Selection of Multiresistant Hepatitis B Virus during Sequential Nucleoside-Analogue Therapy

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Hepatitis B virus (HBV) drug resistance to lamivudine is always accompanied by mutations in the viral polymerase gene at position 550, termed group 1 (M550V with L526M) or group 2 (M550I) mutations. The latter mutation has not been associated with famciclovir resistance. Thus, the addition of famciclovir to lamivudine therapy in persons with group 2 lamivudine resistance may lead to virus suppression. The effect of lamivudine/famciclovir combination therapy on HBV infection was monitored in 5 lamivudine-resistant patients by quantitative polymerase chain reaction and polymerase gene sequencing of serum virus. No patients treated with combination therapy had a decline in HBV load >1 log10. Continual evolution of the viral polymerase was detected in association with virologic resistance to both drugs. Cloning experiments identified the preexistence of these multidrug-resistant virus variants as minority species prior to addition of famciclovir therapy. HBV resistance to lamivudine monotherapy is associated with a complex mixture of variants that limit the efficacy of second-line nucleoside-analogue therapy. First-line potent combination therapy may reduce the emergence of HBV drug resistance.

Lamivudine is a potent inhibitor of hepatitis B virus (HBV) replication that results in an immediate and profound decline of serum virus level that is sustained during short-term treatment. Suppression of viral replication is associated with biochemical and histologic improvement [1, 2]. During prolonged treatment, however, lamivudine-resistant virus species may emerge [1, 3, 4]. Resistance requires a valine or isoleucine substitution for methionine at amino acid position 550 of the HBV polymerase (designated M550V and M550I, respectively). To date, all variants with M550V also encode a leucine-to-methionine change at position 526 (L526M). L526M is not observed in association with M550 isoleucine [5], suggesting that the L526M/M550I variant is at a selective disadvantage. M550V/L526M and M550I (recently termed group 1 and group 2 mutations) frequently exhibit other, but variable, amino acid substitutions, including V519L (valine for leucine at position 519) [6]. Emergence of resistance is accompanied by a rise in serum HBV DNA levels and with renewed hepatic inflammation. At that stage, management options include continuation of lamivudine as monotherapy, lamivudine withdrawal, or addition of a second antiviral. Famciclovir is a candidate antiviral that is active against HBV in vivo [7]. In vitro studies have also shown antiviral synergy of lamivudine and penciclovir (the active metabolite of famciclovir) in HBV-transfected human cell lines [8] and in primary culture of duck HBV–infected duck hepatocytes [9]. Although the polymerase changes associated with HBV resistance to famciclovir are poorly defined, it is believed that important changes associated with famciclovir resistance include L526M and V519L [10]. Thus, the susceptibility of lamivudine-resistant HBV to famciclovir might be predicted by the specific resistance mutations of the lamivudine-resistant species.

We therefore tested the hypothesis that group 2 lamivudine-resistant variants (M550I) retain sensitivity to famciclovir, by adding famciclovir to existing lamivudine therapy in 5 lamivudine-resistant patients with the virus variant in whom therapy was failing. We reasoned that continuing lamivudine treatment would maintain the selective pressure for the M550I mutation.
Patients and Methods

Five patients were studied. All were male; 4 were Asian. All had previously received interferon (IFN)–α as monotherapy and had participated in a phase 2 study that evaluated combination IFN/ lamivudine therapy over 4 months. None achieved HBV e antigen (HBeAg) seroconversion during antiviral treatment. Subsequently, after \( \geq 6 \) months without antiviral therapy, lamivudine monotherapy was commenced (provided on a compassionate basis by GlaxoWellcome, Greenford, UK). The data presented here begin at the time that lamivudine monotherapy was started.

Lamivudine therapy (100 mg once a day) was begun between June and December 1995. At that time, all 5 patients were serum HBeAg positive and HBV DNA positive. All had histologic evidence of chronic HBV without development of cirrhosis. HBV serology and biochemistry were monitored prospectively in the outpatient clinic on a regular basis. In each case, lamivudine effected a reduction in serum HBV DNA of >2 log \(_10\) copies/mL, as determined by quantitative polymerase chain reaction (PCR; Roche Amplicor, Branchburg, NJ). Resistance was defined by a rebound serum HBV DNA with a resistance-associated genotype. Lamivudine treatment was sustained after emergence of the resistant species and famciclovir (500 mg twice a day) was added. Nucleic acid sequencing of the HBV polymerase gene was undertaken at multiple time points for each patient, including before lamivudine, during lamivudine monotherapy, and after \( \geq 3 \) months of combination therapy.

Results

Virus load and liver function changes. Serum HBV DNA levels were \( >40 \times 10^4 \) copies/mL in all patients before the start of lamivudine therapy. Lamivudine effected an immediate and profound fall in virus load, to a median nadir of 77,000 copies/mL (range, \( 3.2 \times 10^3 \)–\( 2.4 \times 10^4 \)). Between 11 and 22 months after the start of therapy, virus load rose by \( 2-4 \log \_\text{10} \)–fold. Famciclovir (500 mg twice a day) was added to lamivudine, and combination treatment was continued for \( \geq 3 \) months, during which time virus load did not vary by \( >1 \log \_\text{10} \) (–0.61 to +0.35 log \_\text{10} \).

Serum alanine transferase (ALT) levels fluctuated during lamivudine therapy in a fashion typical of chronic viral hepatitis. No hepatitis "flare" occurred at the time of lamivudine resistance, and serum ALT did not decline during famciclovir treatment of lamivudine-resistant virus.

Genotypic analysis. Table 1 displays the amino acid residues (deduced from population sequencing of PCR products) for each patient at three time points: start of lamivudine treatment, addition of famciclovir, and after \( \geq 3 \) months of combination therapy. At commencement of lamivudine, all patients had wild-type virus with a valine at position 519 (V519), leucine at position 526 (L526), and methionine at position 550 (M550) of the viral polymerase.

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<thead>
<tr>
<th>Patients and treatment status</th>
<th>$519$</th>
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<tbody>
<tr>
<td>Pretreatment genotype (all patients)</td>
<td>V</td>
<td>L</td>
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<tr>
<td>Patient 1 LAM failure</td>
<td>V</td>
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<td>LAM + FAM failure</td>
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<td>Patient 2 LAM failure</td>
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<td>LAM + FAM failure</td>
<td>L</td>
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<td>V</td>
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<tr>
<td>LAM + FAM failure</td>
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<td>LAM + FAM failure</td>
<td>V</td>
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<tr>
<td>Patient 5 LAM failure</td>
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<td>L/M</td>
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<tr>
<td>LAM + FAM failure</td>
<td>L</td>
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NOTE. V, valine; L, leucine; M, methionine; I, isoleucine.

* Genotypic changes were identified by population sequencing [12]. Amino acid numbering system from [13].

For patient 1, lamivudine resistance was associated with M550I. After 3 months of combination therapy, population sequencing showed persistence of M550I and V519, but amino acid residue 526 had become a mixture of leucine and methionine. Therefore, at least two viral species could be identified, V519/L526/M550I and V519/L526/M550I. For patient 2, lamivudine resistance was associated with V519L and M550I. After combination therapy, population sequencing showed persistence of V519L and M550I and identified a mixture of leucine and methionine at position 526. Therefore, 12 clones derived from the pre-famciclovir virus were examined: 13 were V519/L526/M550I and 1 was V519/L526/M550I. For patient 3, lamivudine resistance was associated with M550I. After 3 months of combination therapy, V519/L526/M550I persisted. For patient 4, population sequencing suggested that lamivudine resistance was associated with M550I, but residue 526 appeared to be a mixture of leucine with methionine. Of 12 clones examined during lamivudine failure, 9 were V519/L526/M550I, 2 were V519/L526/M550V, and 1 was V519/L526/M550I. During combination therapy, population sequencing showed little change—namely, persistence of V519, a mixture of leucine and methionine at position 526, and dominance of M550I. For patient 5, lamivudine resistance was associated with V519L and with stable mixtures at positions 526 (leucine/methionine) and 550 (valine/isoleucine) over 6 months (2 samples). Twenty clones were examined from the latter sample: 9 were V519/L526/M550I, 7 were V519L/L526/M550V, 3 were V519L/L526/M550I, and 1 was V519/L526/M550V. After addition of famciclovir, population sequencing showed that the predominant species was V519L/L526/M550V and that the level of isoleucine at position 550 and leucine at position 526 had declined markedly.
Discussion

There are few data on the use of famciclovir monotherapy following emergence of lamivudine resistance [13]. All lamivudine-resistant variants encode M550V or M550I within the viral polymerase. It has been proposed that the M550I mutant (group 2) may retain susceptibility to famciclovir [10]. We tested this hypothesis by adding famciclovir to existing lamivudine therapy in 5 patients with failing lamivudine monotherapy who had the M550I mutation (mixture of M550I/V in 1 case). None responded to combination therapy as defined by a $>1\log_{10}$ fall in serum HBV DNA.

The heterogeneity of variants from patients with failing lamivudine monotherapy was evident: 2 patients had the V519L change and 2 had methionine/leucine mixtures at position 526. Only 2 patients had the M550I change alone. In patient 5, the mixture of isoleucine and valine at position 550 coexisted in qualitatively equal proportions for $\geq 6$ months of lamivudine monotherapy, indicating an equivalent “fitness” for each species. After the addition of famciclovir, 3 of the 5 patients showed further evolution by population sequencing, such that all but 1 patient had a detectable population of L526M with or without V519L. The classical group 1 variant (L526M, M550V) appeared as the majority population in only 1 patient. Sequencing of 12–20 clones from each of 3 patients with failing lamivudine monotherapy demonstrated the preexistence of these species as a minority population that emerged following addition of famciclovir. The L526M/M550V and M550I mutants have previously been suggested to be mutually exclusive [5]. By contrast, we detected the presence of mutations coding for methionine at 526 and isoleucine at 550 on the same genome in each of the 3 cases.

These observations suggest that HBV drug-resistant variants are more complex than previously recognized and that resistance to lamivudine in chronically infected patients is associated with infection of the liver with multiple species of mutated virus. Groups 1 and 2 (L526M/M550V and M550I, respectively) probably represent extremes of a spectrum, with the predominant species observed by chance [14]. Addition of a further selective pressure (famciclovir) then promotes the emergence of a previously minority species. Thus, in 3 patients, famciclovir/lamivudine cross-resistance was associated with L526M and M550I dual mutations. We are currently characterizing the viral variants described in this study by using enzymatic assays on purified polymerase and cell culture assays.

Other viral and host factors may also determine nucleoside-analogue drug susceptibility, such as mutations outside the area of polymerase studied, drug bioavailability, and the degree of intracellular drug phosphorylation. In 1 patient with the M550I mutation alone, combination therapy failed, with no apparent change after addition of famciclovir. One explanation of the lack of famciclovir effect could have been the low dose used (500 mg twice a day rather than 500 mg 3 times a day), although this was higher than the 250-mg, twice daily dose previously shown to elicit an antiviral response [7]. In addition, the fact that evolution of polymerase drug resistance mutations occurred in most patients receiving double therapy suggests that the drug was exerting a selective pressure on the virus.

Our study demonstrates that dual combination therapy with lamivudine and famciclovir after failure of lamivudine monotherapy is ineffective at suppressing HBV replication in chronically infected patients, despite the presence of the M550I mutation. We show the complex quasispecies nature of HBV genotypes under drug-selection pressure and the difficulty in predicting drug susceptibility on the basis of the dominant virus species in serum. Lamivudine monotherapy appears to “prime” the rapid emergence of famciclovir resistance following addition of this second drug. We suggest that the risk of emergence of nucleoside-analogue resistance will be reduced by initial use of potent drug combinations, rather than sequential therapy, as has been illustrated for human immunodeficiency virus–infected patients [15]. Trials of such combinations are urgently required.

References

13. Wolters LMM, Honkoop P, Niesters HGM, de Man RA. Efficacy of fam-
