Original article

Does treatment of hepatitis B virus (HBV) infection reduce hepatitis delta virus (HDV) replication in HIV–HBV–HDV-coinfected patients?

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Background: Hepatitis delta virus (HDV) has a unique replication process that requires coinfection with hepatitis B virus (HBV). Treatment is currently limited to interferon therapy. The role of potent nucleos(t)ide analogues active against HBV has not been well examined in chronic delta hepatitis (CDH).

Methods: HIV-positive patients with CDH attending our hospital were identified and longitudinally studied. Serum HBV DNA, HDV RNA and HIV RNA, treatment regimens, and biochemical and serological markers were assessed at yearly intervals. Liver fibrosis was measured by transient elastography during the last 2 years.

Results: Sixteen patients were identified and treated with anti-HBV therapy (median time 6.1 years). The majority were male and previous intravenous drug users. Median baselines were: HDV RNA 7 log_{10} copies/ml, HIV RNA 1.7 log_{10} copies/ml, HBV DNA 1.1 log_{10} IU/ml and alanine aminotransferase (ALT) 98 IU/ml. A significant correlation was found between HDV RNA and HBV DNA (r=0.226, P=0.015), aspartate aminotransferase (r=0.430, P<0.0001), ALT (r=0.441, P<0.0001) and hepatitis B surface antigen (HBsAg) (r=0.557, P<0.0001). Overall, 13 patients showed a reduction in HDV viraemia and ALT levels, and three of them achieved undetectable HDV RNA and normal ALT levels.

Conclusion: Patients undergoing successful anti-HBV therapy with potent nucleos(t)ide analogues seem to indirectly benefit from suppression of HDV replication, albeit not very efficiently. Hypothetically, a significant and sustained reduction in serum HDV RNA might only be seen when a reduction in HBV covalently closed circular DNA or HBV surface antigen is achieved, which may require long periods of successful anti-HBV therapy. To our knowledge, this is the first evidence of the benefit of potent anti-HBV nucleos(t)ide analogue therapy in CDH.

Introduction

Hepatitis delta virus (HDV) is a subviral satellite of hepatitis B virus (HBV). HDV is a replication-defective HBV-dependent single-stranded (ss)RNA virus that requires the surface antigen of HBV (HBsAg) for the encapsidation of its own genome. Approximately 5% of chronic HBV carriers are also coinfected with HDV, resulting in nearly 15 million individuals infected with HDV worldwide [1]. Two different epidemiological patterns have been described for HDV infection. Endemic regions include southern Europe, central Asia and west Africa, where transmission is generally by percutaneous and non-percutaneous routes [2]. Non-endemic areas include northern Europe and north America where the main route of HDV transmission is by infected blood and infection is confined to intravenous drug users, a substantial proportion of whom are coinfected with HIV [3].

The outcome of HDV infection largely depends on whether the HDV and HBV infect simultaneously (coinfection) or whether the new HDV infection occurs in a chronic HBV carrier (superinfection). Coinfection usually results in an acute self-limited illness (>95% of cases) that increases the risk of developing fulminant hepatic failure, whereas superinfection generally causes an exacerbation of hepatic disease in a chronic HBV carrier. HDV superinfection frequently causes severe and rapidly progressive liver disease and may increase the mortality due to end-stage liver disease 10-fold [4].
Studies conducted before the introduction of highly active antiretroviral therapy (HAART) for HIV infection regarding the effect of HDV infection on patients with HBV–HIV co-infection have shown inconsistent results. Some authors have suggested that HIV co-infection leads to a more severe course of chronic hepatitis delta (CHD) [5,6], whereas others have shown that long-term HDV infection is not influenced by concomitant HIV infection [7,8].

HDV has a unique replication process that uses the host cellular polymerases [9,10]. For this reason, HDV is a challenging target for antiviral therapy. No specific inhibitor of HDV has so far been developed and treatment is currently limited to interferon-α therapy. However, the role of nucleos(t)ide analogues active against HBV (particularly drugs other than lamivudine [3TC]) used against HDV has not been fully examined. The aim of this study was to analyse the evolution of HDV and HBV viral loads, as well as the serological markers and liver function tests, in chronic HDV patients co-infected with HIV undergoing HAART regimens with anti-HBV activity.

Methods

Study population
A longitudinal study including all HIV-positive patients chronically infected with HBV and HDV regularly attending the Hospital Carlos III in Madrid, Spain for a minimum of 2 years was performed. Data concerning HAART regimens including anti-HBV active agents were recorded. All patients were monitored for changes in treatment and antiviral response. When possible, two or more pre-treatment samples were also analysed for serum HDV RNA levels.

Serology
Serum HBsAg, HBeAg and anti-HBeAg were analysed by commercial enzyme immunoassays (EIA) using AxSYM HBsAg (v2), AxSYM HBeAg (2.0) and AxSym anti-HBe, respectively (Abbott Laboratories, North Chicago, IL, USA). Specific hepatitis C virus (HCV) antibodies were identified using a commercial screening EIA (AxSYM HCV v3.0; Abbott). Serum HDV antigens, total HDV antibodies and HDV immunoglobulin M (IgM) were analysed using RADIM HDVAg, HDVAb and HDVlgM EIA kits (Radim Iberica, Barcelona, Spain).

HBsAg quantitation
Serum HBsAg titres were determined using serial dilutions of an international HBsAg standard (NIBSC, Potters Bar, UK) and an HBsAg EIA (BioRad, Madrid, Spain). Detection limits ranged from 10 to 10,000 IU/ml.

Viral quantitation
Serum HBV DNA, HIV RNA and HDV RNA for each patient were determined prior to commencing anti-HBV treatment and then at yearly intervals. Serum HCV RNA was similarly measured in subjects with positive serum anti-HCV antibodies. Serum HDV RNA was quantified using an in-house real time-PCR (RT-PCR) based on a modified protocol described elsewhere [11]. Briefly, RNA was extracted from plasma using a Qiagen viral RNA extraction kit (Qiagen GmbH, Hilden, Germany). RT-PCR was performed using specific primers deltaF (5'-TGG CGC CGG CTG GCA ACA TTC-3') and deltaR2 (5'-AAG GAA GGC CTT CGA GAA CAA GAG-3') using avian murine virus (AMV) reverse transcriptase (Promega, Madrid, Spain) and the following conditions: 48˚C 45 min, 94˚C 2 min and (94˚C 30 s, 55˚C 30 s, 72˚C 1 min) × 15 cycles. The RT-PCR product was then purified in a millipore filter (Millipore, Madrid, Spain) and eluted in 20 µl H2O and then quantified using a LightCycler PCR thermocycler (Roche) using primers deltaF, deltaR (5'-TCT CCT CTT CGG GTC GCC ATG G-3') and probe deltaP (5'-6-FAM-ATG CCC AGG TCG GAC CGC G-BHQ1a-3') in the following conditions: 95˚C 10 min and 45 cycles of 55˚C 15 s. Negative controls included RNA extracted from HIV, HCV and HBV positive sera. A standard curve was obtained by cloning a deltaF-R2 PCR DNA fragment into a pGEMT vector (Promega) and then spiked into negative serum in 10 consecutive 10-fold dilutions (10⁶ copies/ml to 10⁻¹ copies/ml). The lower limit of detection was calculated as 100 HDV RNA copies/ml.

Serum HBV DNA and HCV RNA were quantified using the Roche Cobas TaqMan (Roche, Barcelona, Spain), with which the lower detection limits are 12 IU/ml and 10 IU/ml, respectively. Plasma HIV RNA was quantified using the bDNA assay (Quantiplex v3.0; Bayer, Barcelona, Spain).

HBV genotyping and resistance
HBV genotype and antiviral resistance were determined using line immunoassays LiPA HBV genotyping and HBV DR v2, respectively (Innogenetics, Ghent, Belgium).

Liver fibrosis
The extent of hepatic fibrosis was measured longitudinally using a validated new non-invasive imaging procedure called transient elastography (Fibroscan®; Echosens, Paris, France) [12]. Fibrosis stage was evaluated according to the METAVIR score. The results were expressed in kilopascals (kPa) and correspond to the following METAVIR scores: F0–1, <7.1 kPa; F2, 7.1–9.4 kPa; F3, 9.5–12.5 kPa and F4; >12.5 kPa [13–15].
Statistical analyses

All parameters are described as absolute numbers and percentages. Spearman’s rank correlation, Wilcoxon matched-pairs and U Mann–Whitney were performed using SPSS v13 (SPSS Inc., North Chicago, IL, USA). Differences were considered as significant when P-values were <0.05.

Results

A total of 16 HIV-infected patients with CDH were included in the study. The majority of patients were male (81.3%) and had a median age of 33 years. Most subjects (93.8%) were also anti-HCV antibody positive but only three of them showed detectable serum HCV RNA. Specific HDV IgM antibodies were detected in eight (50%) subjects and four (25%) were HBeAg-positive. HBV genotype was available for eight patients (five with genotype D and three with genotype A). Plasma HIV RNA was detectable in 11 patients at baseline; six were receiving antiretroviral drugs with no anti-HBV activity (zidovudine and/or emtricitabine (FTC)). During the follow-up, all subjects were under HAART with regimens including anti-HBV drugs such as lamivudine (3TC) (n=16), tenofovir (TDF) (n=10) and/or emtricitabine (FTC) (n=4) for a median time of 6.1 years (interquartile range [IQR] 4.7–8.7). Unfortunately, data prior to commencing anti-HBV therapy were not available for two patients. The remaining 14 patients presented low or undetectable serum HBV DNA (median 1.1 log_{10} IU/ml) at baseline, while serum HDV RNA was detectable in all cases, with a median of 7 log_{10} copies/ml, and 12 of them had elevated alanine aminotransferase (ALT) levels.

After initiating treatment with anti-HBV drugs, 13 patients demonstrated a decrease in their HDV RNA viral load as well as in aminotransferase levels. Furthermore, three patients reached undetectable HDV RNA and reduced their ALT levels to normal values (<35 IU/ml). A decrease in serum HBV DNA and total HBsAg was observed, although this did not reach statistical significance. These patients showed a preserved immunological status and most of them presented undetectable plasma HIV RNA without a significant change during the follow-up (Table 2).

For six patients (numbers 1, 3, 7, 8, 12 and 15), pre-treatment samples were available for testing serum HDV RNA levels for a median of 1.4 years (IQR 0.9–4.8), with 2–3 samples per patient. A significant increase in median serum HDV RNA was found between pre-treatment and baseline values (5.5 versus 7.1 log copies/ml, P=0.028). Longitudinal records of serum HDV RNA in the study population are depicted in Table 3.

In two patients (3 and 4), 3TC resistance mutations (rtM204I) were selected during 3TC therapy, which coincided with an increase in serum HBsAg and HBV DNA. Inclusion of TDF in the HAART regimen was followed by a decrease of serum HBV DNA with a less evident decline of HBsAg and HDV RNA (Figure 1). Overall, a statistically positive significant association was found between serum HDV RNA and HBV DNA (r=0.226, P=0.015), aspartate aminotransferase (AST; r=0.430, P=0.001), ALT (r=0.441, P=0.001) and HBsAg (r=0.557, P<0.001). No statistical association could be found between serum HDV RNA levels and CD4+ T-cell counts, plasma HIV RNA, age, risk group, HBeAg, HDV IgM antibodies or HBV genotype.

Two patients became HDV IgM negative during the follow-up. One subject demonstrated a fivefold decrease in serum HDV RNA at the point of becoming IgM-negative; whereas the other had undetectable serum HDV RNA in both IgM-positive and -negative samples. In 13 patients, liver fibrosis stage was assessed using Fibroscan®, of which nine were checked at least twice at approximately yearly intervals. The METAVIR score was as follows: F4 in four patients, F3 in two, F2 in three and F0–1 in one. A further two patients showed an increase in liver fibrosis (one from F0–1 to F2 and the other from F2 to F3). In both cases this coincided with an increase in ALT levels and in one with
Table 2. Follow-up of 14 hepatitis delta patients under anti-HBV therapy

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline</th>
<th>End of follow-up</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T-cell count, cells/ml</td>
<td>360 (159.5–470.5)</td>
<td>362 (262.5–761)</td>
<td>0.753</td>
</tr>
<tr>
<td>Plasma HIV RNA, log10 copies/ml</td>
<td>1.7 (1.7–4.3)</td>
<td>1.7 (1.7–2.9)</td>
<td>0.735</td>
</tr>
<tr>
<td>Serum HBV DNA, log10 IU/ml</td>
<td>1.1 (1.1–4.6)</td>
<td>1.1 (1.1–1.1)</td>
<td>0.116</td>
</tr>
<tr>
<td>Serum HDV RNA, log10 copies/ml</td>
<td>7 (6.2–7.8)</td>
<td>5.8 (2–6.3)</td>
<td>0.011*</td>
</tr>
<tr>
<td>Serum HBSAg, IU/ml</td>
<td>6,899 (1,792.5–20,085.5)</td>
<td>4,428 (406–6,885)</td>
<td>0.424</td>
</tr>
<tr>
<td>Serum ALT, IU/ml</td>
<td>98 (66.5–147)</td>
<td>63.5 (32.8–110.8)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Serum AST, IU/ml</td>
<td>87 (59.5–102)</td>
<td>60 (35–90.3)</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

All values are median (interquartile range). *Statistically significant. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBSAg, surface antigen of HBV; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus.

Table 3. Pre-treatment, baseline and post-treatment serum HDV RNA levels in 16 HIV-infected patients with CHD under HAART

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time on treatment, years</th>
<th>Pre-treatment</th>
<th>Serum HDV RNA, copies/ml (log10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>24,280,000 (7.3)</td>
<td>128,700,000 (8.1) 48,600 (4.7)</td>
</tr>
<tr>
<td>2*</td>
<td>5</td>
<td>&lt;100 (&lt;2)</td>
<td>160,300 (5.2)</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>40,190,000 (7.6)</td>
<td>164,600,000 (8.2) 2,989,000 (6.4)</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>&lt;100 (&lt;2)</td>
<td>1,664,000 (6.5)</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>7,190,000 (6.9)</td>
<td>3,468,000 (6.5)</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>26,740,000 (7.4)</td>
<td>169,500 (6.2)</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>3,040 (3.4)</td>
<td>13,170,000 (7.1) &lt;100 (&lt;2)</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>29,700 (4.4)</td>
<td>1,722 (3.1)</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>5,579,000 (6.8)</td>
<td>169,500 (6.2)</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>6,366,000 (6.9)</td>
<td>4,820 (3.7)</td>
</tr>
<tr>
<td>11*</td>
<td>4</td>
<td>55,400,000 (7.7)</td>
<td>315,300 (5.4)</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>34,960 (4.5)</td>
<td>315,300 (5.4)</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>53,510,000 (7.7)</td>
<td>89,060 (4.9)</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>2,846,000 (6.4)</td>
<td>630,000 (4.8)</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>274,800,000 (8.4)</td>
<td>685,300 (5.8)</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>33,010,000 (7.5)</td>
<td>33,010,000 (7.5)</td>
</tr>
</tbody>
</table>

*No baseline samples available: first available specimen for patient 2 was 2 years after starting lamivudine (3TC); first available sample for patient 11 was 5 months after starting 3TC. CHD, chronic hepatitis delta; HAART, highly active antiretroviral therapy; HDV, hepatitis delta virus.

Figure 1. Serum HBV DNA, HDV RNA and HBsAg in a patient (number 4) during antiviral treatment and selection of 3TC resistance (M204I)

3TC, lamivudine; HBV, hepatitis B virus; HBSAg, HBV surface antigen; HDV, hepatitis delta virus; TDF, tenofovir.
increased serum HDV RNA levels as well. Another patient showed a decrease in liver fibrosis (from F3 to F2) and this coincided with a decrease in ALT, but not in decreased serum HDV RNA. Overall, no association could be made between the stage of liver fibrosis and serum HDV RNA or ALT levels, although an elevation in serum HDV RNA was seen with increased degrees of liver fibrosis at least up to F3, with a decrease in F4 patients (Table 4).

### Discussion

In this study, 16 patients with HIV–HBV–HDV coinfection were followed during their HAART regimen, which included anti-HBV active agents, for a median time of 6.1 years. Of these patients, 13 demonstrated a decrease in plasma HDV RNA, HBV DNA and ALT levels, and three of them reached undetectable delta viraemia and normal aminotransferase levels.

Previous studies have shown that 3TC, a nucleoside analogue that potently inhibits HBV replication, has little effect on HDV replication [16,17]. Similarly, treatment with famciclovir (FCV) was not effective against HDV infection [18]. In these reports, patients treated with 3TC or FCV were followed up for 6–18 months, which may not have been sufficient time to observe significant serum HDV RNA reductions. However, in our study we analysed HIV-coinfected patients with HDV undergoing long-term antiretroviral therapy including potent active anti-HBV agents such as TDF and FTC, and followed up for longer periods. The fact that these patients were coinfected with HIV offered a unique opportunity to observe the activity of nucleoside analogues more potent than 3TC against HBV (for example, TDF) that are not yet approved for HBV-monoinfected patients. We were able to recognize that most patients who achieved undetectable serum HBV DNA levels as a result of successful therapy also showed a significant reduction in delta viraemia, which eventually became undetectable in three patients, with complete normalization of liver enzymes. As none of these patients has discontinued anti-HBV agents, it is not known whether they may have permanently cleared HDV or if the virus could still rebound following treatment withdrawal. The risk of severe flares and liver injury makes it difficult to plan this clinical investigation [19]. We were able to analyse several pre-treatment HDV RNA levels in six patients. All of them showed a steady increase in serum HDV RNA in the absence of anti-HBV therapy, which further supports the effect of anti-HBV therapy on HDV RNA viraemia.

A number of studies have demonstrated viral interference between HBV, HCV and HDV [7,20–23]. Our results at baseline (prior to anti-HBV therapy) support these findings because most patients had either low or undetectable serum HBV DNA or HCV RNA (those with anti-HCV antibodies) and conversely high HDV RNA titres, suggesting HDV to be the predominant virus replicating in the liver.

It is important to remember that HDV is a defective virus that requires HBV to support its replication. In fact, it is the HBsAg that is necessary for viral packaging, export and spread of HDV infection to other cells. HBsAg is expressed efficiently in enormous quantities from two mRNAs transcribed from the covalently closed circular DNA (cccDNA). The cccDNA is the major transcriptional template that uses the host RNA polymerase and the HBV core promoter. It has a long half-life and is the reason for persistent HBV infection [24].

In conclusion, to our knowledge, this is the first evidence of the benefit of potent anti-HBV nucleos(t)ide analogue therapy for CHD. In HIV-infected patients undergoing antiretroviral therapy including anti-HBV agents there seems to be an indirect benefit of suppression of HDV replication, albeit not a very efficient one. Treatment with clevudine, a novel nucleoside analogue currently in clinical development, has been shown to rapidly reduce serum HBV DNA and, unlike other inhibitors, it seems to reduce HBsAg and cccDNA levels more potently [27]. Moreover, in a woodchuck model, clevudine inhibited HDV RNA [28], which supports our findings that only potent anti-HBV therapies that successfully reduce HBsAg and cccDNA might reduce HDV replication.
Acknowledgements

This work was partially funded by Fundación Investigación y Educación en Sida (IES), Red de Investigación en SIDA (RIS, ISCIII-RETIC R06), the Spanish CIBER on Hepatitis, the European Commission NEAT project, the VIRGIL European Network of Excellence on Antiviral Drug Resistance (LSHM-CT-2004-503359) and Agencia Lain Entralgo.

Disclosure statement

The authors declare no competing interests.

References