MAJOR ARTICLE

Genotypic Analysis of HIV-1 Drug Resistance at the Limit of Detection: Virus Production without Evolution in Treated Adults with Undetectable HIV Loads

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Human immunodeficiency virus (HIV) production continues in patients receiving highly active antiretroviral therapy (HAART) with undetectable (<50 copies/mL) virus loads. Our initial cross-sectional study showed that this viremia is composed of viruses that lack new resistance mutations to the HAART regimen. Here we describe a longitudinal, clonal genotypic analysis of plasma virus loads in treated adults who had undetectable virus loads. We document a continuous production of virus in 8 HIV-1–infected adults who maintained suppression of viremia for up to 15 months. Using analytical approaches for distinguishing selected resistance mutations from nonselected mutations and polymerase chain reaction errors, we detected no evolution of resistance in the reverse-transcriptase and protease genes. Sporadic resistance mutations were detected in some viral clones that were not selected for subsequently. Thus, in some patients, HAART suppresses replication to a level that does not allow the evolution of drug resistance over a time frame of years.

In HIV-1–infected patients who respond optimally to HAART, plasma virus loads fall below the limit of detection of "ultrasensitive" clinical assays (50 copies of HIV-1 RNA) and remain there [1–3]. However, even in such patients, a low level of free virus can be detected in the plasma by more sensitive assays [4–7]. Additionally, many patients receiving HAART have occasional positive plasma virus measurements, termed "blips," after which virus levels fall back to <50 copies/mL [5, 8, 9]. This and other evidence for continuing virus production in patients receiving HAART [10–17] raises concerns regarding the eventual evolution of drug resistance despite apparently effective treatment. Testing for resistance mutations directly through genotyping is an obvious approach for detecting the evolution of drug resistance. However, when virus loads are <50 copies/mL, the number of virions that can be analyzed is limited, and the physiological significance of resistance mutations found can be unclear. In an initial cross-sectional study [7], we showed that this low-level viremia is composed of drug-sensitive viruses that lack new resistance mutations to drugs in the HAART regimen. The results of an analysis of protease sequences in children receiving HAART supported this conclusion [18]. However, other studies have provided evidence for the evolution of drug resistance during HAART [15, 17–26]. The present study was designed to determine whether drug-resistance mutations could be detected in the plasma virus of patients whose HIV-1 RNA levels were <50 copies/mL and, if so, to examine the significance of these mutations.

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PATIENTS AND METHODS

Patients. We studied asymptomatic adults with HIV-1 infection who had shown excellent responses to HAART, with suppression of viremia to <50 copies/mL. Patients were not excluded for isolated blips if subsequent measurements returned to <50 copies/mL without adjustments to therapy regimens. Informed consent was obtained from all study participants.

HIV-1 plasma RNA quantification. Plasma HIV-1 RNA assays were performed using the ultrasensitive Amplicor Monitor System (version 1.5; Roche Diagnostic Systems), which has a detection limit of 50 copies/mL.

Amplification and sequencing of the* pol *gene from HIV-positive plasma samples. The genotypic analysis of low-level viremia was done by nested reverse-transcriptase polymerase chain reaction (RT-PCR) amplification of the *pol* gene from pelleted plasma virions, as described elsewhere [7]. Plasma was filtered (in a 0.8-μm cellulose nitrate membrane; Nalgene) to remove any contaminating cells before the virions were pelleted. A 1.5-kb segment of the *pol* gene, including protease and a portion of RT, was amplified by RT-PCR. Control reactions that did not include an RT enzyme were performed to exclude amplification from HIV-1 DNA templates; these results were invariably negative. A more sensitive RT-PCR procedure was used in some experiments. In that case, a 639-bp segment of protease and a 720-bp segment of RT were amplified separately in nested RT-PCRs [18]. Complete details of the PCR primers and cycling conditions are available on request from the authors. For both procedures, PCR products were separated on agarose gels, purified, cloned, and sequenced [7].

Sequence analysis and quality control. Sequences were analyzed for known drug-resistance mutations using the Los Alamos [27] and Stanford [28] HIV sequence databases. Initial phylogenetic analyses were performed using algorithms from PHYLIP 3.5 [29] and implemented in NimbleTree version 2.0 (available from S.C.R. at http://sray.med.som.jhmi.edu/). Specifically, distance matrices were calculated using the F84 model (transition:transversion ratio, 2.0), trees were inferred using the neighbor-joining algorithm [30], and support for clustering was assessed using bootstrap analysis [31]. The GenBank accession numbers for the sequences that we used are AY455314–AY455655.

Analysis of sporadic nonsynonymous substitutions. Sporadic substitutions (i.e., those observed in a single clone from a study subject) were classified as nonsynonymous or synonymous. The number of nonsynonymous sites in the region analyzed (protease residues 1–99 and RT residues 1–229) was estimated from the number present in the HXB2 sequence (GenBank accession no. K03455), using the method of Nei and Gojobori [32]. Nonsynonymous substitutions or sites were classified as resistance associated, according to the method of Parikh et al. [27].

RESULTS

Analytical issues associated with low-level genotyping. As viremia falls to <50 copies/mL, the population-level analysis used to determine sequences of high levels of viremia is no longer possible. Instead, sequence information must be obtained through the analysis of a small number of viral RNA molecules. This presents obvious problems with sampling. In addition, extensive amplification by PCR is required, which raises the possibility of PCR-generated errors and cross-contamination in the viral sequences obtained.

To address these problems, we performed longitudinal genotypic analysis at the clonal level. Pelleted virions from plasma were lysed, diluted, and amplified by RT-PCR in replicate reactions, such that only a fraction of the reactions showed positive results. Products were then cloned, and multiple clones from individual reactions were sequenced. The clones obtained were considered to represent distinct viral RNA templates in the original sample if they were derived from independent amplifications or if they were derived from a single reaction but differed from one another to a degree that would rule out template resampling [33]. This approach allowed comparisons of independent viral clones from the same individual and provided an indication of the degree of sequence diversity present in the population of circulating virions. Longitudinal analysis allowed us to sample larger numbers of viral sequences and to observe major changes in resistance patterns. Each new set of sequences from a given patient was compared with other sequences from the same patient and with sequences from other patients. Using phylogenetic analysis, we observed patient-specific coclustering of all sequences obtained from a single patient, which verified the expected origin of the sequences (figure 3).

By comparing all clones derived from the same individual, we could classify mutations as shared (those seen in >1 independent clone) or sporadic (those seen in 1 clone). Potential explanations for each class of mutations and an estimation of their probabilities are given in table 1. Shared mutations cannot be caused by PCR error and are unlikely to represent resampling of virions produced by the same cell. Rather, they are likely to represent polymorphisms or selected viral variants that are present in multiple infected cells in vivo and that are subject to further selection. On the other hand, sporadic mutations could represent the sampling of rare virus variants, the detection of new RT errors that have not been propagated, or PCR-induced mutations. Under the conditions that we used, PCR-induced errors were a more likely cause of sporadic mutations than new,

Table 1. Patterns of mutations observed in clonal analysis of low-level viremia.

Definition of mutation, potential explanation	Probability of observing a specific mutation x times in n clones[a]	Comment	Sample calculation of P[b]	Change over time
Shared: observed in x sequences of a set of n sequences from 1 patient ($1 < x \leqslant n$)				
Resampling of virions derived from 1 infected cell with unique mutations	$c(n,x)(1/T^{*})^{x}[1-(1/T^{*})]^{n-x}$	Probability of resampling virions from the same cell x times in n samples depends on T^{*}, the no. of productively infected cells, and is extremely low because, even in patients with <50 copies/mL, T^{*} is large[c]	.000015	↔
Polymorphism unrelated to drug resistance	$c(n,x)f^{x}(1-f)^{n-x}$	Depends on the frequency *(f)* of mutation in the population of actively replicating virus	.19	↔, ↑, ↓[d]
Mutation selected by antiretroviral drugs	$c(n,x)f^{x}(1-f)^{n-x}$	Depends on the frequency *(f)* of mutation in the population of actively replicating virus	.19	↑
Sporadic: observed in 1 sequence of a set of n sequences from 1 patient ($x = 1$)				
Polymorphism unrelated to drug resistance	$nf(1-f)^{n-1}$	Depends on the frequency *(f)* of mutation in the population of actively replicating virus	.39	↔, ↑, ↓[d]
Mutation selected by antiretroviral drugs	$nf(1-f)^{n-1}$	Depends on the frequency *(f)* of mutation in the population of actively replicating virus	.39	↑
New single-cycle RT error	$\approx n(E_{RT}/3)$	Extremely unlikely: depends on the in vivo RT error rate (E_{RT})[e]	.00011	↔
PCR-induced mutation in a cloned PCR product	$\approx n(E_{Taq}/3)c$	Depends on the polymerase error rate (E_{Taq}) and no. of cycles *(c)*[f].	.0036	↔
PCR error in a directly sequenced PCR product from reaction that contained only 1 template	$\approx 2n(E_{Taq}/3)$	Special case of the previous formula in which $c = 2$, because only errors made in the 1st or 2nd cycle would have sufficient representation in the product to be detected	.00011	↔

NOTE. ↑, increased; ↓, decreased; ↔, remained the same; PCR, polymerase chain reaction; RT, reverse transcriptase.

[a] *c(n, x)* represents the binomial coefficient *(n* choose *x)*.

[b] As an example, consider that the frequency *(f)* of a polymorphism or resistance mutation in the population of virus-producing cells is 0.1 and that this mutation is observed as a shared mutation in 2 of 10 clones or as a sporadic mutation in 1 of 10 clones.

[c] The no. of virus-producing cells *(T*)* can be estimated using the standard model of viral dynamics, which states that $T^{*} = cV/N\delta$, where c is the rate constant for free virions (23 virions/day), V is the level of viremia, N is the burst size, and δ is the decay rate constant for productively infected cells (1 cell/day) [35]. Under the assumption of an extracellular fluid volume of 15 L for converting V and T^{*} from concentrations to absolute numbers of virions and productively infected cells and a burst size of 10^4 virions/cell, $T^{*} = 1725$.

[d] The frequency of a particular allele could increase, decrease, or remain the same, depending on other types of selective pressure, including immune responses.

[e] The measured in vivo RT error rate is 3.4×10^{-5} substitutions/nucleotide/cycle [36]. For one cycle, the probability of observing a new specific RT mutation once in n clones analyzed is one-third of the probability of observing any substitution at that position, or $1.13 \times 10^{-5}n$.

[f] The high-fidelity polymerase here has an error rate of 1.7×10^{-5} substitutions/nucleotide/cycle. For 64 cycles, the probability of observing a specific *Taq* error once in n clones is $3.6 \times 10^{-4}n$.

single-cycle RT errors, despite the use of a proofreading polymerase combination (table 1).

Longitudinal genotyping in patients receiving HAART. We studied 10 patients receiving HAART who had suppression of viremia to <50 copies/mL, except for occasional blips (tables 2 and 3). Eight of 10 patients were monitored for 10–15 months. Of the 10 patients, 6 had been treated with HAART for long periods before study entry; the average duration of HAART was 34 months (range, 16–57 months). The other 4 patients (patients 57, 79, 81, and 83) had begun receiving HAART 5–11 months before the study. All patients maintained the suppression of viremia throughout the study period, except for occasional blips.

Molecular characterization of low-level viremia in patients with no prior nonsuppressive therapy. The analysis of drug resistance is simplest in the case of patients who have had no prior nonsuppressive therapy with 1- or 2-drug regimens before starting HAART. Five patients in this category were studied (table 2). Profiles for 2 representative patients are shown in figure 1. After beginning their first HAART regimen and reaching undetectable virus loads, they maintained plasma HIV-1 RNA levels below the limit of detection, except for occasional blips or short periods of low-level viremia, which resulted in a change in regimen that again suppressed viremia.

A total of 133 independent RT or protease clones were obtained when the virus load was <50 copies/mL. Another 27 clones were obtained during blips to 63–143 copies/mL. The overwhelming majority of these independent viral clones lacked recognized drug-resistance mutations. As shown in table 2, 75 of 78 RT sequences and 81 of 82 protease sequences were completely wild type (wt). Most importantly, no shared drug-resistance mutations were detected. These results confirm and extend the results of a previous cross-sectional study, which suggested that low-level viremia in patients receiving HAART does not require the development of new drug-resistance mutations [7]. The absence of drug-resistance mutations was particularly surprising in the case of patient 82, who, before study entry, had periods of persistently detectable viremia that required adjustments in his regimen, and patients 81 and 83, in whom viremia declined very slowly after the initiation of HAART. Even sequences obtained during blips were predominantly wt. In 26 of 27 clones obtained during 3 blips to 63–143 copies/mL, only wt virus was found (table 2). Thus, despite previous reports that low-level viremia [19, 20, 25, 26] and blips [23] represent the replication of drug-resistant virus, our results show that low-level viremia with wt, drug-sensitive virus is a common pattern in patients receiving HAART.

As shown in table 2, sporadic resistance mutations were detected in 4 of 160 clones studied. In patient 83 (figure 1 and table 2), 1 of 5 clones contained a lamivudine resistance mutation (M184V) after 16 months of lamivudine-containing regimens. During this period, the virus load declined slowly toward the limit of detection, and the development of lamivudine resistance in this setting would not be surprising. Nevertheless, genotypes analyzed at 11 and 15 months after the detection of this mutation remained wt, which indicates that the M184V mutant did not become dominant in the plasma, despite continued therapy with lamivudine. Another clone contained the V82A mutation, which confers some degree of resistance to multiple protease inhibitors. However, 4 months later, all 6 clones analyzed contained wt protease sequences. Similarly, patient 80 had a K103N mutation that conferred high-level resistance to efavirenz in 1 of 7 clones and a D67N mutation that conferred resistance to zidovudine in 1 of 7 clones, but neither mutation was observed on subsequent analysis 7 months later, despite continued treatment with these drugs. In patient 85, a secondary nonnucleoside RT inhibitor resistance mutation (Y188H) was detected initially, but it was not found at later time points over the next 12 months. In summary, the only resistance mutations detected were sporadic mutations seen in 1 clone from a given patient. The sporadic nature of these mutations and their failure to appear on subsequent analysis raises questions about their pathophysiological significance (see below).

Molecular characterization of low-level viremia in patients with prior nonsuppressive therapy. The remaining 5 patients had been previously treated with nonsuppressive therapy and had preexisting resistance mutations (table 3). Representative profiles of patients are shown in figure 2. A consistent pattern emerged. Only resistance mutations that were attributable to prior nonsuppressive therapy could be detected. No new shared mutations developed, and none of the existing mutations significantly increased in frequency during the study period. In 3 of 5 patients, a new class of drugs was initiated in the current, suppressive regimen, but no mutations to these new classes were observed. In 1 patient (patient 57), a single sporadic mutation (Y188H) conferring resistance to efavirenz (1 of 11 clones analyzed) was detected at an early time point but was not seen 2, 4, or 10 months later (table 2).

Of interest, in all of these patients, we detected mutations that conferred some level of cross-resistance against at least 1 of the drugs in the current regimen. Viruses carrying such mutations might be expected to have some replicative advantage and would be expected to become dominant. However, we found that sequences of varying predicted drug susceptibility were maintained in the plasma. For example, resistance to lamivudine (M184V) was found in patient 79 (figure 2*A*), and, although this drug remains in that patient's regimen, cocirculating virions did not contain the mutation. These results are consistent with the release of archival virus from stable reservoirs [34]. A similar explanation can be offered for the wt sequences detected in the plasma of some patients who have co-

Table 2. Genotypic analysis in patients with no prior nonsuppressive therapy.

Patient	Age, years	Total months receiving HAART	HAART regimen at time of sampling[a] (months received)	Months since initiation of HAART	Plasma HIV RNA level, copies/mL	No. of clones analyzed		Shared resistance mutations[b]		Sporadic resistance mutations[c]	
						Pro	RT	Pro	RT	Pro	RT
80	48	51	AZT + 3TC + TDF + EFV (18)	41	<50	1	1	…	…	…	…
				44	<50	2	2	…	…	…	D67N[d], K103N
				51	<50	3	4	…	…	…	…
81	32	22	AZT + 3TC + ABC + TDF + EFV (15)	9	<50	3	3	…	…	…	…
				10	<50	1	1	…	…	…	…
				11	<50	3	3	…	…	…	…
				13	<50	2	2	…	…	…	…
				16	143	5	3	…	…	…	…
				17	<50	4	3	…	…	…	…
				22	<50	5	5	…	…	…	…
82	38	70	AZT + 3TC + TDF + LPV/r (13)	57	<50	1	1	…	…	…	…
				59	<50	3	3	…	…	…	…
				61	<50	6	6	…	…	…	…
				66	<50	2	2	…	…	…	…
				70	<50	6	4	…	…	…	…
83	40	26	AZT + 3TC + NVP + IDV/r (12)	11	63	5	5	…	…	…	M184V
			AZT + 3TC + NVP + TDF + IDV/r (5)	22	<50	6	6	…	…	V82A	…
			AZT + 3TC + NVP + TDF + LPV/r (4)	26	<50	6	6	…	…	…	…
85	54	28	AZT + 3TC + ABC + TDF + EFV (12)	16	<50	7	7	…	…	…	Y188H
				19	<50	1	1	…	…	…	…
				21	68	4	5	…	…	…	…
				28	<50	6	5	…	…	…	…

NOTE. Patients noted in this table were male and white. None had received drugs as part of 1- or 2-drug regimens before the advent of highly active antiretroviral therapy (HAART). 3TC, lamivudine; ABC, abacavir; AZT, azidothymidine; EFV, efavirenz; IDV, indinavir; LPV, lopinavir; NVP, neviparine; Pro, protease; /r, regimen including ritonavir; RT, reverse transcriptase; TDF, tenofovir.

[a] In each case, patients were receiving other suppressive HAART regimens before switching to the regimen noted.
[b] Resistance mutations seen in >1 clone from a given patient.
[c] Resistance mutations seen only in 1 clone from a given patient.
[d] Present in the same clone.

Table 3. Genotypic analysis in patients with prior nonsuppressive therapy.

Patient	Age, years/race	Prior nonsuppressive therapy[a] (months received)	HAART regimen at time of sampling (months received)	Months since initiation of HAART	Plasma HIV RNA level, copies/mL	No. of clones analyzed		Resistance mutations in RT/protease selected by prior nonsuppressive therapy[b]		New resistance mutations[c]	
						Pro	RT	Shared[d]	Sporadic[e]	Shared[d]	Sporadic[e]
57[f]	46/A	AZT (15); ddI (10); d4T + 3TC + IDV/r (50); d4T + 3TC + EFV (10)	3TC + ABC + EFV (10)	10	139	1	1	…	…	…	…
				13	<50	3	4	…	…	…	Y188H
			3TC + ABC + EFV + TDF + LPV/r (13)	15	<50	2	1	…	…	…	…
				17	<50	5	3	…	…	…	…
				23	<50	3	2	…	…	…	…
79[g]	56/W	AZT (20); AZT + ddI (24); AZT + d4T (60); AZT + 3TC (5); 3TC + ddC + IDV/r (52)	EFV + IDV + LPV/r (5); EFV + APV + LPV/r (7); EFV + APV + LPV/r + 3TC + TDF (6)	16	<50	6	6	M41L, D67N, T69D, M184V, L210W, T215Y	K219Q	…	…
				18	<50	3	3	M41L, D67N, T69D, M184V, L210W, T215Y	…	…	…
				20	128	1	1	M41L, D67N, L210W, T215Y	…	…	…
			EFV + APV + LPV/r + 3TC (10)	22	<50	6	6	M41L, D67N, T69D, M184V, L210W, T215Y	…	…	…
				24	<50	6	6	M41L, D67N, T69D, M184V, L210W, T215Y	…	…	…
				28	<50	6	6	M41L, D67N, T69D, M184V, L210W, T215Y	…	…	…
84	43/W	AZT + ddC (10); AZT + ddC + IDV (4); AZT + ddC + RTV (7); d4T + 3TC + NFV (27)	ddI + EFV + IDV/r (22)	18.5	68	3	3	M41L, M184V, L210W, T215Y/L90M	K219Q	…	…
				19.5	<50	6	6	M41L, E44D/V118I, M184V, L210W, T215Y/L90M	…	…	…
				22	68	4	4	M41L, T69N, M184V, L210W, T215Y/M46I, L90M	…	…	…
			ddI + EFV + LPV/r (7)	23.5	<50	5	5	M41L, E44D/V118I, M184V, L210W, T215Y/L90M	…	…	…
				24.5	<50	3	3	M41L, T69N, M184V, L210W, T215Y/M46I, L90M	V82A	…	…
				27.5	<50	5	6	M41L, M184V, L210W, T215Y	…	…	…
				29	88	5	3	L90M	…	…	…
			ddI + EFV + LPV/r + IDV/r (5)	30	159	6	6	M41L, M184V, L210W, T215Y	…	…	…
				34	<50	4	6	M41L, M184V, L210W, T215Y/ L90M	…	…	…
87	62/A	AZT (12); ddI (24.5)	d4T + 3TC + EFV (44)	44	<50	5	5	K70R	…	…	…
90	44/W	AZT (5); 3TC (9); EFV + NFV (12)	d4T + ddI + ABC + EFV (37)	37	<50	4	4	D67N, K70R, K219Q	…	…	…

NOTE. All patients were male. 3TC, lamivudine; A, African American; ABC, abacavir; APV, amprenavir; AZT, azidothymidine; d4T, stavudine; ddC, zalcitabine; ddI, didanosine; EFV, efavirenz; HAART, highly active antiretroviral therapy; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; Pro, protease; /r, regimen including ritonavir; RT, reverse transcriptase; RTV, ritonavir; TDF, tenofovir; W, white.

[a] Drugs received as part of prior regimens that did not suppress viremia or maintain viremia below the limit of detection.

[b] Mutations that could be attributed to selection by drugs in prior regimens that did not suppress viremia.

[c] Mutations that could not be attributed to selection by drugs in prior regimens that did not suppress viremia.

[d] Resistance mutations seen in >1 clone from a given patient.

[e] Resistance mutations seen only in 1 clone from a given patient

[f] Started receiving d4T + 3TC + IDV and sustained long-term suppression with occasional "blips." Treatment was interrupted, and viremia rebounded to 2×10^6 copies/mL. Initiation of treatment with ABC + EFV + 3TC 2 months later was able to suppress viremia to undetectable levels once again.

[g] Started receiving 3TC + ddC + IDV/r and continued this for 52 months. However, suppression of viremia was not maintained; there was early failure with detectable viremia in the range of 100–1000 copies/mL for the last 30 months. After a brief treatment interruption, suppression was achieved on a series of EFV-based HAART regimens.

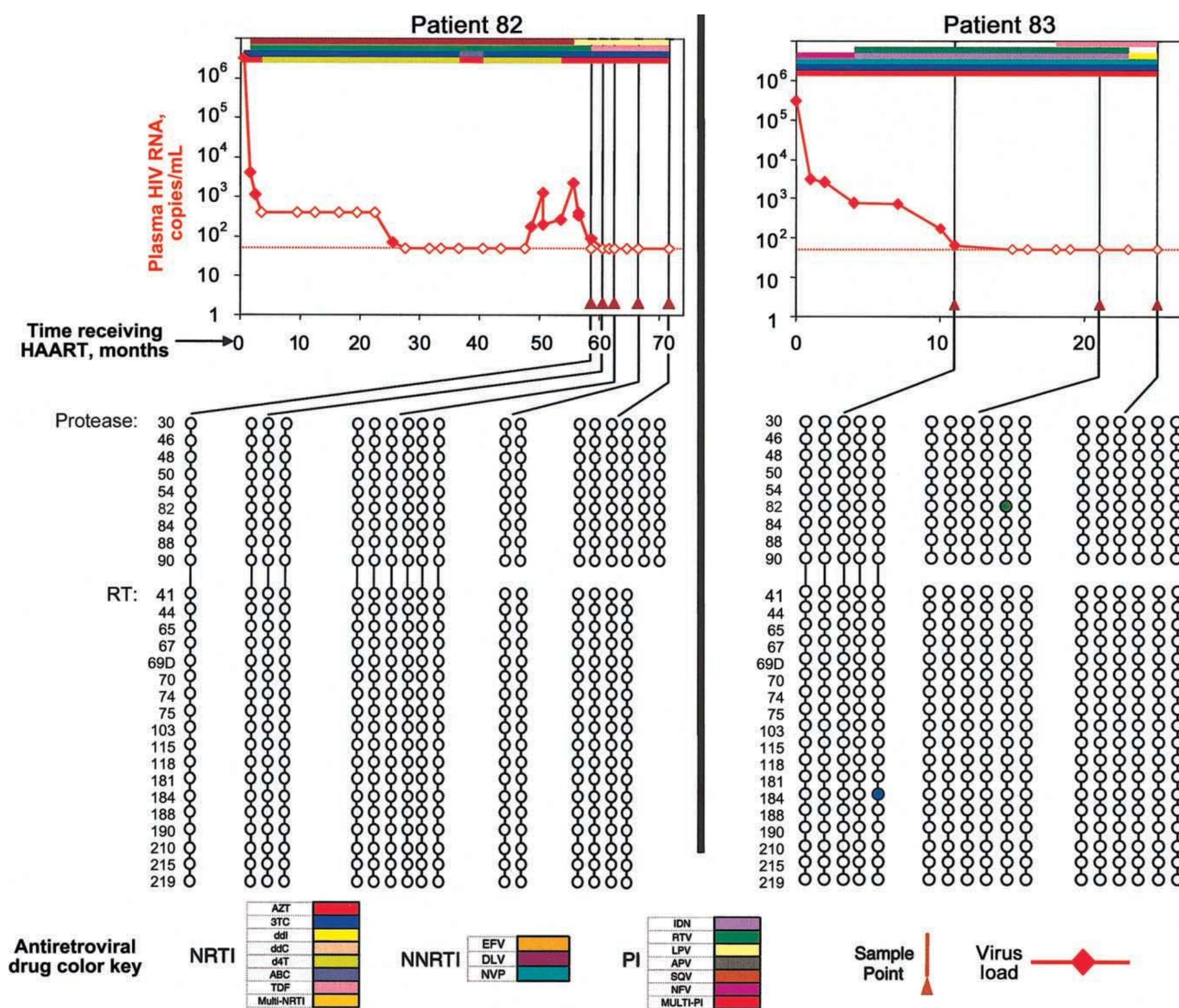

Figure 1. Genotypic analysis of HIV *pol* sequences of viruses isolated from the plasma of representative patients (82 and 83) who had suppression of viremia to below the limit of detection. Treatment history is shown by color-coded bars found along the tops of the graphs, which show plasma HIV-1 RNA levels over time. *White symbols,* plasma HIV-1 RNA level <50 or <400 copies/mL; *dotted horizontal red line,* the limit of detection of the assay used (50 copies/mL); *vertical lines,* sampling times. Genotypes are given for each time point, with resistance mutations colored according to the drug to which they confer resistance. Each sequence was derived from an independent polymerase chain reaction. Lines connecting the protease and reverse transcriptase (RT) sequences indicate linkage. Linked sequences were obtained from amplicons containing both protease and a portion of RT. Unlinked sequences were obtained by separate amplification of protease and RT. 3TC, lamivudine; ABC, abacavir; APV, amprenavir; AZT, azidothymidine; d4T, stavudine; ddC, zalcitabine; ddI, didanosine; DLV, delavirdine; EFV, efavirenz; IDN, indinavir; LPV, lopinavir; NFV, nelfinavir; NNRTI, nonnucleoside RT inhibitor; NRTI, nucleoside RT inhibitor; NVP, neviparine; PI, protease inhibitor; Pro, protease; RTV, ritonavir; SQV, saquinavir; TDF, tenofovir.

circulating resistant virus and who continue to receive HAART. An example of this situation is shown in figure 2*B*. Both wt and highly resistant RT and protease sequences were detected in the plasma at some time points. Thus, in some patients with suppression of viremia to <50 copies/mL, there does not appear to be strong selection for viruses with partial resistance to the current regimen. This suggests that a component of the plasma virus is made up of virions that are released from latently infected cells that have become activated or from some other site where drug selection is not fully operative.

Phylogenetic analysis of plasma viral sequences from patients with well-suppressed viremia receiving HAART. The phylogenetic relationship of these plasma sequences also supports the idea that the activation of latently infected cells contributes to ongoing viremia. If the virus found in plasma is generated by the activation of cells in the latent reservoir, then

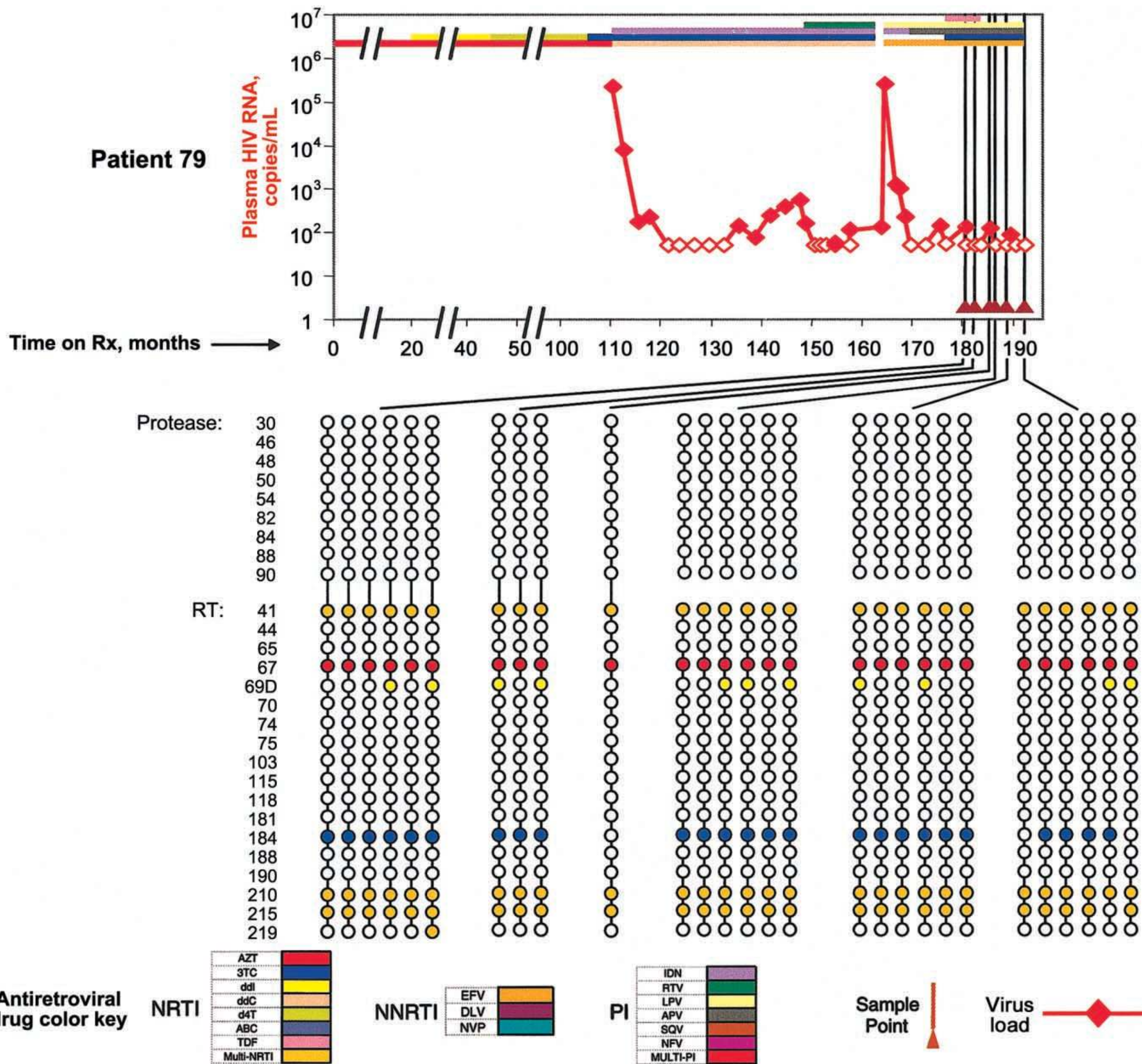

Figure 2. Genotypic analysis of HIV *pol* sequences of viruses isolated from the plasma of representative patients 79 *(A)* and 84 *(B),* both of whom had prior nonsuppressive therapy. Treatment history is shown by color-coded bars found along the top of the graph, which shows plasma HIV-1 RNA levels over time. *White symbols,* plasma HIV-1 RNA levels <50 copies/mL; *dotted horizontal red line,* the limit of detection of the assay (50 copies/mL); *vertical lines,* sampling times. Genotypes are given for each time point, with resistance mutations colored according to the drug to which they confer resistance. Each sequence was derived from an independent polymerase chain reaction. Lines connecting the protease and reverse-transcriptase sequences indicate linkage.

the characteristics of the low-level plasma virus should resemble virus in the reservoir. The latent reservoir is extremely stable, represents an archive of previously replicating viruses, and lacks temporal structure [34]. In the patients we describe, sequence analysis of plasma virus also demonstrated a remarkable lack of temporal structure, which suggests that these sequences were not evolving during the study period. Figure 3 shows all of the sequences obtained from patient 84 over a 15-month period. The most divergent sequence was obtained at an early time point, whereas the most ancestral sequence was obtained from a late time point. In contrast to the continuous pattern of evolution seen in patients with high levels of viremia, with divergence from a common ancestor over time [35], our data show an intermingling of viruses acquired from early and later time points. This result suggests a profound inhibition of virus evolution in patients who receive HAART.

Statistical analysis of sporadic mutations in patients with low-level viremia. As was discussed above, rare, sporadic

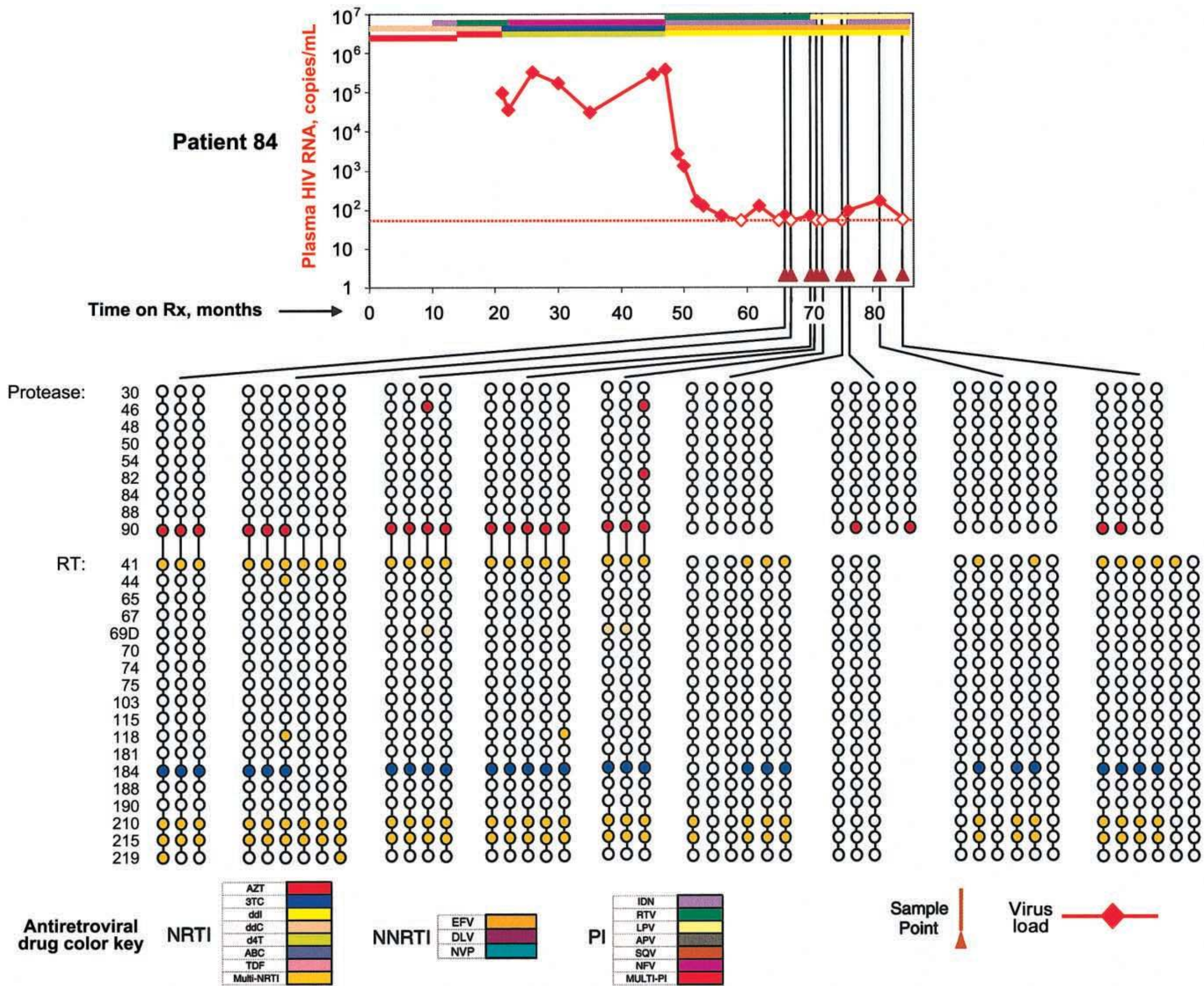

Figure 2. *(Continued.)*

drug-resistant mutations were seen in the viral sequences of patients with virus loads below the limit of detection. These mutations could potentially indicate the initial development of drug-resistant viruses that are present at low frequency in a replicating pool of viruses and are sampled only once (table 1). This possibility can be excluded by repeated sampling. If the variant is selected for by the regimen, it should increase in frequency with each replication cycle. Alternatively, these mutations could represent actual errors introduced by HIV-1 RT during a single cycle when a cell is initially infected without further selection. As is shown in table 1, the probability of detecting such a mutation is low.

The most likely explanation for the sporadic drug-resistance mutations observed is PCR error. To demonstrate this, we measured the frequency of sporadic mutations associated with drug resistance and those that were not associated with drug resistance (table 4). For the 328 codons (984 nt) analyzed per clone, the total number of nonsynonymous sites (where a substitution changes the predicted amino acid encoded at that site) was 775 (see Patients and Methods for details of this analysis). Of these sites, 35 were associated with primary drug resistance. Therefore, if such substitutions are randomly distributed, then 4.52% of nonsynonymous substitutions in this region would be associated with drug resistance. We then calculated the total number of sporadic nonsynonymous substitutions observed in all the patient sequences. In 165 clones representing 49,005 bp sequenced in the protease gene and 141 clones representing 89,394 bp sequenced in the RT gene, there were a total of 198 sporadic nonsynonymous substitutions. Of those sporadic mutations, 4.5% were associated with drug resistance. Thus, the sporadic drug-resistant mutations occurred at a frequency that was not higher than what would be consistent with polymerase errors that are not selected for, such as new single-cycle RT errors, or, more likely, mutations generated by PCR in vitro. This conclu-

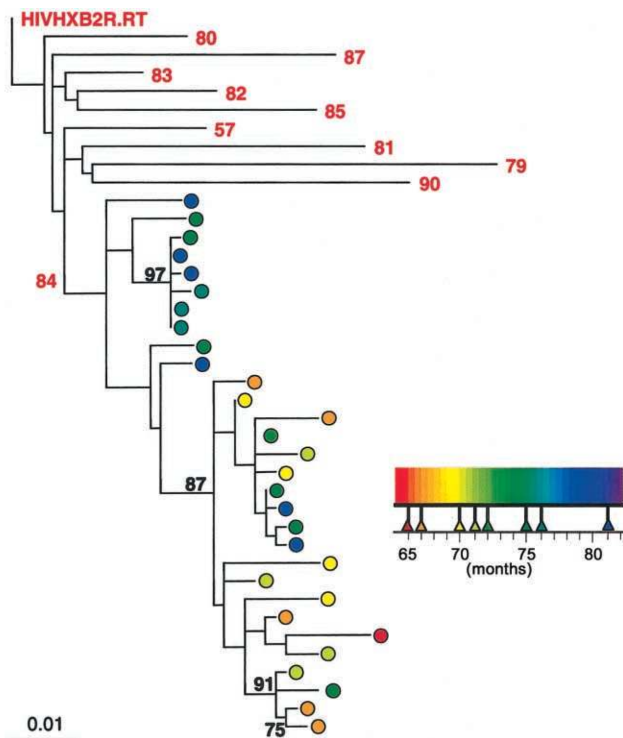

Figure 3. Maximum-likelihood phylogenetic analysis of patient HIV reverse transcriptase sequences isolated from the plasma of patients with virus loads below the limit of detection. Sequences are labeled in red with patient identification nos. Patient-specific clustering was seen. The colored tree represents all the sequences obtained from 1 patient (84). The sampling time is indicated by coloring corresponding to the timescale key. The lack of temporal structure is demonstrated by the fact that genetic distances (horizontal scale) do not correlate with sampling time. The reference sequence HXB2 was used as an outgroup. Bootstrap values are given in black.

sion is supported by the failure of these resistant variants to persist or to increase in frequency on subsequent analysis.

Mathematical analysis of emerging resistance in patients with low-level viremia. In a further effort to explain the continued release of drug-sensitive viruses in these patients, we estimated the number of times that specific drug-resistance mutations would have arisen in each patient during the time the patient was receiving HAART, under the assumption that the low-level viremia was due to continuous cycles of viral replication (table 5). This calculation was done for selected drugs for which a single point mutation can lead to high-level resistance. We first estimated the number of replication cycles that would occur each day in a patient with a level of viremia of 50 copies/mL, using a standard model of viral dynamics [36] and assuming that all of the plasma virus is generated by newly infected cells with a short half-life. Using published values for the mutation rate of HIV-1 RT (3.4×10^{-5} mutations/bp/replication cycle) [37], we then estimated that each mutation would arise between 1.96 times/day (burst size, 100) and once every 51 days (burst size, 10,000). These rates, multiplied by the length of time that a patient was receiving a given drug, gave an approximation of how many times a resistance mutation would have arisen during treatment. We could then compare this value with the number of times the mutation was actually observed in the patient (table 5). Of significance, mutations for most of the drugs these patients were receiving emerged at least 10 (burst size, 10,000) to 1000 (burst size, 100) times during the course of therapy (range, 11–4234 times). Nevertheless, we did not observe these mutations in the sequences of plasma virions at the final time point in any of these patients.

DISCUSSION

A central problem in the treatment of HIV-1 infection is that drug-resistant variants will emerge if the drugs used do not adequately suppress viral replication. Therefore, it is important to know whether patients with plasma HIV-1 RNA levels below the limit of detection have suppressed replication sufficiently to preclude the emergence of drug resistance. As genotypic analysis is extended to lower and lower levels of viremia in an attempt to address this question, many potential concerns arise with regard to the interpretation of the results. Current genotypic assays work well only when viremia is >1000 copies/mL. We developed a sensitive method of sequencing at the clonal-level virus load found in the plasma of patients receiving HAART who have virus loads <50 copies/mL. Our study evaluated the analytical issues inherent in low-level genotyping and provided evidence for adequate suppression to preclude the evolution of resistance, at least in some patients.

With respect to analytical issues, the central problem was that only a small number of virions are present in each sample. We obtained multiple independent clones from each patient at many time points, generating a more comprehensive sampling of virions in the plasma. Another analytical problem was that the detection of a sporadic mutation could lead to the false assumption of emerging resistance. Clinical genotypes are not performed at the clonal level but can detect variant sequences present at frequencies >10%–20%. At low levels of viremia, sequences may be obtained from the amplification of ⩽5 viral RNA molecules. If one of these is copied incorrectly during the early cycles of PCR or contains a new RT error that has not yet been selected for in vivo, the resulting mutation may be detected and incorrectly reported as evidence of resistance. We calculated that, in the region of *pol* sequenced, 4.5% of random nonsynonymous mutations would be at sites associated with drug resistance. In the sequences we obtained, the observed frequency of sporadic mutations associated with drug resistance was the same (4.5%). Thus, the sporadic drug-resistance mutations observed may not have pathophysiological significance. This conclusion is supported by the results of lon-

Table 4. Statistical analysis of sporadic mutations in patients with low-level viremia.

Gene, type of mutation	Observed mutations in sequences from individual patients											NS sites within gene,[a] no. (%)
	80	81	82	83	85	57	79	84	87	90	Total (%)	
Protease												
NS with resistance[b]	0	0	0	1	0	0	0	1	0	0	2 (2.8)	12 (5.3)
NS without resistance	7	12	11	4	4	9	15	8	0	7	70 (97.2)	216 (94.7)
Total NS	7	12	11	5	4	9	15	9	0	7	72 (100)	228 (100)
RT (codons 1–229)												
NS with resistance	2	0	0	1	1	1	0	2	0	2	7 (5.6)	23 (4.2)
NS without resistance	10	13	14	9	14	15	13	30	0	10	119 (94.4)	524 (95.8)
Total NS	12	13	14	10	15	16	13	32	0	12	126 (100)	547 (100)
Both genes combined												
NS with resistance	2	0	0	2	1	1	0	3	0	2	9 (4.5)	35[c] (4.5)
NS without resistance	17	25	25	13	18	24	28	38	0	17	189 (95.5)	740 (95.5)
Total NS	19	25	25	15	19	25	28	411	0	19	198 (100)	775 (100)

NOTE. NS, nonsynonymous substitution; RT, reverse transcriptase.

[a] The no. of nonsynonymous sites in the region analyzed was estimated from the number present in the HXB2 sequence (GenBank accession no. K03455), using the method of Nei and Gojobori [32].

[b] Nonsynonymous substitutions or sites were classified as "with resistance" if the resulting amino-acid substitution was classified as conferring resistance in vivo to antiretroviral therapy, according to the method of Parikh et al. [26].

[c] The no. of mutations associated with resistance to each drug class was calculated using the total number of nucleotide changes that result in a primary drug resistance mutation for all 3 drug classes: protease inhibitor (12), nucleoside RT inhibitor (15), and nonnucleoside RT inhibitor (8) (total = 35).

gitudinal genotypic analysis, which showed that sporadic mutations detected at <50 copies/mL did not become fixed. Therefore, the overinterpretation of resistance mutations detected in genotypic analysis of low-level viremia is a potential problem.

The longitudinal nature of our study allowed insights into the dynamics of low-level viremia. No new shared mutations associated with drug resistance were detected at any time point. Sporadic resistance mutations were seen in a subset of patients, but, as was discussed above, they may not have been selected for in vivo and did not persist. Particularly striking was the fact that 6 patients who were receiving lamivudine did not develop resistance to this drug during the 12–13 months they were monitored. This drug has a low mutational barrier to resistance, given that a single point mutation (M184V) can generate high levels of resistance [38–40]. In summary, despite ongoing low-level viremia, we did not detect any accumulation of new mutations clearly that was attributable to the current suppressive HAART regimen in any of these well-suppressed patients. These results indicate a strong inhibition of replication to a level that prevents the selection of resistance mutations over a time frame of months to years. Furthermore, no existing mutations were shown to increase in the plasma virus population over the 10–15-month study period. On the contrary, in patients who had developed resistance while receiving nonsuppressive therapy, both drug-sensitive and drug-resistant viral sequences remained in the plasma. The presence of viruses with differences in relative replicative capacity in the presence of the current drugs indicates a lack of selection for the most-fit variants and therefore implies that low-level viremia is not composed of only viruses produced by newly infected cells.

Other studies of the evolution of drug resistance in patients receiving HAART have documented the development of resistance mutations at low levels of viremia [19–21, 23–26]. It is clear from these studies that resistance can develop in a setting of ongoing replication with virus loads between 50 and 1000 RNA copies/mL. Unfortunately, none of these studies described the changes in drug-resistance mutations in patients who have virus suppression to <50 RNA copies/mL. In fact, one study suggested that HIV-1 replication differs dramatically between patients who maintain virus loads <400 copies/mL and those who maintain virus loads <50 copies/mL [19]. Taken together, the current evidence suggests that the limit of detection of current ultrasensitive clinical assays for viremia, 50 copies of HIV-1 RNA/mL, may be fortuitously close to an important biological threshold below which significant evolution of the virus does not occur, at least in some patients receiving HAART.

Our results are also consistent with those of a study that demonstrated that blips were not indicative of imminent treatment failure [9]. In contrast, the results of a recent study by Cohen-Stuart et al. [23] suggested that blips involve resistant virus in the majority of cases. However, that study did not perform baseline genotype analysis to exclude the presence of these mutations before the suppression of viremia during HAART. Of the 11 patients analyzed in the study by Cohen-Stuart et al. [23], 8 showed evidence of drug resistance during blips, whereas the other 3 had wt virus. Another study of

Table 5. Estimated no. of times selected mutations have arisen.

Patient, drug	Months receiving drug	Mutation	Expected no.[a] of times mutation has arisen on therapy, assuming N = 100 virions/cell	10,000 virions/cell	No. of clones with mutation at last time point
80					
3TC	42	M184V	2470	25	0/4
EFV	48	K103N	2822	29	0/4
81					
3TC	18	M184V	1058	11	0/5
EFV	22	K103N	1294	13	0/5
82, 3TC	72	M184V	4234	43	0/4
83					
3TC	26	M184V	1529	16	0/6
NVP	26	K103N	1529	16	0/6
85					
3TC	22	M184V	1294	13	0/5
EFV	22	K103N	1294	13	0/5

NOTE. 3TC, lamivudine; EFV, efavirenz; NVP, neviparine.

[a] Calculated using a standard model of viral dynamics. In patients with constant virus loads, the rate of change in the total number of free virus particles *(V)* over time *(dV/dt)* equals zero. Therefore, the rate of new infections, $T^*\delta$, (where T^* is the number of productively infected cells and δ is the clearance rate of virus-producing cells) is equal to cV/N (where c is the viral clearance rate, N is the burst size, and V is the concentration of viral particles in the plasma). We used recent estimates [49] for the clearance rates of free virus ($c = 23$ cells/day) and infected cells ($\delta = 1$ cells/day). N was taken to be either 100 or 10,000 virions/cell, representing the extremes of current estimates [50]. We used a level of viremia of 50 copies/mL, yielding a total body viral burden of 7.5×10^5 virions (assuming that the viral volume of distribution is 15 L). Using the no. of new cells infected per day and published values for the mutation rate of HIV-1 reverse transcriptase (3.4×10^{-5} mutations/bp/replication cycle) [37], we then estimated the no. of times each mutation would arise per day. These rates, multiplied by the length of time a patient was receiving a given drug, gave an approximation of how many times a resistance mutation would have arisen during treatment.

blips [24] found that 31% of patients had drug-resistant mutations, whereas 69% were genotypically wt. Consistent with our findings, some patients with well-suppressed viremia receiving HAART who experience blips do not evolve drug resistance. Further longitudinal studies are required to establish the long-term consequences of these blips.

In standard evolutionary theory, selection drives changes in allele frequency. In this regard, our data show no evidence for evolution occurring in the low-level viremia that is found in patients whose virus loads are suppressed below the limit of detection during HAART. It remains possible that evolution is occurring on a protracted timescale that would preclude the observation of drug resistance in these studies. Additionally, the small sample size may not have allowed for the detection of a very small but progressive increase in the frequency of drug-resistant viruses over the study period. Because stable reservoirs of HIV-1 exist in all patients [41–48], replication-competent virus can be continually released from these reservoirs into the plasma, which would provide an explanation for virus production without evolution. Whether virus originates from the small pool of latently infected memory $CD4^+$ T cells or from cells harboring it in drug-sanctuary sites, plasma is apparently never fully clear of HIV-1. Two of the possible sources of low-level viremia—stable, long-term reservoirs and ongoing replication—are not mutually exclusive; in fact, both processes are most likely occurring, and both probably contribute, to differing degrees, to plasma viremia. What is important is that antiretroviral drugs can, under optimal circumstances, reduce viral replication to a level that does not allow the selection of drug-resistant virus. Resistant viruses that do arise do not seem to be able to establish a foothold and to expand within the population. This finding suggests that, with the appropriate monitoring of viremia, patients may be able to continue receiving therapy indefinitely without failure caused by drug resistance and that treatment may be primarily limited by problems related to drug toxicity and adherence.

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