Subtype-Dependent Response of Hepatitis B Virus during the Early Phase of Lamivudine Treatment

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We conducted a 12-month longitudinal investigation of the subtype-dependent response of hepatitis B virus (HBV) to lamivudine treatment in 43 consecutive patients with chronic hepatitis B. HBV subtype ayw appears to respond better to lamivudine monotherapy than does HBV subtype adw (P = .005). This might be the reason for the lower incidence of lamivudine-resistant strains observed in persons infected with HBV subtype ayw during follow-up.

Lamivudine, which has been shown to be highly effective against hepatitis B virus (HBV) [1, 2], is used widely for treatment of chronic hepatitis B. In patients with hepatitis B e antigen (HBeAg)–positive chronic hepatitis B, lamivudine has been reported to yield seroconversion from HBeAg to antibody (anti-HBe) in 16% [1] and 17% [2] of patients after 12 months of lamivudine therapy, compared with 4% and 6% of control subjects, respectively. However, the use of lamivudine is hampered due to the selection of drug-resistant HBV variants. Strains of HBV with lamivudine resistance–associated mutations have been detected in 14%–32% of patients after 1 year of treatment [1–4], with the frequency increasing to 38%, 57%, and 66% after 2, 3, and 4 years of treatment, respectively [5–7].

Factors that determine the rate of seroconversion from HBeAg to anti-HBe or that influence the selection of lamivudine-resistant strains of HBV have great clinical impact. HBV can be divided into 4 major subtypes (adw, ayw, adr, and ayr); adw and ayw are the predominant subtypes in the United States and Europe. Only limited data are currently available on whether the HBV subtype affects the effectiveness of lamivudine therapy. In a pilot study, we recently showed that the HBV subtype adw appears to have a high risk of developing lamivudine resistance during the first 18 months of treatment [8]. Here, we report the first study of the influence of the HBV subtype on the viral response during the early phase of lamivudine therapy in a larger cohort of patients by longitudinal measurements of the HBV DNAemia and alanine aminotransferase (ALT) levels.

Patients and methods. Forty-three consecutive patients (5 female and 38 male patients) with chronic HBV infection were included in the study. The decision to treat these patients with lamivudine was made independent of the study protocol, which was approved by the local ethics committee (Hamburg Medical Association). Informed consent was obtained from all participants. All patients received 100 mg of lamivudine per day for 12 months. Follow-up data were available for 26 patients who received lamivudine treatment for an additional 6 months. Adherence to lamivudine therapy was high in all patients, as estimated by pill counts deduced from prescription intervals. The outcome variables were achievement of normal ALT levels (defined as an ALT level of ≤22 U/L achieved at least once during the observation period), achievement of undetectable levels of HBeAg, and emergence of lamivudine-resistant HBV.

For every serum sample obtained, levels of HBV surface antigen (HBsAg), HBeAg, anti-HBs, anti-HBe, and anti-HBc were determined by means of EIA (AxSYM; Abbott Laboratories). HBV copy numbers were quantified at 3-month intervals by real-time PCR (LightCycler-DNA Master SYBR GreenI; Roche Diagnostics) that targeted a conserved region of the HBV genome that overlaps the genes encoding the X protein and DNA polymerase [9]. The primer pairs HBV1F (5′-CGGTCTCTGGCCCTTCATCTG-3′) and HBV1R (5′-AGTCAAAGTGYCTCTTATGAAACGCTT-3′) were used, and amplicons were quantified by fluorescent activities (LightCycler DNA Master) compared with serial dilutions of an external standard preparation (cloned HBV, plasmid pHBV991), which was calibrated with 2 Eurohep reference samples [10]. The detection limit of the PCR was shown to be 10 HBV copies per mL of serum (40 EuroHepU per mL of serum) with a dynamic range of up to 105 copies per mL of serum.

For positive PCR results, amplicons were sequenced for lamivudine resistance–associated mutations. The polymerase re-
Table 1. Baseline characteristics of 43 patients enrolled in a study of the subtype-dependent response of hepatitis B virus (HBV) to lamivudine therapy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient group, by HBV subtype carried</th>
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<tbody>
<tr>
<td></td>
<td>adw</td>
<td>ayw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All patients (n = 22)</td>
<td>Follow-up group (n = 13)</td>
<td>All patients (n = 21)</td>
<td>Follow-up group (n = 13)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>Median 43</td>
<td>40</td>
<td>40</td>
<td>38</td>
<td></td>
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<tr>
<td></td>
<td>Range 31–65</td>
<td>31–53</td>
<td>10–69</td>
<td>10–69</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female 2</td>
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<td>3</td>
<td>2</td>
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<tr>
<td></td>
<td>Male 20</td>
<td>11</td>
<td>18</td>
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<td>Region of origin</td>
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<td>12</td>
<td>9</td>
<td>6</td>
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<td>5</td>
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<td></td>
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<td>Northern Africa 1</td>
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<td>HBeAg positive</td>
<td>19</td>
<td>10</td>
<td>14</td>
<td>9</td>
<td></td>
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<tr>
<td>Serum HBV DNA level, log_{10} copies/mL</td>
<td>Median 6.9</td>
<td>7.6</td>
<td>6.3</td>
<td>6.4</td>
<td></td>
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<tr>
<td></td>
<td>Range 3.95–8.47</td>
<td>5.3–7.77</td>
<td>3.0–7.69</td>
<td>3.5–7.69</td>
<td></td>
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<tr>
<td>Serum ALT level, U/L</td>
<td>Median 73</td>
<td>80</td>
<td>75</td>
<td>61</td>
<td></td>
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<tr>
<td></td>
<td>Range 25–135</td>
<td>25–135</td>
<td>15–300</td>
<td>36–262</td>
<td></td>
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</tbody>
</table>

**NOTE.** ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.

gion was amplified by a nested PCR with primers 252 (5′-AGACTCGTGGTGACCTTCTCT-3′)/1309 (5′-AGAATGTGTGCTCCAGACC-3′) as external primers and 377 (5′-GGATGTGTCCTGCGGCGTGT-3′)/840 (5′-ACCCCATCTTTTGTGTGTAG-3′) as internal primers, spanning the polymerase region from codon 451 to codon 592 [11]. Both strands of the amplification products were sequenced for lamivudine resistance–associated mutations (L528M, M552I, and M552V) with use of the BigDye termination sequencing kit (Applied Biosystems) and an automated sequencer. The identical region and procedure were also used for the determination of HBV subtypes [12].

For statistical analysis of quantitative virus loads and ALT values, data for HBV subtype adw–infected patients were compared with data for HBV subtype ayw–infected patients by use of the Mann-Whitney U test. This test was also used in a univariate analysis to determine the influence of sex and baseline HBeAg status on the response to lamivudine. The changes over time of median HBV copy numbers within each group were analyzed by use of the Wilcoxon rank-sum test. In addition, median levels of HBV DNA and ALT were compared by use of a multiple-comparison test with a 2-way analysis of variance (the split-plot repeated-measures model). Fisher’s exact test was used to calculate P values for the proportion of patients with undetectable HBV DNA levels, lamivudine-resistant HBV infections, and ALT-level normalization in each group. A Kaplan-Meier estimate was performed by use of the WinStat software package, and the P value was calculated by use of the Cox-Mantel log-rank test. However, because of the small sample size, the statistical power was <80% for a non-significant result. A P value of <.05 was considered statistically significant.

**Results.** Twenty-two patients carried HBV subtype adw (the adw group; 21 patients carried subtype adw2 and 1 patient carried subtype adw1) and 21 carried the HBV subtype ayw (the ayw group; 17 patients carried subtype ayw2/3, 2 carried ayw1, and 2 carried ayw4). The adw and ayw groups did not differ with regard to mean age, sex, median HBV DNA titer, rates of HBeAg positivity, and median ALT levels at baseline (table 1). Also, for the 26 patients who participated in the follow-up, the baseline characteristics of patients in the adw group were similar to those of patients in the ayw group, as well as with the total cohort (table 1). Therefore, the results of the follow-up were not biased by selection.

The mean decrease in HBV DNA level from baseline was significant in both subtype groups at month 3 (adw group: mean decrease, 2.9 log_{10} copies/mL [95% CI, 1.8–3.9 log_{10} copies/mL]; P < .001; ayw group: mean decrease, 3.4 log_{10} copies/
Figure 1. Mean serum levels of hepatitis B virus (HBV) DNA in 43 patients during lamivudine treatment for chronic hepatitis B. Bars indicate SD of the mean value. \( P \) values are provided for significant differences between carriers of HBV subtype adw and carriers of HBV subtype ayw. NS, not significant.

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\text{mL [95% CI, 2.2–4.6 log}_{10}\text{ copies/mL]; } P < .001.
\]

In the ayw group, HBV DNA levels continued to decrease significantly between months 3 and 6 of lamivudine therapy (mean decrease, \( 1.8 \log_{10}\text{ copies/mL, respectively; } P = .007 \)), month 9 (2.3 vs. 4.9 \log_{10}\text{ copies/mL, respectively; } P = .006 \), and month 12 (1.8 vs. 4.1 \log_{10}\text{ copies/mL, respectively; } P = .033 \). A subgroup analysis showed that these significant differences were independent of sex and baseline HBeAg status. By use of a multiple-comparison test, the difference in HBV DNA levels between the adw group and the ayw group was also significant \( P = .005 \). The respective proportions of patients in the adw and ayw groups with HBV DNA levels that were less than the lower limit of detection of the PCR test were 3 (14%) of 22 patients versus 9 (43%) of 21 patients at month 3 \( (P = .045) \), 4 (18%) of 22 versus 14 (67%) of 21 at month 6 \( (P = .002) \), 4 (18%) of 22 versus 10 (50%) of 20 at month 9 \( (P = .049) \), and 4 (19%) of 21 versus 10 (50%) of 20 at month 12 \( (P = \text{not significant [NS]}) \).

While they were receiving lamivudine therapy, patients in the ayw group had significantly lower mean ALT levels than did patients in the adw group at month 3 (27 vs. 93 U/L; \( P = .003 \)), month 6 (16 vs. 73 U/L; \( P = .006 \)), month 9 (17 vs. 35 U/L; \( P = .009 \)), and month 12 (12 vs. 39 U/L; \( P = .007 \)). These results were also significant in the multiple-comparison test \( (P = .045) \). There was no correlation between HBV copy numbers and ALT levels during lamivudine treatment. Normal ALT levels were achieved in 12 (55%) of the 22 patients in the adw group and in 17 (81%) of the 21 patients in the ayw group \( (P = \text{NS}) \).

A total of 18 patients (42%) were infected with isolates that exhibited lamivudine resistance–associated mutations at month 18. The mutants detected were M552I \( (n = 4; 1 \text{ in the adw group and } 3 \text{ in the ayw group}) \), L528M/M552I \( (n = 2; 1 \text{ in the adw group and } 1 \text{ in the ayw group}) \), and L528M/M552V \( (n = 12; 10 \text{ in the adw group and } 2 \text{ in the ayw group}) \). The proportions of HBV-infected patients infected with isolates that exhibited lamivudine resistance in the adw group and the ayw group were as follows: 7 of 21 patients versus 2 of 20 patients at month 12 \( (P = \text{NS}) \), 11 of 13 versus 4 of 13 at month 15 \( (P = .015) \), and 12 of 13 versus 6 of 13 at month 18 \( (P = .03) \), respectively. The mean time to lamivudine resistance was 12.4 months in the adw group and 16 months in the ayw group. A Kaplan-Meier estimate revealed a significantly higher probability for HBV strains to remain susceptible to lamivudine in the ayw group during the period of lamivudine therapy \( (P = .014; \text{figure 2}) \).

At month 18 of therapy, 3 of 13 patients in the adw group and 5 of 13 in the ayw group \( (P = \text{NS}) \) had seroconversion from HBeAg to anti-HBe. Seven of these patients had sustained suppression of HBV DNA at levels less than the detection limit of the PCR from month 6 until the end of follow-up, and these patients had no relapse of HBeAg. One patient in the ayw group had a rebound in HBV DNA level after seroconversion to anti-HBe due to the emergence of a M552I mutation. This rebound...
in the HBV DNA level was followed by the reappearance of HBeAg in serum. One patient in the adw group and 1 patient in the ayw group achieved undetectable levels of HBsAg after 5 months and 10 months of treatment, respectively.

**Discussion.** The efficiency of lamivudine for the treatment of chronic hepatitis B has been shown in large clinical trials. These studies were performed with either Asian [1, 5–7] or North American/European [2–4] cohorts. In these trials, the incidences of lamivudine resistance after 12 months of treatment were 14% in the Asian cohorts and 32% in the American/European cohorts. We performed a meta-analysis of 5 of these studies [1–5], which included 277 Asian patients and 132 North American/European patients, all of whom received 100 mg of lamivudine per day for 1 year. In this meta-analysis, we found that, after 12 months of monotherapy, the incidence of infection with lamivudine-resistant strains in North American/European patients (41 of 132 patients) was significantly higher than that in Asian patients (40 of 277 patients; \( P < .001 \), determined by the \( \chi^2 \) test; data not shown). Although it was not determined in these studies, the prevalence of HBV subtypes varies considerably in Asia and North America/Europe [13]. Therefore, in addition to other possible factors, the prevalent HBV subtype may be an important reason for the different incidences of lamivudine resistance reported in these studies. A correlation of certain subtypes with sustained response to antiviral treatment has already been shown for the treatment of hepatitis C virus infection [14]. In HBV infection, the subtype adw seems to be associated with an increased risk of chronic outcome [15] and development of hepatic cellular carcinoma [16].

In an earlier pilot study [8], we observed a significantly elevated risk of developing lamivudine-resistant strains in patients who carried the HBV subtype adw, as compared with patients who carried HBV subtype ayw. This study was possible because HBV subtypes adw and ayw are equally distributed in our cohort, owing to a high proportion of Turkish patients, all of whom carried HBV subtype ayw2/3. To further study the selection mechanisms of lamivudine-resistant HBV subtypes, we investigated the early response to lamivudine treatment and found that, between months 6 and 12 of therapy, the mean HBV load in the adw group was significantly higher than that in the ayw group. This was followed by a high incidence of infection with lamivudine-resistant strains in the adw group from month 12 to the end of follow-up. This finding is in line with that of a recent study [17] in which we showed that the incidence of lamivudine resistance was significantly higher in patients who had consistently detectable levels of HBV DNA during the first 12 months of treatment, as compared with patients who had undetectable levels of HBV DNA after 6 months of treatment. On the basis of our results, we conclude that selection of lamivudine-resistant HBV variants occurred mainly between months 6 and 12 of lamivudine monotherapy and was strongly dependent on the background replicative capacity of HBV during antiviral treatment. Because the response of HBV DNA during the first months of lamivudine therapy seems to predict the probability of selecting for lamivudine-resistant HBV variants, the effectiveness of the initial antiviral hit against HBV seems to be essential. Therefore, treatment with a combination of lamivudine with new antiviral drugs, such as adefovir or entecavir, which are currently in phase III clinical trials, may be superior to lamivudine monotherapy, particularly for HBV subtype adw–infected patients. Another alternative is the combination of lamivudine with IFN-α, which has already been shown to significantly reduce the incidence of lamivudine-resistant HBV variants after 12 months of treatment [3].

The underlying mechanism for the different response to lamivudine in patients infected with HBV subtypes adw and ayw is unclear. Our in vivo data suggest that the HBV polymerases of certain subtypes may be inhibited to different degrees by lamivudine. Another explanation is that, because of the overlapping reading frames of the polymerase and the HBsAg of HBV, the immune system may play an important role in the suppression and selection of wild-type and mutant HBV. It has been shown recently that lamivudine treatment can overcome T cell hyporesponsiveness to HBV in patients with chronic HBV carriage, most likely as a result of the decrease in HBV load [18, 19]. The low HBV DNA titers in HBV subtype ayw–infected patients during lamivudine therapy might result in selection processes of HBV variants that are different from those that occur in HBV subtype adw–infected patients. In our investigation, we categorized the genotypes adw1 and adw2 as “subtype adw” and the genotypes ayw1, ayw2/3, and ayw4 as “subtype ayw.” Therefore, one could speculate that the immunodominant regions responsible for the selection of HBV variants may be located within the \( d \) or \( y \) domains of HBsAg. However, further in vitro studies are necessary to investigate the selection mechanisms of wild-type and mutant HBV during lamivudine treatment.

In conclusion, according to our data, HBV subtype ayw appears to respond significantly better to lamivudine treatment than does HBV subtype adw. Insufficient suppression of the adw subtype during the early phase of treatment may lead to the high incidence of lamivudine resistance in HBV subtype adw. Analogous to treatment of HIV infection [20], lamivudine resistance may be detrimental for subsequent combination regimens of antiviral therapy for chronic hepatitis B and, therefore, may play an important role, particularly in HBV subtype adw–infected patients. Because this would have great therapeutic impact, further studies that involve larger cohorts are urgently required to investigate the role of HBV subtypes in antiviral treatment.
References