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Two-Year Assessment of Entecavir Resistance in Lamivudine-Refractory Hepatitis B Virus Patients Reveals Different Clinical Outcomes Depending on the Resistance Substitutions Present▽

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Entecavir (ETV) is a deoxyguanosine analog approved for use for the treatment of chronic infection with wild-type and lamivudine-resistant (LVDr) hepatitis B virus (HBV). In LVD-refractory patients, 1.0 mg ETV suppressed HBV DNA levels to below the level of detection by PCR (<300 copies/ml) in 21% and 34% of patients by Weeks 48 and 96, respectively. Prior studies showed that virologic rebound due to ETV resistance (ETVr) required preexisting LVDr HBV reverse transcriptase substitutions M204V and L180M plus additional changes at T184, S202, or M250. To monitor for resistance, available isolates from 192 ETV-treated patients were sequenced, with phenotyping performed for all isolates with all emerging substitutions, in addition to isolates from all patients experiencing virologic rebounds. The T184, S202, or M250 substitution was found in LVDr HBV at baseline in 6% of patients and emerged in isolates from another 11/187 (6%) and 12/151 (8%) ETV-treated patients by Weeks 48 and 96, respectively. However, use of a more sensitive PCR assay detected many of the emerging changes at baseline, suggesting that they originated during LVD therapy. Only a subset of the changes in ETVr isolates altered their susceptibilities, and virtually all isolates were significantly replication impaired in vitro. Consequently, only 2/187 (1%) patients experienced ETVr rebounds in year 1, with an additional 14/151 (9%) patients experiencing ETVr rebounds in year 2. Isolates from all 16 patients with rebounds were LVDr and harbored the T184 and/or S202 change. Seventeen other novel substitutions emerged during ETV therapy, but none reduced the susceptibility to ETV or resulted in a rebound. In summary, ETV was effective in LVD-refractory patients, with resistant sequences arising from a subset of patients harboring preexisting LVDr/ETVr variants and with approximately half of the patients experiencing a virologic rebound.

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) (32); and many will ultimately develop severe liver disease, including cirrhosis, hepatocellular carcinoma, and liver failure. Significant improvements in patient outcomes have been realized since the use of antiviral therapy for HBV. Due to the poor efficacies of these therapies and the emergence of viral resistance, however, additional therapies are needed (16). Prior to 2005, HBV therapies included parenteral regimens containing interferon alfa and the oral nucleoside/nucleotide analogs lamivudine (LVD) and adefovir dipivoxil (ADV). However, interferon alfa shows poor response rates and poor sustained efficacy (~30 to 40% [reviewed in reference 18]), has low tolerability, and is contraindicated in patients with decompensated liver disease. LVD and ADV are associated with the development of viral resistance. LVD resistance (LVDr) is reported to occur in 24% of patients treated for 1 year, and this rate increases to 70% after 4 years (19). The rate of ADV resistance (ADVr) in nucleoside-naïve HBeAg-negative HBV patients has been reported to be 0% after 1 year and increases to 28% after 5 years (24). Increased rates of ADVr occur in LVD-refractory patients, ranging from 0 to 18% in 1 year and 22 to 25% in 2 years (14, 22, 39). ADV therapy can also be associated with suboptimal treatment responses in up to 50% of patients (15).

Entecavir (ETV) displays greater in vitro potency than LVD or ADV against wild-type (WT) and resistant HBV strains (3, 23, 26, 34, 35). Results from clinical studies revealed that the efficacy of ETV was superior to that of the direct comparator LVD in both nucleoside-naïve (5, 20) and LVD-refractory (4, 33) HBV patients. A meta-analysis revealed the more potent suppression of HBV DNA levels by ETV than by LVD or ADV (J. Dienstag, L. Wei, D. Xu, A. Cross, B. Kreter, and R. Wilber, 40th Annu. Meet. Eur. Assoc. Study Liver, abstr. 481 [J. Hepatol. **42**(Suppl. 2)**:**174, 2005]). Additionally, in a direct comparative study, ETV therapy resulted in a greater reduction in the HBV DNA level than ADV therapy did after just 10 days of treatment (N. Leung, C.-Y. Peng, J. Sollano, L. Lesmana, M.-F. Yuen, L. Jeffers, H.-W. Han, M. Sherman, J. Zhu, K. Mencarini, R. Colonno, A. Cross, R. Wilber, and J.-C. Lopez-Talavera, 57th Annu. Meet. Am. Assoc. Study Liver, abstr. 982, 2006). Importantly, ETV therapy is associated with a high genetic barrier to resistance. Entecavir resistance

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(ETVr) in nucleoside-naïve patients over time is rare, with less than 1% viral rebound due to resistance occurring by 96 Weeks (8). These results are consistent with the finding that ETVr did not emerge during 3 years of ETV treatment in the woodchuck hepatitis virus infection model (9).

The presence of substitutions that result in LVDr results in essentially complete cross-resistance to telbivudine (LdT), emtricitabine (FTC), and clevudine [1-(2-fluoro-5-methyl-β-L-arabinofuranosyl) uracil (L-FMAU)], while the presence of such substitutions decreases the susceptibility to ETV by eight-fold in cell culture. LVDr also facilitates the emergence of ADVr HBV (15). Despite the partial cross-resistance to ETV, 48 weeks of 1.0 mg ETV therapy for LVD-refractory patients reduced HBV DNA levels by a mean of 5 $\log_{10}$ copies/ml (4, 33). The barrier to resistance in this population was not as impervious as that in nucleoside-naïve patients, as revealed in the profiles of two LVD-refractory, phase II patients who experienced a virologic rebound during ETV therapy (34). The sequences of the rebound isolates from these patients identified the key substitutions found in all subsequent patients who have experienced a virologic rebound due to ETVr. Both patients had LVDr HBV (M204V and L180M) at study entry, and the isolates exhibited decreased ETV susceptibility following the acquisition of the primary ETVr-encoding substitutions T184G and S202I or M250V. Isolates from these patients were also considerably replication impaired in vitro. Therefore, at least three substitutions consisting of LVDr-encoding substitutions M204V and L180M plus an additional ETVr-encoding change were required to achieve clinically meaningful levels of ETVr, which likely contributes to the high resistance barrier observed in nucleoside-naïve patients. In patients with isolates with preexisting M204V and L180M LVDr-encoding substitutions, however, only a single additional HBV reverse transcriptase (RT) substitution is required for the virus to achieve high resistance levels and overcome ETV inhibition.

Emerging viral resistance is a recognized complication of antiviral therapy and is most prevalent when prolonged chronic drug administration is required and viral replication is not fully suppressed. Therefore, a comprehensive program was carried out to monitor HBV resistance to ETV at study entry and during therapy in patients treated with 1.0 mg ETV. Here we report on the frequency of RT changes at positions known to affect ETV susceptibility, novel substitutions that emerged on ETV therapy, the rate of virologic rebound due to resistance, as well as the effect of ETVr-encoding substitutions on patient outcome and isolate susceptibility to ETV.

MATERIALS AND METHODS

Study samples. Serum samples were obtained from patients in three studies designed to test the safety and efficacy of ETV in LVD-refractory patients. Written informed consent was obtained from all patients, and each study site obtained ethics committee/institutional review board approval. Since only the 1.0-mg ETV, once-daily (QD) dosage has been approved for use in patients with LVD-refractory HBV disease, only these patients were included in resistance assessments. LVD-refractory patients had evidence of LVDr signature substitutions or continued viremia, despite ≥24 weeks of LVD therapy. Study AI463026 (study 026) compared 1.0 mg QD ETV with 100 mg QD LVD for at least 52 weeks in LVD-refractory patients and is described in detail elsewhere (33). At the end of 52 weeks of dosing, patients stopped therapy or continued blinded treatment, based on protocol-defined decisions. "Responders" were patients achieving serum HBV DNA reductions to less than 7×10^5 copies/ml (the limit of detection of the Chiron Quantiplex branched DNA [bDNA] assay) and a loss of HBeAg at week 48 and discontinued therapy. "Virologic responders" were those who displayed HBV DNA reductions below the limit of detection by the bDNA assay but without a loss of HBeAg and continued blinded treatment up to week 96. Patients whose HBV DNA was detectable by the bDNA assay ($>7 \times 10^5$ copies/ml) were "nonresponders," discontinued therapy, and were offered alternative anti-HBV DNA therapy or continued ETV therapy through enrollment in an ETV rollover protocol, including study AI463901, described below. Study AI463014 (study 014) compared the antiviral efficacy of switching to one of three ETV QD doses (0.1, 0.5, and 1.0 mg) with that of continued 100 mg QD LVD treatment for up to 76 weeks in LVD-refractory patients (4). Only study 014 patients treated with the approved 1.0-mg QD ETV dosage and the LVD comparator arm were included in this analysis. Treatment outcome was determined at Weeks 24 and 48 and is described in detail elsewhere (4). Patients in study AI463015 (study 015) were clinically stable orthotopic liver transplant recipients who were at >100 days posttransplantation and who had recurrent chronic HBV infection, despite anti-HBV prophylaxis with LVD and hepatitis B immunoglobulin. They received open-label ETV at 1.0 mg coadministered with tacrolimus or cyclosporine for at least 12 weeks. Some patients from each of these studies continued treatment in rollover study AI463901 (study 901), in which they received QD treatment with either 1.0 mg ETV or a combination of 100 mg LVD with either 0.5 mg or 1.0 mg ETV. Patients from study 901 were included in resistance analysis only if the treatment was considered to be continuous, with protocol-designed treatment interruptions (such as those used to assess sustained efficacy off treatment) lasting no more than 5 weeks (35 days). Samples designated with the term "Week" are those that were collected in the "windowed" time points: for Week 48, samples were collected at the visits from ≥42 to ≤58 weeks of therapy, and for Week 96, samples were collected at the visits from ≥90 to ≤102 weeks of therapy.

HBV DNA assays. HBV DNA levels were determined by using the Roche COBAS AMPLICOR PCR method, which has a lower limit of quantification/detection of 300 HBV genome copies/ml. Virologic rebound was defined as a confirmed or last on-treatment ≥1-$\log_{10}$ rise in HBV DNA levels from the nadir. Confirmed HBV DNA rises were those in which the next treatment visit also had a ≥1-$\log_{10}$ rise in HBV DNA levels from the nadir.

HBV polymerase sequencing. HBV DNA was extracted from serum samples by using commercial kits (QIAGEN, Valencia, CA); and the HBV polymerase RT domain, which encodes amino acids 1 to 344, was amplified by PCR and sequenced directly, as described elsewhere (34). Nucleotide mixtures were reliably detected when they were present at a level of approximately 25% or greater. Sequencher software (GeneCodes) and MegAlign software (DNAStar, Inc.) were used to align the sequences to determine changes that emerged during therapy. The sequences were also compared to those in a database prepared from the alignment of 250 WT HBV genomes from GenBank, including all phylogenetic HBV genotypes, to reveal conserved residue positions (residues that varied in ≤1% of the sequences), naturally occurring polymorphic changes (residues that varied in four or more sequences), and the phylogenetic genotype of each patient isolate (by using the ClustalW method of the MegAlign software). When isolates contained multiple viruses with different substitutions, a mixture of residues was reported as residue/residue. When mixtures of residues emerged at a position known to be involved in ETVr in such a way as to obscure the identity of the encoded amino acid sequence, the PCR product was cloned directly by using the TA cloning technology (TOPO TA cloning kit; Invitrogen), and the identities of the specific residues from 12 to 24 clones were determined. Ultrasensitive real-time PCR-based single-nucleotide-polymorphism (SNP) detection of LVDr M204 substitutions was performed essentially as described previously (30) by using 10^6 copies of HBV DNA and SNP allele-specific primers that incorporate a locked nucleic acid at the 3′ position (21). Real-time PCR was performed with a Power SYBR green PCR master mix (Applied Biosystems), and SYBR green incorporation was measured on an Applied Biosystems 9700HT instrument. WT and LVDr mutant plasmids derived from patients were amplified and used as SNP PCR standards.

Antiviral compounds. ETV was prepared at Bristol-Myers Squibb (BMS). The triphosphates of ETV and LdT were prepared by TriLink Biotechnologies, Inc. (San Diego, CA); LVD triphosphate, LVD, and ADV were purchased from Moravek Biochemicals (Brea, CA); and the diphosphate of ADV was prepared at BMS.

Cells and viruses. HepG2 human hepatoma cells were maintained as described previously (34). The laboratory HBV genotype D *ayw* serotype clone was kindly provided by Steven Goff (Columbia University, New York, NY) in plasmid pCMV-HBV (13). The laboratory LVDr-encoding plasmids containing the M204V and L180M or M204I substitution engineered into the WT clone were prepared by site-directed mutagenesis (23).

Plasmids for HBV phenotyping. Amplified patient RT DNAs were digested with XhoI and BssHII and cloned into a similarly cut laboratory plasmid derived from pCMV-HBV (p180B3) (34). Individual clones were prepared from isolated colonies grown on Luria-Bertani agar plates containing 200 μg/ml ampicillin. To generate population RT plasmids representing patient quasispecies, plasmid DNA was isolated from the transformation culture inoculated directly into 100 ml of Luria-Bertani broth. In all cases, the DNA sequence of the final plasmid preparation was verified. Full-length HBV used for phenotyping assays was amplified and cloned by methods similar to those reported previously (38).

HBV cell culture susceptibility. HepG2 cell culture susceptibility assays were performed by transfecting cells with the HBV phenotyping plasmids in the presence of a titration of antiviral compounds. The cells were transfected in bulk and were then seeded into individual wells. Dimethyl sulfoxide vehicle-treated control wells, as well as a cloned laboratory wild-type HBV isolate, were tested in parallel in each experiment. At 5 days posttransfection, the virions released from the cells were treated with Nonidet P-40 to remove the envelopes, captured from the medium by using anti-HBV core antibody, and quantitated as described elsewhere (34) to determine the amounts of replicated, extracellular virus. The concentration of antiviral compound that resulted in 50% inhibition of HBV was reported as the 50% effective concentration (EC_{50}).

In vitro HBV polymerase assay. Endogenous polymerase assays used intracellular HBV nucleocapsids isolated from HepG2 cells and were carried out as described previously (34). The concentration associated with 50% inhibition of HBV DNA synthesis was reported as the 50% inhibitory concentration (IC_{50}).

Intracellular nucleotides. The concentrations of intracellular nucleotide triphosphates were determined as described previously (23, 37) by using HepG2 cell volumes of 2.6 picoliters per cell (27). The average plasma concentration (C_{ave}) exposure levels for ETV, LVD, ADV, and LdT were determined by dividing the area under the curve by the 24-h dosing period, according to the instructions in the respective package inserts for ETV, LVD, and ADV and as described by Zhou et al. (40) for LdT.

RESULTS

In vitro efficacy. While low levels of cross-resistance to ETV are seen among isolates with LVDr-encoding substitutions, ETV remains active and potent against LVDr viruses at the predicted clinical concentrations. To examine the potential clinical efficacy in vitro, we used the intrinsic potency and the pharmacologic levels of the active triphosphate achieved in patients. The concentration of ETV triphosphate (ETV-TP) that yielded a 50% inhibition of the WT or LVDr HBV RT in vitro (IC_{50}) was determined, along with the levels of active ETV-TP in cultured cells exposed to the predicted clinical concentrations (C_{ave}) of ETV. The relative potency was expressed as the ratio of the concentration of intracellular triphosphate divided by the IC_{50} (Fig. 1). These ratios predicted greater efficacies for both the 0.5-mg and the 1.0-mg ETV dosages than those for LVD, ADV, or LdT against both WT and LVDr HBV containing the M204V and L180M substitutions.

Clinical efficacy. A total of 192 LVD-refractory patients treated with 1.0 mg ETV in three clinical studies were evaluated for antiviral efficacy and resistance. ETV treatment resulted in mean reductions of 5.06, 5.11, and 3.90 $\log_{10}$ HBV DNA copies/ml at week 48 in studies 014 (4), 026 (33), and 015 (unpublished data), respectively. In contrast, continued LVD treatment of 190 patients in these studies resulted in modest HBV DNA reductions (1.37 and 0.48 $\log_{10}$ HBV DNA copies/ml at 48 weeks in studies 014 and 026, respectively). Figure 2A shows a composite bubble chart of the HBV DNA levels at specific time points for patients treated with ETV and LVD in these studies. An analysis showed that the percentages of patients exhibiting PCR-undetectable HBV DNA levels (<300 copies/ml) while they were receiving ETV were 8% at Week 24, 21% at Week 48, and 28% at Week 96 (Fig. 2A). Importantly, overall HBV DNA levels continued to decrease over time, with the cumulative percentage of ETV-treated patients achieving undetectable HBV DNA levels during treatment continuing to increase over time to 21% and 34% of all ETV-treated LVD-refractory patients by Weeks 48 and 96, respectively (Fig. 2B). In contrast, continued LVD therapy resulted in only 1% of patients reaching undetectable HBV DNA levels by Week 96.

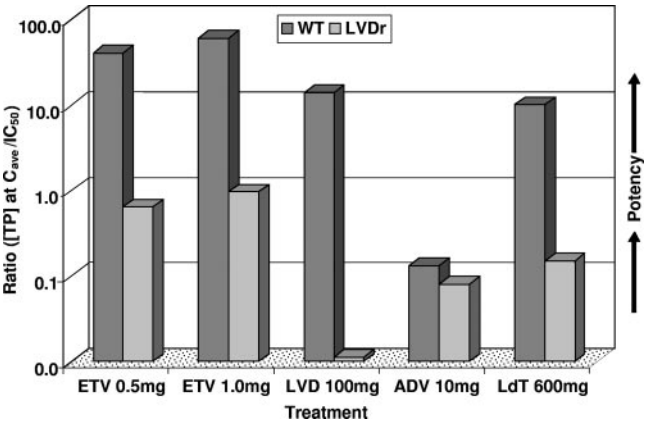

FIG. 1. Relative potencies of anti-HBV agents against WT and LVDr HBV. The relative potency was expressed as a function of the average in vitro HBV RT IC_{50} and intracellular cell culture levels of triphosphates (diphosphate for ADV) at clinical exposure levels. IC_{50}s ($n \geq 3$) and intracellular triphosphate levels at clinical exposures ($n = 2$) were determined as described in Materials and Methods. The higher the number is, the greater the level of potency was. WT, wild-type HBV polymerase; LVDr, lamivudine-resistant HBV polymerase with M204V and L180M substitutions.

Monitoring of resistance-encoding sequences. Because the presence of LVDr-encoding substitutions results in reduced susceptibility to ETV, we monitored patient isolates for both LVDr- and ETVr-encoding substitutions as well as any novel amino acid changes that emerged while the patients were receiving therapy. On-treatment resistance analyses were confined to those patients who received 1.0 mg ETV and had HBV DNA assessments at the baseline and on treatment at Week 24 of therapy or later. The number of patients and the results of the analyses are summarized in Table 1. Baseline samples included those from all 466 LVD-refractory patients from the ETV and the LVD comparator treatment arms and from patients from a dose-ranging study (4). On-treatment sequences were derived from samples from the 192 ETV-treated patients, 187 of whom had on-treatment PCR HBV DNA measurements at Week 24 or later. Ninety-three percent (179/192) of ETV-treated patients had an on-treatment visit at Week 48, and 84% of these patients (151/179) continued treatment into the second year and had HBV DNA measurements. In contrast, only 84% of the LVD-treated patients reached Week 48, and the study protocol dictated that the vast majority of these patients not continue therapy into year 2.

Impacts of ETV on LVDr-encoding substitutions. At study entry, 85% (397/466) of the patients enrolled in the 1.0-mg ETV and 100-mg LVD arms of studies 014, 015, and 026 had the primary LVDr-encoding substitution M204I or the substitution M204V with or without the L180M substitution, detect-

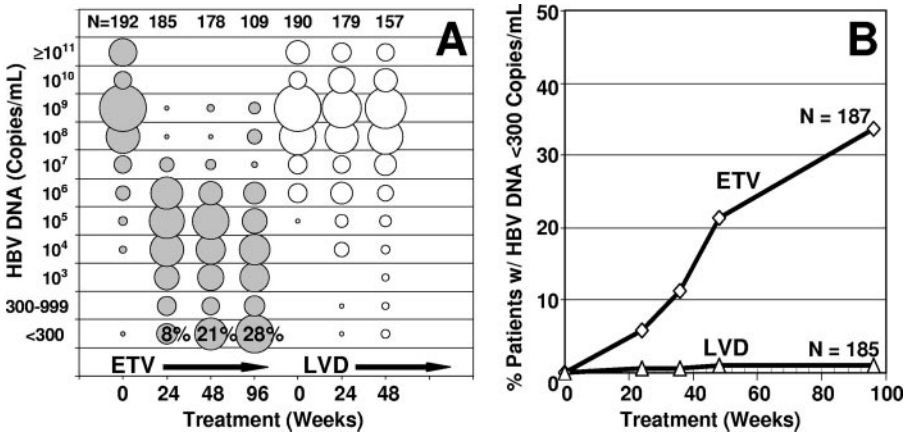

FIG. 2. HBV DNA levels in LVD-refractory patients treated with ETV or LVD. (A) Time point analysis. Gray and white circles, all 1.0-mg ETV- and 100 mg LVD-treated LVD-refractory patients, respectively. The size of the circles at each $\log_{10}$ interval is reflective of the percentage of patients (each column adds up to 100%) with the indicated HBV DNA levels at that treatment time point (week 0, baseline; Week 24 treatment period, weeks 12 to 30; Week 48 treatment period, weeks 42 to 72; Week 96 treatment period, weeks 90 to 102). The lowest circle represents patients with HBV DNA levels below the level of detection (300 copies/ml). *N*, number of patients included in the analysis at each time interval. (B) Cumulative percentage of ETV-treated (diamonds) and LVD-treated (triangles) patients who experienced HBV DNA reductions to undetectable levels (<300 copies/ml) by the week indicated. *N*, number of patients included in each data set.

TABLE 1. LVD-refractory patients analyzed for resistance

Patient population[a]	No. (%) of patients			
	Study 015/901	Study 014/901	Study 026/901	Total
LVD refractory at baseline	9	181	276	466
Evidence of LVDr and ETVr	1	8	18	27 (6)
Treatment with 1.0 mg ETV				
Yr 1 monitored	9	40	138	187
Yr 1 rebounds	0	0	5	5
Rebounds with preexisting LVDr and ETVr	0	0	0	0
Rebounds with emerging LVDr and ETVr	0	0	2	2 (1)[b]
Total rebounds with LVDr and ETVr	0	0	2	2 (1)
Emerging LVDr and ETVr without rebound	1	0	8	9 (5)
Biochemical failures[c]	0	0	0	0
Yr 2 monitored	9	25	117	151
Yr 2 rebounds	2	2	17	21
Rebounds with preexisting LVDr and ETVr	2	0	6	8 (5)
Rebounds with emerging LVDr and ETVr	0	1	5	6 (4)
Total rebounds with LVDr and ETVr	2	1	11	14 (9)
Emerging LVDr and ETVr without rebound	1	0	5	6 (4)
Biochemical failures[c]	0	0	0	0

[a] The baseline includes all LVD-refractory patients, irrespective of treatment, including LVD and ETV at 0.1, 0.5, and 1.0 mg for study 014 (4); ETV and LVD for study 026; and ETV for study 015. The patients monitored during treatment included those with an HBV DNA measurement beyond week 24 for year 1 and within the second year for year 2. Data were collected in the windowed time point indicated or for the sample obtained at the end of dosing. Year 2 data from rollover study 901 were included if treatment gaps did not exceed 5 weeks.

[b] Patients with isolates with a substitution at T184, S202, or M250. The frequency is the number of patients infected with isolates with sequence changes divided by the number of patients monitored.

[c] Biochemical failure, alanine aminotransferase levels 10 times the upper limit of normal or 2 times the reference level (at the baseline or the end of treatment).

able by nucleotide sequencing of the RT gene within population quasispecies. While sequencing routinely detects nucleotides representing ≥25% of the population, a more sensitive SNP PCR method with a detection limit of 0.1% was used with samples from patients randomized to the ETV treatment arm, revealing that an additional 7% of patients had detectable LVDr-encoding substitutions at residue M204. Therefore, patients with detectable LVDr-encoding substitutions were enrolled at an overall frequency of at least 92% and were randomized to receive ETV. The remaining patients may have had lower, undetectable levels of LVDr HBV as a result of their time off of LVD therapy, which results in the reemergence of a predominantly WT HBV population (6). The effect of ETV therapy on various LVDr changes was determined by comparing the sequences at baseline and at Week 48 for patients in both the ETV and LVD treatment arms. This comparison showed either the maintenance of the LVDr-encoding changes present or fluctuations among these residues (data not shown), as previously observed during LVD therapy (28), thus establishing that LVDr-encoding substitutions are maintained during ETV therapy. This is to be expected, given the selective advantage that LVDr HBV isolates would have in the presence of ETV.

Baseline ETVr-encoding substitutions in LVD-treated patients. Initial sequence analysis focused on the identification of amino acid changes at T184, S202, or M250, since they were previously identified as positions encoding primary ETVr. Sequencing of all available baseline patient isolates resulted in the unexpected finding of substitutions at these positions at study entry, prior to treatment with ETV, in 27 (6%) of 466 LVD-refractory patients analyzed (Table 1). Isolates from 10 of the 192 (5%) patients who were randomized to the 1.0-mg ETV arm had ETVr-encoding substitutions at the baseline. The specific changes are detailed in Table 2. We reasoned that if these changes could be detected by standard sequencing in 6% of patients, there was a high likelihood that a greater number of patients could harbor these variants at lower con-

TABLE 2. HBV RT substitutions at T184, S202, or M250

Substitution group	Substitutions (no. of patients)[a]	LVDr backbone substitutions
Baseline T184, S202, or M250 substitution[b]	T184A (2), I (2), S (13), A/S (1)	L180M, M204I/V, or M204I
	S202C (1), G (2)	L180M, M204I/V
	M250I (1), L (4)	L180M, M204I/V, or M204I
	T184A and S202G (1)	L180M, M204V
Emerging T184, S202, or M250 substitution during ETV therapy	T184A (1), I (2), S (1), A/S (1), L/S (1), I/L (1), F/L/M (1)	L180M, M204I/V
	S202G (12)	L180M, M204I/V
	M250L (1)	M204I
	T184A/S and S202G (1), T184A and S202G (1)	L180M, M204V

[a] The sequences of the ETVr- and LVDr-encoding positions most often included mixtures with WT residues, but only the substitutions are shown. Numbers in parentheses indicate the number of patients whose isolates were found to have the indicated substitution.

[b] Baseline patients were those subsequently randomized to any treatment arm; only 10 of the 27 patients were randomized to the 1.0-mg ETV treatment arm. Emerging substitutions were those in the 1.0-mg ETV treatment arm.

TABLE 3. Novel substitutions emerging during HBV therapy

Substitution	Background[a]	Frequency[b]	ETV EC_{50} (nM [SD])[c]
A200V	WT	1	7.6 (3.4)
A200V	M204I	1	33.0 (27.4)
A200V	L180M, M204I	1	10.2 (2.8)
L80V	L180M, M204V	2	11.0 (7.1)
V27A	L180M, M204V	1	NR[d]
I53F	V173F, L180M, M204V	1	22.3 (11.8)
S78T	WT	1	2.4 (1.2)
L80I	L180M, M204I	1	45.3 (20.5)
K168E	M204I	1	NR
A181T	V173L, L180M, M204V	1	14.2 (7.5)
C188S	L80I, L180T, M204V	1	31.2 (20.8)
V191A[e]		1	
V224A	V173L, L180M, M204V	1	NR
L228P	M204I	1	NR
L229W	L180M, M204V	1	32.9 (4.7)
D263G	WT	1	5.8 (1.7)
Q267Stop	L180M, M204V	1	NR
G295S	L80I, M204I	1	NR
M309L	WT	1	4.0 (0.4)

[a] Background resistance-encoding substitutions in the isolates tested.

[b] Frequency indicates the number of patients in whose isolates the substitution emerged.

[c] EC_{50}s (standard deviation) are the averages of three or more tests (only two tests for S78T; A200V, M204I, and L180M; and M309L). Substitutions were tested in the background (WT or LVDr) in which they arose. Parallel assays with either WT or LVDr (with the M204V and L180M or M204I substitution) reference clones yielded ETV EC_{50}s of 5.5 nM and 45 nM, respectively.

[d] NR, no replication (HBV replication was <10% of that for the WT) and unable to measure the phenotype.

[e] V191A was not found in 24 cloned isolates from the patient, and therefore, the isolates were not tested.

centrations. By using a sensitive SNP PCR assay (30), many of the patients with "emerging ETVr-encoding substitutions" while they were receiving ETV therapy (see below) were found to actually contain variants with these substitutions prior to ETV therapy at levels ranging from 10% to 0.1% (data not shown). These results suggest that the vast majority of ETVr-encoding substitutions may well have been generated during prior LVD therapy and that replication impairment likely prevented their proliferation prior to the additional selective pressure exerted through ETV treatment.

A comparative analysis of baseline RT sequences from nucleoside treatment-naïve, HBeAg-positive patients without evidence of M204 substitutions (n = 329) and LVD-refractory patients with an M204 LVDr-encoding substitution at the baseline (n = 229) revealed that LVD-refractory patients also had additional substitutions at residues L180 (84.2%), L80 (49.8%), V173 (21.8%), and V84 (5.7%), in addition to changes at primary ETVr-encoding residues T184 (5.2%), S202 (1.3%), and M250 (1.8%) at the baseline. The L80, V173, and T184S substitutions (1, 2, 10) have previously been reported to be secondary substitutions encoding resistance to LVD. These results confirm that LVD therapy selects for a variety of secondary substitutions, which includes the changes that result in ETVr.

Emerging substitutions at T184, S202, and M250. As indicated above, isolates from some patients exhibited the emergence of substitutions at primary ETVr-encoding residue T184, S202, or M250 during therapy (Table 2). There were 11/187 (6%) additional patients by Week 48 and 12/151 (8%) additional patients by Week 96 (Table 1). Virus isolate populations often showed more than one ETVr-encoding substitution; however, these substitutions were not linked within individual isolates, except in an isolate from a single patient (patient 001; see below) characterized previously (34). In addition, no obvious HBV phylogenetic genotype developed ETVr-encoding substitutions more frequently than others. The genotypes of the isolates from the patients enrolled in the studies were 27% genotype A, 15% genotype B, 20% genotype C, 33% genotype D, 2% genotype F, and 2% indeterminate, while the genotypes of isolates with ETVr-encoding substitutions through 2 years of therapy were distributed similarly, with frequencies of 12% genotype A, 15% genotype B, 33% genotype C, and 39% genotype D.

Novel substitutions emerging during ETV therapy. In addition to the primary substitutions known to confer resistance to LVD and ETV, all emerging HBV RT substitutions absent from an assembled database of 250 WT HBV sequences or found in patients with evidence of primary ETVr-encoding substitutions were selected for phenotyping. Only 18 patient samples had a total of 17 novel changes at 16 different conserved or polymorphic HBV RT residues (Table 3). None of the substitutions were found in more than three patients analyzed (<2%) or correlated with virologic responses, suggesting that they likely arose as a result of random genetic drift rather than as a result of resistance selection. Importantly, none of these 17 substitutions decreased ETV susceptibility beyond the range normally observed for isolates with LVDr-encoding substitutions. The isolate from one patient had evidence of a change at residue A181, which has been associated with virologic rebound while the patient was receiving ADV therapy (14). Here the change emerged in the presence of LVDr-encoding substitutions. Isolates from this patient did not show a further decrease in ETV susceptibility relative to that of the LVDr control (ETV EC_{50}s, 14 nM versus 31 nM for the LVDr control).

TABLE 4. Virologic outcomes of ETV therapy for patients infected with isolates with ETVr-encoding substitutions

Outcome	No. of patients at the following time of ETVr detection or emergence:			
	Baseline (n = 10)	Wk 48 (n = 11)	Wk 96 (n = 12)	Total
HBV DNA level <300 copies/ml	3	2	1	6
No rebound[a]	4	2	5	11
Yr 1 rebound	0	2	0	2
Yr 2 rebound	3[b]	5	6	14

[a] No virologic rebound to the end of the week 96 window (Weeks 90 to 102); does not include patients in the "HBV DNA-undetectable" category.

[b] Rebound isolates had ETVr-encoding substitutions different from those found at study entry.

Virologic outcomes for patients with isolates with ETVr-encoding substitutions. Variables related to specific ETVr-encoding substitutions, ETV susceptibility level, genetic background, and replication capacity likely contribute to whether a patient infected with isolates with ETVr-encoding substitutions eventually fails ETV therapy. An analysis of the timing and the clinical outcomes for ETV-treated patients with ETVr-encoding substitutions for up to 2 years showed a significant delay between the appearance of ETVr-encoding sequences and an observed virologic rebound, supporting the concept that these viruses are indeed replication impaired (34) (Table 4). The majority of virologic rebounds coinciding with ETVr-encoding substitutions did not occur until year 2, suggesting that these variants are very replication impaired and require extended periods of time of ETV treatment before they dominate the circulating HBV population. None of the 16 patients that exhibited a virologic rebound due to ETVr have experienced a subsequent flare in alanine aminotransferase levels (Table 1). ETVr-encoding substitutions alone do not appear to be sufficient to cause a virologic rebound, since three patients with these substitutions at the baseline and two patients with emerging ETVr-related changes by week 48 actually proceeded to have reductions in their HBV DNA levels to <300 copies/ml on continued ETV therapy. It is likely that the variables indicated above and other factors, such as immunologic competence, may play a role in restricting or eliminating this subpopulation of resistant variants. These relationships remain to be examined fully, although results have suggested that each ETVr-encoding substitution imparts a unique level of phenotypic susceptibility and replication capacity and that only infection with those viruses with higher levels of resistance and replication may result in virologic failure during ETV therapy (unpublished observation).

Sequence analysis of rebounds. Analysis of patients experiencing a virologic rebound while they were receiving ETV was particularly emphasized, since resistance is most likely to be found in such patients. By week 48, five ETV-treated patients had experienced a virologic rebound (Table 1). All five patients were infected with isolates with LVDr-encoding substitutions, but only 1% (2/187) of the patients were infected with isolates with evidence of ETVr-encoding substitutions at either S202 or T184. During the second year of therapy, the incidence of rebounds due to ETVr increased to 9% (14/151), as isolates from 14 of 21 patients experiencing a virologic rebound were found to have a substitution(s) at residue T184 and/or S202, in addition to preexisting LVDr-encoding substitutions. Among the 16 patients with virologic rebounds with ETVr-encoding sequences observed over a 2-year period, 9 had mixtures of both ETVr-encoding and WT sequences at ETVr-encoding positions (Table 5), consistent with some degree of replication impairment. Baseline samples from three of these patients (patients 251, 122, and 001) had evidence of T184 substitutions prior to ETV therapy. However, further changes at position T184 that preceded virologic rebound emerged during ETV therapy. Some patients were found to have additional ETVr-associated changes that emerged at time points beyond the time of rebound, suggesting further selection of secondary or compensatory changes. Importantly, isolates from all 16 patients had an M204V LVDr-encoding substitution, either alone or in combination with the M204I change. To date, no patients with ETVr-encoding changes and just the M204I substitution have had a rebound during ETV therapy.

Phenotypic analysis of rebounds. Cell culture susceptibility assays were performed with recombinant viruses derived from patient isolates to confirm that the presence of primary ETVr-encoding substitutions was responsible for the observed virologic rebound. These analyses used cloned populations from patient isolates in order to test the susceptibilities of the circulating virus quasispecies present at the time of rebound. Only those rebound isolates from patients with evidence of ETVr-encoding substitutions at T184 and/or S202 in LVDr backgrounds displayed substantially reduced susceptibilities to ETV relative to the susceptibilities of the baseline isolates (Fig. 3A), with a median ETV EC_{50} change of 7-fold from that for baseline LVDr virus and 64-fold from that for the reference WT virus. Importantly, the relative susceptibility to ADV did not change substantially in isolates from any of the patients following a virologic rebound, even though the isolates were resistant to both LVD and ETV (Fig. 3B). These and previous (34) results showing that ETVr HBV remains susceptible to ADV complement observations that ADVr HBV remains fully susceptible to ETV in vitro (3, 35) and clinically (14) and confirm a lack of cross-resistance between ETV and ADV.

There were no findings from sequence or phenotypic evaluations of isolates from the 10 remaining rebound patients to suggest that these rebounds were due to resistance emergence (Table 5). In fact, isolates from three of these patients did not even have detectable LVDr-encoding substitutions and had average EC_{50}s of 5.3 nM (EC_{50} range, 4.8 to 6.1 nM) and 5.7 nM (EC_{50} range, 4.3 to 6.5 nM) at the baseline and the time of rebound, respectively, which are similar to the EC_{50} for WT HBV. Isolates from the seven other rebound patients with LVDr-encoding substitutions at the baseline and at the time of rebound but no ETVr-encoding substitutions displayed ETV susceptibility levels relatively unchanged from those at the baseline (average EC_{50}, 26.9 nM [EC_{50} range, 8.1 to 52.2 nM]) and at the time of rebound (average EC_{50}, 33.5 nM [EC_{50} range, 14.2 to 85.9 nM]).

HBV DNA profiles of rebound patients. The HBV DNA profiles of all patients with virologic rebounds during ETV therapy are shown in Fig. 4. Patients experiencing a virologic rebound coinciding with the presence of ETVr-encoding substitutions (Fig. 4A to C) had initial reductions in HBV DNA levels before they exhibited gradually increasing viral DNA

TABLE 5. Sequence analysis of isolates from rebound patients

Patient no.	Phylogenic genotype[a]	Baseline substitutions[b]	Rebound substitutions[b] (wk)	Additional resistance substitutions[b] (wk)
251	D	L180M, M204I/V, T184T/S	L180M, M204V, I169I/T, T184T/S/F/C (82)	S202S/G (98)
122	C	L180M, M204M/V, T184T/I	V173V/M, L180M, M204V, I169I/M, T184T/I/M/S, S202S/G (84)	
001	D	L180M, M204V, T184T/S	L180M, M204V, T184G, S202I (76)	I169I/T (92)
069	C	L180L/M, M204I/V	L180M, M204V, S202S/G (36)	T184T/A (48)
256	A	L180L/M, M204M/V	L180M, M204V, T184T/S/A (48)	
113	D	L180M, M204I	L180M, M204V, S202G (68)	
174	B	L180M, M204V	L180M, M204V, S202S/G (68)	
204	C	L180L/M, M204I	L180L/M, M204I/V, T184I/L (96)	
007	D	L180M, M204V	V173V/M, L180M, M204V, T184T/A (64)	
130	C	L180M, M204M/V	L180M, M204V, S202S/G (65)	T184T/I/M (80)
163	D	L180L/M, M204I/V	L180L/M, M204I/V, T184T/S (66)	L180M, M204V, T184T/S/A, S202S/G (94)
235	D	L180M, M204V	L180M, M204V, S202G (85)	
162	D		L180M, M204V, S202G (90)	
386	D	L180L/M, M204I	L180M, M204V, S202G (66)	
136	C	M204I	L180M, M204V, S202G (100)	
206	C	L180M, M204V	L180M, M204V, T184T/L/F/M (77)	
055	C	L180L/M, M204I/V	L180M, M204I/V (36)	
224	C		(36)	
310	C	M204I	M204I (51)	
277	D		(60)	
051	D		(68)	
058	B	L180L/M, M204I/V	L180M, M204V (68)	
401	D	L180L/M, M204I	L180L/M, M204I (88)	
169	A	L180M, M204V	L180M, M204V (89)	
314	D	M204M/I	M204I (96)	
158	B	L180M, M204V	L180M, M204V (84)	

[a] The phylogenetic HBV genotype was determined from the alignments of the RT sequences.
[b] The substitutions noted are primary LVDr-encoding substitutions at L180 and M204; secondary LVDr-encoding substitutions at V173; primary ETVr-encoding substitutions at T184, S202, and M250; and secondary substitutions at I169.

levels to several $\log_{10}$ copies/ml above the nadir. In contrast, the majority of patients with a rebound in the absence of ETVr-encoding substitutions (Fig. 4D) displayed fluctuating HBV DNA levels, occasionally with decreasing HBV DNA levels following the rebounds or with relatively small increases in viral DNA levels. An exception was patient 169, who displayed a rebound profile characteristic of emerging resistance, despite the lack of ETVr-encoding substitutions. While it is unlikely that resistance could arise from changes in sequences outside the RT domain of HBV polymerase, the fact that ETV inhibits priming of HBV DNA synthesis (31) makes this a possibility. To test whether the sequence encoding ETVr mapped outside of the RT domain, the HBV DNA isolated from patient 169 was used to amplify, clone, and phenotype a full-length HBV DNA population isolate (38). Phenotypic analysis failed to show any meaningful reduction in ETV susceptibility when full length clones isolated at rebound (EC_{50}, 38 nM) and baseline (EC_{50}, 27 nM) were used. On the basis of

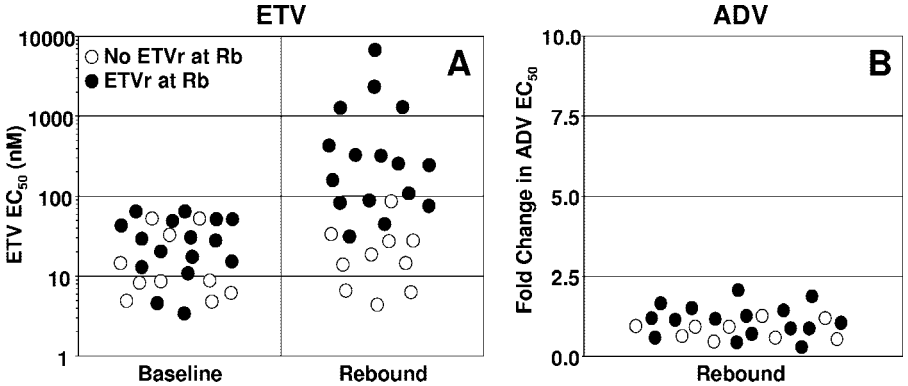

FIG. 3. Phenotypic susceptibilities of population isolates from patients with virologic rebounds. Population phenotypes of baseline and rebound isolates were determined as described in Materials and Methods. (A) ETV EC_{50}s for isolates from patients at baseline and the time of rebound (Rb); (B) fold change in ADV susceptibilities of rebound isolates. Open circles, patient isolates with WT or LVDr-encoding sequences at the time of rebound; filled circles, patient isolates with ETVr-encoding substitutions at the time of rebound. The median ADV susceptibilities (EC_{50}s) at the baseline and the time of rebound were both 2.7 μM. Isolates from patient 158 were not tested for their susceptibilities to ADV.

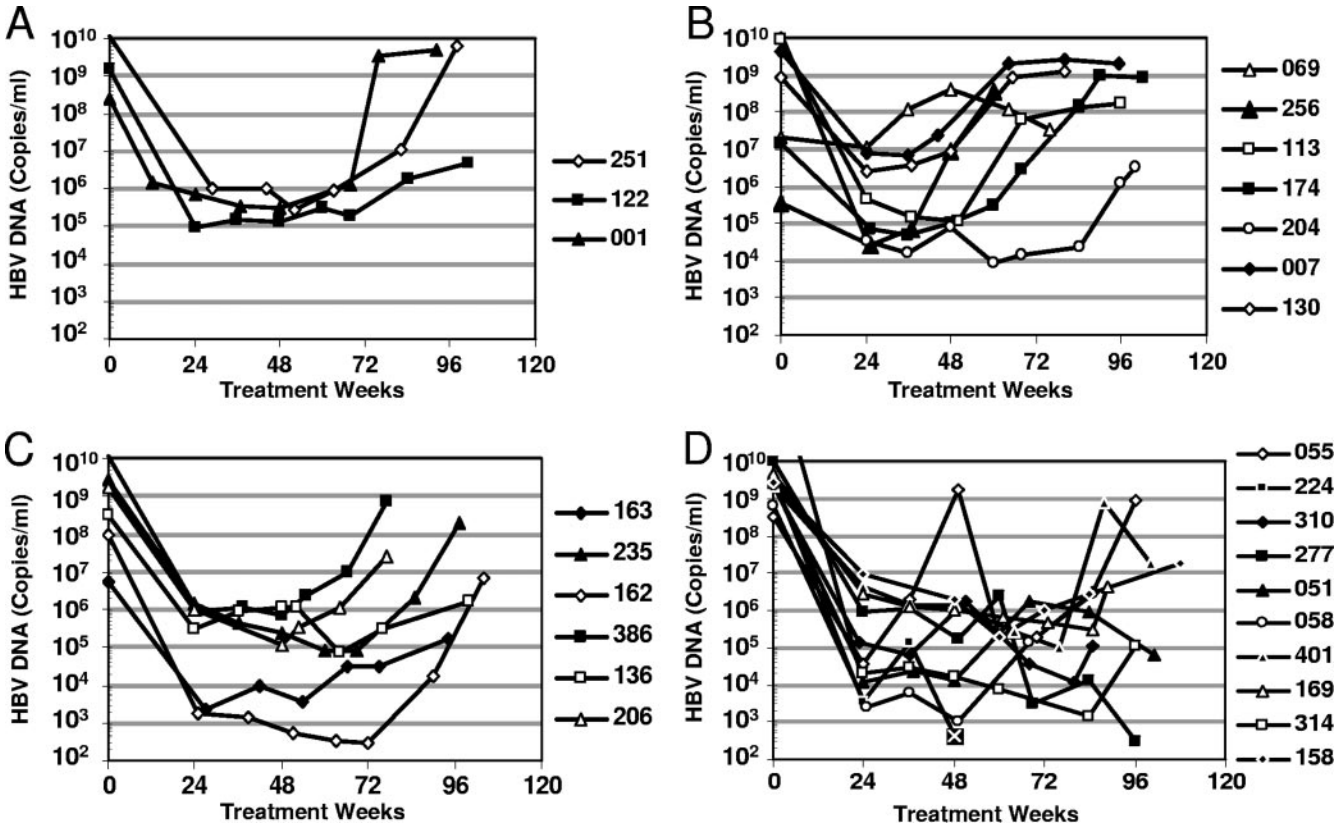

FIG. 4. HBV DNA profiles for patients with virologic rebounds while receiving ETV. Patient HBV DNA levels over time are shown for patients exhibiting virologic rebounds while receiving ETV. Patient isolates had sequence evidence of ETVr-encoding substitutions at baseline (A), substitutions that emerged by Week 48 (B), substitutions that emerged by Week 96 (C), or no ETVr-encoding substitutions or reduced ETV susceptibility (D). Time points marked with a boxed X in panel D represent off-treatment visits.

these findings, the underlying cause for the observed rebound in these patients is more likely due to a lack of adherence to dosing schedules, which results in inconsistent drug exposure, than to viral resistance. Other studies have also identified HBV virologic rebounds during LVD or ADV treatment in the absence of resistance (29, 36). This finding is common in human immunodeficiency virus-infected patients and is typically attributed to a lack of patient adherence rather than viral resistance (12, 17). On the basis of the finding that ETV susceptibility was not reduced in patients without ETVr HBV, the underlying cause for the observed rebound in these patients is more likely due to a lack of adherence, although experiments that can be used to test this hypothesis directly have not been performed.

DISCUSSION

While LVDr-encoding substitutions result in high levels of cross-resistance to LdT (Fig. 1) (34), FTC (11), and L-FMAU (7), ETV continues to exhibit potent activity against LVDr HBV RT in vitro (23, 34), in cell culture (23, 34), and in HBV-infected patients (4, 33) (Fig. 1 and 2). Here a comprehensive resistance surveillance program was undertaken to monitor isolates from LVD-refractory patients treated with 1.0 mg ETV for resistance. Sequence analysis was performed at baseline and Week 48 for isolates from all ETV- and LVD-treated patients and all patients who continued to have detectable HBV DNA levels in the second year of ETV therapy. The sequence analysis was coupled with phenotypic susceptibility testing of isolates from patients experiencing a virologic rebound, irrespective of the presence of resistance substitutions, as well as isolates with novel substitutions, regardless of the clinical outcome.

The frequency of virologic rebounds due to ETVr in LVD-refractory patients was 1% at the end of the first year and increased to 9% in the second year. To date, none of these patients experienced a subsequent biochemical failure (flare in alanine aminotransferase levels) following their virologic rebounds. All rebounds correlated with the presence of various substitutions at position T184 and/or S202 in a background of LVDr-encoding substitutions. Changes at residue M250, a third site known to encode ETVr, did not result in rebound in the patients initially treated with 1.0 mg ETV through year 2. A previous report, however, described a rebound patient who developed an M250V ETVr-encoding substitution during treatment with 0.5 mg ETV (34).

These studies unexpectedly identified isolates from 6% of LVD-refractory patients at baseline with substitutions at T184, S202, or M250 by standard sequencing. Nevertheless, only 3/10 (30%) patients with ETVr-encoding substitutions at baseline and randomized to an ETV treatment arm experienced a virologic breakthrough during the first 2 years of ETV therapy.

The isolates from all three of these patients who subsequently rebounded in the second year were found to have ETVr-encoding substitution patterns different from those found at baseline (Table 4). Among the seven patients with baseline isolates with ETVr-encoding substitutions who did not experience a virologic rebound, three had undetectable viral DNA by PCR in year 2, while four continued to have detectable viral DNA at year 2. These results support previous findings that LVDr/ETVr variants are significantly replication impaired and that the presence of genetic changes alone does not necessarily guarantee subsequent virologic rebound.

Substitutions at the three ETVr-encoding residues were subsequently noted in another 6% and 8% of patients in the first and second years of therapy, respectively. Many of these emerging ETVr-encoding changes could be detected at the time of study entry by more sensitive PCR detection methodologies. In contrast to patients with ETVr-encoding changes at baseline, patients with changes that emerged in year 1 (7/11) and year 2 (6/12) had less favorable HBV DNA outcomes (Table 4). However, three patients with emerging ETVr-encoding changes also proceeded to achieve HBV DNA levels that were undetectable by PCR in year 2 of continued ETV therapy. This may be a result of the fact that the majority of those with ETVr isolates with T184 or M250 substitutions at baseline did not show substantial phenotypic resistance to ETV and that further substitutions are required for actual breakthrough. Furthermore, patients with isolates with the M204I LVDr-encoding change in the absence of the M204V substitution have not been found among those who rebound due to ETVr. This is coupled with the notion that the M204I changes can evolve to M204V upon continued LVD therapy.

In addition to the M204I and the M204V-L180M LVDr-encoding changes, which have different effects on ETVr, various substitutions at T184, S202, and M250 produce a wide range of ETV susceptibilities from virtually no change to >100-fold greater resistance relative to that of LVDr HBV. The assorted changes also result in various levels of replication impairment. These factors and their influence on the clinical outcome with various ETVr-encoding substitutions are still being actively investigated. This, along with longer-term monitoring of ETV-treated patients, will be required to further understand the potential and impact of isolate development of ETVr upon extended treatment.

Sequence surveillance and phenotypic testing of all novel substitutions that emerge during ETV therapy failed to identify changes, other than those at T184, S202, and M250, in LVDr HBV that reduce susceptibility to ETV. These results suggest that the changes at these three positions define the major pathways to clinically relevant ETVr in LVD-refractory patients. However, further testing is warranted, as viral resistance can emerge from several different pathways that often become apparent only after years of study of a large number of patients.

ETVr patient isolates at the time of virologic rebound displayed, in general, ETV EC_{50} values of ≥100 nM, and all remained susceptible to ADV (median EC_{50} at both baseline and rebound, 2.7 μM). These results suggest that ADV is a valid treatment option for patients with virologic breakthrough while they are receiving ETV. Indeed, a limited number of patients ($n = 17$) harboring ETVr variants have subsequently been treated with ADV for various durations, as of the time of preparation of this report (average, 30 weeks; range, 6 to 54 weeks). Each one of these patients displayed reductions in HBV DNA levels (mean and median reductions, 3.1 and 2.7 $\log_{10}$ copies/ml, respectively) consistent with the levels previously reported for patients receiving ADV (mean reduction, 3.6 $\log_{10}$ copies/ml LVDr HBV DNA in 48 weeks) (25). However, the presence of LVDr-encoding substitutions alone has previously been reported to facilitate the more rapid emergence of ADVr (14), and therefore, combination therapy may be the more appropriate treatment option for patients with LVDr-encoding substitutions. These findings, along with the observation that the other L-nucleoside analogs FTC (11), LdT (34), and L-FMAU (7) are not active against LVDr HBV, suggest that ETV should have the greatest utility as a first-line agent.

In conclusion, ETV exhibits potent activity against HBV in vitro and for the treatment of patients infected with LVDr HBV. Phenotypic resistance required the emergence of substitutions at previously defined resistance-associated residues T184, S202, and M250 in HBV isolates containing preexisting LVDr-encoding substitutions (M204V and L180M but not M204I) and not other novel substitutions that emerged during therapy. Substitutions at positions T184, S202, and M250 were found to arise as a result of LVD therapy in 6% of patients, with the subsequent emergence of these changes detected during ETV therapy in 6% and 8% of patients after 1 and 2 years of treatment, respectively, likely due to the enrichment of preexisting variants. Virologic rebound due to ETVr occurred at frequencies of 1% and 9% after 1 and 2 years of ETV therapy, respectively.

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