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Residual Human Immunodeficiency Virus (HIV) Type 1 RNA and DNA in Lymph Nodes and HIV RNA in Genital Secretions and in Cerebrospinal Fluid after Suppression of Viremia for 2 Years

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Residual viral replication persists in a significant proportion of human immunodeficiency virus (HIV)–infected patients receiving potent antiretroviral therapy. To determine the source of this virus, levels of HIV RNA and DNA from lymphoid tissues and levels of viral RNA in serum, cerebrospinal fluid (CSF), and genital secretions in 28 patients treated for ≤2.5 years with indinavir, zidovudine, and lamivudine were examined. Both HIV RNA and DNA remained detectable in all lymph nodes. In contrast, HIV RNA was not detected in 20 of 23 genital secretions or in any of 13 CSF samples after 2 years of treatment. HIV envelope sequence data from plasma and lymph nodes from 4 patients demonstrated sequence divergence, which suggests varying degrees of residual viral replication in 3 and absence in 1 patient. In patients receiving potent antiretroviral therapy, the greatest virus burden may continue to be in lymphoid tissues rather than in central nervous system or genitourinary compartments.

The seeding of lymphoid tissues and the propagation of virus by CD4+ cells (principally T lymphocytes) is established early in the course of infection for both human immunodeficiency virus (HIV) and experimental models of simian immunodeficiency virus infection [1–4]. Throughout subsequent years of

infection, the lymphoid tissues support most HIV replication, and trapping of virus within the follicular dendritic cell (FDC) network represents the bulk of the virus burden in the bodies of untreated patients [5, 6]. The response to antiviral therapy of virus and provirus present in this critical tissue compartment is of paramount importance to the long-term success of any treatment strategy. Thus, previous demonstrations of the rapid initial reduction of lymphoid tissue virus load in response to highly active antiretroviral therapy (HAART) over periods of 6 months to 1 year were encouraging [7–10].

However, several studies now suggest that incomplete suppression of replication occurs in ≥50% of patients who achieve targeted reductions of plasma virus load to <50 HIV RNA copies/mL, as ascertained by evidence of sequence evolution [11, 12], detection of spliced mRNA [13], or unintegrated viral DNA [14, 15]. In 2 studies, evidence for incomplete suppression, based on sequence evolution or failure of reduction in the frequency of latently infected cells, seemed to correlate with the sporadic reappearance of very low levels of detectable viremia while receiving therapy or with a less rapid decay of plasma viremia during initiation of therapy [11, 16]. Of importance, with the exception of one study that examined lymph node (LN) samples from 2 patients [12], the studies that suggested incomplete viral suppression on HAART were based primarily on studies of peripheral blood and did not directly examine the possibility that replication occurs in anatomically isolated sites, although such a scenario has been suggested [17]. Two candi-

date sites are the central nervous system (CNS) and genitourinary (GU) tract, which may harbor genetically discrete virus variants with the added complexity of variable access for antiviral compounds [18–21]. In the formulation of more effective treatment strategies, it will be necessary to determine whether complete suppression of viral replication will depend on treatments with greater activity in the blood and lymphoid compartments or on the introduction of new drugs with better CNS or GU tract penetration or both.

The present study was undertaken to ascertain whether the early phase clearance of virus from lymphoid tissues, cerebrospinal fluid (CSF), and genital secretions are sustained during long-term therapy and to determine which, if any, of these tissue reservoirs contribute to residual viral replication. Viral RNA and DNA levels from LN biopsy specimens and viral RNA from genital secretions and CSF were evaluated in patients who had achieved reductions of virus load to <50 copies/mL for ≤2.5 years while receiving potent antiretroviral therapy ($n =$ 25). An additional 3 patients had sustained virus loads >500 copies/mL at the time of study during treatment with indinavir (Idv), zidovudine (Zdv), and lamivudine (3TC). In a subset of the LN biopsy specimens, we used in situ hybridization (ISH) with quantitative image analysis (QIA), to identify residual viral RNA in individual cells or the FDC network. For 4 patients, partial HIV-1 *env* sequences from baseline plasma and year 1 or year 2 LN biopsy specimens or both could be evaluated for evidence of sequence evolution as an indicator of ongoing viral replication. We believe that the present work represents the first comprehensive study that attempts to simultaneously investigate virus load in 4 different compartments in a well-characterized study population receiving successful long-term antiretroviral therapy [22] with validated assays [23].

Methods

Patients. This study cohort comprised 28 participants from the Merck 035 study [22, 24, 25]. Patients were randomized initially to either Idv ($n = 7$), Zdv plus 3TC ($n = 3$), or Idv, Zdv, plus 3TC ($n = 18$). After 6–12 months, patients were switched to open-label Idv, Zdv, plus 3TC. Patients were asked to volunteer for the present study when serum HIV RNA levels were <500 copies/mL for ≥72 weeks. Patients had study visits at least every 4 weeks through week 52 and every 8 weeks thereafter. At the time of lumbar puncture, LN biopsy specimens or genital secretions were obtained, and 3 patients showed therapeutic failure with high serum RNA levels (group 3; table 1). LN biopsy specimens were obtained from 20 patients, according to published procedures [8]. A subset of these patients ($n = 10$) and an additional 3 patients had CSF evaluations ($n = 13$). For 16 patients with LN biopsy specimens and for an additional 7 without LN biopsy specimens, we evaluated virus loads in genital secretions ($n = 23$). Ten patients participated in all 3 substudies (LN, CSF, and GU).

Quantitation of HIV RNA and DNA. Serum HIV RNA levels were measured by the Amplicor HIV Monitor test (Roche Diagnostic Systems), as described elsewhere [22]. If the result from this assay was <500 copies/mL, we did the Roche ultrasensitive assay, with a detection limit of 50 HIV RNA copies/mL [25]. Tissue RNA and DNA quantitation and extraction methods have been described in detail elsewhere [8]. The Roche ultrasensitive assay was adapted to RNA extracted from LN tissues. RNA extracts were treated with DNase and RNA was re-extracted. HIV RNA was normalized to T cell receptor (TCR) Cα mRNA. LN HIV proviral DNA was quantitated by a semiquantitative polymerase chain reaction (PCR) assay for HIV-1 *gag* after RNase treatment and was controlled by using PCR assay for β-actin. LN samples were deemed to be inadequate for RNA quantitation when TCR Cα RNA was <12,500 RNA copies, which is the equivalent of 500 T cells. CSF RNA was measured by applying the ultrasensitive version of the Amplicor assay to 500 μL of CSF, according to the manufacturer's protocols for serum [23, 28]. Genital secretions were processed, as described in published protocols [29–31], and HIV-1 RNA was quantitated on cell-free fractions by the NucliSens assay (Organon Teknika). We assayed seminal fluid from male patients and vaginal swab, vaginal lavage washings, and cervical swab specimens from female patients. Among the 3 patients with detectable virus load in GU secretions, 1 was from seminal plasma, 1 was from vaginal lavage, and 1 was from a cervical swab. Paraformaldehyde-fixed LN samples were analyzed by ISH with QIA [9].

Clonal sequencing. Amplification of *env* sequences was attempted from LN specimens of 8 patients. PCR amplification was positive in 6 of the 8; however, we excluded 2 of these 6 patients from further evaluation because baseline sequences were contaminated with pNL4-3 sequences in 1 and because a rebound of serum HIV RNA occurred just before the LN biopsy in the other. Four patients were included for final analysis (3 LN biopsies at years 1 and 2 and 1 LN biopsy at year 1). After reverse transcription, a 350-bp fragment that included the C2 and V3 region of HIV-1 *env* was PCR amplified in quadruplicate from baseline serum and RNA extracts from LN biopsy specimens [11]. PCR amplifications on serum and LN extracts were done in separate experiments, to minimize intrapatient contamination, which would be difficult to exclude by phylogenetic analysis. Quadruplicate nested PCR products were pooled and cloned into the TOPO TA cloning system (Invitrogen), and 10–18 clones per pooled PCR sample were selected and amplified in culture. Plasmid DNA was extracted and was bidirectionally sequenced by use of Big Dye dideoxy terminator cycle sequencing [11].

Sequence analysis. For each patient, the total diversity within each population and the total and net divergence (total divergence minus the average within-population diversity) between each sampled population was calculated by assuming a Tamura and Nei [32] model of substitution, with SEs calculated from 1000 bootstrap replicates with the SENDBS computer program [33]. Maximum likelihood trees under a Tamura-Nei model were obtained by using a heuristic search implemented in PAUP 4.0 [34] and other methods.

Estimation of residual viral replication. Residual viral replication was expressed as the net area under the curve (AUC) of serum HIV RNA over time for patients 2, 3, 19, and 27. AUC net was calculated by subtracting a predicted AUC, according to the long-lived infected cell model of Perelson et al. [35], from the AUC observed, as calculated by summation of trapezoids implemented in the program PRISM 2.0. (Graphpad), as described elsewhere in detail [11].

Nucleotide sequence accession numbers. All sequences reported

Table 1. Patient characteristics and results of bulk human immunodeficiency virus (HIV) RNA and DNA measurements in lymph nodes (LNs), cerebrospinal fluid (CSF), and genitourinary (GU) secretions.

Group, patient[a]	Therapy[b]	Log_{10} HIV RNA copies/mL of serum at baseline	Log_{10} HIV RNA copies/mL of serum at year 2	Time suppressed at year 2	Log_{10} HIV RNA copies/ 4×10^6 LN/TCR Cα mRNA at year 2	HIV DNA copies/100 ng in LN at year 1[c]	HIV DNA copies/100 ng in LN at year 2	HIV RNA copies/mL of CSF at year 2	HIV RNA copies/mL of GU secretions at year 2
Group 1									
1 (A)	Zdv/3TC/Idv	5.15	<1.69	2	2.20	++	++	NA	<400
2 (C)	Zdv/3TC/Idv	5.23	<1.69	2	3.10	+++	++	NA	<400
3 (B)	Zdv/3TC/Idv	4.74	<1.69	2	2.40	+++	+	NA	470
4 (N)[d]	Zdv/3TC/Idv	4.43	1.88	2	2.11	NA	++	NA	NA
5	Zdv/3TC/Idv	4.38	<1.69	2	2.50	NA	++	<50	<400
6	Zdv/3TC/Idv	5.09	<1.69	2	0[e]	NA	++[f]	<50	<400
7	Zdv/3TC/Idv	4.73	1.77	2	2.74	NA	++	<50	<400
8	Zdv/3TC/Idv	4.17	<1.69	2	2.87	NA	++	<50	<400
9 (L)	Idv	4.14	<1.69	2	2.43	NA	++	NA	NA
10	Zdv/3TC/Idv	4.52	<1.69	2	2.87	NA	NA[g]	<50	<400
11	Zdv/3TC/Idv	4.95	1.92	2	4.73	NA	NA[g]	<50	<400
12	Zdv/3TC/Idv	4.79	<1.69	2	4.19	NA	NA[g]	<50	<400
13	Zdv/3TC/Idv	4.41	<1.69	2	0[e]	NA	NA[g]	<50	<400
14	Idv	3.84	<1.69	2	NA	NA	NA	<50	NA
15	Zdv/3TC/Idv	5.34	2.93	2	NA	NA	NA	<50	1600
16	Idv	4.21	<1.69	2	NA	NA	NA	<50	<400
17	Zdv/3TC/Idv	4.44[h]	<1.69	2	NA	NA	NA	NA	<400
18[d]	Zdv/3TC/Idv	4.98	<1.69	2	NA	NA	NA	NA	NA
Group 2									
19 (I)	Zdv/3TC	5.12	<1.69	1	3.18	+++	++	NA	450
20 (O)	Zdv/3TC	4.35	<1.69	1	3.40	NA	++	NA	<400
21	Idv	5.00	<1.69	1	0[e]	NA	NA[g]	NA	<400
22	Idv	4.36	<1.69	1	0[e]	NA	NA[g]	<50	<400
23	Zdv/3TC/Idv	3.88	<1.69	1	NA	NA	NA	NA	<400
24	Idv	5.00	<1.69	1	NA	NA	NA	NA	<400
25[i]	Zdv/3TC/Idv	4.01	<1.69	2	NA	NA	NA	NA	<400
Group 3									
26	Idv	4.15	3.38	0	5.77	NA	++	<50	<400
27 (G)	Zdv/3TC	4.95	3.25	0	6.38	++++	+++	NA	NA
28	Zdv/3TC/Idv	4.65	3.31	0	6.76	NA	NA	NA	NA

NOTE. Results of LN DNA quantitation are expressed as follows: +, <1 HIV DNA copy/100 ng of genomic DNA (but detectable on analysis of 400 ng of genomic DNA); ++, 1–10 copies; +++, 10–50 copies; ++++, >50 copies. 3TC, lamivudine; Idv, indinavir; NA, not available; TCR, T cell receptor; Zdv, zidovudine.

[a] Capital letters indicate patient designations used elsewhere [8, 11, 26, 27].

[b] Therapy during initial 6–12 months of study. After 6–12 months of study, treatment was changed to Zdv/3TC/Idv for all patients.

[c] HIV DNA from year 1 from patients 1, 2, 3, 19, and 27 were reported elsewhere, corresponding to designations A, B, C, G, and I [8].

[d] At week 28, patient 4 showed a transient serum HIV RNA level of 35,140 copies/mL, but serum RNA was not detectable 5 days later. Patient 18 had a transient RNA level of 7850 copies/mL. Both transient RNA levels probably were erroneous.

[e] LN samples from these patients had no measurable TCR Cα message (<500 T cell equivalents), rendering HIV quantitation invalid.

[f] Despite insufficient cellularity of HIV RNA, HIV DNA was detected from different tissue section of same LN.

[g] HIV DNA was not assessed because of insufficient tissue for DNA extraction.

[h] At baseline, no serum RNA level was available; value was obtained at week 2 after initiation of therapy.

[i] In patient 25, HIV RNA values at weeks 28, 32, 36, 40 were 53,840, 1480, 600, and 128 copies/mL, respectively. Viral rebound was due to therapy interruption. Subsequent RNA levels up to week 124 were undetectable, apart from 149 copies/mL at week 68.

Table 2. Comparison of year 1 and year 2 human immunodeficiency virus type 1 (HIV-1) RNA concentrations between bulk reverse transcription–polymerase chain reaction and in situ hybridization with quantitative image analysis.

| | | | In situ hybridization | | | |
| | | | Log HIV-1 RNA copies/g of lymphoid tissue | | Log frequency of mononuclear cells/g of lymphoid tissue | |
Patient[a]	Approximate years of negative viremia	Bulk quantitation, log HIV-1 RNA copies/g of lymphoid tissue	LN FDC	LN MNC	With >20 RNA	With few RNA
1 (A)	2	3.50	<4		—	4.4
3 (B)	2	4.20	<4		—	3.7
2 (C)	2	4.80	<4		—	<3
9 (L)	2	3.69	<4		—	<3
4 (N)	2	3.19	<4		—	<3
Mean 2 year		3.88				
1 (A)[b]	1	4.85	<4		—	3.1
3 (B)[b]	1	4.85	<4		—	<3
2 (C)[b]	1	5.19	<4		—	<3
19 (I)	1	4.50	<4		—	<3
21 (O)	1	5.89	<4		—	<3
Mean 1 year		5.06				
27 (G)[b]	0	8.28	7.3	6.2	4.6	—
27 (G)	0	7.63	5	5.3	3.7	—
19 (I)[b]	0	6.57	NA	NA	NA	NA
Mean unsuppressed		7.49				

NOTE.   FDC, follicular dendritic cells; LN, lymph node; MNC, mononuclear cells; NA, not available.
[a] Capital letters indicate patient designations reported elsewhere [8, 11, 26, 27].
[b] Samples analyzed in earlier phase of study and reported elsewhere [8].

were deposited in GenBank (accession nos. AF185840–AF185849, AF185863–AF185872, and AF337215–AF337318).

Results

Patient classification.   For the analysis, patients were stratified into 3 groups, according to the level of suppression of serum viremia: group 1 included 18 patients with suppression of serum viremia to below the level of detection (<50 copies/mL) for ⩾2 years before the present study. At the approximate time that LN biopsy specimens and CSF and GU secretions were obtained, 4 group 1 patients had detectable viremia at low levels (table 1); these subsequently became undetectable. Each of these 4 and 6 additional group 1 patients had the blood virologic response pattern of good long-term suppression but with intermittent low-level viremia that has been described elsewhere [16, 36]. Group 2 comprised 7 patients who had undetectable serum HIV RNA levels for ∼1 year before the study. Group 3 consisted of 3 patients with detectable (range, 1500–2400 HIV RNA copies/mL) serum viremia at year 2.

LN HIV-1 RNA.   After 2 years of therapy, LN biopsy specimens were obtained from 20 patients (table 1). Samples from 4 patients (group 1, 2; group 2, 2) had insufficient cellularity for reliable quantitation, as measured by TCR Cα mRNA (<12,500 copies or ∼500 T cell equivalents). In 3 group 1 patients, LN biopsy specimens had been obtained previously after 1 year of therapy [8].

HIV RNA was quantifiable from LNs with sufficient cellularity from all 16 patients, including those with the most sustained suppression of serum viremia (range, 2.2–6.8 $\log_{10}$ copies/

4×10^6 copies of TCR Cα mRNA). HIV RNA levels were similar among patients with suppression of viremia (<50 copies/mL) for 1 year (mean HIV RNA level, 3.3 $\log_{10}$ copies/4×10^6 copies of TCR Cα mRNA) and 2 years (mean HIV RNA level, 2.9 $\log_{10}$ copies/4×10^6 copies of TCR Cα mRNA; table 1). Furthermore, LN HIV RNA levels did not change in patients sampled at both years 1 and 2 (table 2). Among group 1 patients, LN HIV RNA concentrations did not differ significantly between those with or without intermittent viremia after initial reduction of viremia to <50 copies/mL (data not shown).

ISH and QIA demonstrated no FDC-associated virus at a limit of detection of 4 $\log_{10}$ copies/g of tissue for patients with suppression of viremia for 1 or 2 years. However, rare mononuclear cells associated with low copy numbers of HIV RNA were detectable in 2 of 5 patients with <50 copies/mL of serum for ⩽2 years and in 1 of 5 patients with undetectable viremia after 1 year (table 2). These cells were shown previously to be of lymphocytic lineage [3, 8].

LN HIV-1 DNA.   Proviral HIV DNA was detectable in all LN biopsy specimens with sufficient cellularity (range, 1–147 HIV DNA copies/100 ng of total genomic DNA). A cross-sectional analysis between patients with serum viremia suppressed for 1 or 2 years revealed no difference in proviral DNA levels (table 1). Yet, in 2 of 3 group 1 patients (patients 2 and 3; table 1) from whom sequential LN biopsy specimens were obtained at both years 1 and 2, second-year HIV DNA levels were lower than first-year levels.

CSF HIV RNA.   In CSF of 13 patients at year 2 after initiation of potent therapy, none had detectable HIV RNA

(<50 copies/mL). In 9 of these patients, HIV serum RNA was suppressed below the limit of detection (50 copies/mL) at the time point when CSF samples were obtained.

Genital secretion HIV RNA. Genital secretions were examined from 23 patients (21 men and 2 women). Between years 1 and 2, 22 of these patients had undetectable serum viremia (<50 copies/mL). In 1 patient, HIV serum RNA was intermittently detectable at low levels. Three of 23 patients had detectable HIV RNA in cell-free genital secretions (1 of 21 men and 2 of 2 women) in initial collections. The man had undetectable viremia for 1 year, whereas both women had suppression of viremia for 2 years. Repeat assays on samples from these 3 patients obtained 4–6 months later did not reveal detectable HIV RNA.

Evolution of HIV env. To characterize further the residual HIV RNA from LNs at years 1 and 2, the C2-V3 region of HIV-1 *env* of RNA extracted from LNs from 3 patients with undetectable HIV serum RNA at year 2 (patients 2 and 3 in group 1; patient 19 in group 2) was amplified and sequenced. In addition, patient 27, who had detectable RNA during most of the observation period, served as a positive control for continuous viral replication. Figure 1 shows longitudinal HIV serum RNA levels of these 4 patients.

We did phylogenetic analysis by using the neighbor-joining method with all sequences generated, including a number of laboratory strains to screen for contamination (NL4-3, YU2, JRFL, SF 162, NY5, BAL, JRCSF, HXB2, and ADA; data not shown). To determine the extent of evolutionary divergence of *env* between sampled populations during HAART, we estimated net nucleotide divergence between the subpopulations (total divergence minus average within-sample diversity). Net divergence more accurately reflects the amount of evolutionary divergence between populations than total divergence. When there is within-sample diversity, total divergence may be >0, even if no evolution has occurred. Nucleotide divergence between baseline clonal sequences and LN sequences obtained at years 1 and 2 revealed no discernible evolution for patient 3 (table 3). However, patient 2, who had a slower RNA decay and 1 intermittently detectable HIV RNA level during the first year of therapy showed evidence of evolution by total divergence but none when net divergence was analyzed. Patient 19 showed evolution between baseline plasma sequences and year 1 and an increase of net divergence at year 2 in the absence of any discernible serum viremia. A maximum likelihood tree revealed, however, that LN year 2 sequences were phylogenetically older than plasma sequences, which suggests that these
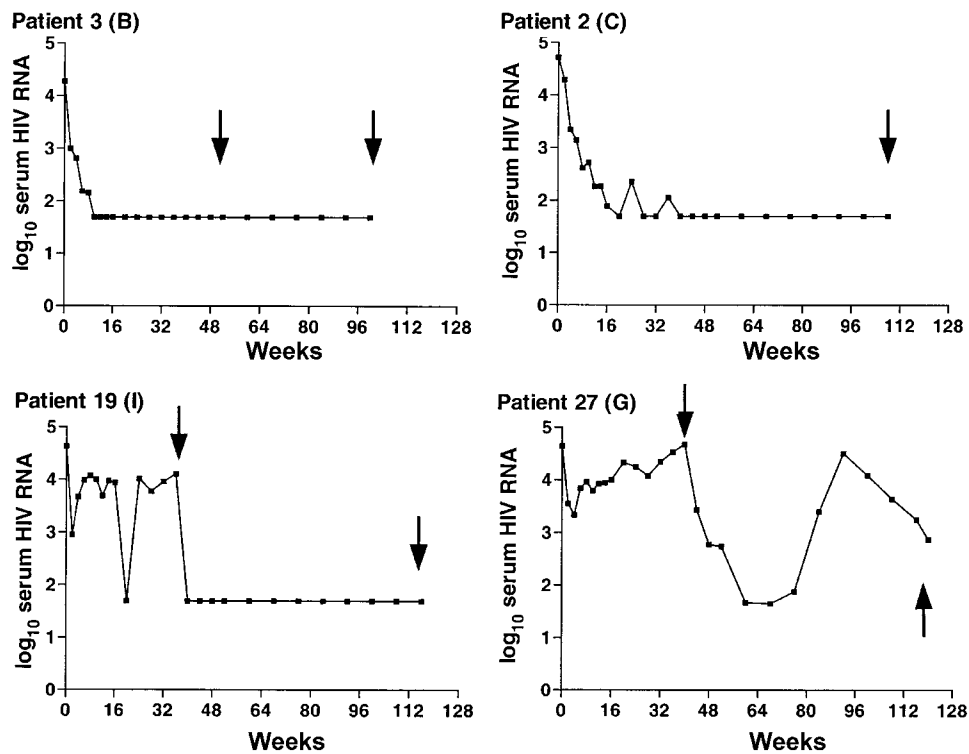
Figure 1. Serum human immunodeficiency virus type 1 (HIV-1) RNA from 4 patients with available clonal HIV *env* sequences plotted against time of treatment with indinavir (Idv), zidovudine (Zdv), and lamivudine (3TC), according to Merck 035 study protocol. Serum HIV RNA concentrations were measured by Roche ultrasensitive assay (limit of detection, 50 copies/mL). Antiretroviral treatment was initiated at week 0. Arrows indicate time points when inguinal lymph node biopsy specimens were obtained. Patient designations 3, 2, 19, and 27 correspond to patients B, C, I, and G, respectively, as reported elsewhere [8, 11, 26, 27]. Patients 19 and 27 received Zdv/3TC for the first 40 weeks and then Zdv/3TC/Idv for the remaining study.

Table 3. Total and net nucleotide divergence from human immunodeficiency virus (HIV)–1 *env* sequences obtained from plasma and lymph nodes (LNs) from 4 patients, by the Tamura-Nei model of substitution.

Patient, populations compared[a]	Percentage total divergence (SE)	Percentage net divergence (SE)
3 (B)		
Plasma year 0 vs. LN year 1	0.45 (0.16)	−0.03 (0.10)
Plasma year 0 vs. LN year 2	0.49 (0.20)	0.03 (0.20)
LN year 1 vs. LN year 2	0.42 (0.18)	0.09 (0.17)
2 (C)		
Plasma year 0 vs. LN year 2 (a)	3.40 (0.63)	0.00 (0.10)
Plasma year 0 vs. LN year 2 (b)	3.72 (0.61)	0.01 (0.08)
LN year 2 (a) vs. LN year 2 (b)	3.21 (0.58)	0.04 (0.08)
19 (I)		
Plasma year 0 vs. LN year 1	3.23 (0.55)	0.39 (0.15)
Plasma year 0 vs. LN year 2	2.43 (0.60)	1.12 (0.63)
LN year 1 vs LN year 2	3.29 (0.67)	1.38 (0.68)
27 (G)		
Plasma year 0 vs plasma year 2	2.98 (0.65)	1.18 (0.45)
Plasma year 0 vs LN year 1	3.47 (0.65)	0.42 (0.17)
Plasma year 0 vs LN year 2	4.01 (0.73)	1.39 (0.45)
LN year 1 vs. plasma year 2	3.76 (0.81)	1.89 (0.57)
LN year 2 vs plasma year 2	1.54 (0.38)	0.10 (0.15)
LN year 1 vs. LN year 2	4.83 (0.87)	2.14 (0.59)

NOTE. Nos. "1" and "2" indicate no. of years after initiation of potent antiretroviral therapy when LN biopsy or plasma sample was obtained. "(a)" and "(b)" indicate clonal sequences obtained from different extractions from 2 different fragments of patient C LN at year 2.

[a] Patient designations 3, 2, 19, and 27 correspond to B, C, I, and G, respectively, as reported elsewhere [8, 11, 26, 27].

RNA transcripts originated from a cell population containing archival HIV variants, compared with baseline plasma sequences (figure 2). As expected, in patient 27, who had detectable serum viremia for almost 2 years, there was significant evolution between sequences at each time point (baseline, year 1, and year 2). Mean pairwise distances were positively correlated with net AUC HIV serum RNA (figure 3).

Discussion

Two major obstacles for the elimination of HIV in patients receiving potent combination therapy have been defined: a pool of latently infected resting CD4+ T cells [26, 37, 38] and ongoing low level viral replication in some patients with undetectable or very low levels of serum viremia for prolonged periods [11, 12, 15–17, 39–41]. Evidence for these 2 mechanisms has been based primarily on studies of peripheral blood mononuclear cells (PBMC) [11, 13, 16, 17, 40, 42] and plasma [39, 41]. Recent speculation has suggested that sanctuary sites, such as the CNS and the genital tract, might account for such low level replication because of lower accessibility for drugs or because of differences in cell turnover of infected cell types [42–44]. Yet, published studies addressing compartmentalization issues of the CNS and of genital secretions rarely investigated more than 2 different compartments simultaneously [23]. To investigate whether the CNS, the genital tract, or lymphoid tissue might be the sites of persistent viral replication in patients receiving

potent long-term antiretroviral therapy, we undertook the present study to assess residual HIV in these compartments.

The persistence of HIV DNA in LN of persons with suppression of viremia was expected, on the basis of the reported longevity of latently infected cells and persistence of defective genomes [16, 26, 37, 38, 45, 46]. However, detection of viral RNA in LNs was uncertain. Given earlier estimates of the clearance rate of lymphoid tissue associated viral RNA from ISH-QIA studies for the first 6 months of therapy, our expectation had been that, for patients with plasma HIV RNA levels consistently <50 copies/mL, HIV RNA in lymphoid tissues should have been reduced further during year 2 of treatment, in some cases to undetectable levels.

Analysis of the RNA data by normalization of TCR Cα mRNA permitted the exclusion of samples with inadequate cellularity and corrected for differences in RNA extraction or PCR amplification efficiency. Calculated in this manner, there was little
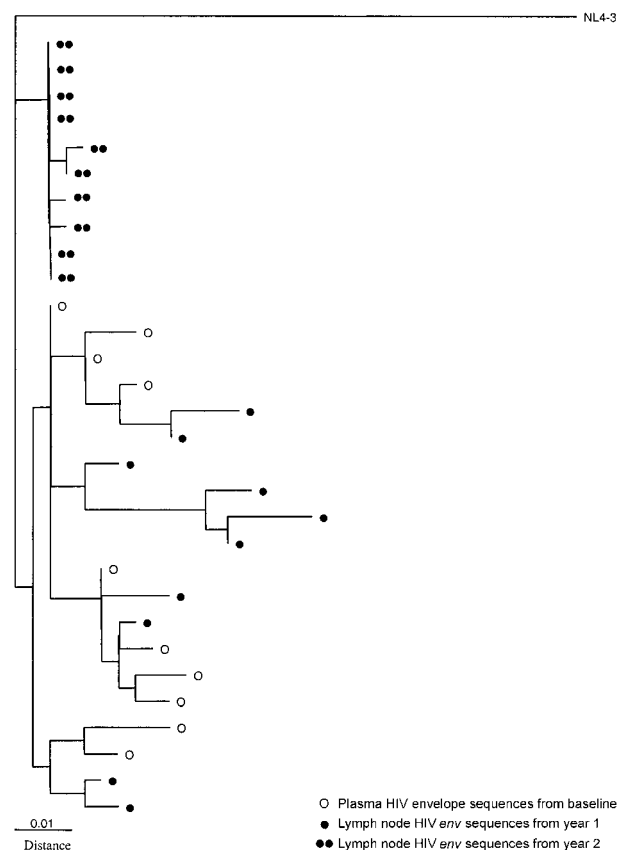
Figure 2. Maximum-likelihood phylogenetic tree for patient 19 shows sequence relationships of human immunodeficiency virus (HIV) *env* clones derived from serum collected at start of therapy and from sequences derived from lymph node HIV RNA at years 1 and 2. Individual clones were compared with NL4-3 reference sequence. Genetic distances (indicated by horizontal branch lengths) correspond to approximate percentage nucleotide differences. Clonal sequences from year 2 clustered with shorter branch lengths to the inferred "most recent common ancestor" (nearest node basal to all patient clones), which suggests that the year 2 clones are ancestral to baseline and year 1 clones.
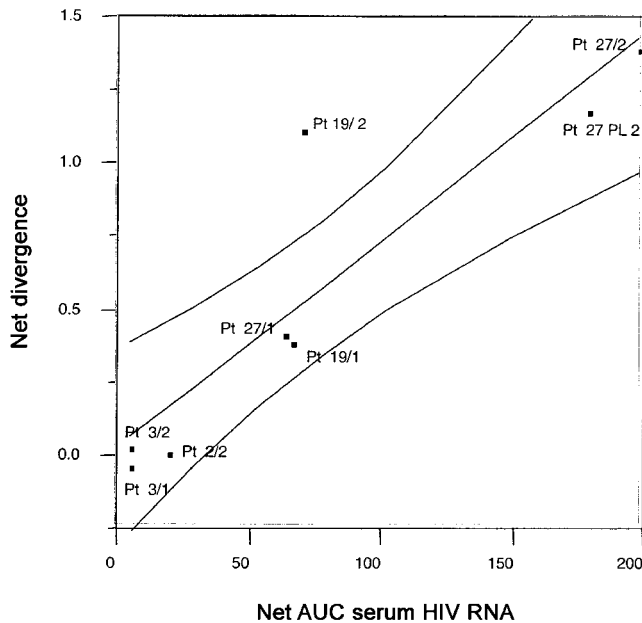
Figure 3. Regression analysis of area under curve (AUC) net and net divergence between clonal human immunodeficiency virus (HIV) *env* serum sequences obtained from baseline serum RNA and lymph node or plasma (PL) sequences at years 1 or 2 from 4 patients (Pts). AUC_{net} was calculated as the difference between RNA concentration × time product observed and RNA concentration × time product predicted (based on ideal decay characteristics) and is used to provide a measure of residual viral replication under potent therapy, as described in detail elsewhere [12]. Patient designations 3, 2, 19, and 27 correspond to B, C, I, and G, respectively, as reported elsewhere [8, 11, 26, 27]. No. after slash is time after initiation of antiretroviral therapy (1, year 1; 2, year 2). PL indicates divergence obtained between plasma sequences. If not otherwise stated, divergence was generated between baseline plasma and lymph node sequences. $r^2 = .8222$, $P = .0019$ (pairwise correlation); Spearman $\rho = 0.9$, $P = .0002$; Kendall $\tau = 0.79$, $P = .0065$.

difference in viral RNA levels between years 1 and 2 either in the 3 patients for whom biopsies were done at both time points or in a cross-sectional comparison of all LN biopsy specimens after 1 and 2 years of suppressive therapy. When RNA levels were simply normalized to the mass of tissue extracted (but with exclusion of samples with inadequate levels of TCR Cα mRNA), a trend toward slightly lower RNA concentrations was noted at year 2, compared with year 1; nevertheless, the apparent decay of HIV RNA levels from year 1 to year 2 appears to be slower than that previously observed for the first 6 months of therapy as the second phase of decay [9]. A tendency toward lower HIV DNA levels at year 2 also was observed in patients who underwent sequential LN biopsies. However, few patients had serum viremia <50 copies/mL with serial biopsies, and cross-sectional comparisons may be suboptimal.

The persistence of viral RNA in LNs from patients with <50 copies/mL of HIV RNA for ≥2 years may reflect ongoing viral replication in all patients, as other investigators have postulated

[12, 13, 15, 39–41], or a change in the rate of clearance of LN-associated RNA following the initial reduction over the first 6–12 months of therapy. The data presented argue against the possibility that the residual lymphoid HIV RNA originates from sanctuary sites such as the CNS or the GU tract. No HIV RNA was detected in the CSF in any of the 13 patients tested (of whom 10 underwent LN biopsies; table 1). No HIV RNA was detected in GU secretions from 20 of 23 patients tested for genital secretions (15 underwent LN biopsies; table 1). In the 3 patients with genital secretions positive for HIV RNA, detection was only transient. However, our assays might not have been sensitive enough to detect very low levels of HIV RNA in CSF and genital secretions. No baseline levels from these 2 compartments were available, and brain parenchyma and cellular components of genital secretions could not be assessed.

In all patients with undetectable serum RNA levels that were examined by ISH with QIA, no FDC-associated signal was detectable with a sensitivity of detection of 10^4 copies/g of tissue. Because this is approximately the concentration of viral RNA that was detected by bulk RNA analysis, the ISH data do not exclude the FDC network as the reservoir of residual viral RNA in the form of trapped virus. Hlavacek et al. [47] recently suggested that FDC-bound virus might decay very slowly, by taking into account a model that assumes binding of HIV by ligand-receptor interactions. Such low levels of FDC-bound virus may well be below the level of detection of ISH methods, as described by several groups who used different detection systems [9, 10, 48].

Cavert et al. [8, 9] observed that a small population of lymphocytes associated with low copy numbers persist after therapy for 6 months to 1 year. A few of the patients studied by ISH in the present study had identifiable cells with detectable virus when we used a prolonged exposure capable of detecting 2 or 3 copies of HIV RNA/cell. These cells might belong to a recently described T cell population that produces viral RNA in a nonactivated state and that persists in patients treated with potent antiretroviral therapy [3]. Such cells might contribute to the residual viral RNA that was detected by bulk RNA quantitation and may represent a reservoir of chronically infected cells or a form of latently infected cells with low-level HIV transcription.

What is the origin of the residual RNA in LN? To address this question, we did phylogenetic analyses from clonal envelope sequences obtained directly from residual LN HIV RNA, to determine whether HIV in lymphoid tissues of patients with undetectable serum RNA levels showed sequence differences as a marker of ongoing replication or as evidence of virus production in distinctive tissue compartments.

For the patient with the highest level of suppression (patient 3; figure 1), no significant evolution of HIV *env* was detected in residual LN RNA from biopsy specimens at years 1 and 2. This finding does not support continuous viral replication, although extremely low levels of replication insufficient to produce sequence change cannot be excluded. However, the se-

quence analyses would be consistent with either virus production from long-lived latently infected cells that were transcriptionally active but did not give rise to new cycles of infection or with the persistence of FDC-trapped virions produced before suppressive therapy.

In theory, HIV RNA could have been produced in a different compartment, and virus could have been trapped in the FDC network at quantities below the detection limit for ISH. Indeed, cervical secretions at year 2 were positive for patient 3 (table 1) but subsequently became negative. However, sequences from different time points and blood and lymphoid tissue sampled were indistinguishable in a maximum likelihood tree that rendered such a possibility improbable, given the effects that differing selective pressures in tissue compartments (e.g., the GU and CNS) would be expected to have on the local virus quasi species [18, 49]. The uniformity of the sequences obtained from patient 3 are unlikely to reflect contamination artifacts. Since *env* PCR amplification and sequencing from baseline plasma were done 1 year before amplification and sequencing from LNs. Sampling artifacts also seem unlikely because 2 different LN biopsies were done 1 year apart and because PCRs were done in quadruplicate to minimize sampling error.

Moreover, envelope sequences derived from in vitro–activated CD8-depleted PBMC at year 2 that presumably were isolated from latently infected CD4 T cells [11, 26] did not differ from sequences derived directly from HIV LN RNA or from baseline plasma sequences in a maximum likelihood tree (data not shown). Additional extensive clonal analysis obtained from year 2 isolates from the latent reservoir from this patient did not reveal any new mutation associated with drug resistance, compared with baseline plasma [50]. Thus, despite rigorous genetic analysis of HIV *env* and *pol* from different compartments, viral replication could not be inferred by evolutionary studies, even though HIV RNA was still detectable in the LN and did not decay significantly over this time.

In contrast to patient 3, patients 2, 19, and 27 showed various degrees of evolution of envelope sequences (table 3), which correlated strongly with the extent of viral replication, as expressed by the AUC_{net} of longitudinal serum HIV RNA measurements (figure 3). Of note, patient 19 had a significant net sequence divergence from baseline to year 1, when serum viremia was detectable and, contrary to expectations, even greater divergence by year 2, despite serum virus load of <50 copies/mL throughout year 2 (table 3). By maximum likelihood phylogeny the year 2 LN sequences appeared to be ancestral to the initial plasma virus (figure 2), which suggests that residual LN RNA resulted from long-lived chronically infected cells with transcriptional activity possibly established before the baseline time point from old FDC-bound virus with very slow decay or from virus produced by activated archival latently infected cells [51].

Chun et al. [42] recently reported that rebounding plasma virus after cessation of potent long-term suppressive antiretro-

viral therapy in HIV-infected patients differed from virus isolated from the latent pool before virus rebound. These differences were found by heteroduplex mobility assay (HMA) and were thought to indicate that rebounding plasma virus originated from sanctuary sites such as the CNS or GU tract [42]. The present data suggest an alternative explanation for their findings. The differences observed by Chun et al. between isolates from the latent reservoir and rebounding virus off therapy detected by HMA may reflect plasma virus produced by activation of latently infected cells harboring ancestral genotypes, compared with those isolated by coculture while receiving therapy [51].

In conclusion, our data show that the lymphoid compartment remains an important reservoir for residual HIV in patients successfully treated with combination antiretroviral therapy who achieve undetectable HIV serum RNA levels for ≤2 years. In the patient without viral evolution of HIV RNA envelope sequences directly obtained from LNs between years 1 and 2, the absence of significant decay of HIV RNA from year 1 to year 2 in these LNs raises the possibility of a slower third phase of viral decay independent of residual viral replication or that ongoing replication with negligible sequence evolution is occurring. In the quest for more effective suppression of viral replication, these findings indicate a more immediate need for treatment strategies with greater antiviral potency in all compartments rather than for specialized regimens that target the CNS or GU tract.

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