to detect WHV core antigen, was a mixture of two mouse anti-HBV core monoclonal antibodies (10E11 and 14E11) which are known to react with the core of WHV (1, 2). The third antibody was goat anti-rabbit immunoglobulin G (IgG) polyclonal antibody conjugated with fluorescein isothiocyanate (Cappel). The fourth antibody was goat anti-mouse immunoglobulin G plus immunoglobulin M conjugated with Texas red (Cappel). In the final incubation the cellular DNA was stained by the addition of 2 μg of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) per ml. After being mounted, the samples were viewed with a Zeiss Axiophot microscope with a 40× objective and specific filter blocks.

RESULTS

Woodchuck infections. The following studies were undertaken to compare and contrast HDV infections initiated in woodchucks. As the inoculum, we used sera from HDV-infected woodchucks. The amount used contained 2×10^{11} particles of HDV and around 1,700 times less WHV. Since the liver of a woodchuck contains around 10^{10} hepatocytes, the multiplicity of HDV infection could have been as high as 20 per cell. Even though the inoculum contained much less WHV, we calculate that as many as 1% of the hepatocytes could have been coinfected directly by it.

To study superinfection, we used woodchucks whose sera tested positive for WHV DNA. To study coinfection, we used animals that were negative for all markers of prior WHV infection (DNA, surface antigen, antisurface antibody, and anticore antibody). Woodchuck infections were initiated by the injection of the inoculum into the leg vein. Both prior and subsequent to this injection we examined both liver biopsy samples and serum samples for evidence of replication of HDV and WHV.

Liver markers. HDV was used to establish superinfections and coinfections. Liver biopsy samples taken from animals with these infections were subjected to RNA analysis to detect HDV genome replication. Total RNA was extracted and then treated with glyoxal prior to agarose gel electrophoresis and Northern transfer to detect HDV-related RNA species. Results of assays for HDV genomic and antigenomic RNA are presented in Fig. 1A and B, respectively. There was a clear time-dependent increase in unit-length 1.7-kb HDV genomic and antigenomic RNAs; between 10 and 21 days the increase was 5.6-fold. A quite different result was obtained for the coinfection of an animal with no prior exposure to WHV. The HDV genomic and antigenomic RNAs were readily detected at day 5 (lane 6). The amounts decreased significantly by day 25 (lane 7) and were almost at background levels by day 62 (lane 8). From quantitation, the decrease was determined to be 5.6-fold for days 5 to 25. It should be noted that 10 times more RNA was analyzed in lanes 6 to 8 than in lanes 3 to 5. Since the amount of detected genomic RNA decreased with time, one could argue that we were detecting not genome replication but simply the progressive clearance of inoculum virus. However, since we were also able to detect antigenomic RNA, which is not present in the inoculum, our data support the interpretation that the HDV genome replicated. As discussed below, additional data were needed to determine whether this genome replication was associated with coinfection of hepatocytes by WHV, so as to allow the assembly and release of progeny HDV particles.

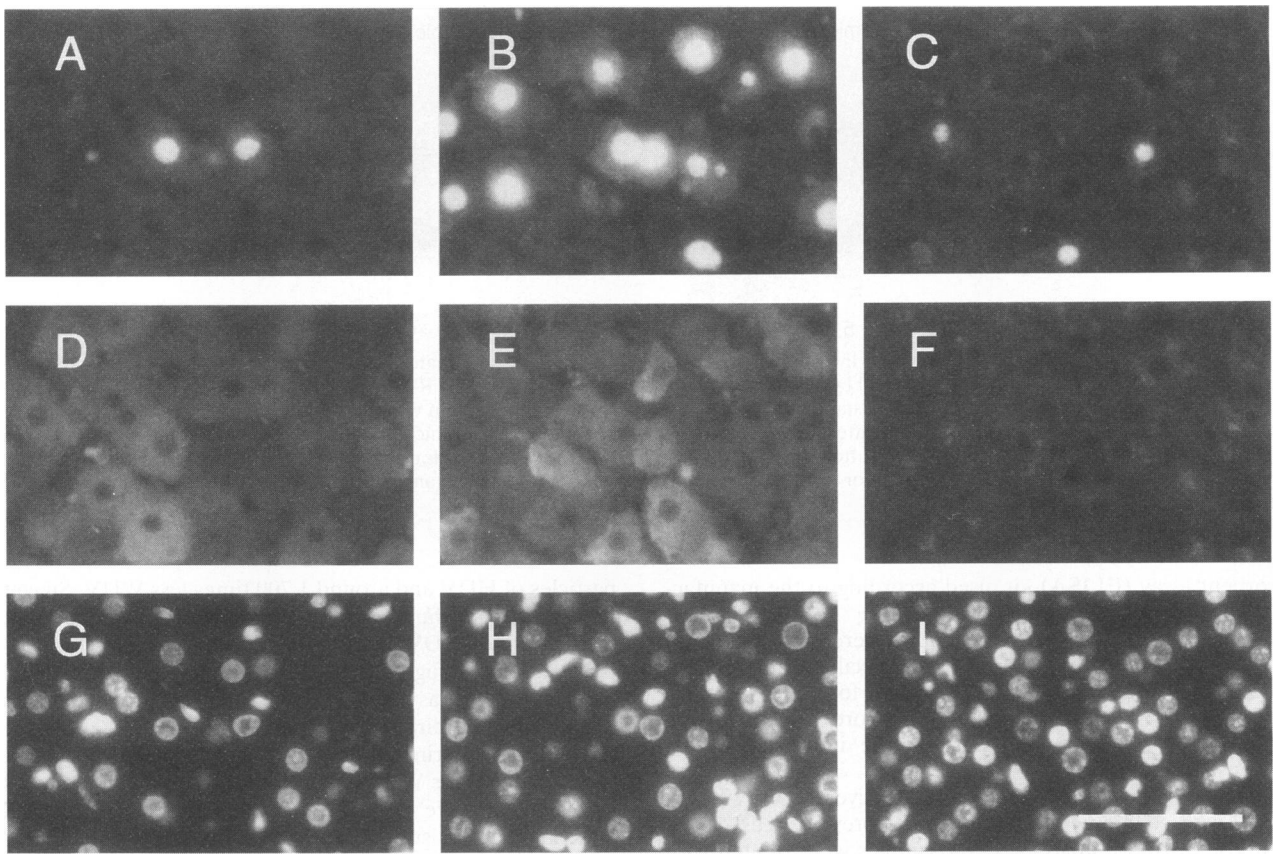

FIG. 2. Immunofluorescence of liver sections. Each liver biopsy sample was triple stained to detect HDV antigens (A to C), WHV core antigen (D to F), and DAPI staining of nuclear DNA (G to I). This was done for biopsy samples taken at 10 days after HDV superinfection of an animal chronically infected with WHV (A, D, and G), at 21 days after HDV superinfection of an animal chronically infected with WHV (B, E, and H), and at 5 days after HDV coinfection of a woodchuck without prior exposure to WHV (C, F, and I). Each column shows triple staining from the same field. Bar, 50 μm.

As a second approach to study virus replication in the liver biopsy samples, we used immunofluorescence. For these studies we examined each liver section in three ways: for delta antigen and for WHV core antigen by immunofluorescence and also by staining for total DNA. As shown in Fig. 2, it was possible to view a single field and to separate these three sources of information. Ten days after the HDV superinfection we detected delta antigen in the nuclei of a small fraction of the hepatocytes (Fig. 2A), while the WHV core antigen was present, as expected, in almost all hepatocytes (Fig. 2D). With DAPI staining we could detect each of the nuclei in the field (Fig. 2G). For this and other liver biopsy samples, we determined the percentage of HDV antigen-positive cells. Such data, summarized in Table 1, indicated that for this animal at 10 days after superinfection, 0.6% of the hepatocytes contained HDV antigen.

An autopsy sample was taken from the superinfected animal at day 21, a time at which we expected the HDV infection to have substantially spread. Consistent with this expectation, we observed HDV antigen in around 18% of the hepatocytes (Fig. 2B and Table 1), with WHV core antigen still in most hepatocytes (Fig. 2E).

We also examined the biopsy samples from three animals with no prior exposure to WHV that were coinfected with HDV and WHV. At day 7 a small fraction of hepatocytes did contain HDV antigen (Fig. 2C), and no WHV core was detected (Fig. 2F). From a quantitation of this biopsy sample and those from two other similarly infected animals, assayed at 5 and 7 days, we found, as summarized in Table 2, that 0.7 to 2.2% of the hepatocytes were positive for HDV antigen. These values were not significantly more than what was obtained for

TABLE 1. Immunofluorescence detection of delta antigen in liver sections of HDV-infected woodchucks

Woodchuck	No. of days after HDV injection	% Liver cells positive for delta antigen (no. of positive cells/total)[a]
A, with prior WHV chronic infection		
	10	0.59 (18/3,100)
	21	17.27 (125/720)
B, without prior exposure to WHV		
	5	0.95 (40/4,200)
	7	0.70 (18/2,600)
	7	2.21 (63/2,900)
	25	0.18 (20/11,000)
	62	<0.02 (<1/4,300)
	126	<0.02 (<1/4,700)

[a] Liver biopsy samples were processed as for Fig. 2 and in addition stained with hematoxylin. In this way we quantitated the total cells and determined the percentage of cells staining positive for delta antigen.

TABLE 2. Quantitation of serum markers in HDV-infected woodchucks[a]

Woodchuck	No. of days after HDV injection	No. of HDV RNA molecules/ml, 10^9	HDV antidelta antigen	No. of WHV DNA molecules/ml, 10^9	WHV surface antigen	WHV antisurface antigen	WHV anticore antigen
A, with prior chronic WHV infection							
	0	<0.05	0.3	5.10	45.7 (+)	ND[b]	28.4 (+)
	7	<0.05	0.5	2.44	56.2 (+)	ND	28.9 (+)
	14	0.21	0.5	6.91	52.3 (+)	ND	27.2 (+)
	21	0.81	0.6	6.70	56.8 (+)	ND	25.6 (+)
	28	5.5	0.5	6.42	42.9 (+)	ND	26.1 (+)
	35	65	0.6	4.96	42.3 (+)	ND	29.2 (+)
	41	180	0.5	3.84	49.3 (+)	ND	29.1 (+)
	49	340	0.6	2.51	48.4 (+)	ND	29.0 (+)
	56	430	1.0	1.75	50.7 (+)	ND	27.6 (+)
	63	170	7.5 (+)	1.68	50.3 (+)	ND	28.9 (+)
	70	120	15.0 (+)	1.88	55.0 (+)	ND	28.4 (+)
	73	100	12.0 (+)	1.12	55.4 (+)	ND	26.8 (+)
B, without prior exposure to WHV							
	5	<0.05	0.4	<0.005	0.2	0.5	3.5
	19	<0.05	0.4	<0.005	0.0	0.1	0.8
	36	<0.05	0.5	<0.005	0.0	2.6	0.6
	62	<0.05	0.6	<0.005	0.2	1.7	21.6 (+)
	92	<0.05	0.4	<0.005	0.3	1.2	13.0 (+)
	109	<0.05	0.4	<0.005	0.4	0.7	9.5 (+)
	126	<0.05	0.4	<0.005	0.3	0.9	9.3 (+)
	152	<0.05	0.4	<0.005	0.1	2.0	7.1 (+)
	165	<0.05	0.5	<0.005	0.2	0.7	7.1 (+)
C, without prior exposure to WHV but superinfected with WHV at day 7							
	0	<0.06	0.7	<0.003	0.0	0.5	1.2
	7	<0.06	0.7	<0.003	0.0	0.3	1.5
	15	0.19	0.6	0.08	5.3 (+)	0.4	17.2 (+)
	20	1.05	0.7	0.21	7.0 (+)	1.0	13.3 (+)
	33	<0.06	0.8	0.14	0.8	0.4	6.7 (+)
	42	<0.06	0.5	<0.003	0.3	2.2	5.6 (+)
	50	<0.06	0.6	<0.003	0.4	23.8 (+)	7.1 (+)
	61	<0.06	0.6	<0.003	0.4	22.4 (+)	17.8 (+)
	75	<0.06	0.8	<0.003	0.4	11.5 (+)	15.4 (+)
D, without prior exposure to WHV but superinfected with WHV at day 33							
	0	<0.09	0.4	<0.0008	0.3	0.0	0.7
	20	<0.09	0.4	<0.0008	0.3	0.0	0.9
	42	<0.09	0.4	0.35	9.7 (+)	0.0	16.9 (+)
	50	<0.09	0.5	1.46	30.5 (+)	1.8	10.9 (+)
	82	<0.09	0.5	0.43	36.9 (+)	0.8	16.8 (+)
	95	<0.09	0.5	1.00	31.1 (+)	0.8	20.0 (+)
	102	0.46	0.5	1.04	29.7 (+)	1.3	19.7 (+)
	116	<0.09	0.5	0.82	28.3 (+)	1.3	24.4 (+)
	125	<0.09	0.6	0.69	30.1 (+)	1.0	25.9 (+)

[a] The four animals (A to D) were infected with HDV as described in Results. The serum assays were performed as described in Materials and Methods. The serology for WHV markers was performed at least in duplicate; values considered positive are >3.1 and are indicated with a plus sign in parentheses. The antidelta values that are >1.0 are considered positive.

[b] ND, not determined.

the animal superinfected with HDV (Fig. 2A and Table 1). Also as summarized in Table 2, when biopsy samples were taken at later times, we observed the decline of the levels of delta antigen to lower (0.18% at day 25) and ultimately undetectable levels (<0.02% at days 62 and 126).

In summary, these results from both the Northern analyses and immunofluorescence support the interpretation that the HDV genome replicated in the livers of both superinfected and coinfected animals. At 5 to 10 days after HDV injection these infections were apparently qualitatively and quantitatively very similar, at least as judged by these assays. However, these assays demonstrated only genome replication. In order to assay for virus assembly, we examined serum samples taken from the infected animals.

Serum markers. It was possible to take serum samples from the infected animals virtually every week. These were assayed for the presence of HDV RNA, antidelta antibody, WHV DNA, WHV surface antigen, antisurface antibody, and anticore antibody. Selected data for four animals (A to D) are presented in Table 2.

Animal A exemplifies the superinfection with HDV of an animal already chronically infected with WHV. As expected,

the animal was consistently positive for markers of WHV replication (WHV DNA, surface antigen, and anticore antibody). The HDV RNA reached a peak of around 4×10^{11} particles per ml at around 50 days. These results were as expected for a superinfection on the basis of the previous studies of others (11, 12, 17).

We next examined the serum markers of animals with no prior exposure to WHV that were coinfected with HDV. Some of the data for one such animal are summarized in Table 2. As expected, this animal (animal B) was initially negative for all WHV markers. At no time did this animal show detectable levels of WHV DNA or surface antigen. However, between days 36 and 62 it did become positive for anticore antibody. In a second woodchuck (13), the response similarly appeared between days 21 and 28. This pattern of anticore antibody response is typical of an acute WHV infection. Consistent with this interpretation, a liver biopsy sample taken from the first animal at day 126 showed about 10% of the hepatocytes as staining positive for WHV core antigen (13). These animals nevertheless failed to show detectable levels of HDV RNA in serum ($<5 \times 10^7$ particles per ml). Our data cannot distinguish between two possible interpretations: either HDV assembly did not occur at all, or HDV assembly did occur at a low level but for one or more reasons (including detection sensitivity) was not detectable in serum, even as late as 165 days after infection.

As an additional assay of possible HDV replication, we tested the serum samples for antibody directed against the delta antigen. As indicated in Table 2, animal B failed to develop measurable antidelta antibody. In contrast, animal A, which was superinfected with HDV, did become positive by day 63. However, for another superinfected woodchuck, one that achieved viremia of only 10^{10} particles per ml, the antibody assays never went above background level (13). Apparently, the level of replication and/or sensitivity was insufficient for the development of a measurable level of antidelta response.

Rescue of HDV infection with WHV. Additional experiments were designed in order to better understand the nature of the HDV infection in the animals not previously exposed to WHV. The liver biopsy analyses clearly showed that the HDV had initially infected hepatocytes, leading to genome replication. However, it was not clear whether the helper virus present in the inoculum did or did not ever infect some of these same hepatocytes. Our approach was therefore to challenge animals not previously exposed to WHV with a relatively high titer of WHV helper virus and determine if this could lead to the rescue of HDV and the production of viremia. The results of two such studies are summarized in Table 2. In these studies we waited 7 and 33 days before administering WHV to animals C and D, respectively, and then monitored the animals for serum markers for HDV and WHV replication. As can be seen in Table 2, both animals promptly became positive for WHV surface antigen and anticore antibody. With these markers it was not possible to distinguish between passive acquisition and active replication. However, the levels of WHV DNA in the sera of both animals increased in a time-dependent manner, which could only be explained as the consequences of viremia. Subsequently the courses of these two infections differed. Animal C cleared the viral DNA and seroconverted to WHV anti-surface antibody positivity, consistent with an acute infection. In contrast, animal D did not clear the viral DNA, consistent with a chronic infection.

Our expectation was that the delay of 7 days would be sufficient but that 33 days might be too long to wait and still achieve rescue of HDV. Actually, as can be seen in Table 2, HDV rescue was achieved under both conditions. The maximum detected level of virus was 0.5×10^9 to 1×10^9 particles per ml, which was not an insignificant amount but nevertheless was at least 400 times less than what was achieved when HDV was administered to a woodchuck already chronically infected with the helper WHV (animal A; Table 2). Note also that the time required for HDV rescue was much shorter for animal C ($20 - 7 = 13$ days; Table 2) than for animal D ($102 - 33 = 69$ days; Table 2). Also, rescue of HDV in the animal D may have been facilitated by the WHV infection becoming chronic in this animal.

DISCUSSION

The most efficient transmission of HDV is known to occur by the superinfection of an animal already chronically infected with hepadnavirus (11, 15, 18). Less efficient is a coinfection in which both the HDV and hepadnavirus are transmitted at the same time to an animal with no prior exposure to the hepadnavirus. However, not all coinfections are equal. Since the typical inoculum for the HDV infection is the blood of an animal infected with both HDV and a hepadnavirus, the absolute and relative amounts of HDV to hepadnavirus in the inoculum are both highly variable. In our studies, the ratio was 1,700, within the range of 10 to 100,000, as measured by others (16, 17). One reasonable generalization is that the HDV will be in excess relative to the hepadnavirus. This excess means that early in a coinfection, the chances of a hepatocyte being truly coinfected are low. Also, if the amount of inoculum is sufficiently reduced, it is possible to reach a dose at which HDV infection is achieved in the absence of hepadnavirus (17a). What we have found in this study is that after the coinfection of a woodchuck with a ratio of HDV/WHV of 1,700:1, the animal did not produce detectable HDV viremia. This is not to say that WHV did not initiate an infection; as can be seen in Table 1, animal B did produce antibody to WHV core antigen by day 60, and core antigen was detected in a liver biopsy sample taken at day 126 (13). Our interpretation is that the number of hepatocytes infected with both HDV and WHV was not sufficient for assembly and spread of HDV.

The HDV dose used in our studies was about 20 HDV particles per hepatocyte, and we expected to infect most of the hepatocytes. In contrast, by immunofluorescence we observed that an average of only 0.6% of the hepatocytes became positive for HDV during the first 10 days (Table 1). Consider the three following explanations for this huge discrepancy. (i) The number of particles per infectious unit may be much greater than one. However, on the basis of similar studies of others, the ratio is expected to be around 10 (17). (ii) The delivery of particles from the leg vein to the liver might be inefficient. (iii) It might be that our immunofluorescence assay measures only a minority of cells, those that replicate HDV with very high efficiency. Consistent with this third explanation, we previously found that even with massive doses of HDV injected into the mouse (equivalent to about 1,000 particles per hepatocyte), we could detect HDV replication in the liver but again in only 0.6% of the hepatocytes (14). Intriguingly, yet another example of around 1 to 2% of the hepatocytes being infected with HDV comes from studies in which primary cultures of woodchuck hepatocytes were exposed to as many as 5,000 HDV particles per cell (22).

Even though the inoculum for our studies contained 1,700 times less WHV than HDV, it still should have been possible that some of the hepatocytes were truly coinfected with both viruses. However, two animals, each with no prior exposure to WHV, did not go on to produce detectable HDV viremia

(Table 2). It is possible that the results for two animals do not exclude the possibility of individual animal variation. Nevertheless, for these animals either coinfection did not occur or it occurred but was not sufficient to produce enough progeny HDV to achieve detectable viremia.

What may be most interesting about this study is that when we challenged such animals with a large dose of WHV, HDV was rescued and the animals did develop HDV viremia. This was true for both a delay of 7 days and a delay of even 33 days (animals C and D; Table 2). We do not think that during this delay the HDV was hiding in extrahepatic sites; this is unlikely because Negro et al. (12) and Dourakis et al. (6) have reported that HDV replication cannot be detected in sites other than the liver. Netter et al. have also looked without success for extrahepatic sites in mouse infections (14). We favor the interpretation that HDV genome RNA was maintained by helper-independent infections of a sufficient number of hepatocytes until such time as WHV reached such cells, leading to assembly and virus spread. We estimated the stability of these hepatocytes in two ways: on the basis of quantitation of cells positive by immunofluorescence used to detect delta antigen (Table 1) and by Northern analysis used to detect HDV RNAs (Fig. 1). Both assays indicated that after HDV injection, HDV genome replication could be detected in infected hepatocytes and reached a maximum involvement at 5 to 7 days, after which there was a drop-off, with a half-life of around 3 to 5 days. It is intriguing that we previously saw a similar infection followed by similar drop-off in the number of infected hepatocytes in studies of HDV infections in mice (14). We interpret these mouse infections as also being helper independent. Furthermore, the same rate of clearance was observed for mice with a severe combined immunodeficiency; thus, the drop-off did not seem to be dependent upon the presence of competent T or B cells. For both the mouse and the woodchuck studies, we do not know if this drop-off (i) is due to the natural replacement of hepatocytes, (ii) is induced by HDV infection, or (iii) represents the cells somehow becoming cured of the infection.

Our findings may be relevant to HDV infections associated with liver transplants. As reviewed in the introduction, others have already implicated helper-independent HDV infections to explain why patients with previous exposure to HDV who undergo a liver transplant invariably demonstrate an early HDV infection of their new liver (5, 8, 19, 23, 24). These seem to be helper-independent infections, just as we have studied for the woodchuck. Of the humans who receive transplants, maybe around 50% ultimately are infected with the helper virus HBV and go on to become chronically infected with both HDV and HBV (19).

It should be noted that our studies of helper-independent infections of animals differ somewhat from comparable studies with cultured cells. Primary cultures of human, chimpanzee, and woodchuck hepatocytes can be infected with HDV (20–22), and in one study it was found that in nondividing hepatocytes undergoing what we presume is helper-independent infection, HDV can actually persist for at least 42 days (20). Similarly, recent studies of Bichko et al. (1) have shown that cultured cell lines can be transfected with HDV, and again, the cells with helper-independent infections persist for at least 6 weeks.

We are puzzled that at <10 days there was no apparent difference in the number of antigen-positive hepatocytes for an HDV superinfection relative to that for a coinfection (Fig. 2 and Table 1). Maybe at <10 days most, but not all, HDV coinfections are essentially or even strictly helper independent. The animal chronically infected with WHV prior to superinfection with HDV showed WHV infection in most hepatocytes and produced large amounts of WHV DNA-containing particles. Apparently, neither this WHV infection nor the presence of these particles caused any significant interference with the ability of HDV to infect hepatocytes (at least not even a fourfold effect; Table 1). We point this out because there are data that HBV and HDV may share the same receptor on primate hepatocytes (21).

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REFERENCES

1. **Bichko, V., H. J. Netter, and J. Taylor.** 1994. Introduction of hepatitis delta virus into animal cell lines via cationic liposomes. J. Virol. **68:**5247–5252.
2. **Bichko, V., F. Schödel, M. Nassal, E. Gren, I. Berzinsh, G. Borisova, S. Miska, D. L. Peterson, E. Gren, P. Pushko, and H. Will.** 1993. Epitopes recognized by antibodies to denatured core protein of hepatitis B virus. Mol. Immunol. **30:**221–231.
3. **Chen, P.-J., G. Kalpana, J. Goldberg, W. Mason, B. Werner, J. Gerin, and J. Taylor.** 1986. Structure and replication of the genome of hepatitis δ virus. Proc. Natl. Acad. Sci. USA **88:**8490–8494.
4. **Cote, P., C. Roneker, K. Cass, F. Schödel, D. Peterson, B. Tennant, F. de Noronha, and J. Gerin.** 1993. New enzyme immunoassays for the serologic detection of woodchuck hepatitis virus infection. Viral Immunol. **6:**161–169.
5. **David, E., A. Pucci, J. Rahier, E. Andorno, L. Fassi, M. Fortunato, and M. Rizzetto.** 1993. Histopathology of recurrent delta hepatitis in liver transplant, p. 419–424. *In* S. J. Hadziyannis, J. M. Taylor, and F. Bonino (ed.), Hepatitis delta virus: molecular biology, pathogenesis, and clinical aspects. Wiley-Liss, New York.
6. **Dourakis, S., P. Karayiannis, R. Goldin, M. Taylor, J. Monjardino, and H. C. Thomas.** 1991. An *in situ* hybridization, molecular biological and immunohistochemical study of hepatitis delta virus in woodchucks. Hepatology **14:**534–539.
7. **Fu, T.-B., and J. Taylor.** 1993. The RNAs of hepatitis delta virus are copies by RNA polymerase II in nuclear homogenates. J. Virol. **67:**6965–6972.
8. **Gowans, E. J., and F. Bonino.** 1993. Hepatitis delta virus pathogenicity, p. 125–130. *In* S. J. Hadziyannis, J. M. Taylor, and F. Bonino (ed.), Hepatitis delta virus: molecular biology, pathogenesis, and clinical aspects. Wiley-Liss, New York.
9. **Kuo, M. Y.-P., M. Chao, and J. Taylor.** 1989. Initiation of replication of the human hepatitis delta virus genome from cloned DNA: role of delta antigen. J. Virol. **63:**1945–1950.
10. **Macnaughton, T. B., E. J. Gowans, S. P. McNamara, and C. J. Burrell.** 1991. Hepatitis delta antigen is necessary for access of hepatitis delta virus RNA to the cell transcriptional machinery but is not part of the transcriptional complex. Virology **184:**387–390.
11. **Negro, F., K. F. Bergmann, B. M. Baroudy, W. C. Satterfield, H. Popper, R. H. Purcell, and J. L. Gerin.** 1988. Chronic hepatitis D virus (HDV) infection in hepatitis B virus carrier chimpanzees experimentally superinfected with HDV. J. Infect. Dis. **158:**151–159.
12. **Negro, F., B. E. Korba, B. Forzani, B. M. Baroudy, T. L. Brown, J. L. Gerin, and A. Ponzetto.** 1989. Hepatitis delta virus (HDV) and woodchuck hepatitis virus (WHV) nucleic acids in tissues of HDV-infected chronic WHV carrier woodchucks. J. Virol. **63:** 1612–1618.

13. **Netter, H. J., J. L. Gerin, B. C. Tennant, and J. M. Taylor.** Unpublished observations.
14. **Netter, H. J., K. Kajino, and J. M. Taylor.** 1993. Experimental transmission of human hepatitis delta virus to the laboratory mouse. J. Virol. **67:**3357–3362.
15. **Ponzetto, A., P. J. Cote, H. Popper, B. H. Hoyer, W. T. London, E. C. Ford, F. Bonino, R. H. Purcell, and J. L. Gerin.** 1984. Transmission of the hepatitis B virus-associated δ agent to the eastern woodchuck. Proc. Natl. Acad. Sci. USA **81:**2208–2212.
16. **Ponzetto, A., B. H. Hoyer, H. Popper, R. Engle, R. H. Purcell, and J. L. Gerin.** 1987. Titration of the infectivity of hepatitis D virus in chimpanzees. J. Infect. Dis. **155:**72–78.
17. **Ponzetto, A., F. Negro, J. L. Gerin, and R. H. Purcell.** 1991. Experimental hepatitis delta virus infection in the animal model, p. 147–157. *In* J. L. Gerin, R. H. Purcell, and M. Rizzetto (ed.), The hepatitis delta virus. Wiley-Liss, New York.

17a. **Purcell, R.** Personal communication.

18. **Rizzetto, M., B. Hoyer, M. G. Canese, J. W. K. Shih, R. H. Purcell, and J. L. Gerin.** 1980. δ agent: association of δ antigen with hepatitis B surface antigen and RNA in serum of δ-infected chimpanzees. Proc. Natl. Acad. Sci. USA **77:**6124–6128.
19. **Smedile, A., A. Mangia, M. S. Brunetto, M. Giarin, A. Marzano, A. Ottobrelli, E. Chiaberge, M. Durazzo, F. Bonino, G. Verme, and M. Rizzetto.** 1993. Different aspects of HDV and HBV reinfections and liver disease in orthotopic liver transplant (OLT), p. 393–402. *In* S. J. Hadziyannis, J. M. Taylor, and F. Bonino (ed.), Hepatitis delta virus: molecular biology, pathogenesis, and clinical aspects. Wiley-Liss, New York.
20. **Sureau, C., J. R. Jacob, J. W. Eichberg, and R. E. Lanford.** 1991. Tissue culture system for infection with human hepatitis delta virus. J. Virol. **65:**3443–3450.
21. **Sureau, C., A. M. Moriarty, G. B. Thornton, and R. E. Lanford.** 1992. Production of infectious hepatitis delta virus in vitro and neutralization with antibodies directed against hepatitis B virus pre-S antigens. J. Virol. **66:**1241–1245.
22. **Taylor, J., W. Mason, J. Summers, J. Goldberg, C. Aldrich, L. Coates, J. Gerin, and E. Gowans.** 1987. Replication of human hepatitis delta virus in primary cultures of woodchuck hepatocytes. J. Virol. **61:**2891–2895.
23. **Van Thiel, D. H., S. Fagiuoli, and H. I. Wright.** 1993. Liver transplantation for viral hepatitis: the current situation, p. 377–387. *In* S. J. Hadziyannis, J. M. Taylor, and F. Bonino (ed.), Hepatitis delta virus: molecular biology, pathogenesis, and clinical aspects. Wiley-Liss, New York.
24. **Zignego, A. L., D. Samuel, M. Gigou, C. Feray, J.-L. Arulnaden, M. Monti, P. Gentilini, M. Reynes, J. P. Benhamou, C. Brechot, and H. Bismuth.** 1993. Patterns of hepatitis delta virus reinfection and their evolution during a long term follow-up, p. 409–417. *In* S. J. Hadziyannis, J. M. Taylor, and F. Bonino (ed.), Hepatitis delta virus: molecular biology, pathogenesis, and clinical aspects. Wiley-Liss, New York.