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Evidence that Low-Level Viremias during Effective Highly Active Antiretroviral Therapy Result from Two Processes: Expression of Archival Virus and Replication of Virus

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Episodes of low-level viremia (LLV), with plasma human immunodeficiency virus type 1 (HIV-1) RNA levels ranging from 50 to 400 copies (c)/ml, occur commonly during highly active antiretroviral therapy (HAART). LLV has been associated with virologic failure of HAART in some studies, while in others LLV did not appear to affect the clinical outcome. To understand the processes leading to LLV, genetic analyses were used to determine whether plasma virions emanated from archived or from newly evolved viral genomes. Episodes of LLV (plasma HIV-1 RNA, 50 to 379 [median, 77] c/ml) were detected in 21/37 (57%) HIV-1-infected children with median plasma HIV-1 RNA levels of <50 c/ml during 79 patient years of HAART. Viral sequences were derived by direct sequencing of PCR products from 21 plasma specimens diluted to end point. In phylogenetic analysis, LLV viral sequences grouped with virus from early in the course of infection in 8/11 subjects. Six specimens had multiple identical viral sequences, suggesting origin from clonally expanded infected cells. LLV plasma virus evolved over time, indicating viral replication, in 3/11 subjects. Two of these had frequent LLV, including the selection of drug-resistant mutants. In summary, plasma virus from episodes of LLV during effective HAART appeared to originate from two distinct processes, (i) clonal outgrowth from long-lived HIV-1-infected cells, presumably following activation and proliferation of these cells, and (ii) ongoing viral replication that included the selection of new drug-resistant mutants. These observations provide a plausible explanation for the divergent clinical outcomes previously associated with LLV.

Suppressive combination highly active antiretroviral therapy (HAART) has improved the health and life span of human immunodeficiency virus type 1 (HIV-1)-infected children (34) and adults (23). The long-term efficacy of HAART, however, has been limited in many individuals (11, 16), often due to the selection of drug-resistant viral mutants (2, 48). Transient low-level viremias (LLV), with plasma HIV-1 RNA levels in the range of 50 to 400 copies (c)/ml, have been reported to occur in 25 to 40% of adults in whom viral replication appeared to have been suppressed by HAART (21, 24, 45) and up to 90% of subjects with very frequent determinations of plasma HIV-1 RNA levels (35). LLV have been associated with selection of drug-resistant virus in several (8, 21, 22), but not all, studies (25, 35, 45).

LLV appeared to be clinically insignificant in two studies, as subjects with and without LLV had similar rates of virologic failure of HAART (24, 45). Detailed studies of LLV have demonstrated viral sequences similar to that found early in the course of infection (25, 35, 38). These observations support the hypothesis that activation of latently infected cells is the source of LLV (7, 17, 25, 38). A modification of this hypothesis proposes that there is continuous low-level expression of virus from a stable proviral reservoir, with detectable virus occurring when random biological processes or assay variations increase viral levels above the limit of detection of the assays (12, 13, 35). Importantly, during effective HAART these virions would not perpetuate infection, as protease (PR) inhibitors interfere with the maturation of new virus, rendering it defective and noninfectious, and reverse transcriptase (RT) inhibitors would block infection of additional cells.

In other studies, ongoing viral replication during HAART was either suspected (12, 14, 20) or detected by phylogenetic analysis and by selection of new drug-resistant mutants (8, 21, 22). Virologic failure of HAART has also been associated with LLV, especially when repeated episodes were observed (21, 22).

To better understand their origin and significance, viral sequences derived from LLV plasma specimens were compared to sequences from peripheral blood mononuclear cells (PBMC) collected before and during HAART. The genetic distances of LLV plasma sequences from the inferred most recent common ancestor (MRCA) of infection was used to "date" the LLV virus. The presence of "ancestral" or "archived" virus (genetic distance relatively close to inferred MRCA of infection) in a subject's plasma was taken to suggest that LLV originated from production of viral particles from cells latently infected with virus prior to HAART, whereas "new" or "recently evolved" virus implied that plasma virus resulted from ongoing viral replication.

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MATERIALS AND METHODS

Selection of subjects, specimens, and definitions. The medical charts of all children and adolescents attending the Seattle Children's Hospital and Regional Medical Center with a median HIV-1 plasma RNA level of <50 c/ml after 1 year of suppressive HAART were reviewed for potential inclusion in this study. Entry criteria were one or more episode of intermittent LLV, defined as plasma HIV-1 RNA measurements between 50 and 400 c/ml after at least one viral load measurement of <50 c/ml, with two or more subsequent values returning to <50 c/ml; available plasma and PBMC specimens; and informed consent in accordance with Institutional Review Board guidelines. When subjects had multiple LLV, a single sample within each 6-month interval was selected for analysis. Virologic failure of HAART was defined as two consecutive RNA values of >400 c/ml or a single RNA value of >1,500 c/ml.

Plasma HIV-1 RNA levels. Plasma HIV-1 RNA values were measured at 1- to 4-month intervals after initiation of HAART as part of clinical care using the UltraSensitive Monitor 1.0 (Roche Diagnostics, Montclair, NJ) or, for subjects with non-B subtypes, the Versant HIV RNA 3.0 (Bayer, Emeryville, CA).

PBMC-associated HIV-1 DNA amplification. DNA was extracted from PBMC using the IsoQuick nucleic acid extraction kit (Orca Research Inc., Bothell, WA). Extracted DNA was then diluted so that 30 or fewer of 100 nested PCRs were positive, yielding a >70% probability that a single viral template was in each reaction mixture (42). Regions of *pol* and *env* were amplified in a first-round PCR with primers PRL (19), RT2 (18), ED31 (9), and BH2 (3), followed by separate second rounds of PCR to amplify *pol* and *env*. Second-round *pol* primers were PRC (19) and RT3 (18), and *env* primers were ES7 and ES8 (10). Occasionally, a three-region multiplex reaction was used, with *pol* regions encoding PR and RT, as well as *env*, amplified. In these cases, the first-round multiplex PCR used PRA (19), PR2 (19), RTA (5′-AACTTCTGTATGTCATTGACAG TCCA-3′), and RT1 (18) with the *env* primers listed above. The second-round PCR used PR4 (19), PRB (19) for the PR region of *pol*, RT4 (18) and RTB (5′-CATTTATCAGGATGGAGTTCATA-3′) for the RT region of *pol*, and ES7 and ES8 for *env*. Alternative primers were then substituted when PCRs were negative using the aforementioned primers. These included ED5 and ED12 (10) for the first-round PCR of *env* in subject T2, PRF1 (19) and PR5 for the first-round PCR of *pol* encoding PR, and RTC and RTB for the first-round PCR and RTD and RT3 for the second-round PCR of *pol* encoding RT in subject G1. Cycling conditions for first and second rounds were 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by 72°C for 6 min.

Plasma RNA extraction and reverse transcription. RNA was extracted from 0.5 to 3 ml of plasma. A positive control for extraction was cell-free HIV-1_{RTMC} (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) (32), at a concentration of 100 viral c diluted into 1 ml of HIV-1-negative plasma.

Virions were pelleted by a 2-h centrifugation at 25,200 × *g* and 4°C. The pellet was suspended in 400 μl of Trizol (Life Technologies, Rockville, MD) for RNA extraction, using the manufacturer's protocol. Following a 5-min incubation, 1 μg of tRNA and 80 μl of chloroform were added. The solution was vortexed, incubated on ice for 10 min, and then centrifuged at 13,500 × *g* for 5 min. RNA was precipitated from the aqueous phase by addition of 250 μl of 100% isopropanol equilibrated at −20°C, collected by centrifugation at 13,500 × *g* at 4°C for 20 min, rinsed with 70% ethanol, and then pelleted at 16,000 × *g* for 5 min. The supernatant was discarded, and the RNA pellet was dried for 10 to 15 min at 56°C. The RNA was then immediately suspended in a small volume of nuclease-free water on ice with or without 0.5 U/μl of RNasin RNase inhibitor (Promega Co., Madison, WI).

The RNA from the subject's samples, the extraction control, and a control for reverse transcription (~1,000 c of HIV-1_{RTMC} RNA) were transcribed into cDNA in a reaction with specific primers for three regions of the viral genome: *pol* region encoding PR with PR5 (5′-CCTGGCTTTAATTTTACTGGTACAG TTTCA-3′), *pol* region encoding RT with RTA, and *env* with BH2. The cDNA was then diluted to the end point and PCR amplified with nested primers. The first round was multiplexed, including PR5 and PRA, RTA and RTC (5′-GGC CCAAAAGTTAAACAATGG-3′), and ED31 and BH2. Three separate second-round PCRs were done, each using 2 μl of the first-round product as the template and primers ES7 and ES8 (*env*), PRB and UHGR (5′-CTAATGGGA AAATTTAAAGTGCA) for *pol* encoding PR, or RTB and RTD (5′-GGCCA TTGACAGAAGAAAAAATAA-3′) for *pol* encoding RT. PCR cycling conditions were as for the DNA amplification above, except that 45 cycles were conducted for each round.

Dideoxynucleotide sequencing. PCR products were purified using ExoSAP-IT (Amersham Biosciences, Piscataway, NJ) prior to direct bidirectional sequencing using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) with the second-round PCR primers listed above. Amplicon lengths were 493 bp for the region of *pol* encoding PR (and including a portion of *gag*), 627 bp for the region of *pol* encoding RT, and 627 bp for the C2-V5 region of *env*. Sequencing reaction products were purified with DyeEx columns (QIAGEN, Bothell, WA) prior to analysis on a 377 or 310 automated Applied Biosystems sequencer.

Sequence analysis. Sequences were assembled and error checked using Sequencher (Gene Codes, Ann Arbor, MI). Those with substantial G→A mutational bias as determined using HYPERMUT (31, 43), suggesting hypermutation (51), were omitted from further analyses. Sequence alignments were obtained using ClustalW 1.7 (49) and edited as necessary. Regions of ambiguous alignment were removed from subsequent evolutionary analyses. Neighbor-joining phylogenetic trees were constructed using PAUP* versions 4.0b4 through 4.0b10 (47) with evolutionary models selected using the Akaike information criterion (1) under Modeltest 3.06 (40). Model parameters were as follows: (i) for PR (GTR+I+Γ), equilibrium nucleotide frequencies $f_A = 0.3880$, $f_C = 0.1653$, $f_G = 0.2310$, $f_T = 0.2175$; shape parameter (α) of the Γ distribution reflecting site-to-site rate variability of variable sites, $\alpha = 0.5382$; assumed proportion of invariable sites = 0.1638; *R* matrix values, $R_{A\leftrightarrow C} = 4.2758$, $R_{A\leftrightarrow G} = 16.3460$, $R_{A\leftrightarrow T} = 1.1576$, $R_{C\leftrightarrow G} = 0.2308$, $R_{C\leftrightarrow T} = 20.2003$, $R_{G\leftrightarrow T} = 1$; (ii) for RT (GTR+I+Γ), $f_A = 0.4642$, $f_C = 0.1383$, $f_G = 0.1755$, $f_T = 0.2220$; $\alpha = 0.7765$; assumed proportion of invariable sites = 0.2908; *R* matrix values, $R_{A\leftrightarrow C} = 1.0896$, $R_{A\leftrightarrow G} = 5.2510$, $R_{A\leftrightarrow T} = 0.2570$, $R_{C\leftrightarrow G} = 0.4460$, $R_{C\leftrightarrow T} = 7.7782$, $R_{G\leftrightarrow T} = 1$; and (iii) for *env* (TVM+I+Γ), $f_A = 0.4734$, $f_C = 0.1704$, $f_G = 0.1603$, $f_T = 0.1959$; $\alpha = 0.8087$; assumed proportion of invariable sites = 0.1198; *R* matrix values, $R_{A\leftrightarrow C} = 1.5222$, $R_{A\leftrightarrow G} = R_{C\leftrightarrow T} = 3.9416$, $R_{A\leftrightarrow T} = 0.5910$, $R_{C\leftrightarrow G} = 0.6665$, $R_{G\leftrightarrow T} = 1$. Supplemental data are available at our website (http://ubik.microbiol.washington.edu/HIV/Tobin-1/). Trees were constructed using all available sequences from the children and their mothers, along with outgroup HIV-1 sequences from GenBank. A sequence corresponding to the MRCA of infection was estimated at the basal node for each gene region of each subject using maximum-likelihood estimation and the evolutionary model determined for the respective gene region.

A plasma specimen was defined as having identical sequences when five or more identical *pol-RT* sequences or two or more identical *env* sequences were obtained by end point dilution of the plasma specimen. The region of *pol* encoding PR was not evaluated for identical sequences due to a high level of sequence conservation in this region.

Statistical analysis of viral genetic divergence and LLV frequency. PBMC viral sequences from each subject were evaluated for divergence from their MRCA over time, separately for each gene region, using a linear random-effect regression model with divergence as the outcome and time since the start of HAART as the covariate of interest. The regression model indicated ongoing replication when the estimated coefficient-of-time covariate was positive and significantly different than zero for any of three gene regions. Plasma sequences were not included in the random-effect model due to an imbalance in the quantity of data from the plasma and PBMC.

The frequency of LLV for each patient was analyzed based on an overdispersed negative binomial for the number of LLV that was offset for the number of years of follow-up. An indicator variable for replicator versus nonreplicator was used to test for the difference in the rate of LLV per year between these two groups.

Nucleotide sequence accession numbers. The GenBank accession numbers for the HIV-1 *env* and *pol* sequences derived or analyzed in this study are AY483287 to AY484389 and AY075701 to AY077450 (19).

RESULTS

LLV during HAART. LLV were documented in 21/37 (57%) children followed in our clinic with plasma HIV-1 RNA levels of <50 c/ml for >1 year. Specimens were available from 14 of these children, all of whom acquired infection through mother-to-child transmission. Each child was infected with group M HIV-1 subtype B virus, except for F1, who was infected with subtype D, and T2, who was infected with subtype C virus. The 14 children had a total of 44 episodes of LLV during 1.8 to 6.6 (median, 4.5) years of HAART when their median plasma HIV-1 RNA levels were <50 c/ml (Table 1). Plasma HIV-1 RNA was quantified 2.6 to 10.9 (median, 4.6) times/year, with 1 to 10 (median, 2) LLV detected per subject (Fig. 1). Plasma viral RNA levels during episodes of LLV ranged from 50 to

TABLE 1. Characteristics of subjects with intermittent LLV

Subject (age)[b]	Duration (yr) of HAART	ART therapy[a]		No. of LLV (No. of HIV-1 RNA c/ml plasma)	No. of LLV studied	No. of plasma viral sequences amplified/episode of LLV			Viral sequence divergence in plasma	Drug resistance mutations in plasma virus
		Pre-HAART	ART regimen			PR[c]	RT[d]	*env*[e]		
B2 (15)	4.3	ddI	d4T-3tC-NFV-NVP	1 (51)	1	0	0	0	ND[g]	ND
F1 (5)	2.7	None	d4T-3tC-SQV-RTV d4T-3tC-LPV/r-EFV	10 (55–374)	4	7–15	3–14	0–2	+	RT: G190E
G1 (15)	6.6	ZDV ddI ZDV-ddI-NVP	d4T-3tC-RTV d4T-3tC-RTV-SQV d4T-3tC-LPV/r	1 (139)	1	0	0	0	ND	ND
G2 (17)	6.6	ZDV ddI ddI-NVP	ZDV-3tC-RTV d4T-3tC-SQV-RTV-NVP d4T-3tC-LPV/r-NVP	9 (50–101)	5	0–15	0–14	2–5	+	PR: L10I, I54V, V82A, I84V, L90M RT:K70R, K103N, Y181C, M184V, T215F
H2 (5)	5.1	ZDV	d4T-3tC-RTV-NVP	3 (53–58)	1	7	8	3	−	None
H4 (3)	1.8	None	d4T-3tC-NFV-EFV	5 (55–73)	4	0–12	0–12	0–12	−	None
L1 (11)	5.8	ddI d4T-RTV	d4T-3tC-RTV-NVP	2 (51–71)	2	0–1	0	0	−	None
M2 (10)	5.3	ZDV ZDV-ddI ZDV-3tC	d4T-ddI-RTV-NVP	2 (54–236)	2	0	0	0–1	−	ND
P1 (12)	4.0	ddI	ddI-d4T-SQV-NFV d4T-3tC-LPV/r	2 (100–110)	2	2–8	2–3	0–3	−	None
R1 (8)	6.3	ZDV-ddI ZDV-3tC	ZDV-3tC-RTV d4T-ddI-SQV-RTV	1 (104)	1	0	1	0	−	PR: ND RT: M41L, M184V, L210W, T215Y
S1 (8)	1.9	ZDV ZDV-ddI ZDV-3tC	d4T-SQV-RTV-NVP	1 (82)	1	3	1	1	−	PR: none RT: D67N, T69D
T1 (15)	1.8	ZDV-3tC	d4T-ddI-NFV d4T-ddI-LPV/r	4 (101–217)	2	0	0	0	ND	ND
T2 (7)	1.3	None	d4T-3tC-LPV/r d4T-3tC-LPV/r-EFV	1 (110)	1	1	1	0	+	None
V1 (10)	4.3	ddI	d4T-3tC-NFV-EFV	1 (53)	1	2	4	5	NA[f]	None

[a] HAART regimens may have had temporary variations due availability of drugs or other reasons. ZDV, zidovudine; ddI, didanosine; d4T, stavudine; 3tC, lamivudine; ABC, abacavir; SQV, saquinavir; RTV, ritonavir; NFV, nelfinavir; LPV/r, lopinavir-ritonavir combination; NVP, nevirapine; EFV, efavirenz.
[b] = Subject's age at analysis in years.
[c] PR, region of *pol* encoding PR.
[d] RT, region of *pol* encoding RT.
[e] *env*, region of envelope gene encoding C2-V5 portion of gp120 SU protein.
[f] NA*, pre-HAART sequences not available for comparison.
[g] ND, no data due to negative PCR results.

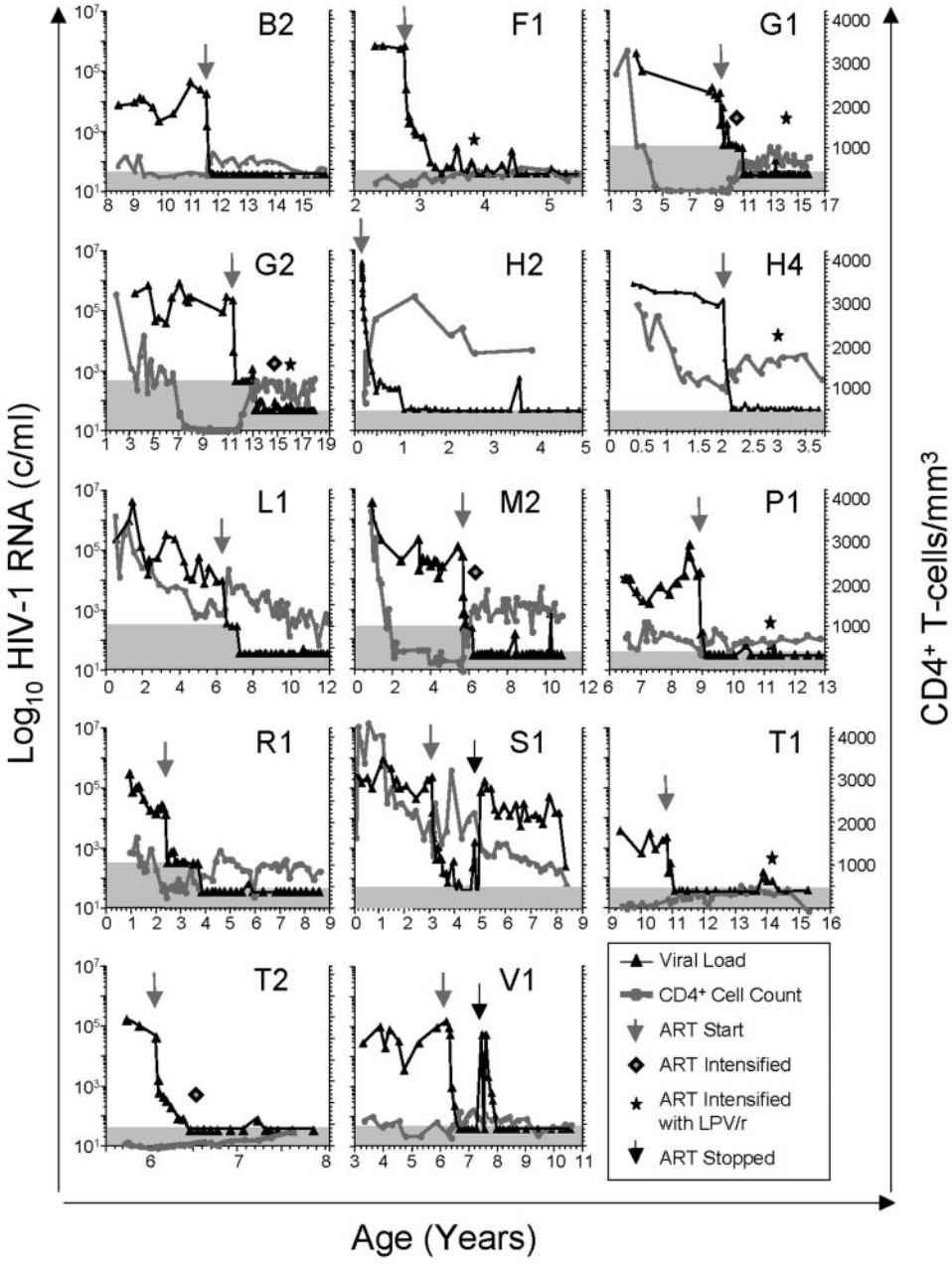


FIG. 1. HIV-1 RNA and CD4 cell levels over time. Plasma HIV-1 RNA and CD4 cell levels of subjects are shown over time. The lower limits of detection of the plasma RNA assays used are shaded. Times at which HAART was initiated, intensified, or stopped are indicated with the symbols defined in the inset. The following very low LLV values of two subjects are not discernible: B2, 51 c/ml at 12.6 years of age; V1, 53 c/ml at 8.5 years of age. LPV/r, lopinavir-ritonavir combination.

374 (median, 72) c/ml. Higher-level viremias were reported in two subjects (1,215 c/ml in M2 and 589 c/ml in H2). However, the latter results could not be confirmed since virus was not amplified from M2's specimen when multiple PCR assays were performed with primers that amplified the subject's PBMC virus, and H2's specimen was not available. Also, LLV were not detected in subsequent specimens from either subject.

Evolutionary divergence of viral sequences. One or more viral sequences were amplified from 21/28 LLV specimens from 11/14 subjects (Table 1). The mean plasma HIV-1 RNA levels were similar in specimens from which virus was (136 c/ml; range, 27 to 330) or was not (149 c/ml; range, 31 to 413) PCR amplified ($P = 0.67$).

Viral sequences from LLV plasma diverged from the inferred MRCA of infection during HAART in 3/11 subjects (Fig. 2, subjects F1, G2, and T2). This divergence continued over time, suggesting ongoing viral replication in two subjects. Viral divergence could not be confirmed in one case (subject T2) because only single plasma PR and RT sequences were amplified from his LLV specimen. Neither LLV plasma nor

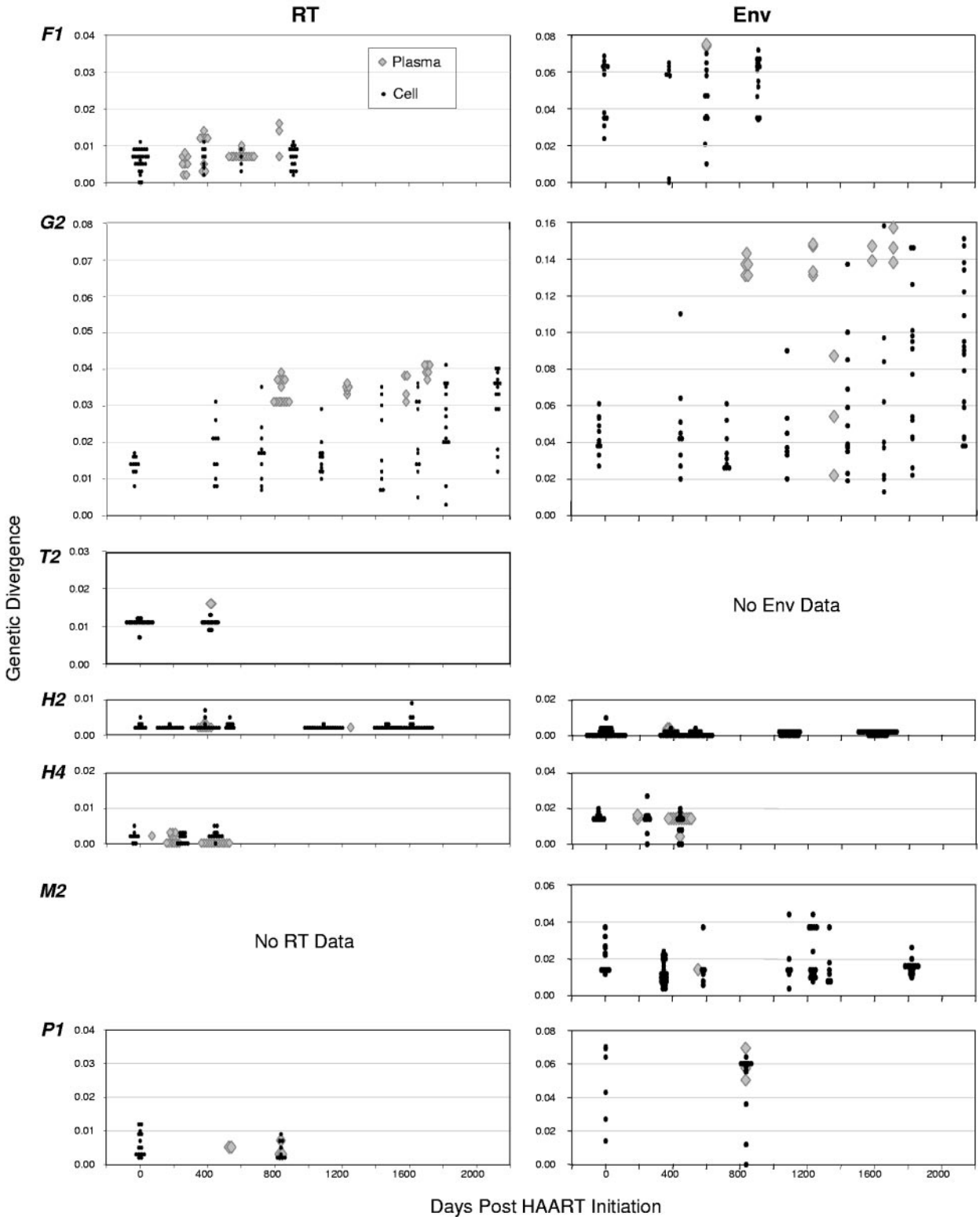


FIG. 2. Genetic divergence from the most recent common ancestor of infection during HAART. Genetic divergence from the MRCA of *pol* encoding RT (left side) and *env* encoding C2-V5 (right side) is shown for representative subjects. The upper three panels (subjects F1, G2, and T2) show viral sequences diverging from the MRCA during HAART in both plasma (gray diamonds) and PBMC (black dots), suggesting ongoing viral replication despite a median plasma HIV-1 RNA level of <50 c/ml. In the lower panels (subjects H2, H4, M2, and P1), plasma and PBMC virus did not diverge from the MRCA, suggesting that viral replication was inhibited by HAART.

TABLE 2. Linear random-effects model to evaluate divergence of PBMC-associated HIV-1 sequence from the MRCA during HAART[a]

Subject	Slope (*P* value)					
	pol PR		*pol* RT		*env C2-V5*	
F1	−0.00012	(0.8)	0.00019	(0.8)	0.00128	(0.5)
G2	0.00210	(<0.00001)	0.00229	(0.00002)	0.00683	(<0.00001)
H2	−0.00022	(0.6)	−0.00008	(0.9)	−0.00107	(0.4)
H4	0.00008	(0.9)	0.00036	(0.7)	−0.00143	(0.6)
L1	−0.00048	(0.3)	−0.00039	(0.5)	−0.00191	(0.2)
M2	0.00002	(1.0)	−0.00036	(0.5)	−0.00076	(0.6)
P1	−0.00023	(0.7)	−0.00026	(0.8)	0.00025	(0.9)
R1	−0.00034	(0.4)	0.00004	(0.9)	−0.00121	(0.4)
S1	−0.00014	(0.9)	−0.00051	(0.6)	−0.00135	(0.6)
T2	0.00010	(0.9)	0.00004	(1.0)	No *env* sequences	

[a] Positive slopes indicate an accumulation of mutations from viral replication, while negative slopes suggest a loss of recently evolved viral sequences from the infected PBMC. In this model, viral replication was detected in the PBMC of G2 only. Positive slopes in *env* suggest immune selection, while positive slopes in *pol* suggest antiretroviral selection.

PBMC viral sequences from the remaining eight subjects diverged from the MRCA during HAART (e.g., subjects H2, H4, M2, and P1 in Fig. 2). The absence of diverging viral sequences in these cases suggests that LLV plasma virus was derived from provirus archived prior to HAART.

Approximately one-third of LLV plasma specimens had multiple identical sequences detected. Identical sequences were found in 5/9 specimens from which five or more RT sequences were derived and 3/11 specimens from which multiple *env* sequences were derived. These sequences were derived by limiting dilution of the specimen and thus not due to resampling of the PCR product. Hence, they are likely to have resulted from virion production after clonal expansion of infected cells.

The divergence of PBMC-associated viral DNA from the MRCA, also indicative of viral replication, was assessed in a linear random-effect regression model. The 10 subjects included in the model had specimens analyzed from immediately pre-HAART and from one or more time points during HAART. Viral divergence during HAART was detected in the PBMC viral DNA of only subject G2 (Table 2) and in all three HIV-1 gene regions evaluated. While the rate of PBMC viral divergence post-HAART in F1 was similar to that in G2 (Fig. 3), the trend in F1 was not statistically significant in the logistic model, presumably due to a shorter period of observation and fewer available sequences.

Relationship between LLV and plasma viral divergence. LLV were most frequent in the two subjects with evolving plasma virus and were detected in sequential specimens of these two subjects. LLV was detected in 9/28 (32%) blood

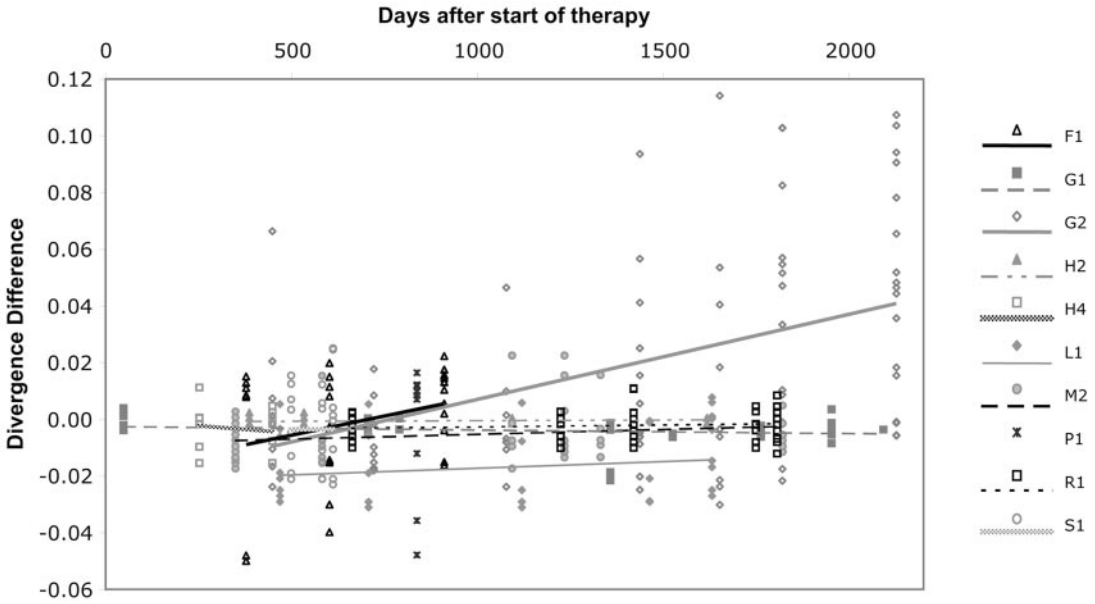


FIG. 3. Divergence of PBMC-associated HIV-1 *env* sequences after initiation of HAART. Viral sequence divergence from the pre-HAART specimen was plotted (vertical axis) for each specimen analyzed during HAART (horizontal axis). The point of reference for calculating divergence was the mean distance of each subject's viral sequences from his or her specimen collected immediately prior to the initiation of HAART. While the divergence slopes for F1 and G2 are similarly positive, suggesting ongoing viral replication, only the slope of G2's data was statistically significant ($P = 0.00002$) in the linear random-effect model, likely due to the greater amount of G2 data and a longer period of observation.

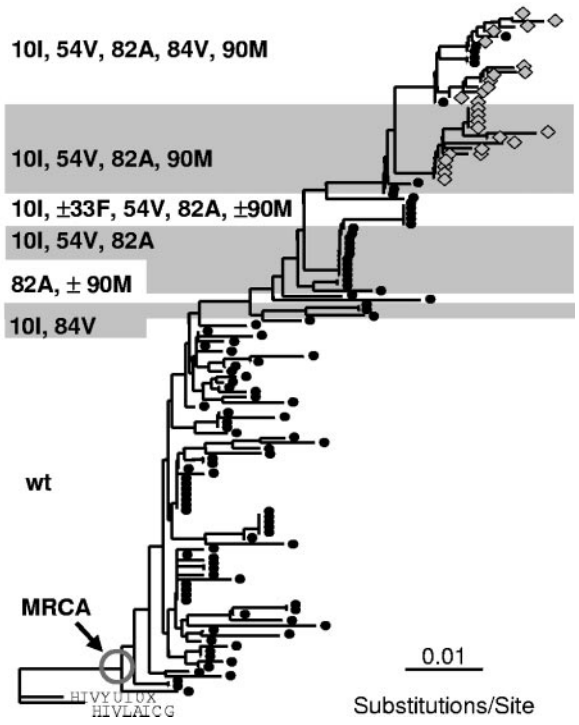


FIG. 4. Neighbor-joining phylogram of *pol* encoding PR with drug-resistant genotypes selected during HAART shown for subject G2. Plasma (gray diamonds) and PBMC (black dots) viral sequence evolution of G2 is shown. Selection of drug-resistant mutants (with codon and encoded amino acid listed) contributed significantly to the evolution from the MRCA (circled). Sequences are rooted to subtype B sequences from GenBank and the percentage of substitutions per site indicated by the horizontal line.

samples from G2 and in 10/24 (42%) samples from F1. LLV were especially common (9/19, 47%) in G2 plasma specimens prior to intensification of his HAART. While these two subjects had higher rates of LLV compared to those of the eight subjects without viral evolution, the difference in LLV frequency was not statistically significant, 2.61 versus 0.77 LLV/year ($P = 0.14$), probably due to the small size of these groups.

Drug resistance mutations in plasma and PBMC viral sequences. Selection of new drug resistance mutations during HAART, a sensitive indicator of ongoing viral replication (19), was detected in the plasma of two subjects, F1 and G2 (Fig. 4). Mutations encoding resistance to nucleoside and nonnucleoside RT inhibitors also appeared to increase in prevalence in G2's PBMC over time (G2's RT sequences are shown in Fig. 5). Drug resistance mutations detected in LLV plasma viral sequences of other subjects (R1 and S1) (Table 1) were all initially selected prior to HAART. (All sequence data are available at http://ubik.microbiol.washington.edu/HIV/Tobin-1/.)

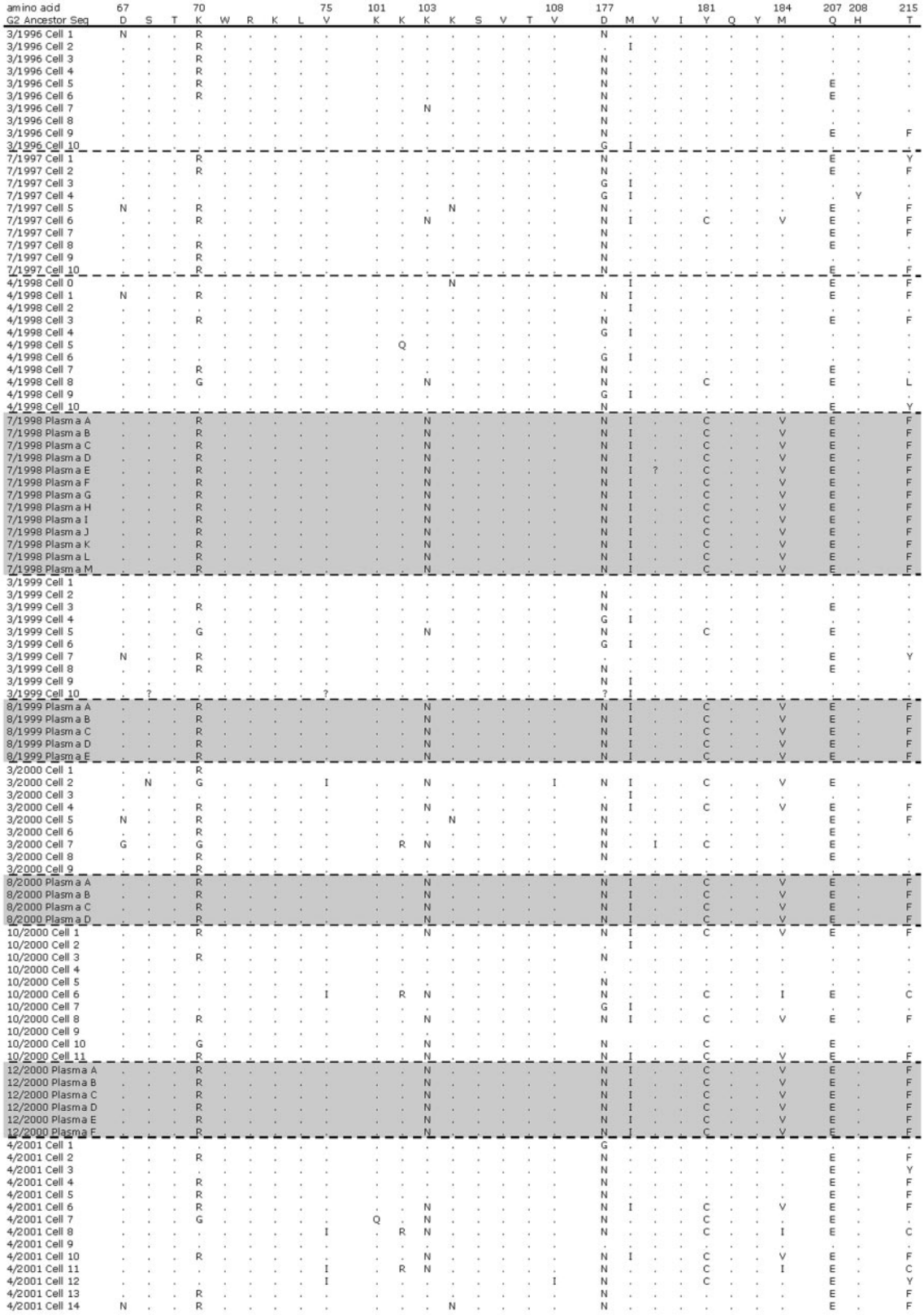
DISCUSSION

The viral genetic studies reported here suggest that LLV can result from two processes, (i) production of virus particles from long-lived cells infected months to years prior to initiation of HAART and (ii) low-level viral replication with selection of new mutants. Only the latter process was associated with outgrowth of HIV-1 resistant to antiretroviral agents.

LLV plasma gene sequences from most subjects in our study were similar to those derived from their PBMC collected prior to the initiation of HAART. Neither viral evolution nor selection of drug-resistant mutants was detected among these subjects. Detection of low levels of virus with genetic sequences typical of that archived ancestral virus during effective HAART have been reported previously (25, 29, 35, 37, 39). However, our unique detection of identical viral sequences during LLV provides strong support for the hypothesis that LLV can result from production of virus following immune activation and clonal expansion of latently infected cells (36). The detection of multiple identical viral sequences within single plasma specimens (e.g., Fig. 2, 11/13 F1 RT sequences at 602 days of HAART and 9/12 H4 *env* sequences at 448 days) suggests that these virions were produced from clonal expansion of activated HIV-1-infected cells. Since derivation of all viral templates was by direct sequencing of the PCR product from plasma diluted to the end point, these identical sequences were not a methodological artifact (33). Importantly, full cycles of infection should be blocked by effective HAART, as PR inhibitors result in production of defective noninfectious virus and RT inhibitors block infection of additional cells. Thus, as observed in several studies (12, 24, 45), LLV during HAART that effectively suppresses viral replication should not increase the risk of virologic failure.

The genetic analyses of LLV sequences from two of our subjects (G2 and F1) demonstrated increases in viral divergence from their MRCA, indicating that viral replication was ongoing during HAART even though their median plasma HIV-1 RNA level was maintained at <50 c/ml. The detection of drug-resistant mutants in all G2's LLV sequences (28/28 RT sequences) but only one of F1's sequences (1/33 RT sequences) suggests that selective pressure from antiretrovirals (4) was greater in G2. Prolonged "nonsuppressive" antiretroviral therapy in G2 prior to the initiation of HAART selected for drug-resistant mutants that conferred partial resistance to his later HAART regimen. Partial resistance to drugs in his HAART probably allowed low-level replication to occur despite unfailing adherence to HAART. In contrast, F1 had periods of nonadherence to HAART. While a sustained viral rebound was not detected, her suspension of HAART presumably allowed viral replication and evolution without selection of drug-resistant mutants. We considered the possibility that F1's median plasma HIV-1 RNA may not have actually been <50 c/ml, due to inaccurate quantification of her subtype D virus by the Amplicor 1.0 RT-PCR assay (28, 50). However, this seemed unlikely given that her HIV-1 RNA was also undetectable in the branched-chain DNA assay sensitive to 75 c/ml plasma, an assay that has been found to adequately quantify HIV-1 subtype D (5).

Two or more serial LLV have been associated with a greater risk of virologic failure of HAART compared to a single LLV (5.8-fold versus 2.0-fold) (21). In our study, serial LLV were detected in the two subjects with evidence of ongoing low-level viral replication (F1 and G2) and in none of the subjects that appeared to express only archived virus. When HAART was intensified in G2 by switching from ritonavir-saquinavir to lopinavir-ritonavir with continuation of the same RT inhibitors, his LLV ceased (0/10 assays with detectable HIV-1 RNA), pre-



amino acid	67			70					75	101	103					108	177				181			184	207	208	215
G2 Ancestor Seq	D	S	T	K	W	R	K	L	V	K	K	K	K	S	V	T	V	D	M	V	I	Y	Q	Y	M	Q	H	T
3/1996 Cell 1	N	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
3/1996 Cell 2	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
3/1996 Cell 3	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
3/1996 Cell 4	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
3/1996 Cell 5	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	.
3/1996 Cell 6	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	.
3/1996 Cell 7	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
3/1996 Cell 8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
3/1996 Cell 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
3/1996 Cell 10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	.	.
7/1997 Cell 1	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	Y
7/1997 Cell 2	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
7/1997 Cell 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	.	.
7/1997 Cell 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	Y	.
7/1997 Cell 5	N	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
7/1997 Cell 6	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
7/1997 Cell 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
7/1997 Cell 8	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	.
7/1997 Cell 9	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
7/1997 Cell 10	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
4/1998 Cell 0	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	E	.	F
4/1998 Cell 1	N	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	I	.	.	.	.	.	.	E	.	F
4/1998 Cell 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
4/1998 Cell 3	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
4/1998 Cell 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	.	.
4/1998 Cell 5	.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4/1998 Cell 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	.	.
4/1998 Cell 7	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	.
4/1998 Cell 8	.	.	.	G	.	.	.	.	.	.	N	.	.	.	.	.	.	N	.	.	.	C	.	.	.	E	.	L
4/1998 Cell 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	.	.
4/1998 Cell 10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	Y
7/1998 Plasma A	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
7/1998 Plasma B	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
7/1998 Plasma C	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
7/1998 Plasma D	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
7/1998 Plasma E	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	?	.	C	.	.	V	E	.	F
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7/1998 Plasma J	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
7/1998 Plasma K	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
7/1998 Plasma L	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
7/1998 Plasma M	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
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3/1999 Cell 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
3/1999 Cell 3	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	.
3/1999 Cell 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	.	.
3/1999 Cell 5	.	.	.	G	.	.	.	.	.	.	N	.	.	.	.	.	.	N	.	.	.	C	.	.	.	E	.	.
3/1999 Cell 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	.	.
3/1999 Cell 7	N	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	Y
3/1999 Cell 8	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	.
3/1999 Cell 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	I	.	.	.	.	.	.	.	.	.
3/1999 Cell 10	.	?	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	?	I	.	.	.	.	.	.	.	.	.
8/1999 Plasma A	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
8/1999 Plasma B	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
8/1999 Plasma C	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
8/1999 Plasma D	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
8/1999 Plasma E	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
3/2000 Cell 1	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3/2000 Cell 2	.	N	.	G	.	.	.	.	I	.	.	N	.	.	.	.	I	N	I	.	.	C	.	.	V	E	.	.
3/2000 Cell 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
3/2000 Cell 4	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
3/2000 Cell 5	N	.	.	R	.	.	.	.	.	.	.	.	N	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
3/2000 Cell 6	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	.
3/2000 Cell 7	G	.	.	G	.	.	.	.	.	.	R	N	.	.	.	.	.	N	.	I	.	C	.	.	.	E	.	.
3/2000 Cell 8	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	.
3/2000 Cell 9	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8/2000 Plasma A	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
8/2000 Plasma B	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
8/2000 Plasma C	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
8/2000 Plasma D	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
10/2000 Cell 1	.	.	.	R	.	.	.	.	.	.	N	.	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
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10/2000 Cell 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
10/2000 Cell 6	.	.	.	.	.	.	.	.	I	.	R	N	.	.	.	.	.	N	.	.	.	C	.	.	I	E	.	C
10/2000 Cell 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	I	.	.	.	.	.	.	.	.	.
10/2000 Cell 8	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
10/2000 Cell 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10/2000 Cell 10	.	.	.	G	.	.	.	.	.	.	.	N	.	.	.	.	.	N	.	.	.	C	.	.	.	E	.	.
10/2000 Cell 11	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
12/2000 Plasma A	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
12/2000 Plasma B	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
12/2000 Plasma C	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
12/2000 Plasma D	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
12/2000 Plasma E	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
12/2000 Plasma F	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
4/2001 Cell 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
4/2001 Cell 2	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
4/2001 Cell 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	Y
4/2001 Cell 4	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
4/2001 Cell 5	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
4/2001 Cell 6	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
4/2001 Cell 7	.	.	.	G	.	.	.	.	.	Q	.	N	.	.	.	.	.	N	.	.	.	C	.	.	.	E	.	.
4/2001 Cell 8	.	.	.	.	.	.	.	.	I	.	R	N	.	.	.	.	.	N	.	.	.	C	.	.	I	E	.	C
4/2001 Cell 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4/2001 Cell 10	.	.	.	R	.	.	.	.	.	.	.	N	.	.	.	.	.	N	I	.	.	C	.	.	V	E	.	F
4/2001 Cell 11	.	.	.	.	.	.	.	.	I	.	R	N	.	.	.	.	.	N	.	.	.	C	.	.	I	E	.	C
4/2001 Cell 12	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	N	.	.	.	C	.	.	.	E	.	Y
4/2001 Cell 13	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F
4/2001 Cell 14	N	.	.	R	.	.	.	.	.	.	.	.	N	.	.	.	.	N	.	.	.	.	.	.	.	E	.	F

FIG. 5. Amino acid alignment of HIV-1 *pol* sequences encoding RT of subject G2. An alignment of a subset of G2 viral sequences is shown to demonstrate the increasing prevalence of mutants over time in PBMC. Sequences are shown in an abbreviated format to feature amino acids that confer resistance to antiretroviral drugs (nucleoside and nonnucleoside RT inhibitors). Dots indicate amino acid identity to the subject's MRCA (G2 Ancestor Seq). Sequences from PBMC are labeled with specimen dates and cell and sequence indicators, and plasma sequences are similarly labeled with dates and plasma and sequence indicators.

cluding determination of whether virologic failure would have eventually ensued under his initial HAART regimen.

Selection of mutant virus during HAART has not always resulted in virologic failure (8, 19). Virologic failure is most likely modulated by multiple interacting parameters, including the genetic and pharmacologic barriers that each regimen imposes on the selection of "fit" drug-resistant mutants. Of note, our subjects with multiple and sequential episodes of LLV, F1 and G2, were treated with four to six antiretroviral agents that should have provided greater genetic and pharmacologic barriers compared to commonly prescribed three-drug HAART regimens. These relatively high genetic and pharmacologic barriers may have contributed to G2 not experiencing virologic failure even though several new primary drug resistance mutations (27) were selected, as detected in his plasma and PBMC during HAART.

LLV could not be confirmed in one-quarter of our subjects' LLV plasma specimens. The LLV in these instances could have been due to false-positive HIV-1 RNA quantification results, usually near the lower assay limits of detection, or incorrect reporting of results by the laboratory. The former appears likely given that the reproducibility of detection of LLV has varied in others studies, with a range of 41 to 97% (26, 41). If a large percentage of LLV are false positives, then clinical studies would be biased to underestimate the risk of virologic failure associated with LLV.

An important caveat to our and others' studies is that following long-term effective HAART an evaluation of sequence divergence could theoretically fail to identify low-level viral replication. The viral genotypes that persist in PBMC after long-term suppression of viral replication date mostly to early infection, with more recently evolved viruses persisting to a relatively lesser degree (19, 39, 44, 46). Virions produced by these archived viral sequences, and infecting additional cells, may not demonstrate sufficient divergence from the MRCA to be recognized as evolving virus (19). Indeed, full cycles of viral replication, even when plasma viremia is undetectable by current assays, may account for the persistence of the latent PBMC reservoir (29, 39). We have shown that sequence divergence and phylogenetic analysis of PBMC-associated virus were less sensitive in gauging low-level viral replication compared to measuring shifts in the size of drug-resistant viral populations in PBMC (19). Prior studies suggest that viral replication may be more readily detected by divergence of plasma viral RNA compared to PBMC-associated viral DNA (19, 39, 44, 46). Here, we did not directly compare the sensitivity of increases in plasma HIV-1 genetic diversity to increases in the size of drug-resistant viral populations in PBMC to gauge low-level viral replication during effective HAART.

Production of identical viral sequences in plasma provided strong evidence that LLV resulted from activation and proliferation of one or more cells infected concomitantly in the past, whereas LLV with ancestral but nonidentical sequences could have resulted from activation of multiple persistently infected PBMC or similar reservoirs of archived virus (6, 15, 30). However, for the reasons mentioned above, we cannot exclude the possibility that the latter instances of LLV resulted from low-level viral replication that was below the sensitivity of our genetic analyses.

The long-term efficacy of HAART would most likely differ, depending on which of the two processes shown here predominate—production of virus from a subset of long-lived infected cells or ongoing viral evolution resulting from new rounds of infection. Furthermore, the inconsistent association of LLV with subsequent virologic failure of HAART in studies of large populations (21, 22, 24, 45) may be due to a greater propensity for one or the other of these processes. Assays that differentiate LLV resulting from expression of ancestral virus from those derived from ongoing productive cycles of viral replication may therefore be of clinical value.

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