HBV load in treated and untreated individuals

Yates, S.C.

Citation for published version (APA):
Chapter 7

Determinants of a beneficial response to lamivudine-containing regimens on HBV replication markers among HIV-1 infected HBsAg, positive individuals

Maarten Penning, Sol Yates, Hans Bogaards, and Jaap Goudsmit

In preparation
Abstract

Lamivudine suppresses both HIV-1 and HBV replication, making lamivudine-containing regimens the therapy of choice for HIV-1 infected HBsAg positive individuals. We studied 47 patients longitudinally during an average period of 2.5 years of lamivudine treatment in order to establish which factors determine the outcome of infection and which laboratory markers respond best to therapy. Newly developed quantitative NASBA-based assays were used to monitor HBV DNA and RNA in serum, while HBeAg was monitored with a routine serologic assay. Of the 28 HBeAg positive individuals, 11 (39%) became HBeAg negative during treatment. Of the 24 HBV DNA positive individuals, 16 (67%) cleared HBV DNA to levels below the quantification limit of the assay. 18 (75%) of the 24 HBV RNA positive individuals cleared HBV RNA to undetectable levels. Response to therapy was first noted when HBV RNA was monitored, HBV DNA second and HBeAg last. Baseline HIV-1 RNA levels and CD4 cell counts as well as the addition of none, single, or multiple anti-HIV drugs to lamivudine, all contributed to the probability that an impact on HBV replication markers of lamivudine-containing regimens would be observed.
Introduction

World wide, 350-400 million people suffer from chronic infection with the hepatitis B virus (HBV), compared to 30 million human immunodeficiency virus (HIV-1) infected persons. Because both viruses have comparable routes of transmission, chronic HBV infection affects about 20% of patients infected with HIV-1. Most coinfected patients receive treatment for their HIV-1 infection and not for HBV. Nowadays, a significant number of patients are treated with highly active antiretroviral therapy (HAART), existing of a cocktail of 3 or more anti-HIV-1 drugs. One commonly included drug is the nucleoside analog lamivudine, which suppresses both HIV-1 and HBV replication. Doses applied in HBV treatment are generally lower than doses used against HIV-1 (100 mg/day vs. 300 mg/day, respectively). In patients without concurrent HIV-1 infection, little difference was found between regimens with 100 or 300 mg concerning the number of patients clearing HBV DNA and HBeAg seroconversion, although inhibition of HBV replication was more rapid with the latter dose. More than 80% of the patients clear HBV DNA from serum within 4-8 weeks upon start of lamivudine treatment, but clearance of HBeAg during this period was only seen in a minority of patients.

We and others reported that besides HBeAg and HBV DNA, HBV RNA levels in the serum or plasma of patients may be related to disease status or progression. For that reason we studied the sequence of three marker responses to lamivudine-containing therapy in HBV/HIV-1 double infected individuals. In the present study 47 patients were included and monitored during the period they received therapy from the moment lamivudine treatment was initiated. NASBA-based quantitative HBV RNA and DNA assays were used for evaluation as well as a routine serological assay for HBeAg.
Materials and methods

Patients
Forty-seven patients were studied, who had chronic HBV infection and were treated at the Academic Medical Center of the University of Amsterdam for HIV-1 infection with lamivudine as part of antiretroviral therapy. Chronic HBV infection was defined as HBsAg expression for at least half a year. Patients were selected for this analysis if (i) prior information on markers of HBV infection was available and (ii) the effect of lamivudine on parameters of HBV replication was monitored subsequently. Only first-time use of lamivudine was considered and observation time ended when either lamivudine therapy ended or patients were lost to follow-up. End of lamivudine therapy was defined as cessation of lamivudine for three weeks or more; cessation for less than three weeks was not considered an end of lamivudine therapy. In analyzing the HBV response to lamivudine, the last measurement in the half-year preceding lamivudine was taken as the baseline value in case no measurement was made on the starting date of lamivudine.

Thirty-five patients provided information on HBV replication markers both prior to and during lamivudine therapy. In this group, starting dates of lamivudine therapy ranged from December 1991 to January 1998. In total, 18 patients (51%) used triple therapy at start of lamivudine. Half of the selected patients had baseline characteristics on HBV infection markers determined within a week before start of lamivudine therapy.

Serological assays
Serum HBsAg, HBeAg and antibodies to hepatitis B c antigen (anti-HBc) were measured using commercially available enzyme immunoassays (EIA; Hepanostika HBsAg/HBeAg/anti-HBc, Organon Teknika bv., Boxtel, the Netherlands) according to the guidelines of the manufacturer.

HIV assays
HIV-1 status was determined by measuring HIV-1 RNA. Three different techniques were used on all samples: HIV-1 RNA QT NASBA (Organon Teknika bv., Boxtel, the Netherlands), Nuclisens NASBA (Organon Teknika bv., Boxtel, the Netherlands), and Roche Amplicor assay (Roche diagnostic systems, Branchburg, New Jersey, USA). CD4+ cells were counted by standard flow cytometry using a FACSCAN flow cytometer (Beckinson, San Jose, CA) and commercially available monoclonal antibodies (Beckinson,
HBV RNA and DNA assays

Nucleic acids were extracted from 100 μl of serum using protocol Y+9. This method is based on the well-known silica-guanidiniumthiocyanate protocol Y, described by Boom et al.10. Protocol Y+ involves addition of 1 ml lysis buffer L7A and 30 μl of size-fractionated silica particles before following the classic Y protocol. L7A is prepared from buffer L6 (5.25 M GuSCN, 50 mM Tris-HCl [pH 6.4], 20 mM EDTA, 1.3% [wt/vol] Triton X-100) by the addition of α-casein to a final concentration of 1 mg/ml. Each sample was eluted in 50 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]).

HBV DNA and HBV RNA were quantified using real-time NASBA (Retina ™; PrimaGen, Amsterdam, The Netherlands). Both assays are developed in-house and are described before11(Penning; JCM). Briefly, Retina ™ reactions were started by mixing 5 μl extracted nucleic acid and 10 μl Retina ™ amplification mix in microtubes. Subsequently, the nucleic acids were denatured for 5 minutes at 65°C for the HBV RNA Retina™, and at 95°C for the HBV DNA Retina™, followed by incubation at 41°C for 5 minutes. Then the enzyme mix was pipetted into the lids of the microtubes. The tubes were centrifuged briefly (10 sec) at 1000 rpm in an Eppendorf 5804 centrifuge (Eppendorf, Hamburg, Germany) to collect the enzyme mix. After gentle mixing by tapping, the tubes were incubated at 41°C in a fluorimeter (Cytoflour 4000, Perkin Elmer, Wellesley MA, USA) for 120 minutes, with measurement of the fluorescent signal every minute. The 20 μl reactions were excited at 485 nm, and fluorescence was measured at 530 nm. Readings were normalized to the background of a reaction containing water instead of template. The amount of HBV DNA or RNA present in samples was calculated using a standard curve generated from HBV plasmid or in vitro RNA standards that indicated the relation between time-to-positivity (TTP) and input amount. The standards were used in 10-fold serial dilutions. Data were processed in an Excel 97/98 spreadsheet to correct for background as measured in the no-template control sample, and to make calibration curves. The HBV DNA or RNA loads of the samples were then interpolated using these calibration curves. The cut-off of each individual assay was set to be equal to the value of the last positive sample in the dilution series. The load of HBV DNA or RNA in the samples was expressed in log copies/reaction.
**Statistical Analyses**

Covariate effects on viral clearance were studied by fully parametric regression techniques, which directly accounted for interval censoring. First, we determined which distribution could best describe residual clearance times in models including coefficients for all baseline variables: lamivudine containing triple therapy, baseline CD4 cell count, baseline HIV-1 viral load and baseline HBV DNA or RNA load, if appropriate. The distribution which provided the best fit to the data, judged by Akaike's Information Criterion, was selected for further analysis. Next, we used univariate models to estimate the independent effects of lamivudine containing triple therapy, baseline CD4 cell count, baseline HIV-1 viral load and baseline HBV DNA or RNA load, if appropriate, on HBV clearance during lamivudine therapy. To study possible effect modifications due to correlation between baseline variables, we also performed multivariate analyses by means of backward variable selection, starting with a full model and repeatedly removing variables with $p>0.10$ until a final model was obtained. Findings of univariate analyses are illustrated by Kaplan-Meier estimates, stratified by the covariate of interest.

The possible bias due to differences in sampling frequency was assessed a posteriori by testing for mean sampling frequency between strata of interest.

Data were analyzed with the SAS system, version 6.12 (SAS Inc., Cary, NC, USA), in particular using the procedures LIFETEST, PHREG and LIFEREG.

Mean sampling frequency during lamivudine therapy was 4.7 samples per year. Sampling frequency was the same for patients with mono/dual or triple therapy (4.7 vs. 4.8 samples per year, $p=0.94$). Sampling frequency was slightly higher for those patients who at baseline (i) had CD4 cell count below 110 cells/mm$^3$ (4.8 vs. 4.3 samples per year, $p=0.53$) or (ii) had HIV-1 viral load above $4.0 \times 10^4$ copies/ml (4.7 vs. 4.2 samples per year, $p=0.73$). In all comparisons, differences were non-significant according to T-tests for mean sampling frequency. Hence, it is unlikely that covariate effects on time to viral clearance are due to biases in sampling frequency related to the variables of interest.

HIV-1 viral load at the start of lamivudine therapy was available for 25 patients, of which 23 (92%) had HIV-1 RNA $>1 \times 10^3$ copies/ml, with a median viral load of $4.0 \times 10^4$ copies/ml.

CD4 cell counts at start of lamivudine therapy were available for 29 patients, with a median CD4 cell count of 110 cells/mm$^3$ and an interquartile range of 40–210 cells/mm$^3$. Median follow-up under first-time use of lamivudine was 2.5 years, with an interquartile range of 1.4–4.0 years.
Results

Baseline characteristics

According to Table 1, 89% of patients were HBsAg+, 80% were HBeAg+, 71% had HBV DNA above the lower quantification limit of the assay (1x10^4 copies/ml) and 69% had HBV RNA loads >1x10^4 copies/ml at baseline. The median HBV DNA load for the HBV DNA positive patients at baseline was 7.9x10^7 copies/ml, and the median HBV RNA load for those positive at baseline was 6.3x10^6 copies/ml. Baseline values of HBV DNA and RNA loads, HIV-1 load (4.0x10^4 copies/ml), and CD4 cell counts (110 cells/mm^3) did not differ significantly between patients receiving mono or dual therapy vs. patients receiving triple therapy.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Mono/Dual</th>
<th>Triple</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease Inhibitor</td>
<td>3 (18%)</td>
<td>14 (78%)</td>
<td>17 (59%)</td>
</tr>
<tr>
<td>HBsAg+</td>
<td>14 (82%)</td>
<td>17 (94%)</td>
<td>31 (89%)</td>
</tr>
<tr>
<td>HBeAg+</td>
<td>13 (76%)</td>
<td>15 (83%)</td>
<td>28 (80%)</td>
</tr>
<tr>
<td>HBV DNA &gt;10E4 cp/ml</td>
<td>11 (69%)</td>
<td>13 (72%)</td>
<td>24 (71%)</td>
</tr>
<tr>
<td>HBV RNA &gt;10E4 cp/ml</td>
<td>10 (59%)</td>
<td>14 (78%)</td>
<td>24 (69%)</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>18</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 1. Patient characteristics at start of lamivudine (N=35).
Only patients with baseline and follow-up information on HBV are included in this analysis.

Response to therapy according to HBeAg monitoring

Out of 28 patients who were HBeAg positive at baseline, 11 (39%) were observed with clearance of HBeAg after initiating lamivudine therapy. All patients who cleared HBeAg, but one, did so in the first half-year during lamivudine therapy. The rest did not clear HBeAg at all for the observed follow-up. Patients using lamivudine triple therapy cleared HBeAg after a mean of 0.73 years.

Response to therapy according to HBV DNA monitoring

Out of 24 patients with positive HBV DNA levels at baseline, 16 (67%) were observed with clearance of DNA to levels below 1x10^4 copies/ml during lamivudine therapy. According to the Kaplan-Meier estimates, the median time to clear DNA was 0.51 years at the left interval (assuming that viral clearance occurred immediately after the last observed
positive measurement since start of lamivudine therapy) or 0.81 years at the right interval (assuming that viral clearance occurred just before the first negative measurement during lamivudine therapy). Estimating clearance time as the midpoint of the two gave a median time of 0.64 years since start of lamivudine therapy to clear HBV DNA. Patients using lamivudine mono or dual therapy cleared HBV DNA after a mean of 2.86 years, compared to 0.36 years for patients receiving triple therapy.

Response to therapy according to HBV RNA monitoring
Out of 24 patients with positive HBV RNA levels at baseline, 18 (75%) were observed with clearance of RNA to levels below 1x10^4 copies/ml during lamivudine therapy. According to the Kaplan-Meier estimates, the median time to clear RNA was 0.51 years at the left interval or 0.62 years at the right interval. Estimating clearance time as the midpoint of the two gave a median time of 0.57 years since start of lamivudine therapy to clear HBV RNA. The median clearance times for patients receiving lamivudine mono/dual therapy vs. triple therapy were 2.41 years and 0.35 years respectively.

Covariate effects on time to viral clearance
We initially considered single covariate effects in fully parametric regression models. Table 2 shows effect estimates according to log-logistic residuals for HBeAg clearance and Weibull residuals for DNA and RNA clearance. These distributions provided the best fit to the full models, including all baseline variables listed.
## Table 2. Effect of triple therapy, CD4 cell count and HIV-1 viral load at baseline on clearance of HBeAg, DNA and RNA during lamivudine therapy.

In models of HBeAg clearance, a log-logistic distribution was assumed for the residual clearance times. In models of DNA and RNA clearance, a Weibull distribution was assumed for the residual clearance times. Distributions were selected on the basis of Akaike’s Information Criterion for models including all baseline variables. Effect estimates of baseline HBV DNA and RNA for HBV DNA and RNA clearance during lamivudine therapy are based on increments per log10 cp/ml. Std Error: standard error of effect estimate; cp/ml: copies/ml.
HBeAg
Clearance of HBeAg was not dependent on use of lamivudine in triple therapy or HIV-1 viral load at baseline. A higher baseline CD4 cell count was associated with a reduced probability of HBeAg clearance, albeit with borderline statistical significance. Patients with baseline CD4 cell count above average experienced a 3.2 times slower clearance of HBeAg, as indicated by a positive coefficient in table 2 (column estimate) and illustrated in figure 1. If CD4 cell count is investigated together with triple therapy (multivariate analysis), the effect is the same. When controlled for baseline HIV-1 viral load, the effect of CD4 cell count is more pronounced.

![Figure 1. Effect of CD4 cell count at start of lamivudine therapy on the probability of HBeAg clearance.](image)

HBV DNA
HBV DNA clearance was not dependent on any of the explanatory baseline variables in univariate models. Interestingly, in multivariate analyses, both the effects of lamivudine in triple therapy and baseline HIV-1 viral load are highly significant, as shown in table 2. Addition of baseline CD4 cell count to the model increased standard errors, but effect estimates remained similar. According to the multivariate model, patients with baseline HIV-1 viral load above average and using lamivudine as part of triple therapy experienced faster clearance of HBV DNA. The sole effect of lamivudine containing triple therapy on HBV DNA clearance is illustrated in figure 2.
Determinants of a beneficial response to lamivudine

**Figure 2.** Effect of triple therapy including lamivudine on clearance of HBV DNA. Kaplan-Meier curves are based on three estimates: event times of viral clearance are assumed at the left or right interval (thin lines) or at the midpoint (thick line) of the censored interval. Therapy switching is not taken into account, except for lamivudine. P values are based on the log-rank test for homogeneity of clearance over strata.

**HBV RNA**

HBV RNA clearance was not dependent on CD4 cell count or HIV-1 viral load at baseline, but use of lamivudine containing triple therapy increased the probability of HBV RNA clearance 1.5-fold (Table 2). Modeling lamivudine containing triple therapy together with HIV-1 viral load and CD4 cell count did not alter the effect estimate or the corresponding standard error of the triple therapy effect. Stratified Kaplan-Meier analysis also yields a significant difference in rate of HBV RNA clearance between patients using lamivudine containing triple therapy and those using lamivudine as mono/dual therapy. The effect of lamivudine containing triple therapy on HBV RNA clearance is illustrated in figure 3.

**Figure 3.** Effect of triple therapy including lamivudine on clearance of HBV RNA. Kaplan-Meier curves are based on three estimates: event times of viral clearance are assumed at the left or right interval (thin lines) or at the midpoint (thick line) of the censored interval. Therapy switching is not taken into account, except for lamivudine. P values are based on the log-rank test for homogeneity of clearance over strata.
Discussion

In this report we studied patients from the start of lamivudine treatment, which was used in different (combination) regimens. Some patients were already treated with other antiretroviral medicines before receiving lamivudine, whereas others were naive. Therefore, the study population is very heterogeneous, but at the same time is a good representation of patients seen in a hospital.

We found that the effect of lamivudine therapy could be detected most quickly by HBV RNA clearance, followed by the clearance of HBV DNA. HBeAg was the last marker to disappear from the serum. In patients receiving lamivudine as mono or dual therapy the clearance times of HBV RNA and DNA were 2.41 and 2.86 years after start of lamivudine treatment, respectively. In patients receiving lamivudine as a part of triple therapy, median clearance times were 0.35, 0.36 and 0.73 years for HBV RNA, HBV DNA and HBeAg, respectively. The fact that HBV DNA containing particles can be detected longer than virions containing HBV RNA may be explained because during HBV replication, DNA containing particles develop from particles with RNA. When the replication of HBV is hampered due to the effect of lamivudine the particles that arise in the beginning of the replication cycle will be the first to be affected. HBeAg can be detected in the serum for a longer time than HBV RNA and DNA. This effect is also seen in a normal resolving acute infection. Also during a chronic infection with HBeAg seroconversion, HBeAg is detected in serum for a longer period of time than viral DNA.

18 of 24 patients (75%) cleared HBV RNA from their serum. The median time to clear HBV RNA was 0.64 years after the start of lamivudine therapy. Patients receiving triple therapy cleared HBV DNA much faster than patients receiving mono or dual therapy (0.35 years vs. 2.41 years respectively). Clearance of HBV DNA was observed in 16 of 24 patients (67%). The median clearance time was 0.64 years after the start of lamivudine administration and 0.36 and 2.86 years for patients using triple therapy vs. mono or dual therapy, respectively. The faster clearance of HBV DNA and RNA in patients receiving triple therapy, is probably due to a more efficient suppression of the HIV-1 infection and therefore the immune system is more competent to suppress HBV. Studies of HBV patients without HIV-1 infection observed that HBV DNA was cleared from serum in 4–8 weeks in more than 80% of patients 5-7. In our study less patients clear HBV DNA and the time it takes is longer, but is confirmed by comparable clearance times of HBV RNA.
These extended periods could be due to the less sensitive assays used in these other studies. At least in one study HBV DNA is cleared within 4 weeks, but when the samples are tested with PCR, HBV DNA is still detectable in the serum of all patients\(^5\). In HIV-1 coinfected patients, HBV DNA is cleared in 30% of the patients at week 12 and 40% at week 52 after start of lamivudine therapy\(^12\), which is comparable to our findings. Furthermore, immune reconstitution with HAART has been reported to shift the spectrum of HBV disease toward an enhanced inflammatory response to hepatitis B followed by decreased viremia and seroconversion\(^13\). This may lead to an extended time period needed to completely clear HBV DNA and RNA compared to patients not infected with HIV-1.

Patients receiving lamivudine as part of triple therapy have a higher chance of clearing both HBV RNA and DNA. As shown in table 2, in the case of HBV RNA this is true in univariate as well as multivariate analysis. In the case of HBV DNA however, this relation is only significant in multivariate analysis. In figure 2, a clear difference in clearance times between patients using mono/dual or triple therapy can be seen. Patients with baseline HIV-1 viral load above average and using lamivudine as part of triple therapy experienced faster clearance of HBV DNA, (Table 2). HBV RNA clearance was not dependent or HIV-1 viral load at baseline nor on CD4 cell count at baseline (Table 2). There was a significant difference in rate of HBV RNA clearance between patients using lamivudine as part of triple therapy and those receiving mono or dual therapy (Fig. 3).

Since HBV RNA in serum seems to be related to chronic HBV disease\(^8\), it would be better to treat HIV/HBV coinfected patients with lamivudine containing triple therapy, rather than a less stringent lamivudine containing regimen, since such a regimen seems to be the best way to fight both viruses at the same time.

We found that of 28 patients with measurable HBeAg at baseline, 12 cleared HBeAg from serum. 11 (39%) did so in the first half-year during lamivudine therapy. The rest did not clear HBeAg during the study period. Although not all our patients receive triple therapy, we find the same percentage HBeAg clearance as reported in a study of den Brinker et al.\(^14\), who reported that 38% of patients with chronic HBV and HIV-1 infection lost HBeAg or developed anti-HBe after initiation of HAART. Clearance of HBeAg was not dependent on use of triple therapy or HIV-1 viral load at baseline, but a higher baseline CD4 cell count was associated with a reduced probability of HBeAg clearance (Table 2). Patients with baseline CD4 cell count above average experienced slower clearance of HBeAg as illustrated in figure 1. This is a remarkable observation, but one has to keep in mind that
only 11 patients clear HBeAg and that the correlation has only borderline significance. All patients studied suffer from chronic HBV infection, which is defined as detectable serum HBsAg for at least six months. This means that in the patients with high CD4 counts at baseline, the immune system was not able to clear the HBV infection during this period. In the patients with low CD4 counts at the start of lamivudine therapy, the immune system just may have been to weak to resolve the HBV infection. Due to the antiretroviral therapy their immune system may improve, resulting in a higher chance of clearing HBeAg. This relation with CD4 cell count is not seen with HBV DNA and RNA clearance, and probably reflects the fact that clearance of HBeAg is only immune mediated, while clearance of HBV DNA and RNA is a result of the immune reaction, combined with the direct influence of lamivudine on the replication of HBV.

In conclusion, we show that the best way to treat HBsAg positive patients with HIV-1 coinfection with a triple therapy regimen including lamivudine. The effect of therapy on HBV can be measured best by monitoring HBV RNA, instead of HBV DNA, since RNA is cleared from the serum in more patients than DNA (18 and 16, respectively) and HBV RNA is cleared faster to levels below $1 \times 10^4$ copies/ml than HBV DNA.
References


