Clevudine Inhibits Hepatitis Delta Virus Viremia: a Pilot Study of Chronically Infected Woodchucks

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In a small controlled study, clevudine, a potent inhibitor of hepadnaviruses, including hepatitis B virus and woodchuck hepatitis virus, suppressed hepatitis delta virus (HDV) viremia in chronically infected woodchucks. Suppression was correlated with the marked reduction of woodchuck hepatitis virus surface antigen in individual animals, consistent with the concept that repression of surface antigen expression may be a useful antiviral strategy for HDV.

Type D hepatitis is caused by infection with hepatitis D (delta) virus (HDV), an obligate subviral satellite of hepatitis B virus (HBV) (9). The HDV virion is composed of a ribonucleoprotein core and an envelope formed by the surface antigen protein (HBsAg) of HBV (1–3). The envelope is the sole helper function provided by HBV, in that HDV is able to replicate within cells in the absence of HBV (13) but requires HBsAg for packaging and release of HDV virions (28, 37) as well as for infectivity (31).

Chronic type D hepatitis, similar to chronic HBV infection, is typically characterized by necroinflammatory lesions, but it is more severe and frequently progresses rapidly to cirrhosis and liver failure, accounting for a disproportionate association of chronic HDV infection with terminal liver disease and an indication for liver transplantation (26, 30). There is currently no generally accepted effective therapy for type D hepatitis (see reference 21 for a review), and liver transplantation is the only option for the associated end-stage liver disease (36). Exceptionally high doses of alpha interferon have been, at best, moderately successful in treating some cases of type D hepatitis (6, 10, 27, 34). Acyclovir enhances HDV replication *in vitro*. Ribavirin does not significantly affect virologic or biochemical parameters, and there were severe side effects in clinical trials. Synthetic analogs of thymosin have been ineffective in the treatment of HDV infection (21).

The dependence of HDV on HBV could suggest that successful treatment of HDV infection would follow successful treatment of the supporting HBV infection. Unfortunately, this does not always appear to be the case. Although treatment of chronic HBV carriers with lamivudine (β -L-2',3'-dideoxy-3'-thiacytidine) leads to decreased levels of HBV in serum and improved liver histology (5, 14, 20), in patients with chronic delta hepatitis prolonged lamivudine therapy neither lowers HDV RNA levels nor ameliorates disease activity, even though

HBV viremia is reduced (15, 35). Similarly, treatment with famciclovir was not effective against HDV infection (38). The most likely explanation for the failure of these treatments to affect HDV is that HDV requires the HBsAg function of HBV, and lamivudine treatment does not typically reduce HBsAg levels. We therefore sought to determine whether HDV could be inhibited by an anti-HBV therapeutic agent that dramatically reduces HBsAg levels.

Woodchuck hepatitis virus (WHV) and its natural host, the Eastern woodchuck, represent a useful model of HBV-induced disease that is predictive of nucleoside analog therapies against chronic HBV infection (11, 33). Like HBV, WHV can support HDV particle formation and infection, and the Eastern woodchuck has been a useful model of chronic HDV infection (18, 23, 25). The nucleoside analog L-FMAU (2'-fluoro-5-methyl- β -L-arabinofuranosyl-uridine [clevudine]) has been shown to have significant activity against WHV replication in chronically infected woodchucks, including sustained reductions in the hepatic WHV covalently closed circular DNA (cccDNA) replication template. Further, this reduction correlated with marked cotemporal reductions in the expression of hepatic WHV RNA, hepatic WHV core antigen (WHcAg), and serum WHV surface antigen (WHsAg) (12, 17, 24, 39). Thus, given the dependence of HDV on the hepadnavirus surface antigen, we tested whether L-FMAU could suppress HDV viremia in chronic carriers.

All animal experiments for the present study were conducted under protocols reviewed and approved by the Cornell University Institutional Animal Care and Use Committee. WHV carrier woodchucks were produced experimentally by administering standardized inoculum cWHV8P1 (4) to neonatal woodchucks born to WHV-negative females. The WHV carrier status of woodchucks was verified by detection of persistent WHV viremia and antigenemia (4). Chronic WHV carriers were infected at 12 months of age with a woodchuck-adapted HDV inoculum derived from an infectious clone, GenBank accession number L22066. Briefly, a 1.26-mer of the HDV cDNA, containing a duplication of sequences from 654 to 1092, was inserted between the PstI and SphI sites of the

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plasmid pGEM3Zf(-) to yield the construct pGDL1x1.2. Transfection of pGDL1x1.2 into cultured Huh-7 cells led to high levels of HDV RNA replication. A woodchuck-adapted HDV pool was created by transfection of pGDL1x1.2 into the surgically exposed liver of a woodchuck (no. 4928) which was chronically infected with WHV. Sera were collected weekly from woodchuck no. 4928 between weeks 7 and 15, during the peak of acute HDV viremia, and were pooled. Analysis of HDV RNA in this serum pool by blot hybridization indicated 10^9 genomes/ml. Twenty-six WHV carriers were inoculated with a dilution of the pooled HDV-positive sera from woodchuck no. 4928 containing 10^8 genomes and were monitored for HDV viremia by reverse transcription-PCR (22, 29). HDV RNA was quantified by radiolabeling PCR products, which were separated by acrylamide gel electrophoresis and analyzed by phosphorimager; serial dilutions of HDV RNA standards were used to generate standard curves for quantification.

Of the 26 WHV carrier woodchucks inoculated with the HDV-positive pool, 25 became viremic (detection limit of 500,000 genomes/ml) for at least one bleed date. Six animals died from causes not directly related to HDV infection. Of the remaining 19 animals, 9 (47%) were determined to have chronic HDV infection as defined by reverse transcription-PCR-detectable HDV viremia for at least 74% of bleed dates for at least 11 months. This chronicity rate is similar to the high rate of chronicity of HDV superinfection in humans. The range of duration of HDV viremia prior to the start of clevudine treatment was 11.4 to 20 months; the range of positive bleed dates was 74% to 100%.

Two groups of age-matched HDV chronic carrier woodchucks were used for the clevudine study. The four animals in the treatment group were given 10 mg of clevudine (L-FMAU)/kg of body weight orally once daily for 20 weeks as previously described (17, 24); the five animals in the control group received a placebo on the same schedule. Whole blood was obtained as described previously (11, 24) immediately prior to the initiation of drug treatment (week 0) and at weeks 2, 4, 8, 12, 16, and 20. Serum HDV RNA, WHV DNA, WHsAg, and anti-WHs and anti-WHc antibodies were measured as described previously (11, 17, 22, 24). Animals were euthanized at week 20 due to the presence of WHV-induced hepatocellular carcinoma, a natural consequence of chronic WHV infection (33). There was no statistically significant effect of clevudine treatment on the severity or incidence of hepatocellular carcinoma. No obvious treatment-related clinical (e.g., body weight loss), hematologic, or serological indications of toxicity, including drug-related hepatic toxicity (alanine transaminase, aspartate transaminase, and sorbitol dehydrogenase) (11, 32), were observed in any of the treated animals.

Both the kinetics and levels of WHV and WHsAg suppression were comparable to those observed in previous studies using larger groups of chronic WHV carrier woodchucks treated with clevudine (12) and indicated that chronic HDV infection did not interfere with the antiviral effect of clevudine on WHV. All treated animals exhibited marked decreases in serum WHV DNA after 4 weeks of treatment ($>10^7$ -fold reduction), as has been previously observed with this compound (17, 24) (Fig. 1A). In previous studies in woodchucks, about 75% of animals treated with a similar dose of clevudine exhib-

FIG. 1. Patterns of serum markers of WHV and HDV replication under clevudine therapy. Triangles, animal no. 4543; squares, animal no. 4883; diamonds, animal no. 4878; circles, animal no. 4879. ud, detection limit: for WHV DNA, 100 genomes/ml; for WHsAg, 0.01 μ g/ml; for HDV, 500,000 genomes/ml. Values shown in panel C are averages of triplicate measurements, with standard deviations indicated by error bars.

ited 100-fold or greater decreases in serum levels of WHsAg, while the remainder exhibited less than a 10-fold reduction (12, 24). Likewise, in the present study, a nearly 1,000-fold decrease in WHsAg levels was observed by 12 weeks in three of the four treated animals (Fig. 1B). Animal no. 4543 exhibited less than a 10-fold change in serum levels of WHsAg over the course of the study. The reason for the different patterns of WHsAg response is not clear but may be related to the effects of clevudine treatment on hepatic levels of WHV cccDNA. Although hepatic WHV cccDNA was not analyzed in this study, Peek et al. (24) observed a correlation between clearance of hepatic WHV cccDNA and sharp declines of serum WHsAg.

HDV RNA became undetectable in three of the four treated animals by 16 weeks of treatment (Fig. 1C). No substantial changes were observed for any of these viral markers in the control group. This decreased HDV viremia correlated with decreased levels of WHsAg. HDV RNA became undetectable following at least 100-fold reductions of WHsAg or reductions to less than 1 μ g/ml (Fig. 1A and C). Of particular note, HDV

FIG. 2. HDV viremia in WHsAg responders (squares) and untreated animals (circles). At week 20, $P = 0.02$. Values shown are geometric means; error bars represent standard deviations of the log of the viremia.

RNA remained at high levels in the one animal in the treated group, no. 4543, that did not exhibit decreased levels of WHsAg (although WHV DNA levels were decreased). When average levels of HDV viremia in the treatment and control groups were compared, there was no statistically significant effect of treatment. However, when the three clevudine-treated animals in which WHsAg declined (WHsAg responders) are grouped together, it is clear that HDV RNA levels declined dramatically compared to pretreatment levels and to levels in the control group during the course of treatment (Fig. 2). Statistical analysis by Student's t test indicates the decline is statistically significant ($P = 0.02$ for a paired, one-tailed comparison of week 0 and week 20 in the WHsAg responders; $P = 0.02$ for an unpaired, one-tailed comparison of week 20 levels in untreated animals and WHsAg responders).

The correlation of the suppression of HDV viremia with the reduction of surface antigen in individual animals is consistent with the concept that targeting surface antigen expression is a useful antiviral strategy for HDV. Thus, we suggest that any therapy that lowers WHV or HBV surface antigen levels sufficiently may be useful as a therapeutic option to control chronic HDV infection. Although HBsAg levels were not measured in previous reports (15, 35), lamivudine therapy of chronic HDV carriers most likely did not inhibit HDV replication because HBsAg was not reduced. Both of the nucleoside analogues currently licensed for the treatment of HBV infection, lamivudine and adefovir dipivoxil, reduce HBV replication sufficiently to induce improvements in HBV-induced disease progression, but neither routinely has a significant effect on circulating levels of HBsAg (5, 8, 14, 20).

This study indicates that the WHV/woodchuck model of experimental chronic hepatitis infection could be applied to therapeutic studies of chronic HDV superinfection. Nevertheless, the relatively rapid progression to hepatocellular carcinoma in WHV-infected woodchucks does pose challenges for the use of this model for evaluating drug efficacy against chronic HDV disease. Further, HDV infection appears to increase the risk of hepatocellular carcinoma in patients with compensated cirrhosis type B (7), and the influence of chronic

HDV on progression of end-stage liver disease in the woodchuck model has not been established. Indeed, many of the woodchucks in this study progressed to hepatocellular carcinoma, which precluded posttreatment follow-up studies. Perhaps these limitations can be overcome by the use of younger WHV carrier animals or use of less-virulent WHV strains. Clearly, further studies of the natural history of HDV disease in this model are needed before many important therapeutic issues can be addressed.

There is no long-standing cellular repository for HDV as there is for HBV (29), and the half-life of HDV-infected cells may be brief; in mice, infected cells survive for as little as 2 weeks (19). While in this study it was not possible to determine the long-term outcome of clevudine therapy on the course of chronic HDV infection or disease, the sustained reduction of HDV to undetectable levels by therapy with this potent nucleoside analog suggests that HDV disease in clinical patients would be reduced and that treatment has the potential to eliminate HDV infection in chronically infected individuals. In this regard, it is important to note that a recent phase II clinical trial indicated potent activity of clevudine in HBV-infected patients, although effects on HBsAg levels were not reported (16).

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