Editing efficiency of hepatitis delta virus RNA is related to the course of infection in woodchucks

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Based on evidence in vitro which shows that the small form of hepatitis delta virus (HDV) antigen (S-HDAg) initiates virus replication, whereas the long form (L-HDAg) encoded by the edited L-genome inhibits replication, we first put forward the hypothesis that HDV RNA editing efficiency, i.e. the intracellular L/S-genome ratio, could be a determining factor on the course of the infection. In order to analyse the precise sequence of events after infection, woodchuck carriers of woodchuck hepatitis virus (WHV) were superinfected with HDV and sequential changes in HDV RNA editing efficiency were analysed in relation to the duration of viraemia. Our findings show that: (1) in both transiently and persistently viraemic woodchucks, the percentage of L-genome is higher at the early stage of onset of the disease than at the late stage; (2) at the early stage of onset the percentage of L-genome is higher in cases with transient viraemia than in those with persistent viraemia; (3) a relatively greater decrease in L-genome is seen later in transiently viraemic animals than in those that remain persistently viraemic. In view of the above findings in vivo and other supporting evidence in vitro, we propose a hypothesis for the pathogenesis of HDV. This hypothesis predicts the outcome of acute infection and we suggest a gene therapeutic approach to this disease based on the intracellular accumulation (or increase) of the L-genome.

Introduction

Hepatitis delta virus (HDV) is a subviral satellite agent whose transmission requires the helper function of hepatitis B virus (HBV) for virion assembly (Bonino et al., 1984, 1986) but not for replication (Kuo et al., 1989). HDV infection results from co-infection with HBV or superinfection of HBV carriers, and is associated with an accelerated course of the underlying chronic hepatitis and with an increased occurrence of fulminant hepatitis (Rizzetto, 1983; De Cock et al., 1986; Hoofnagle, 1989). HDV circulates in the plasma as a 36 nm particle consisting of an HBV surface antigen envelope enclosing the hepatitis delta antigen (HDAg) and the minus-stranded circular RNA genome of about 1700 bases in length (Bonino et al., 1984; Wang et al., 1986). In common with plant viroids, the RNA forms an unbranched rod-like structure by intramolecular base pairing (Kos et al., 1986); its replication occurs in the nucleus of infected hepatocytes, presumably by the rolling circle mechanism (Sharmeen et al., 1988). Unlike viroids, which do not encode any proteins, HDV expresses one protein, HDAg, which exists in the serum and liver of patients as two forms (Bergmann & Gerin, 1986; Bonino et al., 1986): the small antigen (S-HDAg) which contains 195 amino acids, and the large antigen (L-HDAg) which shares the open reading frame (ORF) with S-HDAg but contains 19 additional amino acids at the carboxy terminus of S-HDAg. The S-HDAg is encoded by the genome coding for a small ORF (small genome, S-genome, or wild-type genome) and the L-HDAg is encoded by the genome coding for a large ORF (large genome, L-genome, or edited genome) which is edited at position 1012 (T → C) leading to the replacement of an amber termination codon by tryptophan and resulting in the readthrough of 19 additional amino acids.

In vitro studies have demonstrated that S-HDAg is essential for HDV replication (Kuo et al., 1989; Glenn & White, 1991), whereas L-HDAg inhibits viral replication but is required for virion assembly (Glenn & White, 1991; Chao et al., 1990; Chang et al., 1991; Ryu et al., 1992). Based on these observations, we put forward the hypothesis that the intracellular L/S-genome ratio could be a determining factor on the time-course of infection.
and that the sequence around position 1012, the editing site, may also play a relevant role in the outcome of HDV infection by modulating the RNA editing efficiency.

In a previous in vivo transfection study we first demonstrated that the L/S-genome ratio changes during the course of subsequent infection (Yang et al., 1993). This observation encouraged us to investigate further the functional role of the L/S-genome ratio during natural infection, and to explore its potential significance in pathogenicity.

In order to carry out this study we had to use an experimental animal model so that the precise sequence of events could be analysed after inoculation and throughout infection. Successful infection of woodchucks with HDV was first reported by Ponzetto et al. (1984). We therefore chose the woodchuck as a model to carry out a comparative study between transient and persistent viraemias, and to correlate the L/S-genome ratio with the duration of natural infection. The issues addressed were: (1) whether the L/S-genome ratio is related to the time-course of infection; (2) whether the change in the L/S-genome ratio is different between transient and persistent viraemias; (3) whether the sequence around the editing site has a role in editing efficiency.

### Methods

**Inoculation of animals.** Eight wild-caught woodchucks (Marmota monax; Cocalico Biologicals Inc., Reamstown, USA), housed individually in our isolation facility, were used in the experiments. All these animals were carriers of chronic woodchuck hepatitis virus (WHV) as shown by the presence of WHV surface antigen and WHV DNA. All animal procedures were covered by a project licence from the Home Office issued in accordance with the Animal (Scientific Procedures) Act of 1986.

**HDV inocula.** A woodchuck-adapted HDV serum (1 ml, containing approx. 10^8 HDV genomes) representing the fifth woodchuck passage (from Antonio Ponzetto, Molinette Hospital, Turin, Italy) was used to infect woodchucks (no. 2, 3, 4, 5, 8 and 10) intravenously. Blood collected from woodchuck no. 2 (W2) 3 weeks after infection was found to contain high levels of HDV RNA by slot blot hybridization, and was used subsequently as the standard inoculum for W22 and W26.

Woodchucks W3, W10 and W22 showed transient viraemia, and W4, W5, W8 and W26 showed persistent viraemia (Karayiannis et al., 1990, 1993) (Fig. 1). W2 was put down 3 weeks after infection as described above and serum was used as a positive control.

**Extraction of HDV RNA and RT-PCR.** Extraction of HDV RNA from woodchuck serum and RT-PCR were optimized as follows. Woodchuck serum (50 ml) was incubated in 100 mM-Tris–HCl pH 8.3, 12.5 mM-EDTA, 0.15 mM-NaCl, 1% SDS, 0.2 mg/ml proteinase K at 37 °C for 40 min, followed by phenol–chloroform extraction and ethanol precipitation. HDV RNA was finally dissolved in 10 μl of diethyl pyrocarbonate (DEPC)-treated water.

**HDV RNA (2 μl) was denatured by boiling for 2 min and the reaction was then quenched on ice. The RT-PCR reaction was carried out in 20 mM-DTT, 600 units/μl RNAsin (placental ribonuclease inhibitor, GIBCO BRL), 1 nmol of HDV primer SP (1529–1500, 5’ AGAAAGAAGTTAGAGGAACCTCGAGAGAC 3’), 50 mM-Tris–HCl pH 8.3, 140 mM-KCl, 10 mM-MgCl_2, 0.5 mM-dNTPs and 4 units of avian myeloblastosis virus reverse transcriptase (Bioquote) in a total volume of 20 μl at 42 °C for 90 min. Total cellular RNA from liver tissue was extracted using the method of Sambrook et al. (1989) with homogenization buffer containing 4 M-guanidium thiocyanate. RNA extracted from about 0.5 g of liver tissue was finally dissolved in 50 μl of DEPC-treated water.

The PCR reaction was carried out in a 100 μl reaction mixture containing 5 μl of cDNA mixture, 1 unit of Ampli-Taq DNA polymerase (Perkin Elmer), 10 mM-Tris–HCl pH 8.3, 1.5 mM-MgCl_2, 50 mM-KCl, 0.1 mg/ml gelatin, 250 μM of each dNTP, and 0.5 μM of each of the HDV primers SP and AY1 (877–896, 5’ GAGGTGGAGATGCGCATGCCG 3’). After the initial denaturation at 95 °C for 4 min, the first round was performed over 40 cycles with denaturation at 95 °C for 1 min, annealing at 53 °C for 2 min, and extension at 72 °C for 2 min, followed by a final single extension step at 72 °C for 6 min to ensure that all amplified molecules were completed. Of the 100 μl DNA sample from the first round PCR, 1 μl was used as a template for the second round PCR with the HDV primers AY2 (1350–1332, 5’ CCGGACCTAGGAGGAGGGC 3’) and AY1 under the same conditions as those described for the first round PCR.

**Subcloning and DNA sequencing.** The standard procedures described by Sambrook et al. (1989) were followed. Both second round PCR DNA and pUC19 were cut with PstI and SalI, electroeluted, ligated and transformed into competent Escherichia coli DH5α cells. Recombinants were identified by colony hybridization with 32P-labelled subgenomic HDV cDNA (i.e. PCR DNA amplified with primers SP and AY1). Twenty recombinant clones were then further analysed by means of NcoI digestion, allowing the L-genome to be distinguished from the S-genome (as only the L-genome contains the NcoI recognition sequence 5’ C;CATGG 3’ and can be cut with NcoI). This was confirmed by the dideoxy sequencing method using Bst DNA polymerase (Bio-Rad) and the HDV primer AY4 (1083–1064, 5’ GCAAGGGTAGCCGAGCTGCC CCC 3’).

Direct sequencing of PCR DNA was also performed. The second round PCR DNA was purified by ammonium acetate differential precipitation and sequencing was carried out using the same method as described above with primer AY3 (983–1002, 5’ AGGGAGGGTCCGAGCTGCC 3’).

**Western blot analysis for HDAg.** Serum HDAg was prepared essentially as described by Bergmann & Gerin (1986). The serum sample (0.3–0.5 ml) was layered over 2 ml of 20% (w/v) sucrose in phosphate-buffered saline solution A and centrifuged at 190000 g for 5 h at 4 °C. The pellet was dissolved in 100 μl 0.05 M-Tris–HCl pH 6.8 and 2% SDS, and boiled for 5 min. HDAg was then analysed by 12% SDS–PAGE, blotted onto a nitrocellulose filter and incubated with a 1:200 dilution of rabbit anti-HD (D-280 HDAg, amino acids 12–75; Saldanha et al., 1990), developed with Protein-A–gold (Bio-Rad) and enhanced with silver lactate according to the manufacturer’s instructions.

### Results

**HDV genomic and antigenic profile analyses in both woodchuck serum and liver samples from the same time-point**

Due to the risk involved in performing repeated liver biopsies, our study had to be restricted to the analysis of serum. This being the case we first had to establish that changes occurring in the serum accurately mirrored
events in the liver where replication, packaging and release of virions occurs. In accordance with this we felt that if determination of the L/S-genome ratio was the main objective of our study it had to be demonstrated that serum and liver L/S-genome ratios were identical, i.e. that there was no preferential packaging of either form of genome. To achieve this both serum and liver samples of two woodchucks (W2 and W8) from the same time-point were analysed.

HDV RNA was extracted from the serum samples and total cellular RNA was extracted from the liver samples, followed by RT-PCR (Fig. 2a). After cloning into pUC19, insert-containing clones were identified, and plasmid DNA was digested with NcoI for L/S-genome analysis (Fig. 2b). Confirmatory sequences were also obtained in 3/20 clones (Fig. 2c).

As shown in Table 1 in one woodchuck (W2) the percentage of L-genome was 35% (7/20) in both serum and liver, whereas in the other woodchuck (W8) the percentage of L-genome was 55% (11/20) in serum and 50% (10/20) in liver, a difference which is not significant. These findings demonstrate that the genome ratio in serum is representative of the ratio found in liver and imply that both L- and S-genomes are packaged with equal efficiency.

In addition to the investigation of both forms of the genome in serum and liver, we confirmed the presence of the two forms of HDAg in serum. Immunoblot analysis
Fig. 2. (a) Detection of HDV RNA by the second round PCR with primers AY2 and AY1 (W5 serum). Lane 1, PCR from positive control serum (W2); lane 2, negative control from uninfected woodchuck serum; lanes 3, 4 and 5, PCR of serum HDV RNA from three time-points for W5. (b) Restriction analysis with NcoI to identify recombinants with the L-genome. Lanes 2, 4 and 7, plasmids containing the L-genome were digested to linear form; lanes 1, 3, 5, 6 and 8, plasmids containing the S-genome were not cut and remained in supercoiled form. (c) Sequence confirmation of the NcoI site (5' C\CATGG 3') generated by the T → C mutation at position 1012. Samples 1, sequences from plasmids undigested by NcoI show T at position 1012; samples 2, sequences from plasmids digested by NcoI show C at position 1012. The sequence for potential NcoI recognition is indicated on both sides.

Table 1. Liver and serum distribution of the two forms of HDV genome and HDAg

<table>
<thead>
<tr>
<th>Woodchuck no.</th>
<th>RNA source</th>
<th>L-genome (%)</th>
<th>HDAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>W2</td>
<td>Liver</td>
<td>35</td>
<td>+</td>
</tr>
<tr>
<td>W2</td>
<td>Serum</td>
<td>35</td>
<td>+*</td>
</tr>
<tr>
<td>W8</td>
<td>Liver</td>
<td>50</td>
<td>+</td>
</tr>
<tr>
<td>W8</td>
<td>Serum</td>
<td>55</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Two forms were identified with predicted molecular masses of 24 kDa and 27 kDa. ND, Not determined.

Sequential analysis of the ratio of the two forms of the HDV genome during transient or persistent viraemia in woodchucks

To quantify the L/S-genome ratio in relation to the time-course of infection and the duration of viraemia,
HDV V RNA editing and course of infection

(a)

(b)

kDa M W2 kDa M W5

45.0

31.0

21.5

14.4

Fig. 3. Western blot analysis of serum HDAg in HDV-infected woodchucks (W2 and W5). L-HDAg is indicated by arrow L; S-HDAg is indicated by arrow S.

Table 2. Sequential analysis of the L-genome frequency in serum in relation to the course of HDV viraemia

<table>
<thead>
<tr>
<th>Woodchuck no.</th>
<th>Viraemia*</th>
<th>L-genome (%)†</th>
<th>Change in L-genome (%)</th>
<th>Interval (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W10 Transient</td>
<td>80 35</td>
<td>-45</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>W22 Transient</td>
<td>60 33</td>
<td>-27</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>W3 Transient</td>
<td>70 45</td>
<td>-25</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>W26 Persistent</td>
<td>45 30</td>
<td>-15</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>W8 Persistent</td>
<td>55 50</td>
<td>-5</td>
<td></td>
<td>540</td>
</tr>
<tr>
<td>W4 Persistent</td>
<td>40 25</td>
<td>-15</td>
<td></td>
<td>210</td>
</tr>
<tr>
<td>W5 Persistent</td>
<td>65 45</td>
<td>-20</td>
<td></td>
<td>351</td>
</tr>
</tbody>
</table>

* The P values between the two groups, transient and persistent viraemias, were determined by two-group t-tests: < 0.05 (P = 0.034) for early, > 0.05 (P = 0.035) for late and < 0.05 (P = 0.035) for the percentage change.
† The P value between the early and late stages of onset of infection for all seven woodchucks was determined by a paired t-test: < 0.005 (P = 0.0039).

individual clones were analysed. Out of seven woodchucks, three (W3, W10 and W22) showed transient viraemia whereas four (W4, W5, W8 and W26) had persistent viraemia. For each woodchuck, serum samples from both the early and late stages of the onset of the disease were analysed. Serum HDV RNA was subjected to RT-PCR and cloning, and 20 recombinant clones were then further analysed by means of NcoI digestion followed by confirmatory sequencing of three clones from each time-point.

The results, as summarized in Table 2, showed that in all seven woodchucks, the percentage of L-genome was higher at the early stage (80–40%) than at the late stage (50–25%) of the acute phase of the infection (P < 0.005, i.e. P = 0.0039, determined by a paired t-test). At the early stage of transient viraemia, the percentage of L-genomes ranged from 60–80%, then dropped sharply by 25–45% within a short period of between 7 and 21 days. In contrast, in persistent viraemia at the early stage of the acute phase the percentage of L-genome was significantly lower, 40–65%, and then dropped much more slowly over a period of 30–540 days and by only 5–20%. These results showed that at the early stage of onset, the percentage of L-genome was significantly higher in transient viraemia than in persistent viraemia (60–80% vs 40–65%) (P < 0.05, i.e. P = 0.034, determined by a two-group t-test), and that it showed a significantly greater reduction (25–45% vs 5–20%) (P < 0.05, i.e. P = 0.035, determined by a two-group t-test), although no difference was apparent at the late stage between transient and persistent viraemias (33–45% and 25–50%, respectively, P > 0.05).

Since both W22 and W26 were infected with W2 serum which was known to contain 35% of the L-genome, it was of interest that we had found an increase in the percentage of L-genome after inoculation. This increase was more marked in W22, which developed transient viraemia (60% of L-genome) than in W26, which developed persistent viraemia (45% of L-genome), and a more pronounced decrease was subsequently shown in W22 (27%) compared with W26 (15%) as shown in Table 2.

The HDAg profile was also investigated in one of the woodchucks (W5). As shown in Fig. 3, two forms of HDAg were found, with molecular masses of 27 kDa and 24 kDa, confirming the pattern previously seen in W2.

Sequence analysis around the editing site 1012

The second round PCR DNA amplified with primers AY2 and AY1 was used for direct sequencing. Sequences at three time-points from W10 and W22 (transient viraemia) and from five time-points from W5 (persistent viraemia) were compared (Fig. 4).

Since the PCR DNA was amplified from both L- and S-genomes, the mixed templates made the sequence ambiguous at position 1012 (not shown). The remaining sequence (positions 1007–1100) was clear and showed no significant differences either between different time-points from the same woodchuck, or between woodchucks showing transient or persistent viraemias (Fig. 4). Although some base changes occurred at positions 1037, 1048 and 1057 in W5, the encoded amino acids (Ile → Val at position 1037, Glu → Gly at position 1048, Pro → Arg at position 1057) were either not associated with a significant hydropathic change, or they reverted to the original amino acid at a later stage. Overall, no nucleotide changes between positions 1007 and 1100 were consistently associated with changes in editing efficiency.
Although in vitro data have shown that the two forms of HDAg play a critical role in the HDV life cycle, no significant differences in the serum were found between different groups of patients with hepatitis D (Buti et al., 1987; Zyzik et al., 1987; Pohl et al., 1987) or between the early and late stages of infection in chimpanzees (Bergmann & Gerin, 1986). This could be explained by the fact that circulating anti-HD may form immune complexes with HDAg, making the detection of HDAg inaccurate, and by the poor reproducibility of Western blot analysis for quantification of the two forms of HDAg (Zyzik et al., 1987).

Since the two forms of HDAg are encoded by two forms of the genome, we approached this question by investigating whether the serum (i.e. indirectly the liver) L/S-genome ratios were related to the outcome of the disease. The sensitive PCR and cloning approach made it feasible to determine the L/S-genome ratio in serum samples from different time-points in each infected animal. In addition, it enabled us to analyse the sequence diversity around the editing site 1012 between different samples. The use of the woodchuck animal model offered us the opportunity to analyse the sequence of events (e.g. the sequential L/S-genome ratio and sequence changes) after HDV infection, which we were otherwise unable to carry out in patients.

Sequence analysis of HDV genomic regions flanking the editing site (1007-1100), between isolates from animals with transient or persistent viraemias, was carried out at different time-points. No differences were found in this region, not just between the different time-points in the same woodchuck, but also between animals showing transient or persistent viraemia (Fig. 4). Since we have restricted our analysis to a small region of the genome, we still cannot at this stage exclude the possibility that sequence variation elsewhere in the genome can directly modulate pathogenesis, possibly by affecting genome editing efficiency (see below). Further evidence as to whether the HDV genome sequence plays a role in determining the course of infection is likely to be generated from the comparative analysis of genome sequences in relation to the course of subsequent infection using intrahepatic in vivo transfection.

The observation that the W2 serum inoculum containing 35% of the L-genome generates virus in the infected woodchucks with a higher percentage of L-genome (60% in W22, 45% in W26) not only confirms the observation in vivo that the L-genome can be derived from recombinant S-HDV cDNA in intrahepatically transfected woodchucks (Yang et al., 1993), but also demonstrates that the same mutation occurs in animals infected with woodchuck serum in which both forms of the HDV genome co-exist. This observed relative increase in the percentage of L-genome was present in animals showing both transient viraemia (W22) and persistent viraemia (W26). That both transient and persistent viraemias were seen after infection with the same inoculum clearly illustrates the importance of host factors (which include the underlying WHV infection) in determining the duration of viraemia. The observation that in one woodchuck showing transient viraemia (W22) the increase of L-genome is more pronounced than in the one with persistent viraemia (W26) emphasizes the role of host factors in regulating HDV RNA editing. This

# Discussion

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**Fig. 4. Sequential HDV genome analysis of position 1012 in transient viraemia (W10 and W22) and persistent viraemia (W5) of woodchuck HDV infection by PCR direct sequencing with primer AY3. Month(s) after inoculation with HDV are indicated (m) and 'y' represents T or C.**
conclusion is also consistent with observations after in vivo transfection of woodchucks with the same recombinant HDV cDNA (Yang et al., 1993; Cicciaglione et al., 1993), although differences in transfection efficiency cannot be ruled out. These observations from both infection and in vivo transfection studies, taken together, have led us to postulate that host factors may play an important role in determining the rate of appearance and the relative amounts of L-genome, which in turn may determine the duration of infection and thus pathogenicity.

Significantly, we found that in all seven infected woodchucks, the percentage of L-genome was invariably higher at the early stage than at the late stage of onset of the acute phase of the infection (Table 2), in good agreement with what was previously observed in woodchucks transfected with recombinant S-HDV cDNA (Yang et al., 1993). Furthermore, our results (Table 2) also show that at the early stage of the acute phase in transient viraemia, the percentage of L-genome is significantly higher than that seen in persistent viraemia and that its decrease is more marked and proceeds at a faster rate. These results also support the hypothesis we have proposed, i.e. that the L/S-genome ratio may determine or contribute to the course of infection in vivo.

Since the L-genome codes for L-HDAg which causes inhibition of virus replication, we propose that the outcome of the acute infection may be determined by the ability to generate L-genome and whether a critical intracellular level is reached which causes complete inhibition of virus replication. If this occurs, the viraemia will be cleared, whereas an intracellular concentration of L-genome below a critical level may only achieve a 'partial' inhibitory effect, leading to persistent viraemia. In addition, we speculate that cells expressing higher amounts of L-HDAg may be better targets for cell-mediated immune clearance, which would explain why in self-limiting infections the percentage of L-genome shows a higher rate of decline during the acute phase.

We put forward the hypothesis that after infection of hepatocytes with HDV, host factors have a dominant role in determining the efficiency of the HDV RNA editing process and the critical steady state ratio between the L- and S-genome forms. The following changes occur as a result of a higher intracellular L/S-genome ratio: (1) strong inhibition of virus replication, limiting the number of HDV genomes; (2) efficient assembly and release of virions which contain a larger proportion of the L-genome and are thus incapable of propagating the infection; (3) higher intracellular levels of L-HDAg activate cell-mediated immune responses more efficiently and result in these cells being preferentially lysed. The outcome of this response would be the resolution of the infection. This sequence of events explains why in this study the percentage of L-genomes decreased at a faster rate in woodchucks showing transient viraemia, and in extreme cases this might result in marked liver damage leading to fulminant hepatitis.

Alternatively, if the L/S-genome ratio increases, but does not reach a critical intracellular level at the early stage of infection, the following changes would be expected: (1) virus replication would not be strongly inhibited and many HDV genomes would accumulate; (2) although small amounts of L-HDAg may be sufficient to initiate the assembly of virions, this process would be inefficient; (3) lower levels of intracellular L-HDAg may activate the cell-mediated immune responses less efficiently and thus clearance of infected cells would be inefficient. The overall result of this interplay between virus and host will be the persistence of infection, i.e. chronic hepatitis D. This would explain why the percentage of L-genomes showed a slower rate of decline in woodchucks showing persistent viraemia. Similarly, the liver damage may be expected to be less severe.

Our hypothesis is consistent with the observation that inflammation and HDAg expression are more pronounced in hepatitis D cases that are destined to resolve, although in this study the two HDAg forms, L-HDAg or S-HDAg, were not discriminated (Negro et al., 1989).

Finally, the mechanisms proposed suggest exploration of two therapeutic strategies for this disease: gene therapy designed to increase the intracellular concentration of L-genome and drugs which enhance the cell-mediated immune response.

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