Dynamics of Hepatitis B Virus Resistance to Lamivudine

Coralie Pallier,[1,2]† Laurent Castéra,[2,3]† Alexandre Soulier,[2] Christophe Hézode,[4] Patrice Nordmann,[1]
Daniel Dhumeaux,[2,4] and Jean-Michel Pawlotsky[2]*

*Department of Bacteriology and Virology, Hôpital de Bicêtre, Université Paris XI, Le Kremlin-Bicêtre,[1] Department of Virology,
INSERM U635, Hôpital Henri Mondor, Université Paris XII, Créteil,[2] Department of Hepatology and Gastroenterology,
Hôpital Haut-Lévêque, Université de Bordeaux, Bordeaux,[3] and Department of Hepatology and Gastroenterology,
Hôpital Henri Mondor, Université Paris XII, Créteil,[4] France*

Lamivudine was the first approved inhibitor of hepatitis B virus (HBV) reverse transcriptase (RT). Lami-
vudine resistance develops in 53% to 76% of patients after 3 years of treatment. We extensively characterized
the dynamics of HBV quasispecies variant populations in four HBV-infected patients who developed lamivu-
dine resistance. Virological breakthrough was preceded by 2 to 4 months by the emergence of quasispecies
variants bearing amino acid substitutions at RT position 204, i.e., within the YMDD catalytic motif (rtM204V/
I). Three patients had a gradual switch from a YMDD variant population at baseline to a 100% lamivudine-
resistant variant population, whereas the remaining patient had a fluctuating pattern of resistance variant
dynamics. Careful analysis of amino acid substitutions located outside domain C of HBV RT, including those
known to partially restore replication capacities in vitro, showed that the in vivo replication of HBV variants
is driven by multiple forces, including intrinsic replicative advantages conferred by mutations accumulating
outside domain C and the changing environment in which these variants replicate. Our findings also suggest
that individual treatment optimization will require sensitive methods capable of detecting the emergence of
viral resistance before the relevant variants acquire optimal replicative capacities.

Hepatitis B virus (HBV) infection is a major public health
problem, with approximately 350 million individuals chroni-
cally infected worldwide (19). Chronic HBV carriers are ex-
posed to a risk of complications, such as chronic hepatitis,
cirrhosis, and hepatocellular carcinoma, of which HBV is cur-
rently the most frequent cause (13). Up to one million people
die every year from complications of HBV infection (19).

HBV infection is characterized by high levels of virus pro-
duction and turnover (28, 39), whereas the HBV reverse tran-
scriptase (RT), like the human immunodeficiency virus (HIV)
RT, is an error-prone enzyme lacking 3′-5′-exonuclease proof-
reading capacity (3, 14). As a result, HBV, like other viruses
with error-prone polymerases, such as HIV, hepatitis C virus,
and poliovirus, has a quasispecies distribution in infected in-
dividuals (14). This means that HBV circulates as a complex
mixture of genetically distinct but closely related variants that
are in equilibrium at a given time point of infection in a given
replicative environment. The quasispecies distribution of HBV
implies that any newly generated mutation conferring a selec-
tive advantage to the virus in a given replicative environment
will allow the corresponding viral population to overtake the
other variants, following a classical Darwinian evolutionary
process (10).

Treatment of chronic hepatitis B is aimed at driving viral
replication to the lowest possible level, and thereby to halt the
progression of liver disease and prevent the onset of compli-
cations. However, HBV infection cannot be fully eradicated,
because of covalently closed circular proviral DNA persistence
in host cells. The first HBV RT inhibitor to be approved for the
treatment of chronic hepatitis B was lamivudine (dideoxy-2′,3′-
thiacytidine) (8, 9, 17, 22, 23). Its principal target is the YMDD
catalytic motif of HBV RT, located in domain C of the poly-
merase molecule (5). Lamivudine exerts its anti-polymer-
ase/RT activity by inhibiting the elongation of the HBV DNA
minus strand through competition with the natural polymerase
substrate dCTP and by acting as a chain terminator through its
incorporation in the nascent DNA strand (37, 41). Lamivudine
therapy results in a 3 to 4 log decline in plasma HBV DNA
after a few weeks and also reduces disease activity and im-
proves liver histological status (8, 9, 17, 22, 23). Long-term
lamivudine administration frequently elicits viral resistance
characterized by a reincrease of viral replication in an adherent
patient. The incidence of lamivudine resistance is 14% to 32%
after 1 year of treatment, 38% after 2 years, and 53% to 76%
after 3 years (18). The principal mutations associated with
lamivudine resistance are located in domain C of the YMDD
motif. They include rtM204V (YVDD sequence), rtM204I
(YIDD) (1), and the more recently identified rtM204S
(YSDD) (2, 27). Lamivudine-resistant mutants with amino
acid substitutions in the YMDD motif appear to replicate less
efficiently than the wild-type virus in vitro. However, additional
substitutions that are often coselected with the resistance sub-
stitutions at RT position 204 of domain C, such as rtL180M
and rtV173L, which are located in the B domain, can compen-
sate for this loss of replication efficiency in vitro (1, 6, 12, 36).

A proper understanding of the mechanisms underlying viral
resistance to a specific inhibitor implies the need to unravel the
quasispecies variant dynamics at work during the emergence
and amplification of resistance, together with the role of the
relevant amino acid substitutions in viral replication in vivo. In

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* Corresponding author. Mailing address: Department of Virology,
Hôpital Henri Mondor, 51 avenue du Maréchal de Lattre de Tassigny,
94010 Créteil, France. Phone: 33-1-4981-2827. Fax: 33-1-4981-4831.
E-mail: jean-michel.pawlotsky@hmn.aphp.fr.

† C.P. and L.C. contributed equally to this study.
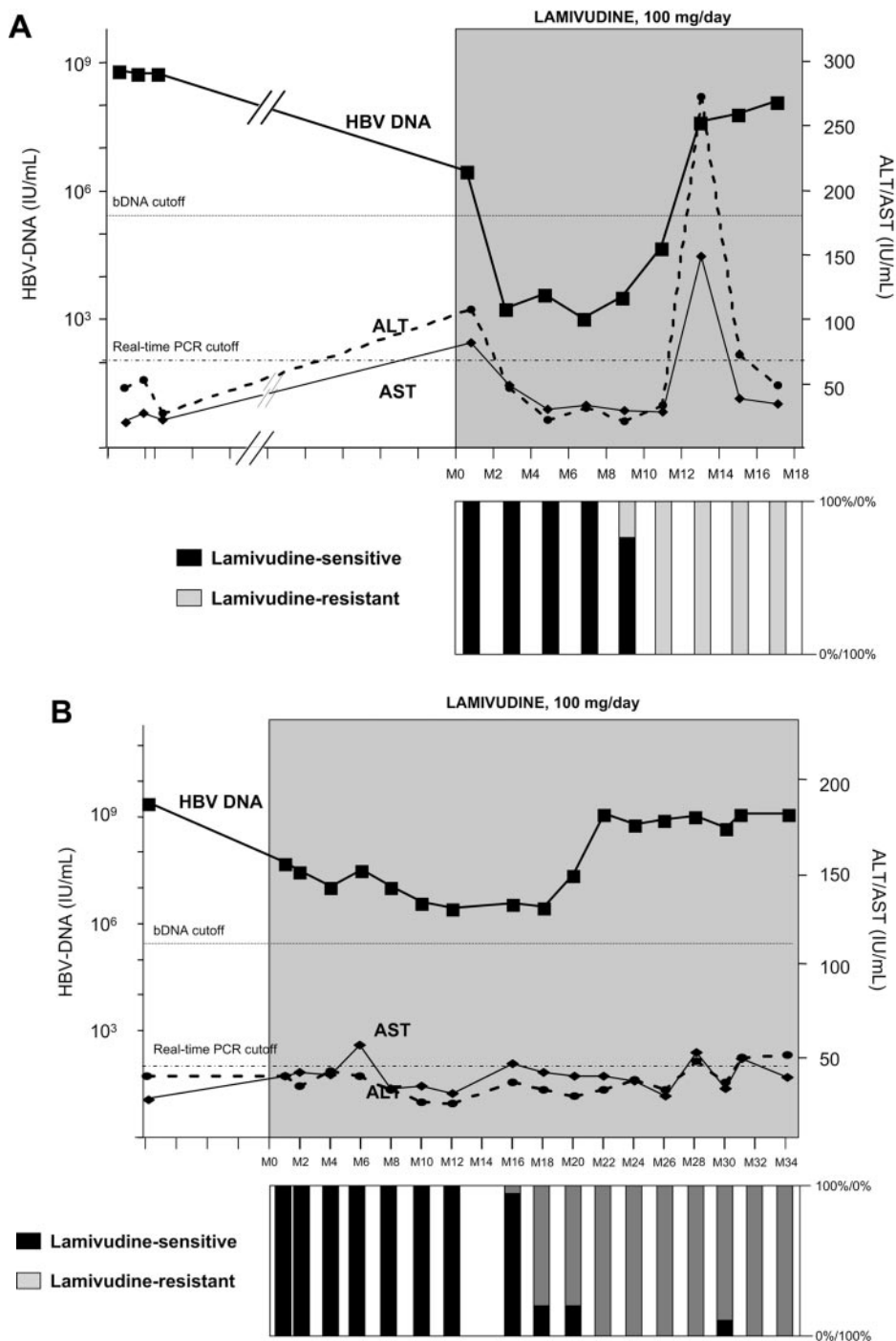
contrast to HIV and hepatitis C virus (4, 31, 32), little such information on resistance to specific RT inhibitors is available for HBV.

MATERIALS AND METHODS

Patients. We studied four male patients, aged 39 to 52 years, who had chronic HBeAg-positive HBV infection (genotype A was present in two cases, genotype C was present in one case, and genotype E was present in one case) and who had developed viral resistance to lamivudine. Resistance was characterized by an initial decline in HBV DNA levels during lamivudine therapy followed by a

reincrease despite full adherence to ongoing lamivudine therapy. All four patients were HIV seronegative and immunocompetent. Patients A, B, and C had not responded to previous treatment with standard alpha interferon, while patient D had not been treated before receiving lamivudine. All the patients received lamivudine monotherapy at 100 mg/day. Serum samples were taken at various time points before and during lamivudine therapy to monitor the virological response to lamivudine and HBV RT quasispecies dynamics (12 samples each for patients A and C, 18 samples each for patients B and D).

Methods. Aspartate aminotransferase and alanine aminotransferase levels were determined before treatment and for each available sample taken during lamivudine therapy. HBV DNA levels were measured at each time point by
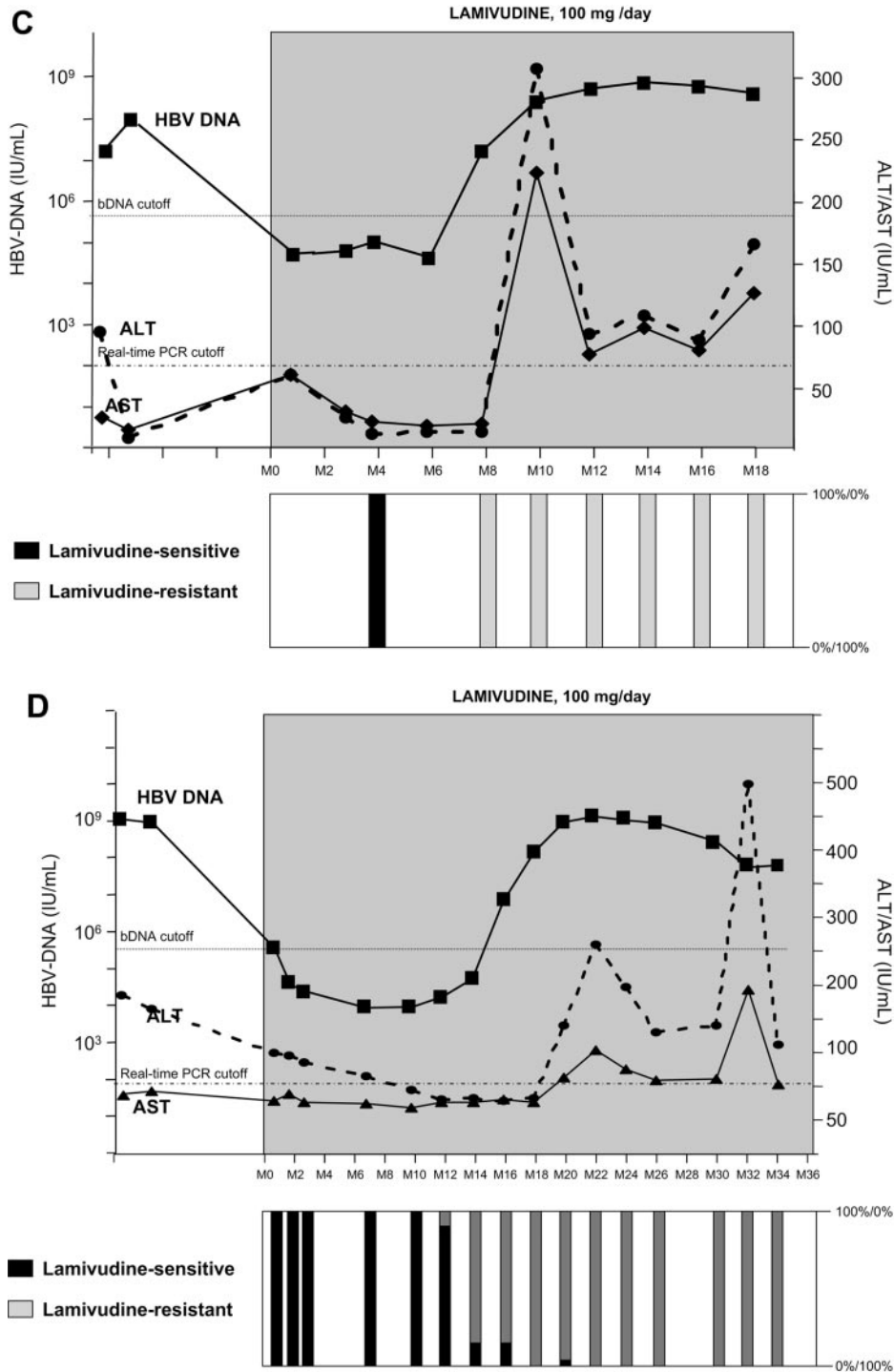
FIG. 1. Dynamics of serum HBV DNA levels, serum aminotransferase activities, and lamivudine-sensitive and -resistant HBV viral populations before and during lamivudine therapy in patients A to D (panels A, B, C, and D, respectively). For each patient, the dynamics of HBV DNA and aminotransferase levels are shown at the top, while the dynamics of HBV variants are shown at the bottom as respective proportions of lamivudine-sensitive and lamivudine-resistant variant populations within the viral quasispecies at each time point. The lamivudine treatment period is shown in gray. The patients continued to receive lamivudine after this study. Abbreviations: bDNA, branched DNA; ALT, alanine aminotransferase; AST, aspartate aminotransferase; M, month.

means of a branched DNA-based assay (Versant HBV DNA 1.0 assay; Bayer Corporation, Tarrytown, N.Y.) with a lower detection limit of 125,000 IU/ml (5.1 log IU/ml). Serum samples testing negative in the branched DNA assay (HBV DNA level, <100,000 IU/ml or 5.0 log IU/ml) were retested with an in-house

quantitative real-time PCR assay based on Lightcycler technology (Roche Molecular Systems, Pleasanton, California) with a lower detection limit of 50 IU/ml.

HBV RT quasispecies were characterized for all available samples. For this, DNA was extracted from 200 μl of serum and a 630-bp fragment encompassing
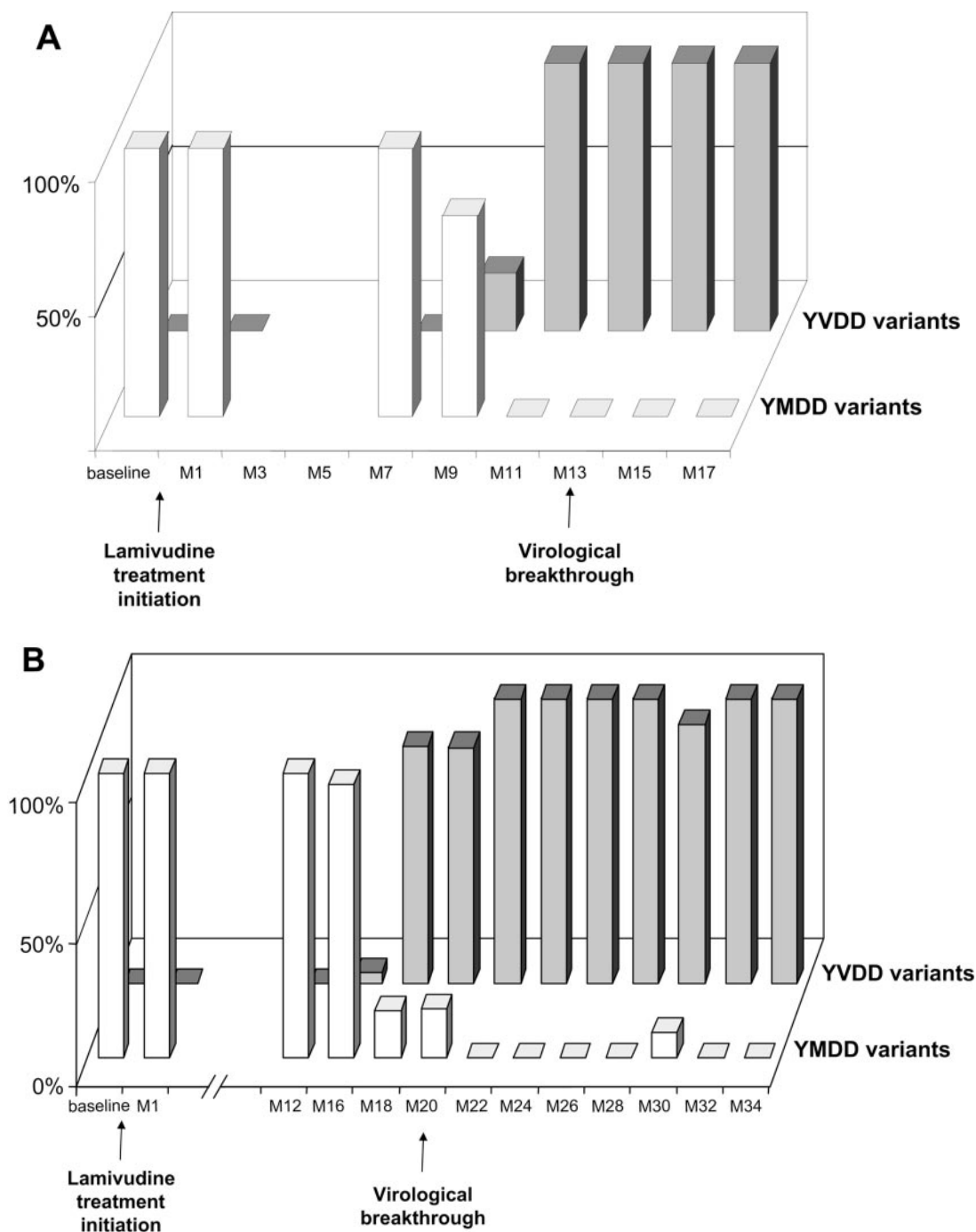
domains A to E of the HBV RT was amplified with primers POL4b and PORV2 as previously described (35). When HBV DNA level was below 100,000 IU/ml, a nested PCR technique was used to amplify a 132-bp region spanning the YMDD catalytic motif of domain C. The first round consisted of the 630-bp amplification. The second round used upstream primer CO1 (5′CTGGGCTTCCGCAA AATACCT3′, nucleotide positions 619 to 639) and downstream primer CO2 (5′ ATAATGGGACTCACGATGCTG-3′, nucleotide positions 772 to 792). Purified PCR products were cloned, recombinant plasmid DNA was transformed into *Escherichia coli* competent cells, and transformants were grown on ampicillin plates. Cloned DNA was reamplified by PCR for sequencing. Approximately 30 clones per sample were sequenced. HBV RT clone nucleotide and amino acid sequences were aligned by using the CLUSTAL W program, version 1.5 (38).

The PHYLIP program, version 3.572, was used to construct phylogenetic trees by means of the neighbor-joining method (11, 33) with a sequence matrix determined by the two-parameter method of Kimura. Phylogenetic trees were constructed for each patient.

Nucleotide sequence accession numbers. Sequence data from this article have been deposited in GenBank under accession numbers AM073272 to AM073968.

RESULTS

Biochemical and virological outcomes. As shown in Fig. 1, lamivudine administration induced a sharp decline in HBV DNA in all four patients, ranging from −2.5 to −6.0 log IU of
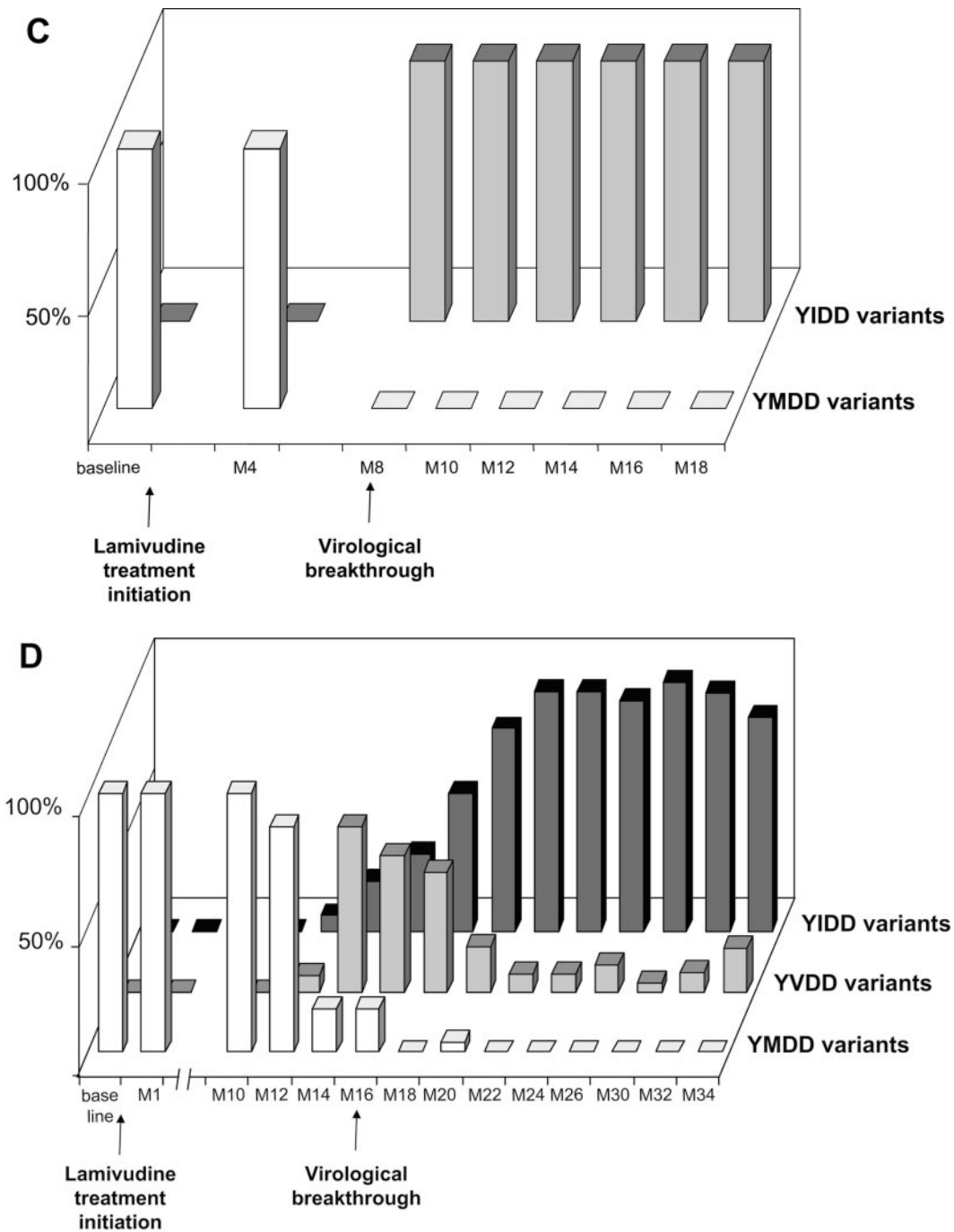
FIG. 2. Dynamics of HBV variant populations before and during lamivudine therapy in patients A to D (panels A, B, C, and D, respectively). This figure illustrates the respective losses and gains of in vivo fitness by the different HBV populations over time, relative to treatment outset and virological breakthroughs. M, month.

HBV DNA/ml. All patients, having started with high viral loads, maintained a relatively high residual level of HBV replication during lamivudine administration (Table 1). A breakthrough characterized by a significant increase in HBV DNA levels by more than 1.0 log above the nadir was observed in all four patients after several months of stable, reduced HBV DNA loads. HBV DNA levels returned to baseline within a few months in all four patients despite continued lamivudine

therapy. Virological breakthrough was followed in three patients by biochemical breakthrough (alanine aminotransferase and aspartate aminotransferase peaks); this occurred when the HBV DNA level had reached baseline level in patients A and C and several months later in patient D. No biochemical breakthrough occurred in patient B (Fig. 1).

Quasispecies distribution of HBV RT. A total of 1,710 sequences were generated and analyzed, allowing us to fully

TABLE 1. Dynamics of HBV populations with mutations located outside the YMDD motif according to the amino acid at position 204 in the four patients during lamivudine therapy[a]

Patient	Time point	Amt of HBV DNA (log IU/ml)	YMDD	M204V	M204V + L180M	M204V + V173L	M204V + V207I	M204V + L180M + V173L	M204V + L180M + V173L + V207I	M204V + L180M + V207I	M204V + V173L + V207I	M204I	M204I + L180M	M204I + V207I	M204I + V173L	M204I + L180M + V173L	M204I + L180M + V207I	M204I + V173L + V207I	M204I + L180M + V173L + V207I
A	Start	8.6	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M1	6.4	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M3	2.1	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M5	3.5	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M7	2.9	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M9	3.4	78.5%	–	21.5%	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M11	3.6	–	–	96.9%	–	–	3.1%	–	–	–	–	–	–	–	–	–	–	–
	M13	7.5	–	–	96.8%	–	–	3.2%	–	–	–	–	–	–	–	–	–	–	–
	M15	7.7	–	–	91.9%	–	–	8.1%	–	–	–	–	–	–	–	–	–	–	–
	M17	8.0	–	–	93.8%	–	–	6.2%	–	–	–	–	–	–	–	–	–	–	–
B	Start	9.4	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M1	7.7	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M2	7.4	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M4	7.0	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M6	7.5	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M8	7.0	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M10	6.6	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M12	6.4	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M16	6.5	96.2%	–	3.8%	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M18	6.4	16.6%	19.8%	9.9%	–	–	53.7%	–	–	–	–	–	–	–	–	–	–	–
	M20	7.3	17.0%	–	20.6%	–	–	62.4%	–	–	–	–	–	–	–	–	–	–	–
	M22	9.0	–	–	10.0%	–	–	90.0%	–	–	–	–	–	–	–	–	–	–	–
	M24	8.7	–	–	13.6%	–	–	86.4%	–	–	–	–	–	–	–	–	–	–	–
	M26	8.9	–	–	8.3%	–	–	91.7%	–	–	–	–	–	–	–	–	–	–	–
	M28	9.0	–	3.6%	14.4%	–	–	82.0%	–	–	–	–	–	–	–	–	–	–	–
	M30	8.6	9.0%	2.2%	4.4%	–	–	84.4%	–	–	–	–	–	–	–	–	–	–	–
	M32	9.1	–	–	3.6%	–	–	96.4%	–	–	–	–	–	–	–	–	–	–	–
	M34	9.0	–	–	8.8%	–	–	91.2%	–	–	–	–	–	–	–	–	–	–	–
C	Start	8.0	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M4	5.0	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M8	7.2	–	–	–	–	–	–	–	–	–	100.0%	–	–	–	–	–	–	–
	M10	8.4	–	–	–	–	–	–	–	–	–	96.9%	3.1%	–	–	–	–	–	–
	M12	8.7	–	–	–	–	–	–	–	–	–	67.9%	32.1%	–	–	–	–	–	–
	M14	8.9	–	–	–	–	–	–	–	–	–	49.9%	50.1%	–	–	–	–	–	–
	M16	8.8	–	–	–	–	–	–	–	–	–	40.1%	59.9%	–	–	–	–	–	–
	M18	8.6	–	–	–	–	–	–	–	–	–	31.3%	68.7%	–	–	–	–	–	–
D	Start	8.8	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M1	5.6	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M2	4.6	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M3	4.4	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M7	4.0	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M10	4.0	100.0%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M12	4.0	87.2%	–	6.4%	–	–	–	–	–	–	3.2%	–	3.2%	–	–	–	–	–
	M14	4.7	16.7%	11.1%	52.8%	–	–	–	–	–	–	8.3%	–	11.1%	–	–	–	–	–
	M16	6.8	16.9%	6.7%	46.0%	–	–	–	–	–	–	6.8%	–	23.6%	–	–	–	–	–
	M18	8.1	–	6.8%	39.7%	–	–	–	–	–	–	36.6%	–	16.9%	–	–	–	–	–
	M20	8.9	3.6%	–	14.2%	–	–	–	–	–	–	53.7%	–	14.1%	7.2%	3.5%	–	3.6%	–
	M22	9.0	–	–	6.8%	–	–	–	–	–	–	69.2%	–	13.8%	10.2%	–	–	–	–
	M24	8.9	–	–	6.8%	–	–	–	–	–	–	48.1%	–	17.3%	20.8%	–	–	7.4%	–
	M26	8.8	–	–	10.4%	–	–	–	–	–	–	31.0%	–	20.6%	20.6%	–	–	–	–
	M28	8.3	–	–	–	–	–	–	–	–	–	–	–	38.0%	25.9%	–	–	–	–
	M30	7.7	–	–	–	–	–	–	–	–	–	11.1%	–	44.5%	25.9%	–	7.4%	–	–
	M32	7.7	–	–	3.7%	–	–	–	–	–	–	11.1%	–	44.4%	26.0%	–	–	7.4%	3.7%
	M34	7.7	6.8%	–	3.7%	–	–	–	–	–	–	7.0%	–	41.5%	20.8%	–	–	–	6.8%

[a] Each possible combination of the L180M, V173L, or V207I mutation with either the M204V or M205I mutation has been taken into consideration. The proportion of each variant population is shown as a percentage of the circulating quasispecies at each time point. The HBV DNA load at each time point is given in log international units/milliliter. M, month of therapy. –, no such population was found within the quasispecies at the indicated time point.

characterize nearly full-length HBV RT quasispecies at each time point before and during lamivudine therapy in the four patients. The risk of resampling errors, which may result from the analysis of quasispecies from a commonly sampled source that contains a small number of viral genomes (generally fewer than 100 viral copies/ml) (16, 24), was minimal in our study, in which HBV DNA levels remained high before as well as during lamivudine therapy (Table 1). Before treatment, the lamivudine-sensitive (YMDD) catalytic motif was fully conserved in all quasispecies variants of the four patients. No minor quasispecies populations were found to bear amino acid substitutions known to confer lamivudine resistance. Note that this did not mean that such variants were absent but that their prevalence in the quasispecies populations was very low. Sporadic amino acid substitutions were found in minor variants at various positions outside the YMDD motif.

Dynamics of HBV variants during lamivudine therapy: amino acid substitutions within the YMDD motif. In all four patients, virological breakthrough was preceded by 2 to 4 months by the detection of quasispecies variants bearing amino acid substitutions within the YMDD motif that are known to confer lamivudine resistance (Fig. 1A to 1D, bottom panels). Gradually, these resistant variants, which initially represented a minority of the quasispecies, fully replaced the wild-type YMDD variants (Fig. 2A to 2D). This switch occurred in parallel to the increase in HBV DNA load. The absolute amount of resistant HBV variants increased exponentially such that they represented 100% of the viral quasispecies by the time the HBV DNA load had returned to baseline. In the case of patient B (Fig. 1B and 2B), HBV DNA load was low and stable at month 16, when 3.8% of circulating variants already bore the rtM204V substitution. Two months later, 83.4% of the variants bore the rtM204V substitution, but the HBV DNA load remained low. In contrast, at month 20, when the proportion of resistant variants was still high, HBV DNA load had increased by approximately 1 log, reflecting the significant increase in the absolute amount of circulating resistant variants. At month 22, the HBV DNA load had returned to baseline, and 100% of the variants bore the rtM204V substitution. The analytical sensitivity of the techniques used here allowed us to observe the last steps of the exponential expansion of preexisting resistant HBV variants in a context in which the replication of wild-type (sensitive) HBV variants was continually inhibited by lamivudine.

The dynamics of the RT quasispecies during lamivudine therapy (Fig. 2A to 2D) showed two distinct patterns. The first pattern, which was observed in patients A, B, and C, involved a simple, gradual switch from a 100% YMDD variant population at baseline and during the first months of lamivudine therapy to a 100% lamivudine-resistant variant population, including rtM204V (YVDD) in patients A and B and rtM204I (YIDD) in patient C (Fig. 1 and Table 1). In contrast, patient D had a much more complex, fluctuating pattern of resistance variant dynamics, in which the sensitive YMDD variant was gradually replaced by a resistant YVDD variant, which started to be detected 4 months before virological breakthrough (Fig. 2D). As the population of this resistant variant increased, another population of resistant variants bearing the YIDD motif also appeared (Fig 3). After month 14, the YVDD population started to decline and was gradually replaced by the YIDD

population, which had apparently gained in replication capacity. A steady state was reached after a few months, with the YIDD and YVDD variants representing approximately 90% and 10% of circulating variants, respectively (Fig. 2D).

Dynamics of HBV variants during lamivudine therapy: amino acid substitutions outside the YMDD motif. The principal substitutions outside the YMDD motif were rtV173L and rtL180M (located outside the C domain), which have been reported to partially restore the in vitro replication capacity of variants with substitutions in the YMDD motif (1, 6, 12, 36), and rtV207I, found within the C domain in patient D. These three substitutions were always associated with rtM204V or rtM204I, except for one minor variant detected at a single time point in patients B and D. The dynamics of the different HBV populations during lamivudine therapy are summarized in Table 1. (i) In patient A, the wild-type YMDD population was rapidly replaced by selected viruses bearing both the rtM204V and rtL180M substitutions. The latter appeared to confer a replication advantage in this patient's replicative environment. The rtV173L substitution did not appear to provide a significant replication advantage in this patient, as variants bearing all three substitutions, although detected early during virological breakthrough, remained present as a minor population until the end of follow-up. (ii) In contrast, in patient B, the HBV population bearing triple substitutions (rtM204V, rtL180M, and rtV173L) rapidly overtook HBV populations with single (rtM204V) or dual (rtM204V and rtL180M) substitutions, suggesting optimal replication capacities in patient B's specific replicative environment. (iii) In patient C, we saw a gradual switch from a situation where most variants bore only the rtM204I substitution to one where most variants bore both the rtM204I and rtL180M substitutions. This switch was not associated with a major gain in replication, as the HBV DNA load had already increased sharply when the single mutant was still predominant (Fig. 2C). (iv) The HBV quasispecies dynamics were far more complex in patient D (Fig. 2D, Fig. 3, and Table 1). Variants with both the rtM204V and rtL180M substitutions quickly overtook the YVDD population. No additional substitutions were selected in this mutated population, whose in vivo replication capacity gradually declined relative to the YIDD population. The gain in replication capacity of the YIDD population was apparently associated with the selection of a population bearing substitutions rtM204I and rtV173L and a population bearing substitutions rtM204I and rtV207I. These populations coexisted, representing the majority of circulating quasispecies variants, but variants bearing both mutations outside domain C were very rare.

In summary, the rtL180M substitution conferred a substantial replicative advantage to lamivudine-resistant variants in patient A but conferred only a moderate advantage in patient C. In patient D, the same substitution conferred a replicative advantage to YVDD variants, but this advantage was lost as YIDD populations gradually took over. The rtV173L substitution apparently had no effect in patient A, but it provided significant added replication capacity to YVDD variants in patient B and to YIDD variants in patient D, in whom the additional rtV207I substitution also contributed to takeover by the corresponding variants. Additional substitutions, such as rtS213T and rtY245S, were found outside known functional

domains of HBV RT. Whether or not they influenced in vivo replication is unclear.

DISCUSSION

Our findings show that extensive analysis of HBV quasispecies dynamics aids understanding of the fine mechanisms of resistance to antiviral drugs. Experience with highly active antiretroviral therapy in HIV-infected patients shows that resistance to HIV RT inhibitors is acquired gradually, through the selection of preexisting resistant variants and gradual accumulation of new amino acid substitutions that confer stepwise increases in the level of drug resistance (reviewed in reference 4). Schematically, partial resistance conferred by a substitution in a preexisting viral population impairs drug efficacy sufficiently to restore a level of replication compatible with the accumulation of further mutations. The latter may restore the in vivo replication capacity of the resistant virus, allowing viral replication to return to pretreatment levels. There are some exceptions to this rule, however, such as the M184V substitution in the HIV RT, which immediately confers full resistance to lamivudine together with a significant fitness hit (34), and single mutations in the hydrophobic pocket of the HIV RT, which also confer strong resistance to nonnucleoside RT inhibitors (15). HBV resistance to lamivudine appears to be more closely related to the latter mechanism, for the following reasons: (i) HBV lamivudine resistance is characterized by a rapid, one-step reincrease in viral replication to baseline levels (18), as confirmed here; and (ii) full resistance can be conferred by a single substitution (V or I) at RT position 204, in the YMDD motif; this results in a more than 1,000-fold increase in resistance in vitro (1), and it led to a return to baseline replication levels in our patients B and C.

Our results suggest that rtM204V and rtM204I are frequent preexisting polymorphisms in HBV-infected patients and that they appear randomly in viral populations which have a replication disadvantage relative to YMDD variants in the absence of lamivudine. Their selection during lamivudine therapy appears to depend principally on their presence at the outset of treatment, as only rtM204V variants were selected in our patients A and B, while only rtM204I variants were selected in patient C. In patient D, who harbored both variants prior to treatment, the two were selected together, and their dynamics fluctuated as additional mutations accumulated. A steady state was reached when YIDD variants overtook YVDD variants, even though both variants were still present at the end of follow-up. It is theoretically possible, however, that YMDD variants could arise after the initiation of therapy, given the relatively high levels of residual replication during lamivudine administration (Table 1).

This study provides new information on the influence of amino acid substitutions located outside the C domain of HBV RT in vivo. Two substitutions, rtL180M and rtV173L, are frequently associated with the C domain during the onset of lamivudine resistance. Both partially restore the replication capacities of C-domain HBV mutants in vitro (1, 6, 12, 36). In our study, the in vivo replicative advantages conferred by these substitutions varied substantially from one patient to another and also from one time point to another in a given patient. Thus, the in vivo replication of HBV variants appears to be driven by multiple forces, including the following: (i) intrinsic replicative advantages potentially conferred to lamivudine-re-

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sistant variants by mutations accumulating outside domain C, such as rtL180M and rtV173L, and (ii) the fluctuating environment in which these variants replicate during lamivudine administration to a given patient, potentially favoring one variant population over another, regardless of their intrinsic replication capacity. Direct host protein-viral protein interactions and the host immune response probably play a key role in determining relative HBV variant replication capacities in vivo. As a result, the influence of specific resistance mutations on the development and establishment of HBV resistance to antiviral drugs in vivo cannot be inferred solely from in vitro replication experiments and cannot be fully predicted by structural analysis in silico.

Our results also have major implications for virological follow-up and diagnosis of resistance in the clinical setting. Currently, the most sensitive method for detecting early selection of resistant variants is to generate large numbers of clones at regular intervals during therapy. By analyzing approximately 30 clones per time point and carrying out a highly sensitive quantitative HBV DNA assay based on real-time PCR technology, we started to detect resistant variants between 2 and 4 months before virological breakthrough. However, this approach is too cumbersome to be used in clinical practice. Reverse hybridization with the line probe assay, which can detect variant populations representing approximately 10% of the HBV quasispecies, is usable in practice and was recently reported to reveal the emergence of resistance variants an average of 2 weeks before the HBV DNA load started to increase (26, 30). The time of virological breakthrough is too late for diagnosis of HBV resistance, because most of the quasispecies variants are already both resistant and highly fit. This is a particularly important issue, because lamivudine resistance is associated with gradual progression of liver disease (7, 20, 21, 23, 25, 29, 40).

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