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Human Immunodeficiency Virus Type 1 Quasi Species That Rebound after Discontinuation of Highly Active Antiretroviral Therapy Are Similar to the Viral Quasi Species Present before Initiation of Therapy

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In an effort to identify the sources of the viruses that emerge after discontinuation of therapy, analyses of human immunodeficiency virus (HIV) quasi species were done for 3 patients with sustained levels of HIV RNA of <50 copies/mL for 1–3 years. The sequences found in the rebounding plasma virus were closely related to those of the actively replicating form of viruses present before the initiation of combination therapy. All quasi species found in the rebounding plasma virus were also present in proviral DNA, cell-associated RNA in peripheral blood mononuclear cells (PBMC), and virion RNA derived from PBMC coculture during periods when plasma HIV RNA levels were <50 copies/mL. These findings suggest that the rapid resurgence of plasma viremia observed after discontinuation of therapy and the viruses cocultured from PBMC are derived from a relatively stable pool of the replicating form of virus rather than from activation of a previously latent pool.

The use of highly active antiretroviral therapy (HAART) has resulted in a decrease in human immunodeficiency virus (HIV) type 1 replication to levels <50 copies/mL for prolonged periods of time in many patients with HIV-1 infection [1, 2]. In late 1997, 3 research groups independently reported that, among patients who have sustained HIV RNA levels of <50 copies/mL of plasma for an extended period of time, replication-competent HIV-1 could still be recovered from resting memory CD4 cells by an enhanced culture technique [3–5]. Recently, residual viral replication was demonstrated in some patients with prolonged suppression of plasma viremia [6–8]. In addition, the persistence of viral transcriptional activity in peripheral blood mononuclear cells (PBMC) has been reported in patients treated with HAART [9–12], and, after discontinuation of HAART, plasma levels of HIV-1 returned to baseline levels in a matter of weeks in the majority of patients [13–16].

In the present study, we attempted to identify the source of the rebounding plasma viruses after discontinuation of HAART, through sequence analysis of changes in virion RNA, cell-associated viral RNA, and proviral DNA for an extended period of time.

Methods

Patient population. Three HIV-infected subjects who had been undergoing HAART for a minimum of 1 year with sustained levels of HIV RNA of <50 copies/mL form the basis of this report. All 3 patients were enrolled in a study examining the consequences of discontinuation of antiretroviral therapy. Detailed clinical characteristics of these patients and the study of discontinuation of therapy have been reported elsewhere [13]. HAART was defined as a minimum of a 3-drug combination, including at least 2 licensed reverse-transcriptase inhibitors and at least 1 licensed protease inhibitor. Plasma virus loads were measured by use of the branched DNA signal amplification assay (Chiron) [17].

HIV-1 virion RNA isolation from blood plasma. Peripheral blood was centrifuged at 400 g for 5 min, and plasma was divided into aliquots of 1 mL and immediately stored at −120°C until use. Plasma was concentrated by centrifugation at 150,000 g for 1 h, and virion RNA was extracted from the subsequent pellet by use of the QIAamp Viral RNA Mini Kit (Qiagen). The isolated RNA was resuspended in RNase-free water, and then reverse transcription was done with Superscript-II reverse transcriptase (Life Technologies). Primers 5′-TTGTTTTACATCATAAGTGTGGGC-3′ (antisense, nt 3627–3650 in HIV-1 HXB2) and 5′-AGTGCTTCCTGCTGCTCCCAAGAA-3′ (antisense, nt 7788–7811) were used for cDNA synthesis of the protease and envelope genes, respectively.

Proviral DNA and cell-associated viral RNA isolation from

Table 1. Characteristics of 3 patients with human immunodeficiency virus (HIV) type 1 infection before, during, and after highly active antiretroviral therapy.

| | | | Sequences detected by sensitive nested PCR[c] | | | | |
Drug regimen	Time, months[a]	HIV RNA, copies/mL[b]	HIV-1 DNA in PBMC	HIV-1 RNA in PBMC	HIV-1 RNA in plasma	Qualitative coculture assay[d]	Total CD4 cells/mL
Patient 1							
AZT + 3TC	−14	27,440	+ (22, 5)	+ (28, ND)	+ (21, 14)		1267
3TC + d4T + Sqv	0	<50[e]	+ (22, 8)	+ (4, ND)	−	+ (5, ND)	1242
3TC + d4T + Nfv + Sqv	6	<50	+ (24, 8)	+ (2, ND)	−	+ (1, ND)	1065
3TC + d4T + Nfv + Sqv	12	<50	+ (33, 6)	+ (5, ND)	−	−	990
Discontinued therapy	17	<50					1078
Discontinued therapy for 4 weeks	18	3495	+ (11, 7)	+ (18, ND)	+ (14, 12)		1138
Patient 2							
3TC + d4T	−5	3016[f]	+ (18, 15)	+ (12, ND)	+ (18, 7)		900
3TC + d4T + Idv	0	<50[e]	+ (25, 11)	+ (4, ND)	−		1005
3TC + d4T + Idv	12	<50	+ (31, 16)	+ (8, ND)	−	+ (4, ND)	1184
3TC + d4T + Idv	18	<500	+ (33, ND)	+ (2, ND)	−		925
Discontinued therapy	28	<50					1021
Discontinued therapy for 3 weeks	29	3902	+ (13, 9)	+ (12, ND)	+ (12, 15)		980
Patient 3							
None	−20	18,000	+ (31, 9)	+ (16, ND)	+ (15, 11)		203
3TC + d4T + Idv	0	<50[e]					507
3TC + d4T + Idv	5	<500			−		740
3TC + d4T + Idv	12	<50	+ (21, 10)	+ (14, ND)	+ (3, ND)		882
3TC + d4T + Idv	17	<500	+ (15, 10)	+ (12, ND)	+ (3, ND)		908
3TC + d4T + Idv	22	<50			−		722
3TC + d4T + Idv	28	<50			−		852
3TC + d4T + Idv	30	<50	+ (16, 5)	+ (15, ND)	+ (8, ND)		901
3TC + d4T + Idv	32	<50	+ (16, 11)	+ (15, ND)	+ (4, ND)		1123
Discontinued therapy	36	<50					767
Discontinued therapy for 2 weeks	36	6166	+ (9, 9)	+ (17, ND)	+ (12, 12)		524

NOTE. Blank spaces indicate that no experiments were done at those time points. 3TC, lamivudine; AZT, zidovudine; d4T, stavudine; Idv, indinavir; ND, not done; Nfv, nelfinavir; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; Sqv, saquinavir.

[a] Time relative to first documented HIV RNA measurement of <50 copies/mL.

[b] Measured by the branched DNA signal amplification assay (Chiron).

[c] Nos. in parentheses represent no. of sequences obtained for protease and for envelope genes. +, Sequence detectable; −, sequence undetectable.

[d] Coculture of 7×10^6 CD8 cell–depleted patient PBMC with CD8 cell–depleted donor PBMC stimulated with anti-CD3 antibody and interleukin-2.

[e] First documented HIV RNA measurement of <50 copies/mL.

[f] Measured by modification of Roche Amplicor assay with detection limit of 50 HIV RNA copies/mL.

PBMC.   PBMC were obtained by ficoll-hypaque density gradient centrifugation of peripheral blood and were viably frozen at −120°C in aliquots of 10^7 cells before use. One aliquot, containing ∼10^7 cells, was divided for proviral DNA isolation and for cell-associated viral RNA isolation. Proviral DNA was isolated from 10^6 cells, as described elsewhere [18]. Cell-associated viral RNA was isolated from 9×10^6 cells by use of TRIzol reagent (Life Technologies) according to the manufacturer's protocol. The isolated RNA was treated with DNase I (Life Technologies), and then reverse transcription was done as described above. A reverse transcriptase–negative control was set up for each reaction to control for the possibility of proviral DNA contamination.

Polymerase chain reaction (PCR) and sequencing.   Single molecules of proviral DNA or viral cDNA, obtained through limiting dilution, were amplified and sequenced directly to minimize resampling errors. Up to 33 separate limiting dilution–PCR amplifications were done for each sample (table 1). The reproducibility of the assay was assessed by 2 independent amplifications of proviral DNA (31 and 35 separate limiting dilution–PCR amplifications for

data sets A and B, respectively) from different cell aliquots of the same sample. The 2 independent data sets yielded comparable quasi species (data not shown). PCR amplification was done with the Expand High Fidelity PCR System (Roche Molecular Biochemicals). Amplification of the HIV-1 protease gene was done in a 50-μL reaction containing 1× Expand High Fidelity buffer 3, 0.2 mM dNTPs, 2 mM MgCl$_2$, 20 pmol of primers, and 1.75 U of Expand High Fidelity PCR System enzyme mix. The forward primer was 5′-CAGAAGAGAGCTTCAGGTTTGGG-3′ (sense, nt 2165–2187) and the reverse primer was 5′-ACTTTTGGGCCATCCATT-CCTGGY-3′ (antisense, nt 2588–2611) in a first-round reaction. One microliter of the PCR product from the first reaction was used in a second-round reaction with a forward primer of 5′-TCAGAA-GCAGGAGCCGATAGAC-3′ (sense, nt 2208–2229) and a reverse primer of 5′-TGGTACAGTCTCAATAGGACTAATGGG-3′ (antisense, nt 2550–2576). Amplification of the C2-V3 region of the HIV-1 envelope gene was done by use of the procedure just described for the protease gene but with the following primers: 5′-TACAATGTACACATGGAATT-3′ (sense, nt 6958–6977) for the

forward primer and 5′-ATTACAGTAGAAAAATTCCCC-3′ (antisense, 7362–7382) for the reverse primer for the first reaction and 5′-TGGCAGTCTAGCAGAAGAAG-3′ (sense, nt 7010–7029) for the forward primer and 5′-CTGGGTCCCCTCCTGAGG-3′ (antisense, nt 7315–7332) for the reverse primer for the second reaction, as described elsewhere [19]. Each round of PCR consisted of 25 cycles, with the initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min, with the final extension at 72°C for 7 min. The PCR products were purified with the QIAquick PCR purification kit (Qiagen). To determine the error rate of Taq polymerase for the protease gene, a recombinant plasmid containing the HIV-1 NL4.3 protease gene underwent end-point dilution and was amplified by PCR (nested PCR, 25 cycles for each round). A total of 20 amplicons of 297 bp of the protease gene were independently obtained for sequencing. The protease sequences of each amplicon were identical to the template protease sequence (data not shown). In addition, no mutational hot spots or nucleotide-change bias (such as G→A hypermutation) were observed. Taken together, the data suggest that the level of noise introduced by Taq polymerase was low. The DNA was sequenced with the dRhodamine Terminator Cycle Sequencing Ready Reaction with AmpliTaq DNA Polymerase (PE Applied Biosystems) and analyzed with an automated sequencing system (model 377; Applied Biosystems) as described elsewhere [20].

Genotypic analyses. Nucleotide sequences were aligned by use of the default settings of the CLUSTAL W program [21] and subsequently were edited by hand with the MASE (multiple-aligned sequence editor) program [22]. Pairwise distances among sequences were estimated by use of the show-pairwise-distances option in the PAUP* (phylogenetic analysis using parsimony and other methods) program [23]. Phylogenetic relationships among the protease and envelope sequences were estimated, by use of the neighbor-joining method [24], with the PAUP* program. The appropriate model of evolution was determined, by use of the likelihood-ratio tests of the model of evolution, with the Modeltest program [25]. The Kimura 3ST model of evolution [26] and the HKY model of evolution [27] were used in the present study. Gaps were ignored for affected pairwise comparisons. Statistical support for various nodes in the neighbor-joining tree was obtained by 1000 replications of the bootstrap procedure [28]. Topology of each phylogenetic tree was statistically tested by use of the Kishino-Hasegawa test [29]. All new sequences reported in the study were compared with 19 known laboratory strains of HIV-1 and a total of 97 molecular clones that were recently obtained in the laboratory from another 6 patients. The phylogenetic tree showed that there was no indication of laboratory cross-contamination during the PCR amplification (data not shown). For signature-type analyses, amino acid sequences were realigned with the CLUSTAL W program and grouped on the basis of sequence similarity. The VESPA (viral epidemiology signature pattern analysis) program [30] was used to detect a set of amino acids that is unique for each subgroup, by use of the HIV-1 NL4.3 sequence as a reference. The groups of sequences that shared unique signature patterns were designated as signature types 1–7 in the analyses. The sequence data used in this study have been deposited in GenBank under accession numbers AF299692–AF299961 (patient 1); AF299427–AF299691 (patient 2); and AF299962–AF300280 (patient 3).

PBMC coculture assays. PBMC cultures were done by means of the procedure described elsewhere [31]. Patient PBMC were separated by ficoll-hypaque density gradient centrifugation and were depleted of CD8 T cells by use of antibody-coated beads (Dynal). Cells were seeded into a 12-well plate at a density of 7×10^6 cells/well in 2 mL of RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, phytohemagglutinin (4 µg/mL; Sigma), and irradiated PBMC (10×10^6 cells per well) from HIV-1–seronegative persons. The next day, the medium was changed to RPMI supplemented with 10% heat-inactivated fetal bovine serum, 20 IU/mL interleukin-2 (Roche Molecular Biochemicals), and 1.5×10^6 CD8 cell–depleted donor PBMC activated with 200 pg/mL anti-CD3 antibody (Ortho Diagnostic Systems) for 3 days. At weekly intervals, about half the cells were split and fed with 1.5×10^6 freshly prepared CD8 cell–depleted donor PBMC activated by anti-CD3 antibody. At days 4–7 and 11, half of the culture medium was collected from each well and replaced with fresh medium containing 20 IU/mL interleukin-2. The collected culture supernatants were centrifuged at 1600 g for 10 min and then were passed through a 0.22-µm filter. The cell-free supernatants were divided into aliquots and stored at −120°C before use. The coculture supernatants were centrifuged at 150,000 g for 1 h, and virion RNA was extracted from the subsequent pellets by use of the QIAamp Viral RNA Mini Kit (Qiagen). The isolated RNA was treated with DNase I (Life Technologies), and reverse transcription was done as described above.

Statistical analysis. All analyses were done with StatView version 4.5 (Abacus Concepts). A paired t test was used to evaluate the significance of differences among within-sample variations and nucleotide distances from baseline consensus.

Results

Patient characteristics. Three patients, aged 38, 40, and 50 years, were studied (table 1). The patients had been infected with HIV-1 for at least 6 years before initiation of combination antiretroviral therapy. For all patients, levels of HIV RNA declined to <50 copies/mL during antiretroviral combination therapy that included a protease inhibitor, and plasma HIV RNA levels were maintained at <50 copies/mL for 17, 28, and 36 months for patients 1, 2, and 3, respectively, before discontinuation of therapy (figure 1).

By use of a nested PCR technique with a sensitivity of ~10 copies/mL, plasma virus could not be detected in patients 1 and 2 but could be detected in patient 3. Proviral DNA, cell-associated HIV RNA, and virion RNA from cell coculture supernatants were present in all 3 patients during the period of treatment when plasma HIV RNA levels were <50 copies/mL.

In all 3 patients, HIV RNA levels in plasma quickly increased to >50 copies/mL within 2–4 weeks after discontinuation of therapy (table 1).

Comparison of plasma viruses obtained at baseline and at the time point of rebound after discontinuation of therapy. To attempt to identify the source of the virus that resurged after discontinuation of therapy, analyses of plasma virus quasi species were done. Sequence analyses were done on the protease
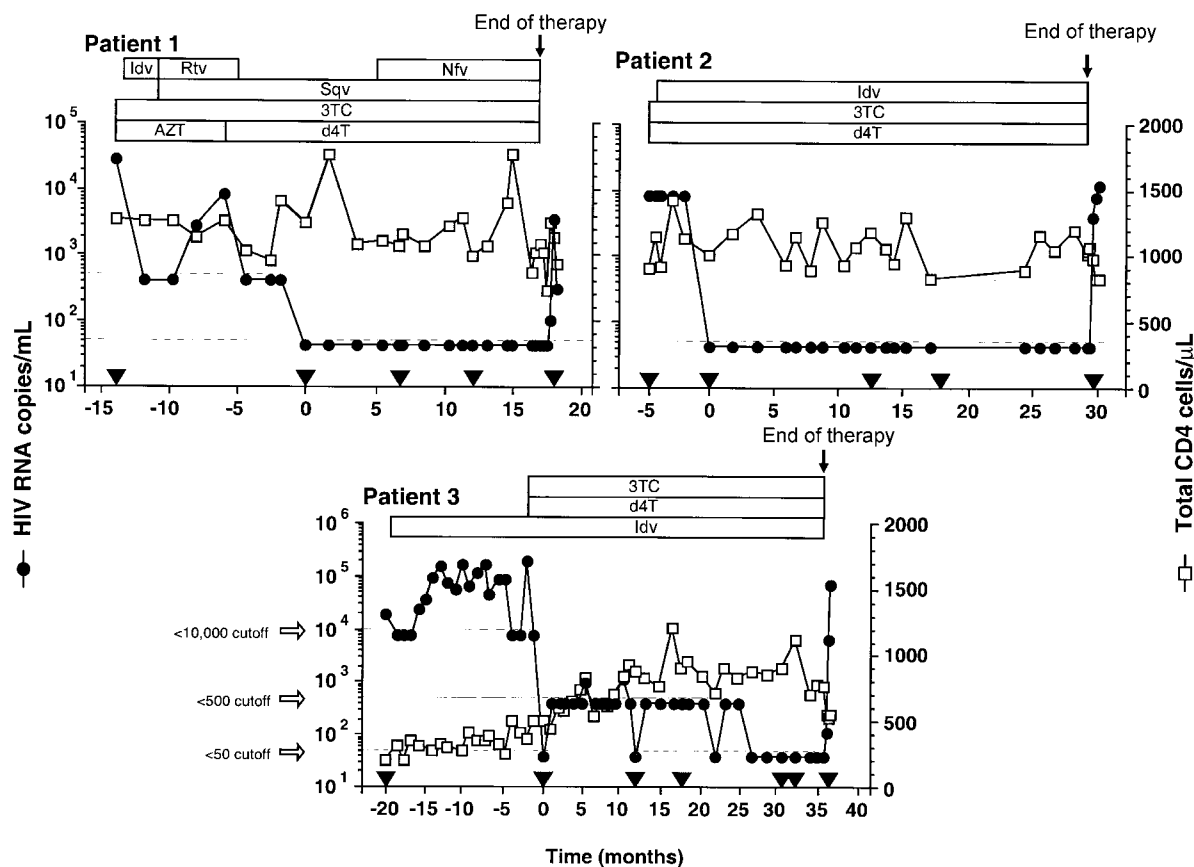
Figure 1. Changes in plasma virus load and CD4 T cell count before, during, and after discontinuation of combination antiretroviral therapy. Plasma virus loads were measured by use of the branched DNA signal amplification assay (bDNA assay; Chiron), with a detection limit of 50 copies/mL at most time points. For some time points, bDNA assays with a detection limit of 500 or 10,000 copies/mL were used. Drug-treatment history is shown at top. Arrows indicate time points at which plasma and peripheral blood mononuclear cell (PBMC) samples were obtained for sequencing analyses. 3TC, lamivudine; AZT, zidovudine; d4T, stavudine; HIV, human immunodeficiency virus; Idv, indinavir; Nfv, nelfinavir; Rtv, ritonavir; Sqv, saquinavir.

and envelope genes. The relationships between plasma virus isolates obtained before the start of combination therapy (−14, −5, and −20 months for patients 1, 2, and 3, respectively; figure 1) and those obtained at the time point of virus relapse after discontinuation of therapy (4, 3, and 2 weeks for patients 1, 2, and 3, respectively) are shown in figure 2. The clustering patterns of the protease and envelope sequences were predominantly patient dependent, with bootstrap supports ranging from 63 to 96 in the protease tree and from 82 to 100 in the envelope tree (figure 2). As noted by the scale for each tree, sequence diversity was greater in the envelope gene.

Overall, the viruses obtained at the time of relapse were indistinguishable from the viruses present in plasma at baseline within an individual patient. To determine whether the rebounded viruses are significantly different from the baseline viruses, the Kishino-Hasegawa test [31] was used. First, a constraint tree forcing the rebounded viruses to be one monophyletic group and the baseline viruses to be another monophyletic

group was constructed on the basis of the null hypothesis of rebounded viruses forming their own monophyletic group separated from the baseline viruses. Second, the constraint tree was compared with the best estimated tree with no constraint (figure 2) by use of the Kishino-Hasegawa test. For both the protease and envelope trees, the null hypothesis of the rebounded viruses forming separate monophyletic groups was strongly rejected (*P* = .0059 for the protease tree; *P* < .0001 for the envelope tree). Thus, the phylogenetic relationship, estimated in figure 2, that the rebounded viruses are indistinguishable from the baseline viruses is supported by this statistical analysis. These data demonstrate that the viruses that emerged after discontinuation of therapy were coming from sources very similar to those giving rise to the viruses present at baseline.

Characterization of PBMC proviral DNA sequences during therapy. Analyses of proviral DNA quasi species were done to determine the relationships between viral quasi species in plasma and PBMC proviral DNA during combination therapy.

Figure 2. Phylogenetic trees for protease and envelope sequences obtained from plasma virion RNA present at baseline and found in rebounding virus in blood, after discontinuation of highly active antiretroviral therapy (HAART). Phylogenetic relationships among the protease and the C2-V3 region of the envelope sequences were estimated using the neighbor-joining method. Neighbor-joining trees for protease and envelope sequences were constructed by use of the Kimura 3ST model of evolution, taking into account (1) unequal base frequencies with a gamma shape parameter of 1.04 and an estimated proportion of invariable sites of .56 and (2) unequal base frequencies with a gamma shape parameter of 0.58, respectively. Bootstrap percentile values from 1000 replications are shown at nodes defining major groupings of sequences from individual patients. Colors identify times when samples were obtained. Phylogenetic trees showed that isolates from 3 patients were well confined within distinct subgroups that were divergent from each other and from known laboratory strains of human immunodeficiency virus (HIV) type 1. HIV-1 U455 (subtype A) was used as an outgroup.

All sequences obtained from an individual patient were found to form a monophyletic group in both the protease and envelope trees (figure 3).

The protease sequence phylogeny for the 3 patients showed complex patterns of provirus relationships. All 3 patients had multiple protease lineages present at baseline, and these persisted throughout the entire sampling period of up to 56 months. Discontinuation of therapy did not have any significant impact on the distribution pattern.

The envelope sequence phylogeny showed a similarly complex pattern. The diverse provirus population present at baseline persisted throughout the period of therapy for all 3 patients. No significant changes in the distribution pattern for provirus quasi species were observed even after the discontinuation of therapy. In addition, no changes were noted in the V3 loop motif.

The average within-sample variations were determined for the envelope and protease proviral nucleotide sequences (table 2). As expected, the envelope genes exhibited a higher variability (average nucleotide variation $\pm$ SE: 1.33 ± 0.90, 1.62 ± 1.23, and 3.07 ± 1.14 for patients 1, 2, and 3, respectively) than did the protease genes (average nucleotide variation $\pm$ SE: 1.05 ± 0.66, 0.86 ± 0.42, and 1.35 ± 0.67 for patients 1, 2, and 3, respectively) at baseline. Relative to the degree of variation at baseline, no significant decreases were observed in the degree of within-sample variation in either the protease or envelope sequences, despite substantial changes in plasma virus load during the sampling period. In addition, the nucleotide distances from the majority of the posttherapy time points (18 of 19) to their respective baseline consensus sequences were similar throughout the duration of study. This was true for both the envelope gene and the protease gene ($P > .05$).

It is noteworthy that in 10 of 13 time points for the protease gene and in 8 of 12 time points for the envelope gene, the average within-sample variation was greater than the average nucleotide distances of these families to the baseline consensus sequence, again indicating persistence of the original virus pools that were present at baseline.

Characterization of protease quasi species during therapy. To search for fluctuation of virus subpopulations selected by antiretroviral combination therapy, the complexity of the protease quasi species was characterized at baseline, during a period of HIV RNA at <50 copies/mL, and at the time point of virus relapse after discontinuation of therapy for each patient. Protease sequences were grouped on the basis of sequence similarity. The groups of sequences that shared unique signature patterns common to ⩾2 sequences were designated as signature types 1–7 (figure 4). In patient 1, there were a total of 14 unique amino acid sequences and 4 different signature types among 112 protease sequences. In patient 2, a total of 11 unique amino acid sequences and 7 different signature types were found among 120 protease sequences. In patient 3, a total of 12 unique

amino acid sequences and 4 different signature types were found among 108 protease sequences.

Table 3 shows the complexity of the protease quasi species derived from proviral DNA in PBMC. About half the nucleotide changes in the protease gene resulted in amino acid substitutions. The overall distribution of the protease quasi species did not significantly change at the amino acid level within an individual patient ($P = .4$, $.8$, and $.2$ for patients 1, 2, and 3, respectively). The number of different signature types observed within a given time point were strikingly stable throughout the entire sampling period. The reproducibility of sampling was demonstrated by independently analyzing 2 aliquots of PBMC (A and B) from the same time point (12 months, patient 2). A total of 31 protease sequences from aliquot A and a total of 35 sequences from aliquot B were obtained. The 2 independent data sets yielded comparable sequences and frequencies (data not shown). The amino acid sequence of the major form was the same, and the complexity of the 2 sets of sequences was comparable (frequencies of the major form were 68% and 77% in aliquots A and B, respectively).

There was a trend toward a decrease in the number of copies of proviral DNA in PBMC as the plasma virus load decreased to <50 copies/mL during combination therapy. However, this decrease in the number of copies of proviral DNA did not lead to significant changes in the degree of complexity of the proviral DNA quasi species ($P = .5$).

Temporal changes in quasi species derived from proviral DNA, cell-associated HIV RNA, plasma HIV RNA, and HIV RNA from coculture supernatants. Having defined and analyzed signature types in the protease region (figure 4, table 3), we used this classification to study the turnover of sequence variants over time (figure 5).

In a comparison of quasi species between proviral and cell-associated RNA populations at baseline, 62% of the provirus variants present in baseline PBMC were found to be expressed in cell-associated RNA from the same aliquots of PBMC. Interestingly, such transcriptionally active variants were also found in plasma virion RNA at the same time point.

During periods when HIV-1 RNA levels were <50 copies/mL, cell-associated viral RNA could still be detected in PBMC from all 3 patients. This HIV RNA in PBMC represented 83% of the variants present in proviral DNA in PBMC at the initiation of combination therapy for the 3 patients.

Results of recent studies that have made use of an enhanced coculture technique suggest the possibility of the persistence of latent HIV-1 reservoirs in patients receiving HAART. To determine whether the presence of transcriptionally active variants in PBMC leads to the production of replication-competent virus in vitro, we conducted coculture assays on PBMC from the 2 patients with plasma virus loads of <10 copies/mL (patients 1 and 2). Replication-competent virus were isolated from both patients (patient 1, 0 and 6 months; patient 2, 12 months). In both patients, the viral sequences derived from the coculture
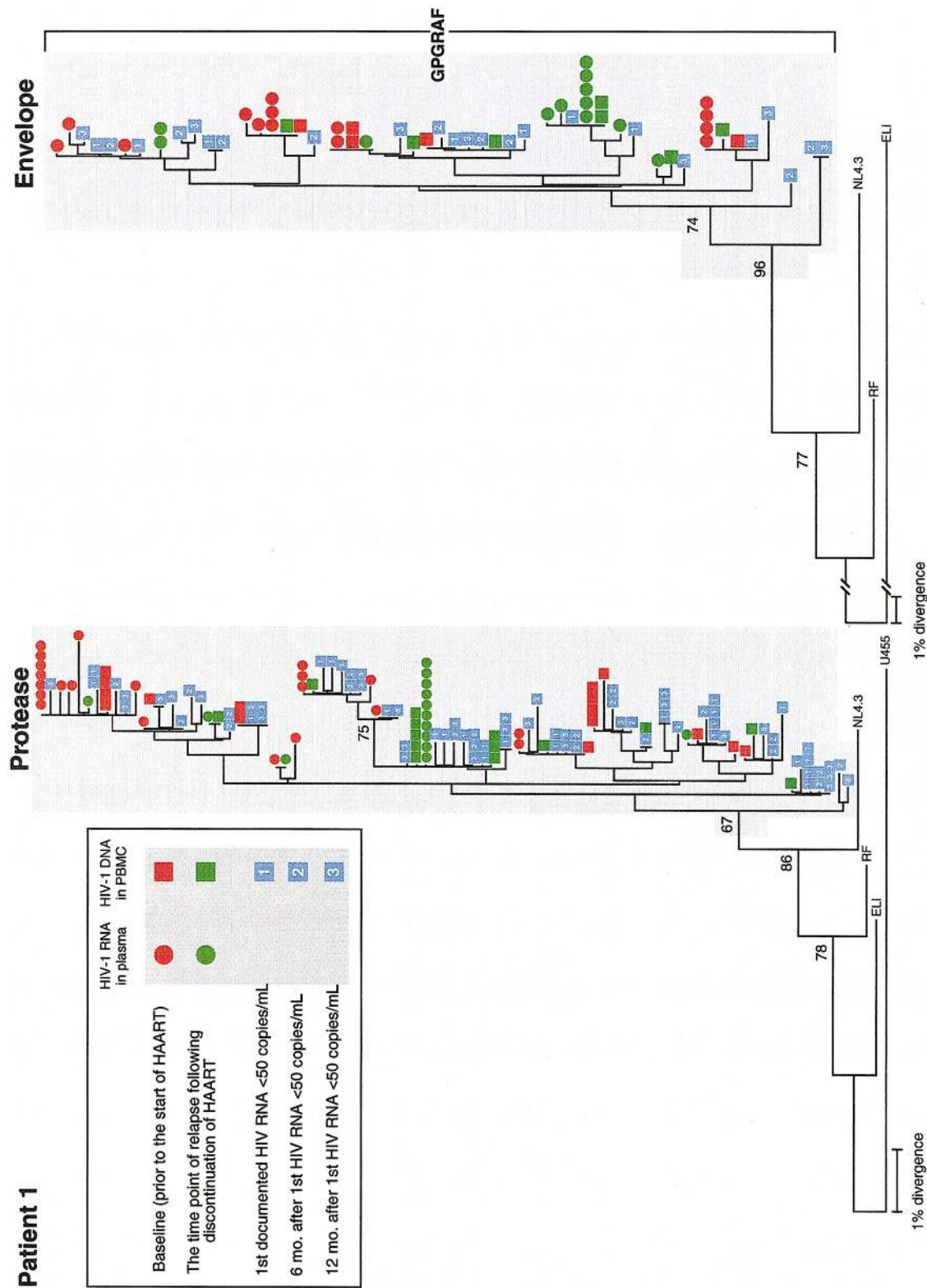
Figure 3. Phylogenetic trees for protease and envelope sequences obtained from proviral DNA in peripheral blood mononuclear cells (PBMC) at baseline, during period when human immunodeficiency virus (HIV) RNA levels were <50 copies/mL, and after discontinuation of highly active antiretroviral therapy (HAART). Phylogenetic trees were obtained as explained in the legend to figure 2. Plasma viral sequences used in figure 2 were also included in these analyses. The V3 loop motif is indicated in the envelope tree. For patient 1, the protease and envelope trees were constructed by use of the HKY+Γ model of evolution, taking into account unequal base frequencies and unequal transition (ti) and transversion (tv) rates (ti/tv = 4.83) with a gamma shape parameter of 0.29 and unequal base frequencies and unequal ti and tv rates (ti/tv = 1.59) with a gamma shape parameter of 0.44, respectively.
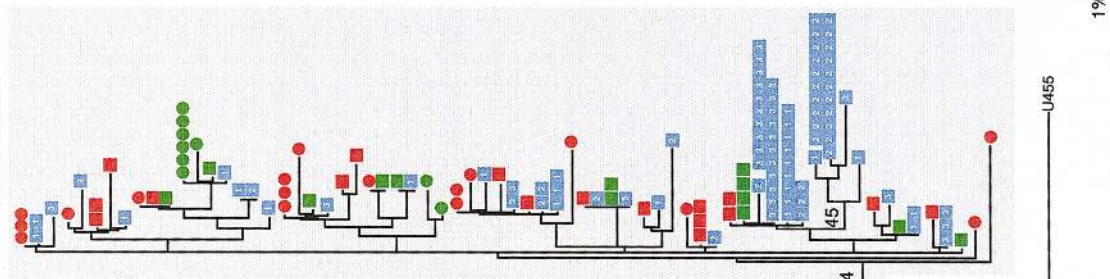
42

Figure 3 (*Continued*). For patient 2, the protease and envelope trees were constructed by use of the Kimura 3ST model of evolution, taking into account unequal base frequencies with a gamma shape parameter of 0.16 and unequal base frequencies with a gamma shape parameter of 0.27.
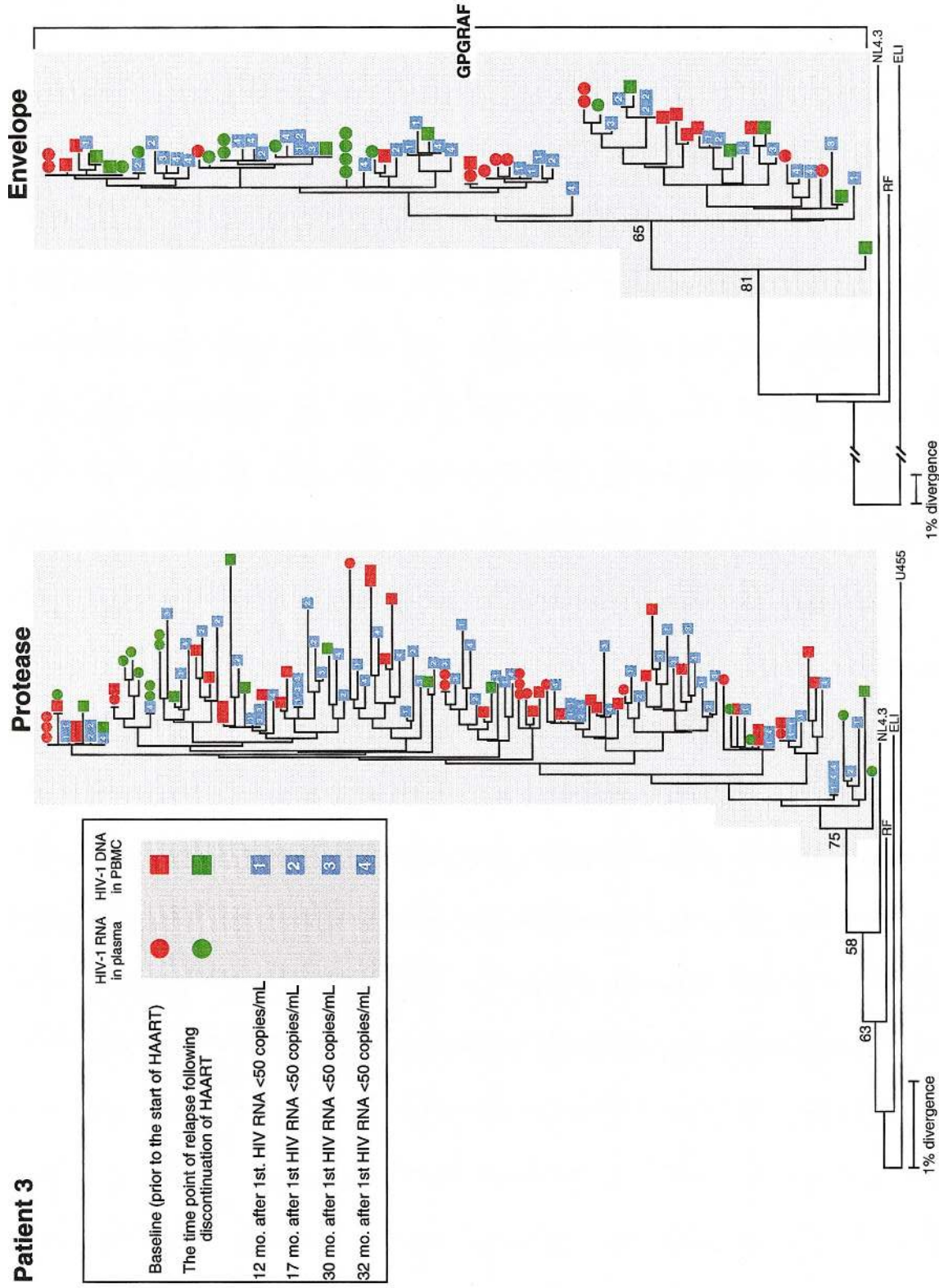
Figure 3 (*Continued*). For patient 3, the protease tree was constructed by use of the HKY+Γ model of evolution, taking into account unequal base frequencies and unequal ti and tv rates (ti/tv = 13.75) with a gamma shape parameter of 0.47 and an estimated proportion of invariable sites of 0.48 and an estimated proportion of invariable sites of .63. The envelope tree was constructed by use of the Kimura 3ST model of evolution, taking into account unequal base frequencies with a gamma shape parameter of 0.48 and an estimated proportion of invariable sites of .30.

Table 2. Within-sample variations and nucleotide distances to baseline sequence in protease and envelope genes of human immunodeficiency virus (HIV) type 1 before, during, and after highly active antiretroviral therapy.

Time, months[a]	Protease gene				Envelope gene			
	Within-sample variation, %[b]	P[c]	Baseline-to-posttherapy isolate distance, %[d]	P[e]	Within-sample variation, %[b]	P[c]	Baseline-to-posttherapy isolate distance, %[d]	P[e]
Patient 1								
−14	1.05 ± 0.66	—	—	—	1.33 ± 0.90	—	—	—
0	1.06 ± 0.58	.835	0.84 ± 0.39	—	1.97 ± 0.81	.251	1.49 ± 0.63	—
6	1.09 ± 0.59	.767	0.86 ± 0.47	.921	1.93 ± 0.90	.493	1.40 ± 0.76	.833
12	1.22 ± 0.59	<.001	0.92 ± 0.49	.585	2.43 ± 0.94	<.001	1.75 ± 1.15	.912
18	0.65 ± 0.49	<.001	0.83 ± 0.33	.896	2.22 ± 1.03	.121	1.45 ± 0.85	.523
Patient 2								
−5	0.86 ± 0.42	—	—	—	1.62 ± 1.23	—	—	—
0	0.75 ± 0.48	.553	0.61 ± 0.33	—	3.32 ± 1.93	<.001	3.50 ± 2.12	—
12	0.63 ± 0.62	<.001	0.90 ± 0.26	.006	1.98 ± 2.06	.079	2.52 ± 1.14	.352
18	0.53 ± 0.52	<.001	0.59 ± 0.29	.999	ND	—	ND	—
28	0.88 ± 0.46	.0130	0.62 ± 0.22	.674	3.68 ± 2.73	<.001	4.09 ± 2.47	.514
Patient 3								
−20	1.35 ± 0.67	—	—	—	3.07 ± 1.14	—	—	—
12	1.21 ± 0.55	.463	0.71 ± 0.43	—	2.51 ± 1.15	.189	2.27 ± 0.80	—
17	1.45 ± 0.57	<.001	0.83 ± 0.47	.236	3.10 ± 1.73	.679	2.37 ± 0.56	.771
30	1.34 ± 0.59	.001	0.90 ± 0.39	.056	3.46 ± 0.99	.272	2.28 ± 0.47	.982
32	1.47 ± 0.56	<.001	0.84 ± 0.46	.289	1.98 ± 0.92	<.001	2.28 ± 0.75	.852
36	1.58 ± 0.71	.001	0.86 ± 0.60	.641	3.30 ± 1.32	.501	2.15 ± 1.04	.788

[a] Time relative to first documented HIV RNA measurement of <50 copies/mL. Final time points for patients 1, 2, and 3 were 4, 3, and 2 weeks, respectively, after discontinuation of therapy.

[b] Data are average nucleotide variation ± SE, at given time point.

[c] Paired 2-sided *t* test; within-sample variation at given time point was statistically compared with respective baseline within-sample variation.

[d] Data are average uncorrected nucleotide distances ± SE, relative to the consensus sequence at baseline.

[e] Nucleotide distances from posttherapy time points to respective baseline consensus sequences were calculated and statistically compared with nucleotide distances from first documented time point of measurement of HIV RNA level of <50 copies/mL to baseline consensus sequence, for each patient.

supernatants of PBMC were of genotypes identical to those of sequences present in both proviral DNA and cell-associated RNA at the same time point. These results indicate a correlation between the presence of transcriptionally active variants in circulating PBMC and the production of replication-competent virus in tissue culture. Consistent with the data obtained from in vitro coculture assays for patients 1 and 2, 58% of transcriptionally active variants in PBMC from patient 3 were found to lead to the production of replication-competent virus in plasma, even during periods when HIV-1 RNA levels were <50 copies/mL.

A comparison of the HIV-1 quasi species at baseline and at the time of virus relapse after discontinuation of therapy showed that virus variants found in the rebounding virus all represented variants that had been actively replicating at baseline.

The protease sequences obtained during periods when HIV RNA levels were <50 copies/mL and after discontinuation of therapy displayed no evidence of resistance to protease inhibitors. Thus, as previously reported by others, the residual replication of HIV-1 does not seem to be due to the emergence of drug-resistant mutants.

Discussion

In the present study, we have provided evidence for the long-term persistence of virus reservoirs and their support of ongoing viral replication in patients receiving HAART. By examining sequence changes in proviral and viral genomes over a period of 18–36 months, we were able to demonstrate that plasma virus and coculture virus are derived from a pool of persistent cell-associated virus despite HAART-associated decreases in HIV RNA levels in plasma to <50 copies/mL for that period of time. The possibility of the persistence of latent HIV-1 reservoirs in patients receiving HAART has been suggested from a number of studies [3–5, 10, 32]. Other studies have presented evidence of ongoing viral replication in HIV-infected persons with prolonged suppression of plasma viremia [6–9]. However, the relationships between these reservoirs, mechanism of persistence of such reservoirs, and their potential role in the maintenance of viral persistence during prolonged suppression of plasma viremia with HAART have been unclear. In the present study, we found that virus pools established before the initiation of HAART acted as persistent sources of replication-competent viruses. These pools were detected in PBMC-associated viral RNA during a period when plasma HIV RNA levels were <50
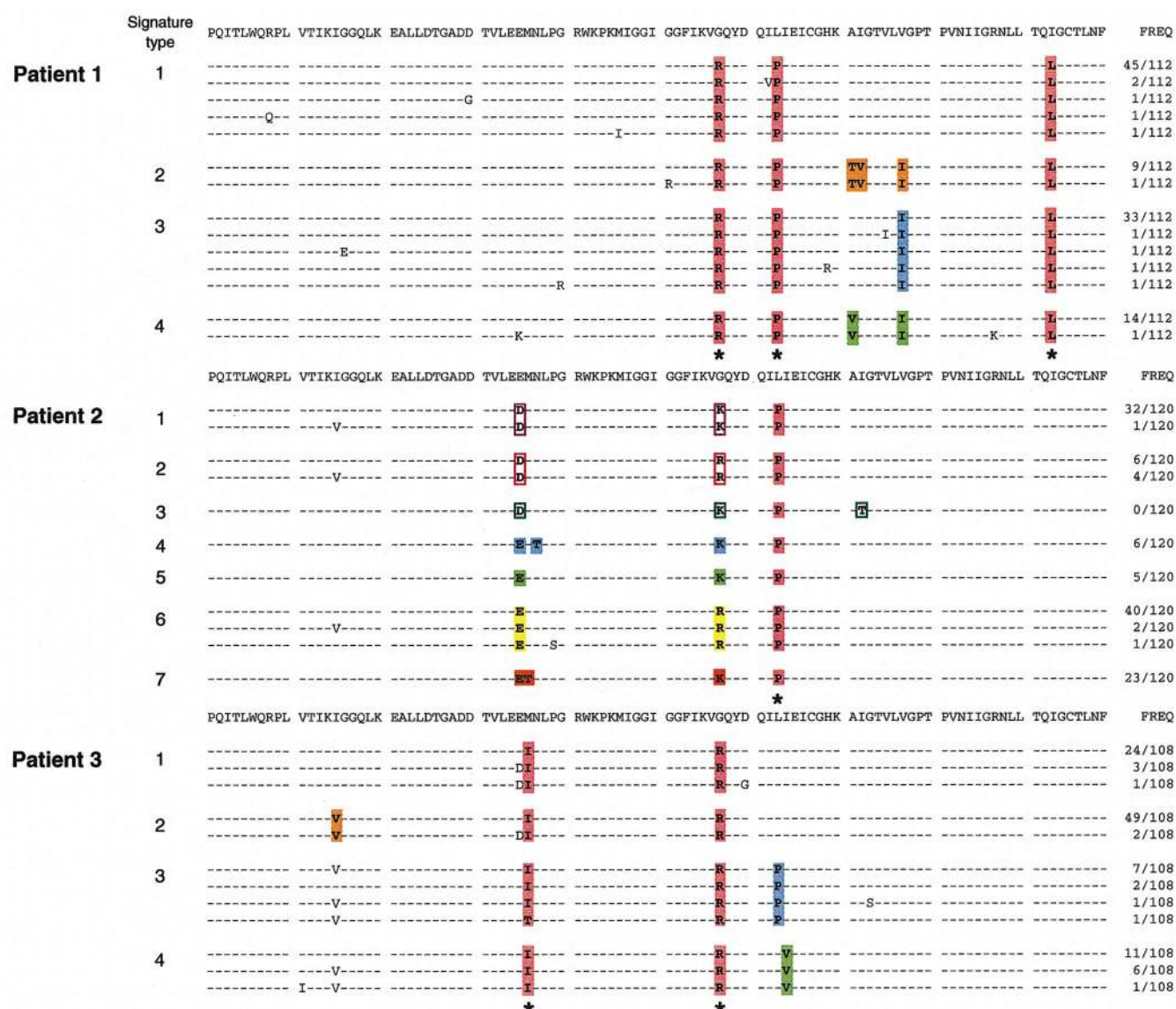
Figure 4. Signature patterns found among different patient protease sequences. All distinct protease amino acid sequences derived from peripheral blood mononuclear cell proviral DNA were aligned with human immunodeficiency virus type 1 NL4.3 as a reference. Only differences are shown. Sequence identity is indicated by a dash. The frequency ("FREQ") of a given amino acid sequence is shown at right. Groups of sequences that shared unique signature patterns common to ⩾2 sequences were designated at left as signature types 1–7. Amino acid positions involved in defining a unique signature pattern within each patient are indicated by different colors; amino acid positions that define a patient's signature pattern are indicated by an asterisk (*).

copies/mL and served as the source of infectious virus on discontinuation of HAART.

Our approach in addressing the question was to genotypically characterize the viral and proviral HIV-1 quasi species found in plasma, circulating PBMC, and coculture supernatants. The sequences obtained from the various sources were sorted on the basis of their genetic characteristics, and the turnover of each variant was carefully tracked in individual patients over

time. This approach allows one to characterize the diversity of viral quasi species, to distinguish between variants that are a latent form and those that are transcriptionally active, and to identify which transcriptionally active variants are giving rise to plasma- or coculture-derived virus.

It can be argued that the presence of PBMC-associated RNA represents virus particles attached to the cells from privileged sites where antiretroviral therapy is ineffective rather than tran-

Table 3. Complexity of human immunodeficiency virus (HIV) type 1 quasi species in protease gene.

Time, months[a]	HIV RNA, copies/mL	HIV DNA, copies/10^6 PBMC[b]	No. of sequences obtained from HIV DNA in PBMC	No. of unique sequences at nucleic acid level	No. of unique sequences at amino acid level	No. of different signature types
Patient 1						
−14	27,440	150	22	11	5	3
0	<50	150	22	14	6	4
6	<50	60	24	16	5	4
12	<50	60	33	22	9	4
18	3495	120	11	7	4	4
Patient 2						
−5	3016	300	18	13	6	5
0	<50	300	25	13	9	5
12	<50	20	31	8	5	4
18	<50	60	33	9	6	6
29	3902	300	13	9	5	5
Patient 3						
−20	18,000	12,000	31	27	5	4
12	<50	3000	21	18	7	4
17	<500	3000	15	14	7	4
30	<50	1500	16	14	8	4
32	<50	1500	16	16	5	4
36	6166	1500	9	9	4	4

NOTE.   PBMC, peripheral blood mononuclear cells.

[a] Time relative to first documented HIV RNA measurement of <50 copies/mL. Final time points for patients 1, 2, and 3 were 4, 3, and 2 weeks, respectively, after discontinuation of therapy.

[b] No. of copies of proviral DNA was estimated by limiting dilution.

scriptional activity of the viral genome in the cells. Either possibility requires the relatively recent transcription of the proviral genome in vivo. In our analyses of the HIV-1 quasi species in patients with prolonged suppression of plasma viremia, the viral quasi species identified in plasma or in coculture supernatants faithfully reflected the corresponding proviral and viral quasi species detected in PBMC. The frequencies of the major forms of viral quasi species in plasma or coculture supernatants were almost identical to the frequencies of the major forms of viral and proviral quasi species in PBMC. Given these observations, it seems unlikely that bound virus particles would be the explanation for the persistence of cell-associated RNA in PBMC. However, because our techniques do not allow us to differentiate between viral genomic RNA and viral mRNA within the cells, future studies are necessary to address this question.

The viruses obtained from cell coculture supernatants on stimulation in vitro have generally been considered to be derived from a latent pool of virus. However, our study and the study by Sharkey et al. [11] indicate that most of the variants derived from coculture supernatants may in fact be reflective of species actively replicating in circulating PBMC rather than an expression of a previously silent proviral DNA pool.

Evidence for ongoing expression of virus in the absence of significant quasi-species evolution is a seemingly paradoxical set of observations. Indeed, a lack of ongoing quasi-species evolution during HAART has been interpreted as evidence for complete shutdown of active viral replication [5]. In addition, any model of viral pathogenesis must take into account the clinical observation that, within 3 weeks of stopping therapy, the virus load tends to return rapidly to pretreatment levels

[13–16]. One possible model to explain these observations is that there is a reservoir of long-lived cells that chronically express low levels of virus. The results of a number of studies have suggested that current HAART regimens are not sufficient for complete suppression of viral replication [6–9]. Thus, a major effect of treatment is to prevent spread of virus through the uninfected CD4 T cells from the pool of chronically infected cells. Cells of this reservoir might be of the monocyte/macrophage lineage. These cells are resistant to the cytopathic effects of HIV. Thus, once infected, they could persistently produce HIV [33]. Furthermore, unlike CD4 T cells, monocytes/macrophages do not require cell proliferation to produce infectious virus particles [34]. The persistence of stable RNA virus pools throughout the entire sampling period supports this type of model. In the absence of effective therapy, there is widespread replication of virus, with additional seeding of the reservoir with variant strains of HIV-1, leading to a higher viral set point and drug resistance. This is somewhat similar to the proximal activation and transmission model of viral replication [35]. In that model, there are periodic bursts of previously infected cells. One prediction of that model (not seen with the current data) is that there would be a decline in diversity over time.

In summary, the current data demonstrate that virus pools detected in PBMC-associated viral RNA persisted even under the setting of suppressive HAART and that these pools can act as persistent sources of replication-competent virus identified in plasma or coculture supernatants during a period when plasma HIV RNA levels are <50 copies/mL and can serve as the source of infectious virus on discontinuation of HAART. Therefore, the persistence of virus reservoirs that sustain viral
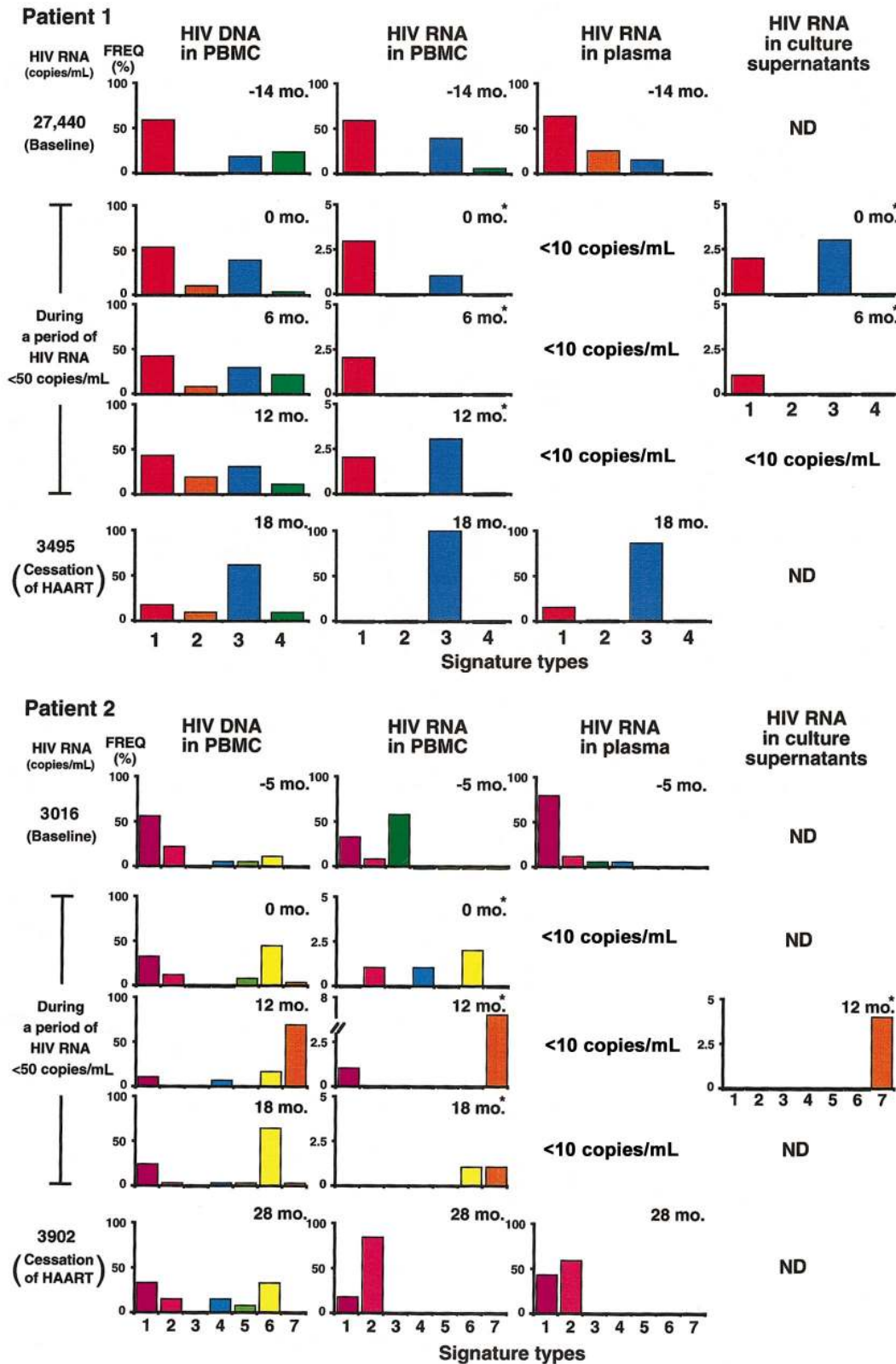
Figure 5. Temporal changes in quasi species for proviral DNA, cell-associated human immunodeficiency virus (HIV) RNA, plasma HIV RNA, and HIV RNA from coculture supernatants, presented vertically as a function of time before, during, and after highly active antiretroviral therapy (HAART). Five serial time points were analyzed for patients 1 and 2, and 6 serial time points were analyzed for patient 3. Corresponding plasma virus loads for those time points are shown at left. Signature types are the same as those shown in figure 4. An asterisk (*) indicates that data on the *X*-axis are no. of sequences observed (instead of frequency). The label "<10 copies/mL" indicates that sequences were not detected by the nested polymerase chain reaction technique with a sensitivity of ~10 copies/mL. ND, not done; PBMC, peripheral blood mononuclear cells.
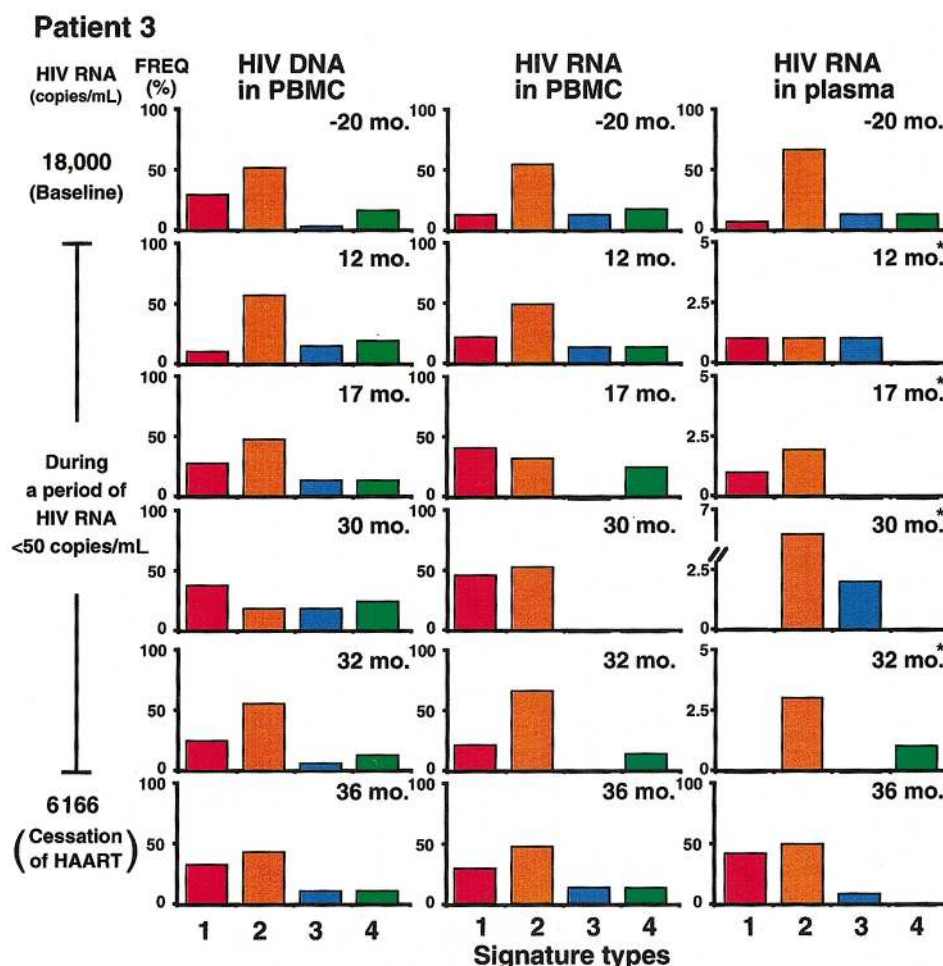
Figure 5 *(Continued).*

replication in aviremic patients undergoing HAART are likely to be the major impediment to the eradication of viral infection. These findings add to the growing body of data addressing the inability of current drug regimens to completely block HIV-1 replication and highlight some of the difficulties being faced in the development of effective long-term therapy for patients with HIV infection.

Acknowledgments

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