Evolution of Hepatitis B Virus Polymerase Gene Mutations in Hepatitis B e Antigen–Negative Patients Receiving Lamivudine Therapy

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Lamivudine has been shown to be effective in patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B, but its long-term efficacy and the rate of resistant mutations in patients with HBeAg-negative chronic hepatitis B is less clear. Twenty-nine patients with HBeAg-negative chronic hepatitis B, who have received lamivudine for at least 1 year were studied to determine the antiviral response, the rate and pattern of lamivudine-resistant mutations, and the effect of lamivudine-resistant mutations on HBeAg status. The mean duration of treatment was 21 ± 7 months. Before treatment, core promoter variant was detected in 16 (55%) patients and precore stop codon variant in 18 (62%) patients. Serum hepatitis B virus (HBV) DNA was detected by solution hybridization assay in 62%, 4%, and 24% and by polymerase chain reaction (PCR) assay in 100%, 31%, and 40% at months 0, 6, and 24, respectively. The cumulative rates of detection of lamivudine-resistant mutations after 1 and 2 years of treatment were 10% and 56%, respectively. In addition to the duration of treatment, core promoter mutation was associated with the selection of lamivudine-resistant mutants. Three patients with lamivudine-resistant mutations had reversion of the precore stop codon mutation; in 2 patients this was accompanied by the reappearance of HBeAg. We found that lamivudine-resistant mutants were detected at similar rates in patients with HBeAg-negative as in patients with HBeAg-positive chronic hepatitis B. Additional changes in other parts of the HBV genome may restore the replication fitness of lamivudine-resistant mutants. (HEPATOLOGY 2000;32: 1145-1153.)

The approval of lamivudine has revolutionized the treatment of chronic hepatitis B. Lamivudine is an orally administered nucleoside analog with an excellent safety profile. Clinical trials in patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B have reported rates of HBeAg to antibody (anti-HBe) seroconversion in 16% and 17% patients after 1 year of lamivudine treatment compared with 4% and 6% in controls.[1,2] Lamivudine has also been evaluated in patients with decompensated cirrhosis[3] or recurrent hepatitis B after liver transplantation (LT)[4,5] although its use in these conditions is still experimental.

The greatest drawback with lamivudine treatment is the selection of drug-resistant hepatitis B virus (HBV) mutants. Genotypic resistant mutations have been detected in 14% to 32% of patients after 1 year of treatment[1,2,6] increasing to 38%, 49%, and 66% after 2, 3, and 4 years of treatment, respectively.[7-9] The most common mutation affects the highly conserved YMDD motif in the catalytic domain of the HBV reverse transcriptase/DNA polymerase (P gene), resulting in a methionine to valine or isoleucine substitution at codon 552 (M552V or M552I).[10] *In vitro* studies showed that either mutation alone can confer resistance to lamivudine but the M552I mutant is significantly more resistant to lamivudine than the M552V mutant.[10,11] Another mutation involving a leucine to methionine substitution at codon 528 (L528M) has been reported. The L528M mutant alone confers very little resistance to lamivudine but the combination of L528M and M552V mutation is more resistant than M552V alone and similar to that of M552I.[10-12] Similar mutations have been found in patients who received lamivudine treatment for human immunodeficiency virus (HIV) infection.[13] In these patients, the M184I mutant is transiently detected during the first few weeks of treatment and then replaced by the M184V mutant[14] because the latter mutant has greater replication fitness.[15,16] The pattern and evolution of lamivudine-resistant HBV mutations is less well described. Published reports of big clinical trials described the rates of detection of "YMDD mutant" but did not specify the type of mutation(s).[1,2,6,17] In many instances, genotypic resistant mutation(s) were tested at one time point, and mutations affecting other regions of the HBV P gene were not reported.

Most clinical trials on lamivudine treatment have focused on HBeAg-positive patients. A few studies have reported a

Abbreviations: HBeAg, hepatitis B e antigen; anti-HBe, hepatitis B e antibody; LT, liver transplantation; HBV, hepatitis B virus; P, reverse transcriptase/polymerase; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; HBsAg, hepatitis B surface antigen; ALT, alanine transaminase.

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TABLE 1. Baseline Characteristics of the Patients Studied

Group	No. of Patients	Sex M/F	Age (yr) Mean ± SD	No. (%) HBeAg Positive	No. (%) Anti-HBe Positive	ALT (IU/L) Mean ± SD	No. (%) HBV DNA Positive	
							Hyb	PCR
I	8	8/0	53 ± 6	0 (0)	7 (88)	164 ± 147	5/8 (63)	8 (100)
II	21	18/3	49 ± 8	0 (0)	21 (100)	190 ± 105	13/21 (62)	21 (100)
Total	29	26/3	50 ± 8	0 (0)	28 (97)	183 ± 116	18/29 (62)	29 (100)

Abbreviation: Hyb, solution hybridization assay.

beneficial effect of lamivudine in patients with HBeAg-negative chronic hepatitis B, but the effect was not maintained when treatment was withdrawn. The rates of genotypic resistant mutations in HBeAg-negative patients are more controversial varying from 0 to 18% after 12 to 18 months of treatment.[17-19] Several factors may account for the differences in rates of lamivudine-resistant mutants in these studies: sample size, ethnic background, viral load, HBV genotype, and prevalence of core promoter and precore HBV variants.

We detected lamivudine-resistant mutations in a high percent of Italian patients who were receiving lamivudine for HBeAg-negative chronic hepatitis B in a pilot study. In addition, we observed that breakthrough infection caused by lamivudine-resistant mutation was sometimes accompanied by re-

TABLE 2. HBV Core Promoter, Precore, and P Gene Sequences Before Treatment and at the Last Follow-Up Visit

		Pretreatment							Last Follow-Up					
							Duration of Rx							
Patient No.	HBV Genotype	HBV DNA Hyb (pg/mL)	ALT (IU/L)	CP (nt) 1,762	CP (nt) 1,764	Precore (nt) 1,896	Pre/Post LT (mo)	Total (mo)	HBeAg	HBV DNA Hyb (pg/mL)	HBV DNA PCR	ALT (IU/L)	Precore (nt) 1,896	P Gene (aa) 528	P Gene (aa) 552
Group I															
1	D	55	273	A	G	G	5/18	23	−	ND	−	13	*	*	*
2	D	7	70	A	G	G	4/18	22	−	ND	−	22	*	*	*
3	A	9	158	A	A	G (TGA)	2/25	27	−	ND	−	14	*	*	*
4	D	ND	103	A	G	A	8/15	23	−	ND	−	11	*	*	*
5	D	ND	52	A	G	A	7/8	15	−	ND	−	41	*	*	*
6	D	6	161	T	A	A	9/12	21	−	ND	−	13	*	*	*
7	D	905	471	T	A	A	0/38	38	+	176	+	80	G	M	V
8	D	ND	21	T	A	A	4/20	24	−	ND	−	19	A†	M‡	V‡
Mean ± SD			164 ± 147					24 ± 7				27 ± 24			
Group II															
9	D	ND	300	A	G	G		34	−	ND	−	27	*	*	*
10	D	ND	153	A	G	G		18	−	ND	−	14	*	*	*
11	A	22	371	T	A	G		13	−	ND	−	30	*	*	*
12	A	ND	169	T	A	G		12	−	ND	−	30	*	*	*
13	D	ND	50	T	A	G		15	−	ND	−	16	*	*	*
14	D	ND	168	A	G	A		12	−	ND	−	23	*	*	*
15	D	33	302	A	G	A		25	−	ND	−	21	*	*	*
16	D	7	189	A	G	A		18	−	ND	−	50	*	*	*
17	C	13	266	A	G	A		22	−	ND	−	16	*	*	*
18	D	5	380	A	G	A		19	−	ND	−	29	*	*	*
19	D	ND	298	T	A	A		12	−	ND	+	33	A	L	M
20	D	54	134	T	A	A		13	−	ND	−	27	*	*	*
21	D	13	104	T	A	A		15	−	ND	−	45	*	*	*
22	D	ND	296	T	A	G		19	−	ND	−	26	*	M‡	V‡
23	C	ND	161	A	G	A		25	−	ND	+	64	A	M	V
24	D	4	106	T	A	A		31	−	ND	+	34	A	M	I
25	D	15	169	T	A	A		32	−§	10	+	90	A†	M	I
26	D	15	109	T	A	A		21	−	36	+	106	A	M	I
27	D	11	206	T	A	A		16	−	ND	+	114	A	L	I
28	D	28	10	T	A	G		20	−	ND	+	6	G	M	V
29	D	10	52	T	A	G		19	+	6	+	37	G	L	I
Mean ± SD			190 ± 105					20 ± 7				40 ± 30			

Abbreviations: Hyb, solution hybridization assay; CP, core promoter; nt, nucleotide; aa, amino acid; ND, not detected; NT, not tested.

* Sequence not determined, sample PCR negative.

† Transient reversion from A to G at nt 1,896.

‡ Transient detection of lamivudine-resistant mutations.

§ Transient reversion to HBeAg positivity.

appearance of HBeAg. These observations prompted us to conduct a systematic study of the entire cohort of HBeAg-negative patients who were receiving lamivudine therapy to determine (1) the antiviral response to lamivudine therapy in patients with HBeAg-negative chronic hepatitis B, (2) the rate and pattern of lamivudine resistance mutations in HBeAg-negative patients receiving lamivudine therapy and the risk factors associated with the selection of these mutants, and (3) the effect of lamivudine-resistant mutations on HBeAg status and sequences in the core promoter and precore regions.

PATIENTS AND METHODS

Patients. Twenty-nine patients with chronic hepatitis B, who have received lamivudine for at least 1 year at the Liver Clinic, Semeiotica Medica, University of Bologna, Italy, were studied. All the patients were HBeAg negative and 28 (97%) patients were anti-HBe positive before treatment. There were 26 men and 3 women, with a mean age of 50 ± 8 years (range, 27-61 years) (Table 1). Group I included 7 patients with decompensated cirrhosis, who received lamivudine pre- and post-LT and 1 patient (patient 7) who received lamivudine for recurrent hepatitis B post-LT. The 7 patients with decompensated cirrhosis also received hepatitis B immune globulin post-LT.[20] Group II included 21 patients with histologically proven chronic hepatitis B with or without cirrhosis. Group I patients received lamivudine in doses of 100 mg daily. Group II patients received 150-mg doses because the 100-mg tablets were not available when this study began. All the patients had detectable serum HBV DNA by polymerase chain reaction (PCR) assay, 18 (62%) patients also had detectable serum HBV DNA by solution hybridization assay before treatment (Table 1). The mean ± SD duration of treatment was 21 ± 7 months (median, 20; range, 12-38 months). One patient in Group I (patient 2) was also positive for hepatitis C and hepatitis D antibodies. All the patients were negative for antibody to HIV.

Materials. Serial sera pretreatment and at 3- to 6-month intervals during treatment were tested for liver biochemistry, HBeAg, anti-HBe, hepatitis B surface antigen (HBsAg), and HBV DNA. Pretreatment samples from all patients and all subsequent samples that were HBV-DNA positive by PCR assay were examined for HBV core promoter and precore as well as polymerase gene sequence variations.

Serology Assays. Serology markers for HBV (HBsAg, HBeAg, and anti-HBe), for hepatitis C (anti-HCV), for hepatitis D (anti-HDV), and for HIV (anti-HIV) were tested by commercially available enzyme immunoassay kits (EIA; Abbott Diagnostics, North Chicago, IL).

HBV DNA Assays. Serum HBV DNA was detected using a solution hybridization assay (Abbott Diagnostics), which has a detection limit of 1.6 pg/mL as well as an in-house PCR assay that has a detection limit of 250 to 500 genome copies/mL.[21]

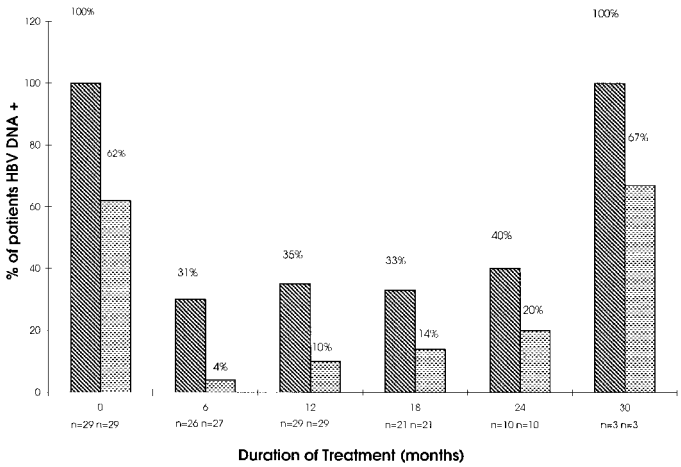

FIG. 1. Antiviral response during lamivudine treatment. (▨) PCR; (▩) solution hybridization assay.

TABLE 3. Factors Associated With the Selection of Lamivudine-Resistant Mutations

Factors		No. (%) With M552V or M552I	P
Pretreatment HBV DNA			
Solution hybridization assay	+	7/18 (39)	NS
Solution hybridization assay	−	3/11 (27)	
Group I		2/8 (25)	NS
Group II		8/21 (38)	
HBV genotypes			
A		0/3 (0)	NS
C		1/2 (50)	
D		9/24 (38)	
Precore eW28X/G_{1896}A variant	+	7/18 (39)	NS
Precore eW28X/G_{1896}A variant	−	3/11 (27)	
Core promoter $A_{1762}T/G_{1764}A$ variant	+	9/16 (56)	.01
Core promoter $A_{1762}T/G_{1764}A$ variant	−	1/13 (8)	
Duration of treatment (months)	≤18	1/10 (10)	.05
Duration of treatment (months)	>18	9/19 (47)	

Abbreviation: NS, not significant.

DNA Extraction, PCR Amplification, and Direct Sequencing. DNA extraction, PCR amplification, and direct sequencing of the core promoter and precore regions were performed as described previously using primers P1, P2, P3, and P4.[22] For amplification of P and overlapping S gene, the external primers used were SS1 and SAS1 and the internal primers were SS2 and SAS2.[23] All necessary precautions to prevent cross-contamination were observed and negative controls were included at each step.

Amplified PCR products were purified by QIAquick spin columns (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. Purified HBV DNA was directly sequenced by the DNA sequencing core facility at the University of Michigan Medical Center using the standard protocol for the Applied Biosystems DNA Sequencer 377 (Perkin Elmer Corp., Foster City, CA) using primers P3 and P4 for the core promoter and precore regions and SS2 and SAS2 for the P gene.

HBV Genotyping. HBV genotyping was determined by comparing the sequences in the HBV S gene with published sequences.[24]

Cloning. To determine if changes in the P gene and the precore region during lamivudine treatment occurred in the same genome, PCR-amplified HBV DNA from serial samples of 3 patients were cloned and sequenced. HBV genome amplification was performed using 10 μL DNA extract in the Expand High Fidelity Assay (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. The reactions were performed with an initial denaturation step at 94°C for 2 minutes; 10 cycles at 94°C for 40 seconds, 60°C for 1.5 minutes, and 68°C for 4 minutes; followed by 30 cycles at 94°C for 40 seconds, 60°C for 1.5 minutes, 68°C for 4 minutes with an increment of 5 seconds per cycle, and a 7-minute extension step at 72°C at the end. The external primers were: P5 (5′-TTTTTCACCTCTGCCTAATCA-3′ 1821-1841) and P6 (5′-AAAAAGTTGCATGGTGCTGG-3′ 1825-1806). The second round PCR used hemi-nested primers P5 and SAS1.

The amplified products were used fresh (within 24 hours) for ligation with the pCR2.1 vector (Invitrogen Corp., San Diego, CA) at 14°C overnight according to the manufacturer's instructions. TOP10F′-competent *Escherichia coli* (Invitrogen Corp.) was used for transformation. Transformed bacteria were selected by plating on Luria-Bertoni agar in the presence of ampicillin (50 μg/mL), X-Gal (1.6 mg/mL), and IPTG (0.4 mmol/L). Clones containing the HBV DNA insert were selected based on their white color and were grown

TABLE 4. Serial Changes in HBeAg Status, HBV DNA and ALT Levels, and Precore and P Gene Sequences in the 10 Patients Who Developed Lamivudine-Resistant Mutations

Patient No.	Duration of Rx (mo)	HBeAg	HBV DNA (pg/mL)	ALT (IU/L)	Precore (nt)	P Gene (aa)*					
					1,896	528	552	Other Changes			
7	0	−	905	471	A	L	M				
	6/6 clones				A	L	M				
	3	−	ND	65	A	L	M	N437Y sL80F			N602I
	7	−	698	60	G	M	V sI195M	N437Y	A548V sL192F	L577M sF220L	N602I
	5/5 clones				G	M	V	N437Y	A548V	L577M	N602I
	14	+	238	173	G	M	V	N437Y	A548V	L577M	N602I
	26	+	204	85	G	M	V	N437Y	A548V	L577M	N602I
	39	+	176	80	G	M	V	N437Y	A548V	L577M	N602I
	5/5 clones				G	M	V	N437Y	A548V	L577M	N602I
8	0	−	ND	21	A	L	M				
	3	−	ND	24	A	L	M	N496Y sX139C			T585P
	10	−	ND	12	A	M	V sI195M	N496Y	W501R	L577A	T585P
	22	−	ND	18	A	M	V	N496Y	W501R	L577A	T585P
	24	−	ND		*	*	*				
22	0	−	ND	296	G	L	M				
	9	−	ND	26	G	L	M				
	11	−	ND	26	G	M	V sI195M				
	15	−	ND	25	*	*	*				
	19	−	ND	26	*	*	*				
23	0	−	ND	161	A	L	M				
	14	−	ND	25	A	L	M				
	16	−	ND	34	*	*	*				
	22	−	ND	38	A	M	V sI195M	N496Y sX139C	K497Q	I572V	
	26	−	ND	64	A	M	V > M	N496Y	K497Q	I572V	
24	0	−	4	106	A	L	M				
	14	−	ND	44	*	*	*				
	19	−	ND	67	*	*	*				
	29	−	ND	34	A	M	I sW196L	F492L	M493L	P585H	
	31	−	ND	35	A	M	I	F492L	M493L	P585H	
25	0	−	15	169	A	L	M				
	10/10 clones				A	L	M				
	2	−	5	48	A/G	L	M				
	4	−	ND	35	G	M	I sW196L		K560T sS204R		
	5/5 clones				G	M	I		K560T		
	10	−	10	30	A	L	I	S491T sF134L	K560T		
	12	+	10	225	G	M	I	S491T	K560T		
	9/9 clones				G	M	I	S491T	K560T		
	20	+	18	104	G	M	I	S491T	K560T		
	26	−	10	90	G > A	M	I	S491T	K560T		
	32	−	5	291	A	M	I	S491T	K560T		
	10/10 clones				A	M	I	S491T	K560T		
26	0	−	15	109	A	L	M				
	7/7 clones				A	L	M				
	6	−	ND	29	A	L	M				
	9	−	ND	47	A	L	I sW196L				
	12	−	5	219	A	L/M	I	N470L sT114S	N472H	Q478P sK122R	
	3 clones				A	L	I	N470L	N472H	Q478P	
	2 clones				A	M	I	N470L	N472H	Q478P	
	15	−	44	90	A	L/M	I	N470L	N472H	Q478P	
	21	−	36	106	A	M > L	I	N470L	N472H	Q478P	

TABLE 4 (Cont'd).

Patient No.	Duration of Rx (mo)	HBeAg	HBV DNA (pg/mL)	ALT (IU/L)	Precore (nt) 1,896	P Gene (aa)* 528	552	Other Changes			
	4 clones				A	M	I	N470L	N472H	Q478P	
	3 clones				A	L	I	N470L	N472H	Q478P	
27	0	−	11	206	A	L	M				
	6	−	ND	62	A	L	I sW196L				
	9	−	8	52	A	L	I	S401N sS45T	Y402H sP46T	L428G	A562E
	16	−	ND	114	A	L	I	S401N	Y402H	L428G	A562E
28	0	−	28	10	G	L	M				
	13	−	ND	15	G	L	M			I591M sX163W	
	16	−	ND	4	G	M	V sI195M	Q497K	R501W	I519M	
	20	−	ND	6	G	M	V	Q497K	R501W	I519M	
29	0	−	10	52	G	L	M				
	9	−	ND	30	G	L	M				
	12	−	ND	26	G	L	I sW196L				
	19	+	6	37	G	L	I				

NOTE. Corresponding changes in overlapping S gene are indicated beneath the P gene changes, changes in P gene that result in reversion to predominant P gene sequence for that particular HBV genotype are underlined. Abbreviations for changes in overlapping S gene are denoted with small letter s to indicate surface gene, e.g., M552V in P gene results in isoleucine to methionine substitution in position 195 of S gene (sI195M).

Abbreviations: nt, nucleotide; ND, not detected; NA, sample not available.

* Sequence not determined, sample PCR negative.

overnight at 37°C in 5 mL of Luria-Bertoni broth containing 50 μg/mL ampicillin. Recombinant plasmids were recovered using the QIAprep-8 miniprep kit (Qiagen Inc.) following the manufacturer's instructions. Purified plasmids were digested with the restriction enzymes *Hin*dIII and *Not*I (Gibco BRL, Grand Island, NY). Plasmids containing the 2.2-kb insert, which spanned the core promoter and precore regions and domains A-E of the HBV reverse transcriptase/polymerase gene were sequenced using the primers M13 Reverse (5′-CAGGAAACAGCTATGACC-3′) andT7 (5′-TAATACGACTCACTATAGGG-3′) on pCR2.1 vector.

Statistical Analyses. Two-tailed Student's *t* test was used to compare continuous variables. χ^2 test with Yates correction was used to compare categorical data.

RESULTS

HBV Genotypes and Baseline Core Promoter, Precore, and P Gene Sequences. Sequence alignment showed that HBV genotypes A, C, and D were present in 3, 2, and 24 patients, respectively (Table 2). Before the initiation of lamivudine treatment, the dual core promoter variant $A_{1762}T$, $G_{1764}A$ was detected in 16 (55%) patients and the precore stop codon variant (G to A change at nucleotide 1,896 in the precore region resulting in conversion of tryptophan to stop codon at position 28 in HBeAg, eW28X/G_{1896}A) in 18 (62%) patients (Table 2). Ten (34%) patients had both core promoter and precore mutations. Another precore stop codon variant eW28X/G_{1897}A was found in 1 patient (patient 3). The predominant sequence at positions 528 and 552 of the HBV polymerase protein was leucine and methionine in all patients.

Antiviral Response. A decrease in viral load was observed in the vast majority of patients. The proportion of patients who had detectable serum HBV DNA decreased from 62% at month 0 to 4% at month 6 ($P < .0001$) when tested by solution hybridization assay and from 100% to 31% ($P < .0001$) when tested by PCR assay (Fig. 1). However, lamivudine-resistant mutations began to emerge after month 4. Thus, the proportion of patients who had detectable serum HBV DNA increased as treatment continued (Fig. 1).

Of the 18 patients who had detectable serum HBV DNA by solution hybridization assay before treatment, most had low serum HBV DNA levels, only one had HBV DNA level greater than 100 pg/mL. During treatment, HBV DNA became undetectable by solution hybridization assay in all patients. However, breakthrough infection defined as reappearance of serum HBV DNA by solution hybridization assay after its initial disappearance was observed in 6 (33%) patients. Serum HBV DNA remained detectable in 4 of these 6 patients at the last follow-up.

In the remaining 11 patients, HBV DNA became undetectable by PCR assay in 10 (91%) patients initially but reappeared in 1 patient as treatment continued (patient 23). None of these 11 patients had detectable serum HBV DNA by solution hybridization assay throughout the study.

None of the patients cleared HBsAg.

Detection of Lamivudine-Resistant Mutations. Lamivudine-resistant mutations involving the YMDD motif were detected in 10 (35%) patients, 7 (39%) of 18 patients who had detectable HBV DNA and 3 (27%) of 11 patients who had undetectable HBV DNA by solution hybridization assay before treatment (Table 3). There was no correlation between HBV genotypes, presence or absence of the precore stop codon variant, and detection of lamivudine-resistant mutants. Lamivudine-resistant mutations were more often found in patients with the dual core promoter variant and in those who had longer duration of treatment (Table 3).

The mean interval from onset of treatment to the first detection of these mutations was 13 months (median, 10.5 months; range, 4-29 months) (Table 4). The cumulative rates

of detection of lamivudine-resistant mutations at the end of 1 and 2 years of treatment were 10% and 56%, respectively (Fig. 2).

The 10 patients with lamivudine-resistant mutations have been followed for a mean of 12 ± 10 months (range, 2-32 months). Despite the emergence of these mutations, only 5 patients (patients 7, 25, 26, 27, and 29) had detectable serum HBV DNA by solution hybridization assay (Table 4), 3 of whom had elevated alanine transaminase (ALT) levels. In the remaining 5 patients, only 1 (patient 23) had elevated ALT levels. All 10 patients with lamivudine-resistant mutations had stable liver disease with no evidence of hepatic decompensation.

Five (50%) patients were found to have the M552V mutant, and all had accompanying L528M mutation, which was detected simultaneously with the M552V mutant (Table 4). Subsequent samples from 2 of these 5 patients (patients 8 and 22) became undetectable for HBV DNA by PCR assay. Five patients were found to have the M552I mutant. In 2 patients (patients 24 and 25), the L528M mutation was detected simultaneously with the M552I mutant. In 1 patient (patient 26), the L528M mutation was detected 3 months after the first detection of the M552I mutant. In the other 2 patients (patients 27 and 29), the L528M mutation was not detected 10 and 7 months after the first detection of the M552I mutant. The M552V and M552I mutations result in amino acid substitutions in the overlapping S gene, isoleucine to methionine at position 195 (sI195M), and tryptophan to leucine at position 196 (sW196L), respectively; whereas the L528M mutation is silent in the S gene.

Changes in Precore and HBeAg Status in Association With the Emergence of Lamivudine-Resistant Mutations. Of the 10 patients with lamivudine-resistant mutations, 3 (patients 7, 8, and 25) had reversion of the precore stop codon mutation to tryptophan at codon 28 (A to G at nucleotide 1896) (Tables 2 and 4). In 1 patient (patient 7), the reversion in the precore sequence was simultaneous with the first detection of the M552V mutant and accompanied by persistent detection of HBeAg and high serum HBV DNA levels (Fig. 3A). In the second patient (patient 25), reversion in the precore sequence was simultaneous with the first detection of the M552I mutant but appearance of HBeAg occurred 8 months later (Fig. 3B). This patient subsequently became HBeAg negative again with reappearance of the precore stop codon mutation. Serum HBV-DNA levels were higher during the period when the patient was HBeAg positive. The third patient (patient 8) had transient reversion in the precore sequence but remained HBeAg negative and serum HBV DNA was persistently undetectable by solution hybridization assay (Fig. 3C). An additional patient (patient 29) became HBeAg positive with reappearance of low serum HBV-DNA levels. This latter patient did not have the precore stop codon mutation initially. No change in precore sequence accompanied the appearance of HBeAg.

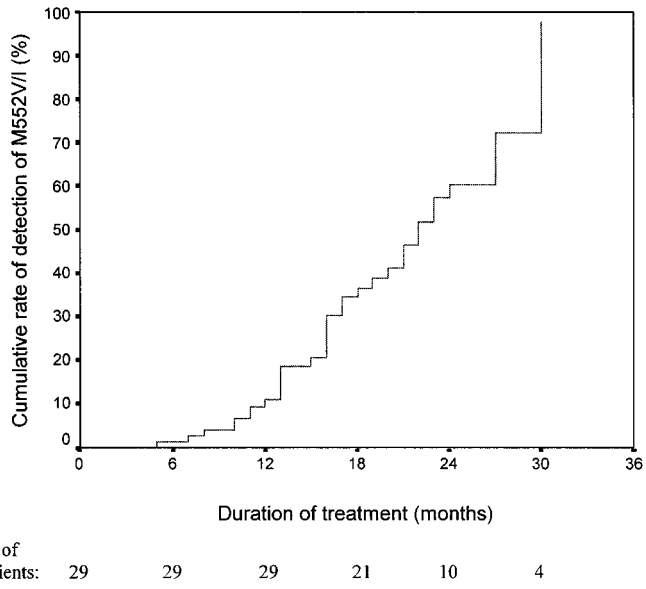

FIG. 2. Cumulative rates of detection of lamivudine-resistant M552V/I mutants. Number of patients at risk are listed at the bottom.

To determine if changes in the precore and P gene sequences occurred in the same genome, serial (3-4) samples from 3 patients (patients 7, 25, and 26) were cloned and multiple (5-10) clones at each time point were sequenced. In all 3 patients, changes in the precore and P gene sequences were detected in the same clones (Table 4).

None of the patients had detectable changes in the core promoter sequence during the study.

Other Changes in the P Gene. Eight of the 10 patients who had mutations in the YMDD motif were observed to have additional changes in the P gene (Table 4). Most of these changes emerged at the same time when the M552V or M552I mutants were first detected. Only one of the 25 changes, N496Y, was detected in more than one patient (patients 8 and 23). In these 2 patients, the pretreatment samples had asparagine at position 496 in the P gene and stop codon at position 139 in the overlapping S gene. During treatment, the sequence at this position reverted back to the predominant/reference sequence, tyrosine at 496 in the P gene and cysteine at 139 in the S gene. Because position 139 in the S gene is located in the "a" determinant, the predominant epitope of HBsAg, multiple clones from the pretreatment samples of these 2 patients were sequenced. Tyrosine at position 496 in the P gene and cysteine at 139 in the S gene were found in 4 of 9 clones in patient 8 and in 2 of 7 clones in patient 23. Ten of the remaining 24 changes in the P gene resulted in changes in the overlapping S gene. Of these, 4 resulted in unique amino acid changes in the S gene, one in the "a" determinant (patient 25, pS491T/sF134), and the other 3 in the transmembrane domain of HBsAg (patient 7, pA548V/sL192F, pL577M/sF220L; patient 25 pK560T/sS204R). The remaining 6 changes were reversions back to the predominant/reference sequence.

DISCUSSION

In this study, we confirmed that lamivudine therapy is effective in inhibiting HBV replication in patients with HBeAg-negative chronic hepatitis B. Although HBeAg-negative chronic hepatitis B in Mediterranean countries has been equated with the presence of precore stop codon mutation, the classic eW28X/G_{1896}A variant was only detected in 62% of our patients, all of whom were Italians. An antiviral response was observed in patients with precore stop codon mutation (eW28X) alone, core promoter mutation (A_{1762}T, G_{1764}A) alone, combined precore and core promoter mutations, or wild-type precore/core promoter sequence. Because of the small number and the heterogeneity of the patients, we cannot determine if there is any correlation between antiviral response and HBV precore/core promoter sequence.

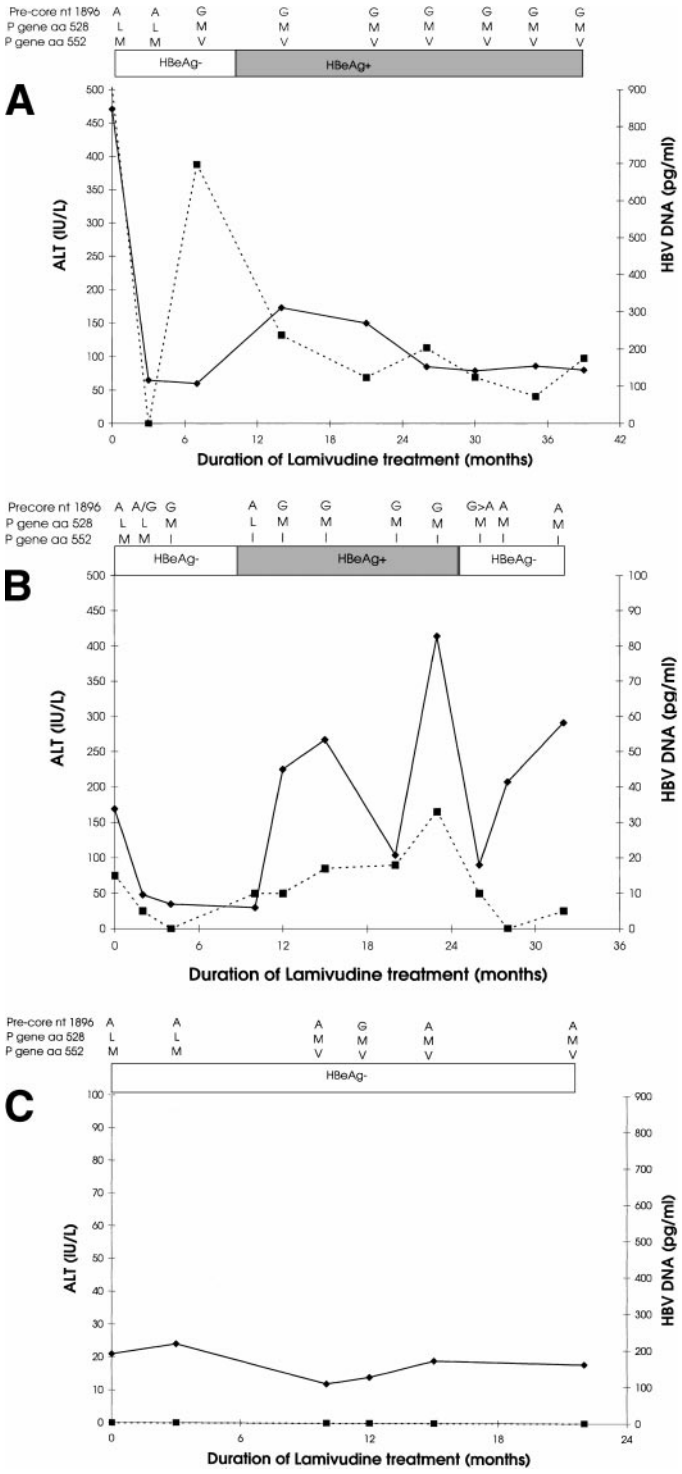

FIG. 3. Serial changes in serum HBV DNA (■) and ALT (◆) levels in 3 patients who had lamivudine-resistant M552V/I with or without L528M mutations and reversion of the precore stop codon to tryptophan at position 28 (A to G at nucleotide 1,896). In one patient (patient 7), reversion in precore sequence was accompanied by persistent reappearance of HBeAg and high serum HBV DNA levels (A). In the second patient (patient 25), reversion in the precore sequence and reappearance of HBeAg was transient. Serum HBV DNA levels were higher when the patient became HBeAg positive (B). The third patient (patient 8) had transient reversion in precore sequence but remained HBeAg negative with undetectable serum HBV DNA by solution hybridization assay (C).

Genotypic resistance to lamivudine was detected in 10 (35%) patients after a mean of 22 ± 7 months of treatment. The cumulative rates of detection of M552V/I mutations after 1 and 2 years of treatment were 10% and 56%, respectively. Compared with other studies on patients with HBeAg-negative chronic hepatitis B, the rate of genotypic resistance after 1 year of treatment was similar to that reported by Santantonio et al.[18] and lower than Tassopoulos et al.[17] Despite the detection of genotypic resistance, only 50% of our patients had detectable serum HBV DNA by solution hybridization assay and only 40% had elevated ALT levels. All 10 patients continued to have stable liver disease after a mean follow-up of 12 ± 11 months. The favorable outcome of these patients may be related to the decreased replication fitness of the lamivudine-resistant mutants.[25,26] Two patients with M552V subsequently had undetectable serum HBV DNA by PCR assay. Disappearance of lamivudine-resistant mutants and HBeAg seroconversion after the detection of genotypic resistance have been reported in HBeAg-positive patients.[27]

In addition to the duration of treatment, we found that core promoter mutations were more likely to be associated with the selection of lamivudine-resistant mutants. The exact reason(s) for this association is not clear. In this study, presence of the precore stop codon mutation did not correlate with the selection of lamivudine-resistant mutants.

Half of our patients with genotypic resistance to lamivudine had M552V and the other half had M552I mutation. Previous studies suggested that M552V was the predominant mutant among patients with lamivudine-resistant HBV mutations and that L528M was only found in association with M552V.[10,28] However, a review of the literature found that M552V and M552I were detected in similar proportions of hepatitis B patients with lamivudine resistance and the combination of L528M and M552I had been reported in 2 patients (Table 5).[29-31] In this study, L528M was detected simultaneously with M552V in 5 patients, simultaneously with M552I in 2 patients, delayed for 3 months in 1 patient with M552I, and not detectable 7 and 10 months after the detection of M552I in 2 patients. The more frequent association of L528M and M552V may be related to the fact that M552V alone confers less resistance to lamivudine than M552I. *In vitro* studies comparing the antiviral effects of lamivudine in transfected hepatocyte cell lines showed increasing order of resistance with L528M < M552V < L528M + M552V and M552I.[10,11] Thus, the addition of L528M to M552V may be necessary for *in vivo* resistance to lamivudine. Several studies have shown a de-

TABLE 5. Patterns of Mutations in the Reverse Transcriptase/DNA Polymerase Domain in Patients Receiving Lamivudine Therapy

	No. of Patients With Lamivudine-Resistant Mutations			
Reference	M552V + L528M	M552V Alone	M552I + L528M	M552I Alone
Liaw et al.[27]	12	0	0	20
McCaughan et al.[33]	6	0	0	0
Hussain and Lok[29]*	28	3	2	15
Current	5	0	3†	2
Total	51	3	5	37

* Review of published data.

† In 1 patient L528M detected 3 months after detection of M552I.

crease in replication fitness of M552V and M552I compared with "wild-type" HBV and a partial restoration in replication fitness with the addition of L528M.[25,26] It is possible that the subsequent selection of L528M in some patients with M552I serve to restore replication fitness rather than to increase resistance to lamivudine. The infrequent report of the combined mutations L528M and M552I may be related to sampling time and the fact that most studies tested for resistant mutants at one time point only. The additional selection of L528M in some patients with M552I during continued treatment with lamivudine was also observed in one recent study.[32]

Three patients with lamivudine-resistant mutations had reversion of the precore stop codon mutation. The exact reason(s) for the reversion of the precore stop codon mutation in these 3 patients is not clear. The time relationship between the detection of the lamivudine-resistant mutations and the reversion in the precore sequence, and the fact that these changes occurred in the same genomes suggest that the changes in polymerase and precore regions may be related. It is possible that the reversion of the precore stop codon mutation restores the replication fitness of the lamivudine-resistant mutants. This accounts for the higher serum HBV DNA levels when the patients reverted from A to G at nucleotide 1,896 and became HBeAg positive.

Eight of the 10 patients who had M552V/I mutations had additional changes in the P gene. However, the significance of these changes is not clear because there was no consistent pattern and most of the changes were detected at the same time as the M552V/I mutants. Approximately half of the changes in the P gene resulted in amino acid substitutions in the overlapping S gene. Previous studies found that M552V/I do not affect HBsAg secretion[25] but the impact of other P gene changes on virion production, HBsAg secretion, and antigenicity is unknown.

In this study, we have systematically characterized the evolution of HBV core promoter, precore, and reverse transcriptase/polymerase sequences in a cohort of patients with HBeAg-negative chronic hepatitis B, who have received lamivudine therapy for at least 1 year. An initial antiviral response was obtained in patients with or without precore/core promoter variants. Genotypic resistance to lamivudine was detected at similar rates as in HBeAg-positive patients. In addition to the duration of treatment, core promoter mutations were more likely to be associated with lamivudine resistance. However, further studies with larger numbers of patients from different geographical areas are needed to confirm this observation and to determine if there is any correlation between HBV genotypes, precore, and core promoter variants and response to lamivudine therapy. Contrary to most published reports, we found that L528M may be found in association with M552I, albeit at a later stage. The most important finding in our study is the reversion of precore stop codon mutation and reappearance of HBeAg in some of our patients who had genotypic resistance to lamivudine. Our findings suggest that in patients with genotypic resistance to lamivudine, additional changes may be selected to restore replication fitness of HBV. These changes may be found outside the polymerase/reverse transcriptase domain of the HBV genome.

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REFERENCES

1. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998;339:61-68.
2. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256-1263.
3. Villeneuve JP, Condreay LD, Willems B, Pomier-Layrargues G, Fenyves D, Bilodeau M, Leduc R, et al. Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. HEPATOLOGY 2000;31:207-210.
4. Perrillo R, Rakela J, Dienstag J, Levy G, Martin P, Wright T, Caldwell S, et al. Multicenter study of lamivudine therapy for hepatitis B after liver transplantation. Lamivudine Transplant Group. HEPATOLOGY 1999;29:1581-1586.
5. Andreone P, Caraceni P, Grazi GL, Belli L, Milandri GL, Ercolani G, Jovine E, et al. Lamivudine treatment for acute hepatitis B after liver transplantation. J Hepatol 1998;29:985-989.
6. Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, et al. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut 2000;46:562-568.
7. Liaw YF, Leung NWY, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, et al. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Gastroenterology 2000;119:172-180.
8. Leung NWY, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, Wu PC, et al. Three year lamivudine therapy in chronic HBV [abstract]. J Hepatol 1999;30(Suppl 1):59.
9. Chang TT, Lai CL, Liaw YF, Guan R, Lim SG, Lee CM, Ng KY, et al. Incremental increases in HBeAg seroconversion and continued ALT normalization in Asian chronic HBV (CHB) patients treated with lamivudine for four years [abstract]. Antiviral Therapy 2000;5(Suppl 1):44.
10. Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, et al. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. HEPATOLOGY 1998;27:1670-1677.
11. Ono-Nita SK, Kato N, Shiratori Y, Lan KH, Yoshida H, Carrilho FJ, Omata M. Susceptibility of lamivudine-resistant hepatitis B virus to other reverse transcriptase inhibitors. J Clin Invest 1999;103:1635-1640.
12. Xiong X, Flores C, Yang H, Toole JJ, Gibbs CS. Mutations in hepatitis B DNA polymerase associated with resistance to lamivudine do not confer resistance to adefovir in vitro. HEPATOLOGY 1998;28:1669-1673.
13. Boucher CA, Cammack N, Schipper P, Schuurman R, Rouse P, Wainberg MA, Cameron JM. High-level resistance to (-) enantiomeric 2′-deoxy-3′-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother 1993;37:2231-2234.
14. Schuurman R, Nijhuis M, van Leeuwen R, Schipper P, de Jong D, Collis P, Danner SA, et al. Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). J Infect Dis 1995;171:1411-1419.
15. Keulen W, Back NK, van Wijk A, Boucher CA, Berkhout B. Initial appearance of the 184Ile variant in lamivudine-treated patients is caused by the mutational bias of human immunodeficiency virus type 1 reverse transcriptase. J Virol 1997;71:3346-3350.
16. Back NK, Nijhuis M, Keulen W, Boucher CA, Oude Essink BO, van Kuilenburg AB, van Gennip AH, et al. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. EMBO J 1996;15:4040-4049.
17. Tassopoulos NC, Volpes R, Pastore G, Heathcote J, Buti M, Goldin RD, Hawley S, et al. Efficacy of lamivudine in patients with hepatitis B e antigen-negative/hepatitis B virus DNA-positive (precore mutant) chronic hepatitis B. Lamivudine Precore Mutant Study Group. HEPATOLOGY 1999;29:889-896.
18. Santantonio T, Mazzola M, Iacovazzi T, Miglietta A, Guastadisegni A, Pastore G. Long-term follow-up of patients with anti-HBe/HBV DNA-positive chronic hepatitis B treated for 12 months with lamivudine. J Hepatol 2000;32:300-306.
19. Lau DTY, Ghany MG, Doo E, Herion D, Kleiner DE, Park Y, Schmid P, et al. Features of response and resistance to lamivudine in patients with chronic hepatitis B with and without HBeAg [abstract]. HEPATOLOGY 1998;28(Suppl):318A.
20. Grazi GL, Mazziotti A, Sama C, Jovine E, Stefanini F, Paladini R, Rossi R, et al. Liver transplantation in HBsAg-positive HBV-DNA–negative cir-

rhotics: immunoprophylaxis and long-term outcome. Liver Transpl Surg 1996;2:418-425.
21. Chan HL, Leung NW, Hussain M, Wong ML, Lok AS. Hepatitis B e antigen-negative chronic hepatitis B in Hong Kong. HEPATOLOGY 2000; 31:763-768.
22. Chan HL, Hussain M, Lok AS. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. HEPATOLOGY 1999; 29:976-984.
23. Ghany MG, Ayola B, Villamil FG, Gish RG, Rojter S, Vierling JM, Lok AS. Hepatitis B virus S mutants in liver transplant recipients who were reinfected despite hepatitis B immune globulin prophylaxis. HEPATOLOGY 1998;27:213-222.
24. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994;198:489-503.
25. Melegari M, Scaglioni PP, Wands JR. Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. HEPATOLOGY 1998;27:628-633.
26. Ono-Nita SK, Kato N, Shiratori Y, Masaki T, Lan KH, Carrilho FJ, Omata M. YMDD motif in hepatitis B virus DNA polymerase influences on replication and lamivudine resistance: a study by *in vitro* full-length viral DNA transfection. HEPATOLOGY 1999;29:939-945.
27. Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. HEPATOLOGY 1999;30:567-572.
28. Locarnini S, Birch C. Antiviral chemotherapy for chronic hepatitis B infection: lessons learned from treating HIV-infected patients. J Hepatol 1999;30:536-550.
29. Hussain M, Lok AS. Mutations in the hepatitis B virus polymerase gene associated with antiviral treatment for hepatitis B. J Viral Hepat 1999;6: 183-194.
30. Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, et al. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. HEPATOLOGY 1998; 27:1711-1716.
31. Petit MA, Buffello D, Roche B, Dussaix E, Aulong B, Bismuth H, Samuel D, et al. Residual hepatitis B virus (HBV) infection in liver transplant patients under treatment with lamivudine (3TC): assessment by quantitation of HBV DNA by PCR and assay of preS antigens [abstract]. HEPATOLOGY 1997;26:746A.
32. Yeh CT, Chien RN, Chu CM, Liaw YF. Clearance of the original hepatitis B virus YMDD-motif mutants with emergence of distinct lamivudine-resistant mutants during prolonged lamivudine therapy. HEPATOLOGY 2000;31:1318-1326.
33. McCaughan GW, Spencer J, Koorey D, Bowden S, Bartholomeusz A, Littlejohn M, Verran D, et al. Lamivudine therapy in patients undergoing liver transplantation for hepatitis B virus precore mutant-associated infection: high resistance rates in treatment of recurrence but universal prevention if used as prophylaxis with very low dose hepatitis B immune globulin. Liver Transpl Surg 1999;5:512-519.