Attenuated and Nonproductive Viral Transcription in the Lymphatic Tissue of HIV-1–Infected Patients Receiving Potent Antiretroviral Therapy

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Human immunodeficiency virus type 1 (HIV-1) RNA that persists in the lymphoid tissue of patients despite treatment with highly active antiretroviral therapy (HAART) may represent extracellular virions or intracellular RNAs residing within HIV-infected cells. To further characterize residual viral transcription, tonsil biopsy specimens from patients receiving long-term HAART, untreated patients, and patients undergoing 2 weeks of structured treatment interruption were analyzed by polymerase chain reaction quantification of virion-encapsidated RNA, intracellular unspliced HIV RNA (HIV UsRNA), multiply spliced HIV RNA encoding *tat* and *rev* (HIV MsRNA), and HIV DNA. Tonsil biopsy specimens from viremic patients harbored high amounts of virions, which primarily stemmed from local production, as indicated by a strong correlation of extracellular tonsillar RNA with intracellular HIV-1 nucleic acid levels but not with plasma viremia, and as shown by phylogenetic analysis of clonal *env* sequences from lymphoid tissue and plasma. In patients receiving HAART, intracellular HIV UsRNA persisted at significantly decreased levels, whereas HIV MsRNA and lymphoid virion levels were depleted. Thus, residual lymphoid HIV-1 RNA in patients receiving HAART indicates attenuated viral transcription in HIV-1–infected cells that lack virion production.

Combination antiretroviral therapy may reduce levels of viral RNA in plasma to levels below detection [1], but low levels of HIV-1 RNA in peripheral blood mononuclear cells (PBMCs) [2–4] or in lymphoid tissue persist for years [4–6]. Such persistent viral transcripts may

arise from newly infected cells as a result of ongoing productive viral replication [2], or they may originate from reactivated HIV-1–infected cells [7]. Alternatively, persistent HIV-1 RNAs may reflect viral transcription in HIV-1–infected cells that do not produce virions and, possibly, do not produce viral antigens [8]. There is evidence that ongoing low-level HIV-1 replication occurs in a fraction of patients, despite treatment with highly active antiretroviral therapy (HAART), as has been shown by sequence evolution of virus quasi species [5, 9] and by reduction of residual viremia on therapy intensification [10]. Nevertheless, even in viremic patients, productively infected cells are the rarest class of HIV-1–infected cells in lymphoid tissue [11, 12] and PBMCs [8], presumably because of their short half-life [13]. Therefore, the main fraction of persistent viral transcripts in patients receiving HAART may be derived from cells with a repressed viral expression pattern [8].

A major difficulty in associating persistent HIV-1 transcription with individual classes of HIV-1–infected

cells is the complexity of HIV-1 transcription represented by >30 different RNAs [14, 15]. One key transcript of HIV-1 is unspliced HIV RNA (HIV UsRNA), because it mediates an array of different functions, serving as (1) the primary retroviral transcript, (2) mRNA for the translation of *gag* and *pol,* and (3) genomic RNA, which is encapsidated into virions [16]. Thus, HIV UsRNA can be found intracellularly and extracellularly in lymphoid tissue [12] and in PBMCs [8, 17]. Quantitative in situ hybridization has suggested that a major fraction of HIV-1 RNA in the lymphoid tissues of HIV-1–infected patients with viremia corresponds to extracellular viral particles bound to follicular dendritic cells (FDCs) [11, 12, 18]. Efforts to characterize the topology of HIV RNAs in the lymphoid tissue of patients receiving HAART have been impeded by the lower sensitivity of in situ hybridization, compared with that of polymerase chain reaction (PCR) analysis [6]. However, novel specific and highly sensitive PCR-based assays now permit detection and quantification of both extracellular virion-encapsidated HIV UsRNA and intracellular HIV UsRNA [8].

In addition to HIV UsRNA, a second class of HIV-1 RNAs—multiply spliced HIV-1 RNAs encoding *tat* and *rev* (HIV MsRNAs)—plays a central role in the retroviral life cycle. The HIV MsRNA gene products that promote the transcriptional activation of the HIV-1 long terminal repeat [19] and the nucleocytoplasmic transport of a majority of HIV-1 RNAs [20] mediate efficient expression of HIV-1 and are essential for viral replication. In addition, expression of HIV MsRNA also occurs early after infection [21–24], and detection of HIV MsRNA in vivo has been associated with newly infected cells [23, 25]. Expression of HIV MsRNA has also been observed in latently infected cells showing posttranscriptionally blocked latency [26]. Because these different classes of HIV-infected cells may coexist in vivo, unambiguous identification of defined cell types that produce HIV MsRNA is difficult. However, absence of HIV MsRNA strongly suggests depletion of ≥1 category of cells—in particular, of HIV-1–infected cells producing virions [8].

To elucidate the origin of persistent HIV-1 RNAs in vivo during treatment with HAART, the present study focused on viral expression patterns in lymphoid tissue, which is the major reservoir of HIV-1 [27], by use of assays for HIV MsRNA and extracellular and intracellular HIV UsRNA. These HIV RNAs were studied in conjunction with levels of HIV-1 DNA mapping to the *gag* gene (HIV DNA). Because of the presence of defective genomes [28–30], episomal viral genomes, and nonintegrated linear viral DNA [31], HIV DNA levels are a maximum estimate of the number of infected cells exceeding the number of replication-competent provirus genomes.

Combined analysis of these virological parameters permitted characterization of productive infection in lymphoid tissue in vivo, under conditions of ongoing viral replication in therapy-naive patients and during structured treatment interruption (STI). Observation of highly repressed viral transcription patterns in the lymphoid tissue of patients receiving HAART suggests that residual HIV RNA mainly resides in latently infected cells.

PATIENTS, MATERIALS, AND METHODS

Patients and specimens from patients. Tonsil biopsy specimens obtained from untreated viremic patients ($n = 13$) and from patients receiving HAART resulting in plasma viremia levels <50 copies/mL for >12 months ($n = 21$) were studied. A subgroup of patients receiving HAART ($n = 11$) underwent a 2-week STI. Samples were obtained from patient 4 before initiation of HAART, during treatment, and after STI.

All 33 patients were enrolled in the Swiss HIV Cohort Study [32] and provided written informed consent, according to the guidelines of the ethics committee of the University Hospital Zurich (Zurich). Tonsil biopsy specimens were obtained during 3 clinical trials. In 2 trials, biopsy specimens were obtained from therapy-naive patients (patients 1–13) [33, 34]. The immunological status of untreated patients included a mean CD4 cell count ($\pm$ SE) of 466 ± 49 cells/μL of blood (median, 505 cells/μL; range, 65–743 cells/μL) and a mean CD8 cell count ($\pm$ SE) of 1014 ± 169 cells/μL of blood (median, 814 cells/μL; range, 365–2400 cells/μL).

Treatment with HAART consisted of 2 reverse-transcriptase (RT) inhibitors plus 1 protease inhibitor (for patients 14–23 [35] and for patients 28, 29, and 33 [36]). Patients 24–27 and patients 30–32 were initially treated with 2 RT inhibitors plus 1 protease inhibitor for ≥6 months and then were switched to a simplified drug regimen consisting of 3 RT inhibitors when the plasma viremia level was <50 copies/mL [36]. Tonsil biopsy specimens from patients undergoing STIs (patient 4 and patients 14–23) were obtained at baseline (i.e, "on-therapy" specimens) and 2 weeks after STI (i.e., "off-therapy" specimens) [35]. The immunological status of patients receiving HAART included a mean CD4 cell count ($\pm$ SE) of 694 ± 56 cells/μL of blood (median, 723 cells/μL; range, 347–1269 cells/μL) and a mean CD8 cell count ($\pm$ SE) of 756 ± 60 cells/μL of blood (median, 342 cells/μL; range, 805–1228 cells/μL). The CD4 cell counts of patients receiving HAART were significantly higher than those of untreated patients ($P = .01$, by t test), whereas differences in CD8 cell counts did not reach statistical significance ($P = .1$).

Plasma samples were obtained by Ficoll-gradient purification (Lymphoprep) or by use of Vacutainer CPT tubes (Becton Dickinson). Tonsil biopsy specimens were snap-frozen at -80°C. Cryosections were prepared using a strategy that ensured optimal homogeneity of the 5 aliquots of tissue for nucleic acid analysis, by use of our previously developed strategy for tissue sectioning [11], with minor modifications: Each biopsy spec-

imen was entirely cross-sectioned, and each series of 18 consecutive sections was defined as a level. Per level, 3 consecutive sections were used for morphologic analysis, to verify that lymphoid tissue was present, and 3 consecutive sections were pooled into 5 separate tubes for nucleic acid extraction.

Differential extraction of particle-associated and total HIV RNA. Differential extraction of particle-associated and total HIV RNA was performed in duplicate, as described elsewhere [8], with minor modifications. For some biopsy specimens, only 1 extraction was performed. Singular extraction of the extracellular fraction was performed for patients 14 (on-therapy and off-therapy specimens) and 15 (on-therapy specimen); extraction of total RNA was performed for patients 4 (off-therapy specimen), 16 (off-therapy specimen), 18 (off-therapy specimen), 19 (on-therapy and off-therapy specimens), 21 (on-therapy and off-therapy specimens), and 22 (on-therapy specimen). For the biopsy specimens of 2 patients (patient 18 [off-therapy specimen] and patient 21 [off-therapy specimen]), extracellular fractions were measured in triplicate.

Nucleic acid quantitation. Plasma HIV RNA was quantified using the Amplicor HIV-1 Monitor Test, version 1.5 (Roche), or an ultrasensitive modification of the test [37]. Cell-associated HIV-1 RNAs were quantified as described elsewhere [8]; HIV UsRNA was quantified using a modification of the Amplicor HIV-1 Monitor Test [8]. HIV MsRNA was measured using a limiting dilution analysis of an ultrasensitive direct RT-PCR assay, followed by a 96-well immunosorbent assay [8] that used PCR primers encompassing the start codon of *rev* and the second coding exon of *tat* and *rev* [38].

HIV DNA was extracted using the QIAmp DNA extraction minikit (Qiagen) and was quantified using the Amplicor HIV-1 Monitor Test, as described elsewhere [39]. Absolute detection limits of HIV RNA and HIV DNA measurements were <2 copies/PCR [8, 39]. Relative detection limits of HIV RNA and HIV DNA in PCR-negative specimens, normalized to the cellular input and expressed as the number of copies per 10^6 cells, were calculated as reported elsewhere [8].

Total cellular RNA and DNA were quantified fluorometrically by use of fluorescent dyes (Ribogreen and Picogreen; Molecular Probes). Residual endogenous cellular RNA was monitored by RT-PCR for glyceraldehyde-3-phosphodehydrogenase mRNA [8, 40, 41].

Phylogenetic analysis. Amplification, cloning, and sequencing of HIV-1 *env* in nucleic acid preparations from plasma samples (RNA) and tonsil biopsy specimens (DNA and extracellular RNA) were performed as reported elsewhere [9], with minor modifications. HIV-1 *env* (410 bp, including *C2* and *V3*) was amplified by nested PCR in duplicate reactions. Combined cDNA synthesis and first-round PCR (RT-PCR kit; Finnzymes) were followed by second-round PCR performed using HotStarTaq (Qiagen) with primers (5′-GAACAGGACCA-

TGTACAAATGTCAGCACAGTACAAT-3′ and 5′-GCGTTAA-AGCTTCTGGGTCCCCTCCTGAG-3′). Pooled products were cloned (with use of the pCR-4 TOPO cloning system; Invitrogen), and 16 clones per sample were selected. Virus inserts were amplified by direct PCR analysis of bacterial colonies and were bidirectionally sequenced (BigDye; Applied Biosystems). The average misincorporation rate under these conditions was <0.2%.

Diversities were calculated using MEGA software, version 2.1 (S. Kumar et al., http://www.megasoftware.net), under the assumption of a Tamura and Nei substitution model. Phylogenetic trees were constructed using the maximum-likelihood method DNAML, by use of transition/transversion ratio 2.0, global rearrangements, and random input order (PHYLIP, version 3.6; J. Felsenstein, University of Washington, Seattle; http://evolution.genetics.washington.edu/phylip.html). Trees were displayed by TreeView (version 1.6; R. Page, University of Glasgow, Glasgow, UK; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The reference sequence used for constructing phylogenetic trees was HXB2 [42]. All sequences reported were deposited in GenBank (for patient 4 [known as "SSITT patient 109"], AY375568–AY375615; for patient 17 [known as "SSITT patient 120"], AY375616–AY375662; for patient 20 [known as "SSITT patient 102"], AY375711–AY375758; and for patient 21 [known as "SSITT patient 118"], AY375663–AY375710).

Calculations and statistics. Copy numbers of extracellular HIV UsRNA were calculated, with the efficacy of nuclease digestion of cellular nucleic acids taken into account, as reported elsewhere [8]. Copy numbers of intracellular HIV UsRNA were calculated as the difference between measurements of total HIV UsRNA and extracellular HIV UsRNA [8]. HIV RNA copy numbers in undigested control samples were normalized to the input of total cellular RNA (expressed as the number of copies per 10^6 cell-equivalents), with a median of 2.68 μg of RNA (range, 0.4–11.4 of μg of RNA; $n = 41$ samples) recovered per 10^6 cell-equivalents. The number of cell-equivalents in a given sample was calculated from the recovery of total DNA, under the assumption that 7 μg of total cellular DNA represents 10^6 cell-equivalents [43]. Copy numbers obtained from nuclease-treated samples were normalized, under the assumption that the input of cell-equivalents per PCR was equal to that of the matched undigested control [8].

Statistical analyses were performed using GraphPad Prism software (AMPL Software). Mean values ($\pm$SE) are indicated. Median values were compared using the Mann-Whitney test. Correlation coefficients (r^2) were calculated by linear regression on $\log_{10}$ values. Alternatively, adjusted r^2 values were calculated by Statview software, version 5.0.1 (SAS; data not shown), resulting in slightly lowered values (mean difference [$\pm$SE] of -0.06 ± 0.008), which almost completely correlated ($P < .0001$; $r^2 = 0.997$) with unadjusted r^2 values.

RESULTS

Plasma viremia in untreated patients receiving HAART and in untreated patients undergoing STI. The following groups of HIV-1–infected patients were studied: untreated asymptomatic patients with detectable plasma viremia levels in the range of 1943–1.02×10^5 HIV-1 RNA copies/mL (median, 42,487 HIV-1 RNA copies/mL; mean [±SE], 43,563 ± 9664 HIV-1 RNA copies/mL; patients 1–13) (table 1) and patients receiving HAART with plasma viremia levels <50 copies/mL (patient 4 and patients 14–33) (table 2). All patients who were receiving HAART continuously had plasma viremia levels <50 copies/mL for >12 months. For a subgroup of patients receiving HAART (patient 4 and patients 14–23), therapy was interrupted for 2 weeks. This resulted in increased plasma viremia levels in 8 of 11 patients (range, 141–213,043 HIV-1 RNA copies/mL; median, 15,134 copies/mL; mean [±SE], 47,330 ± 25,765 copies/mL), whereas, for 3 patients (patients 16, 19, and 22), no rebound of plasma viremia was observed (table 2).

HIV-1 nucleic acid levels in the lymphoid tissue of patients with high-level, suppressed, and rebounding viremia. To assess lymphatic HIV-1 transcription patterns, levels of the following cell-associated HIV-1 nucleic acids were measured in tonsil biopsy specimens: HIV DNA, HIV MsRNA, and HIV UsRNA. The latter was further distinguished as intracellular or extracellular HIV UsRNA. For untreated patients, the ranges of these measurements were 90–5782 copies/10^6 cells for HIV DNA (median, 982 copies/10^6 cells; mean [±SE], 1479 ± 423 copies/10^6 cells), <38–8586 copies/10^6 cells for HIV MsRNA (median, 1029 copies/10^6 cells; mean [±SE], 2316 ± 712 copies/10^6 cells), 747–5.5×10^6 copies/10^6 cells for intracellular HIV UsRNA (median, 1.1×10^5 copies/10^6 cells; mean [±SE], $6.1 \times 10^5 \pm 4.5 \times 10^5$ copies/10^6 cells), and 1198–3.9×10^6 copies/10^6 cells for extracellular HIV UsRNA (median, 1.0×10^5 copies/10^6 cells; mean [±SE], $5.8 \times 10^5 \pm 3.1 \times 10^5$ copies/10^6 cells).

As expected, these levels were generally lower in patients receiving HAART than in untreated patients ($P \le .0001$) (figure 1). However, whereas HIV DNA (range, <22–461 copies/10^6 cells; median, 98 copies/10^6 cells; mean [±SE], 146 ± 31 copies/10^6 cells) and intracellular HIV UsRNA (range, 4–372 copies/10^6 cells; median, 43 copies/10^6 cells; mean [±SE], 76 ± 21 copies/10^6 cells) were present at detectable levels in 83% and 86%, respectively, of all samples, HIV MsRNA (range, <2–10 copies/10^6 cells; median, 4 copies/10^6 cells; mean [±SE], 4 ± 0.5 copies/10^6 cells) and extracellular HIV UsRNA (range, <2–<20 copies/10^6 cells; median, 11 copies/10^6 cells; mean [±SE], 11 ± 1 copies/10^6 cells) were only detectable in 26% and 5%, respectively, of the tested biopsy specimens and, thus, were significantly depleted ($P = .01$ and $P \le .001$, respectively, by Fisher's exact test, in comparison with untreated patients). The levels of the 3 types of cell-associated HIV-1 RNA assessed in the tonsil biopsy specimens obtained from patients with

Table 1. Characteristics of untreated asymptomatic HIV-1–infected patients with detectable plasma viremia.

Patient	Plasma HIV-1 RNA level, copies/mL	Measurement of HIV-1 nucleic acids in tonsil biopsy specimens, copies/10^6 cells[a]				
		Total HIV UsRNA	Intracellular HIV UsRNA	Extracellular HIV UsRNA	HIV MsRNA	HIV DNA
1	12,483	1,149,265	NA[b]	1,176,340	3566	1671
2	7149	8328	747	7581	190	92
3	88,637	2,329,785	658,043	1,671,742	3649	2611
4	42,487	245,868	142,772	103,096	1929	852
5	11,379	9,425,289	5,540,779	3,884,510	8586	5782
6	91,110	440,346	326,263	114,083	89	2420
7	50,527	12,708	3834	8874	<50	90
8	64,916	57,769	56,571	1198	1026	628
9	62,786	11,129	4486	6643	<38	602
10	102,900	225,078	28,185	196,893	780	1036
11	1943	155,162	115,320	39,842	1029	585
12	38,836	576,456	294,351	282,105	4174	982
13	14,560	145,355	101,398	43,957	5007	1871

NOTE. HIV MsRNA, multiply spliced HIV RNA encoding *tat* and *rev*; HIV UsRNA, unspliced HIV RNA; NA, not applicable.

[a] "Less than" symbols (<) denote detection limits of polymerase chain reaction–negative specimens.

[b] Could not be calculated because >99% of total HIV UsRNA in the biopsy specimen was contained in the extracellular fraction.

Table 2. Characteristics of HIV-1–infected patients treated with highly active antiretroviral therapy.

Patient, therapy status	Plasma HIV RNA level, copies/mL[a]	Measurement of HIV-1 nucleic acids in tonsil biopsy specimens, copies/10^6 cells[a]				
		Total HIV UsRNA	Intracellular HIV UsRNA	Extracellular HIV UsRNA	HIV MsRNA	HIV DNA
4						
Receiving therapy	<10	43	43	<19	<3	238
2 Weeks' STI	1876	49	49	<8	<3	142
14						
Receiving therapy	<9	4	4	<18	<5	<22
2 Weeks' STI	74,375	312	312	<79	<20	111
15						
Receiving therapy	25	114	114	<9	<3	331
2 Weeks' STI	213,043	2080	1673	406	11	260
16						
Receiving therapy	<12	7	7	<6	<2	35
2 Weeks' STI	<10	<1	<1	<10	<3	9
17						
Receiving therapy	<22	91	91	<9	2	66
2 Weeks' STI	258	6756	2555	4201	52	166
18						
Receiving therapy	44	7	7	<12	<4	103
2 Weeks' STI	141	6	6	<6	<3	<11
19						
Receiving therapy	47	78	78	<12	<8	56
2 Weeks' STI	36	58	58	<14	<5	57
20						
Receiving therapy	<22	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
2 Weeks' STI	58,678	123,260	105,727	17,534	1191	8917
21						
Receiving therapy	<6	35	35	<10	<7	45
2 Weeks' STI	26,815	24,229	18,057	6172	655	451
22						
Receiving therapy	<11	<5	<5	<20	<5	<32
2 Weeks' STI	<10	<4	<4	<14	<3	<101
23						
Receiving therapy	42	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
2 Weeks' STI	3453	1624	1426	197	<5	140
24, receiving therapy	19	5	5	<17	<3	ND
25, receiving therapy	<5	73	73	<11	<3	59
26, receiving therapy	<5	174	174	<8	4	258
27, receiving therapy	<4	121	121	<8	4	158
28, receiving therapy	<5	17	17	<20	<7	98
29, receiving therapy	<9	19	19	<2	10	ND
30, receiving therapy	<6	57	57	<2	<2	182
31, receiving therapy	<8	211	211	<15	<3	289
32, receiving therapy	<5	376	372	5	3	461
33, receiving therapy	24	13	13	<14	<4	55

NOTE. HIV MsRNA, multiply spliced HIV RNA encoding *tat* and *rev;* HIV UsRNA, unspliced HIV RNA; ND, not determined; STI, structured treatment interruption.

[a] "Less than" symbols (<) denote detection limits of polymerase chain reaction–negative specimens.
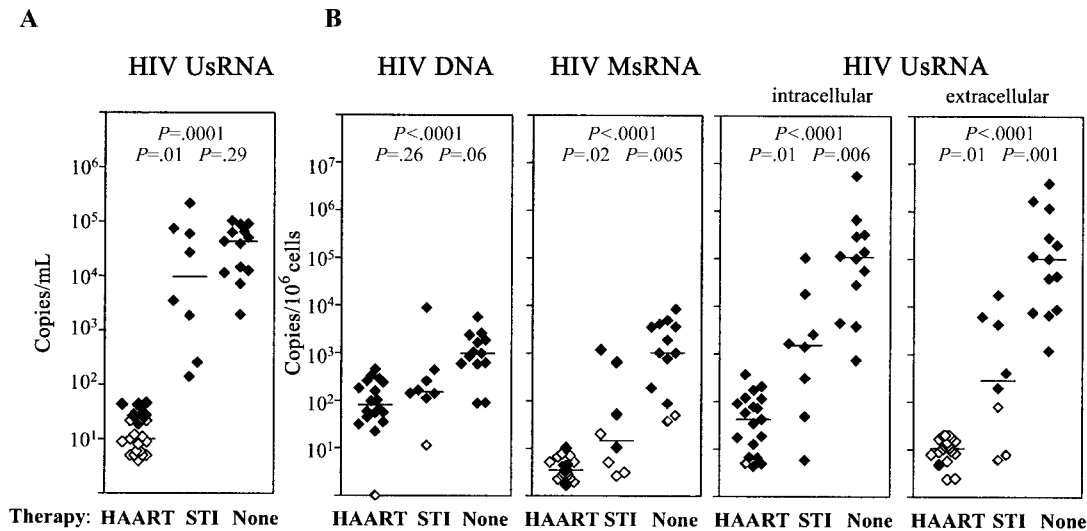
[b] No tonsil biopsy specimen was available.

Figure 1. Levels of HIV-1 nucleic acids in tonsil biopsy specimens and plasma. *A,* Levels of unspliced HIV RNA (HIV UsRNA) in plasma. *B,* Levels of HIV-1 nucleic acids (HIV DNA, multiply spliced HIV-1 RNA encoding *tat* and *rev* [HIV MsRNA], and HIV UsRNA) in tonsil biopsy specimens. Note that only patients who had levels of plasma viremia >50 copies/mL were included (*n* = 8) in the group showing data for patients after 2 weeks of structured treatment interruption (STI). *Closed diamonds,* Values of detectable polymerase chain reaction (PCR) measurements; *open diamonds,* detection limits of PCR-negative specimens; *horizontal lines,* medians. *P* values that indicate the significance of differences between groups are shown within each panel. HAART, highly active antiretroviral therapy.

rebounding viremia during STI appeared to be intermediate (figure 1*B*); they were significantly lower than those in untreated patients ($P \leqslant .006$) and were higher than those in patients receiving HAART ($P \leqslant .02$). Levels of HIV DNA in the tonsils of patients with rebounding viremia trended lower ($P = .06$), compared with samples from untreated patients, but they were not significantly higher ($P = .26$) than such levels in the tonsils of patients receiving HAART.

Correlation analysis of HIV-1 nucleic acids in lymphoid tissue and plasma. To characterize the interdependencies of HIV-1 nucleic acids in lymphoid tissue and their association with plasma viremia, analysis of correlation between each of the parameters tested was performed. Cross-sectional analysis of combined data from untreated patients and from 8 patients with rebounding viremia as a result of STI showed highly significant correlations of all types of cell-associated HIV-1 nucleic acid levels (figure 2). Of note, the extracellular fraction of HIV UsRNA, which, to a great extent, is associated with FDCs [8, 11, 12], was correlated, with high significance ($P \leqslant .002$), with HIV DNA ($r^2 = 0.46$), HIV MsRNA ($r^2 = 0.52$), and intracellular HIV UsRNA ($r^2 = 0.70$), but not with plasma HIV RNA ($P = .22$; $r^2 = 0.01$). Similarly, no correlation was observed between levels of plasma HIV RNA and HIV DNA or HIV MsRNA ($P > .17$). Plasma viremia was weakly but significantly correlated with intracellular HIV UsRNA ($P = .03$; $r^2 = 0.23$), which verified that virus pools in plasma and tonsils, to a limited extent, communicated with each other. Under conditions of fully suppressive HAART, HIV RNA in plasma,

HIV MsRNA, and extracellular HIV UsRNA were predominantly undetectable, which precluded paired analysis, except for intracellular HIV UsRNA and HIV DNA, for which, again, the correlation was significant ($P = .007$; $r^2 = 0.41$).

To corroborate these findings under conditions of restricted viral replication, the relative change, on STI, in each of the parameters measured was calculated (figure 3). This longitudinal analysis yielded results similar to those of the cross-sectional analysis of absolute nucleic acid levels. Within the cellular compartment, paired correlations between levels of HIV-1 nucleic acids were significant ($P \leqslant .01$), and changes in plasma viremia levels were correlated with intracellular HIV UsRNA ($P = .004$) and, to a lesser extent, with HIV DNA ($P = .05$); however, they were correlated neither with HIV MsRNA nor with extracellular HIV UsRNA ($P \geqslant .1$). Of note, the best correlation was observed between changes in extracellular HIV UsRNA and HIV MsRNA ($P < .0001$; $r^2 = 0.88$), revealing an almost complete linkage between increases in these parameters under conditions of rebounding HIV-1 replication. One outlier in this correlation, patient 23, had no detectable HIV MsRNA, despite production of lymphoid viral particles. Insufficient detection of HIV MsRNA in this patient, who was infected with a non-B clade virus, was the result of primer mismatches (data not shown).

Phylogenetic analysis of viral nucleic acids in lymphoid tissue and plasma. The high correlation between levels of tonsillar extracellular HIV-1 RNA and intracellular viral nucleic acids in lymphoid tissue was in marked contrast to the lack of
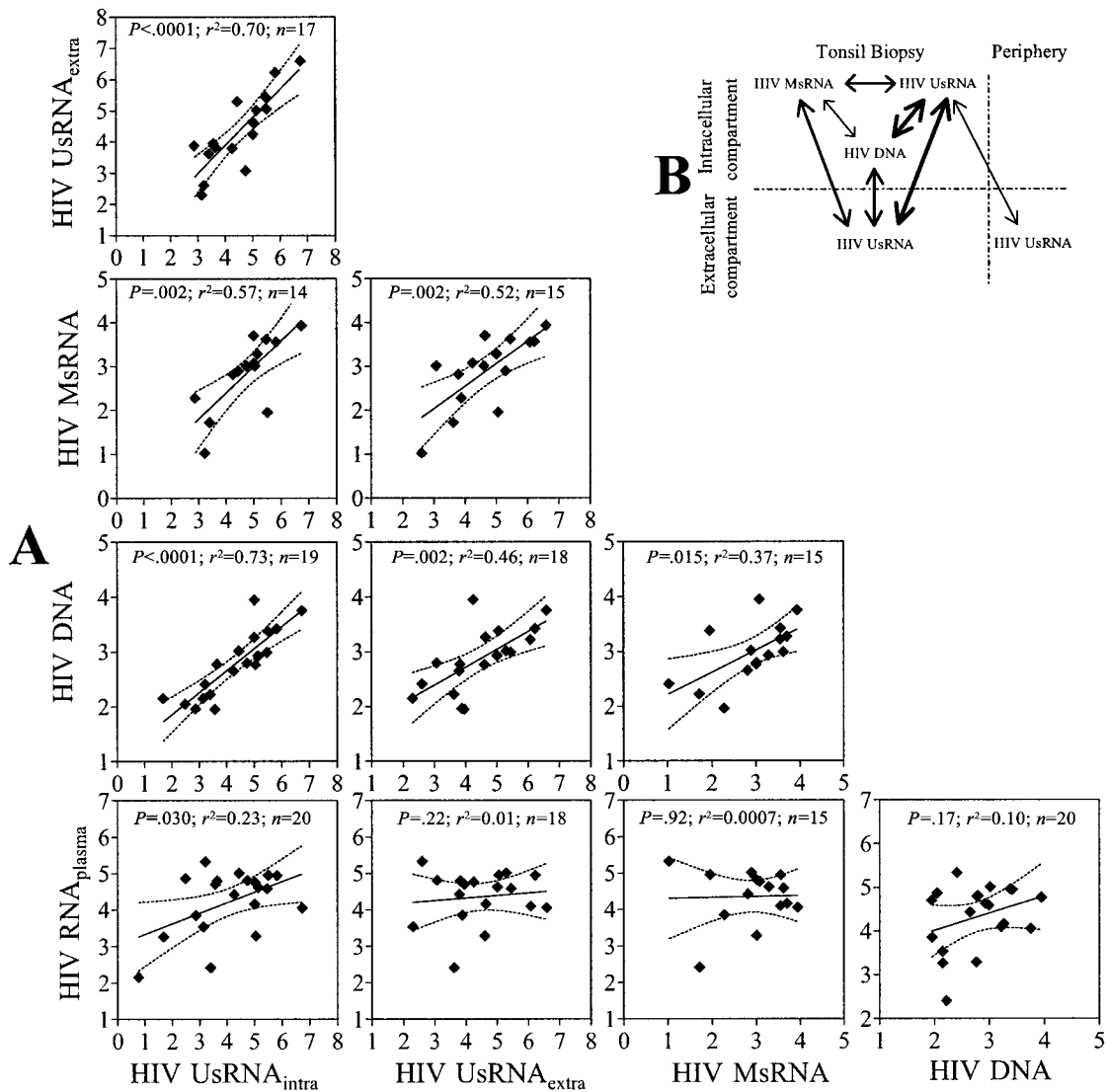
Figure 2. Correlations of HIV-1 nucleic acid levels in the tonsil biopsy specimens and plasma of patients with detectable plasma viremia. Values for unspliced HIV RNA (HIV-UsRNA) in plasma are expressed as $\log_{10}$ copies per milliliter, whereas all cell-associated HIV RNA measurements are expressed as $\log_{10}$ copies per 10^6 cells. *A*, Linear regression analysis of $\log_{10}$ values of extracellular and intracellular HIV UsRNA (HIV UsRNA$_{extra}$ and HIV UsRNA$_{intra}$, respectively), multiply spliced HIV-1 RNA encoding *tat* and *rev* (HIV MsRNA), and HIV DNA in tonsil biopsy specimens and of HIV UsRNA in the plasma of patients with >50 RNA copies/mL (HIV RNA$_{plasma}$). *Straight lines,* Linear regression curves; *broken lines,* 95% confidence intervals. Correlation coefficients (r^2), sample size *(n),* and *P* values are indicated within the graphs. *B,* Summary of correlation analysis. *Arrows,* Interdependencies between parameters; *thickness of the arrow lines,* the magnitude of correlation coefficients.

correlation of tonsillar extracellular HIV-1 RNA with plasma viremia. This finding implies that tonsil-associated viral particles reflected local virus production in a given lymphoid organ, rather than origination from the pool of HIV-1 particles in plasma. To verify this hypothesis, phylogenetic analysis of clonal HIV *env* sequences derived from plasma HIV RNA, tonsillar extracellular HIV UsRNA, and tonsillar HIV DNA was performed for 4 patients (1 untreated patient [patient 4] and 3 patients who had viral rebound in the tonsils during STI [patients 17, 20, and 21]). Relatively high viral diversities of 3.4% and 2.6% were observed in patients 4 and 20, respectively,

whereas lower diversities of 1.4% and 0.6% were observed in patients 17 and 21, respectively. Maximum-likelihood trees (figure 4) and neighbor-joining trees (data not shown) revealed clustering of the majority of virus quasi species (≥56%) of the extracellular tonsillar pool into a monophyletic group, which was designated a lymphoid cluster (table 3). Accordingly, pools of viral nucleic acids could be grouped into 2 classes: the lymphoid cluster and a second class containing the remaining clones (figure 4 and table 3). In patients 4, 17, and 21, we observed distributions of virus quasi species derived from plasma HIV UsRNA within these 2 classes, which were signif-
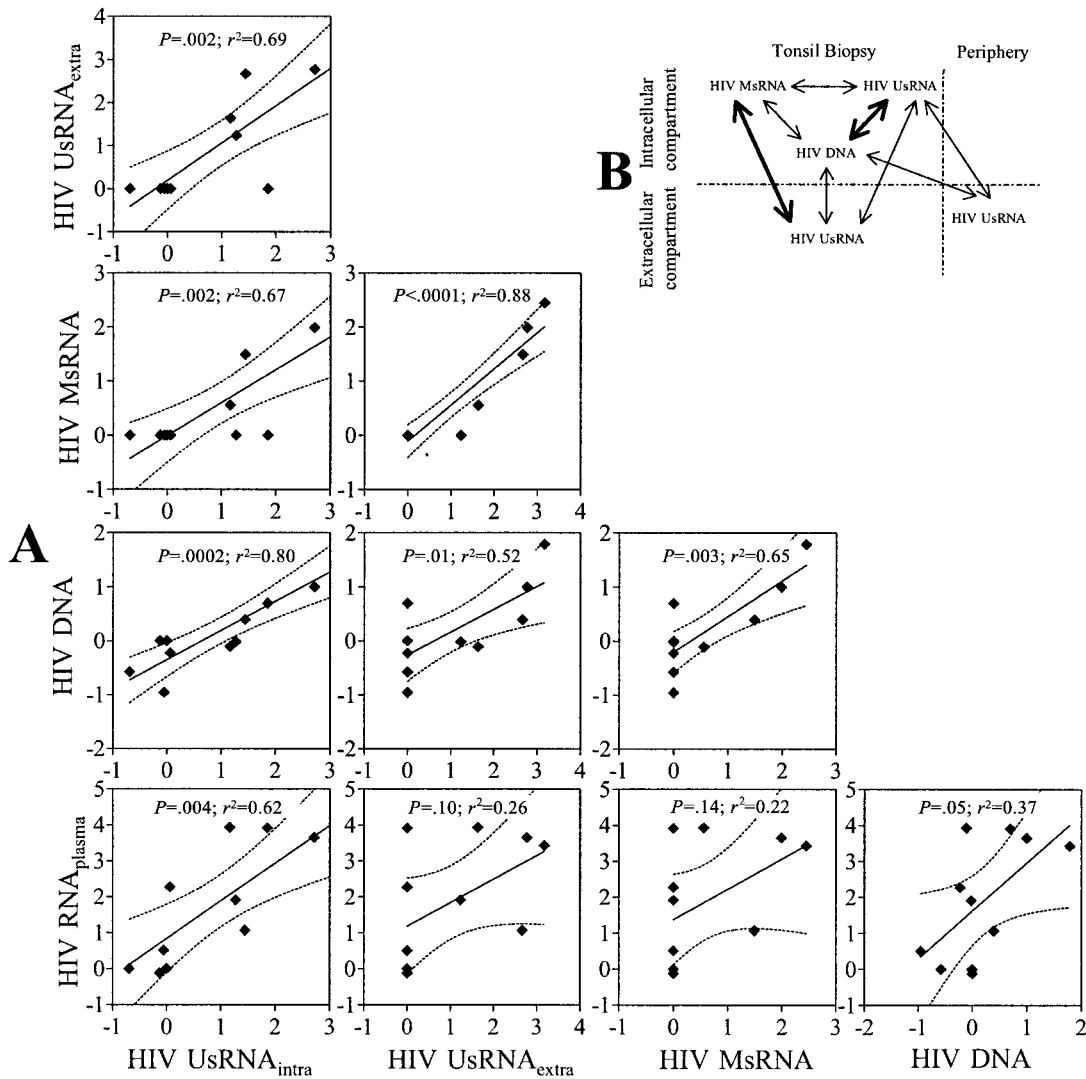
Figure 3. Correlations of relative changes in HIV-1 nucleic acid levels in tonsil biopsy specimens and plasma during structured treatment interruption (STI). Relative increases are indicated as $\log_{10}$ copies during receipt of therapy/$\log_{10}$ copies after STI. When measurements during receipt of therapy and measurements after STI were both below the limit of detection, relative increases of 0 $\log_{10}$ [1] were assigned. Note that such data points can coincide for several patients and that these data points may lie on top of each other. Increases in the viral nucleic acids of 2 patients from whom tonsil biopsy specimens were not collected during receipt of therapy (patients 20 and 24) were calculated by assuming the median measurements in specimens obtained from the remaining 9 patients during receipt of therapy. *A,* Linear regression analysis of changes in tonsil-associated unspliced HIV RNA (HIV-UsRNA [HIV UsRNA$_{extra}$, extracellular HIV UsRNA; HIV UsRNA$_{intra}$, intracellular HIV UsRNA), multiply spliced HIV-1 RNA encoding *tat* and *rev* (HIV MsRNA), and HIV DNA and of HIV UsRNA in plasma (HIV RNA$_{plasma}$). *Straight lines,* Linear regression curves; *broken lines,* 95% confidence intervals. Correlation coefficients (r^2) and *P* values are indicated within the graphs. *B,* Summary of correlation analysis. *Arrows,* Interdependencies between parameters; *thickness of the arrow lines,* the magnitude of correlation coefficients.

icantly different ($P \leqslant .01$) (table 3) from the distributions of clones derived from extracellular tonsillar HIV UsRNA. Remarkably, also, the distribution of provirus quasi species differed from that of plasma virus. This trend reached statistical significance for patient 4 ($P = .003$). Thus, the lymphoid provirus and lymphoid extracellular RNA populations appeared to be more related to each other than to the pool of virions in plasma. Even though such clear clustering into 2 monophyletic groups could not be documented for patient 20, this lack of

documentation did not reflect homogeneity between plasma and tonsillar virus quasi species but, rather, the relatively high overall viral diversity (2.6%) observed in patient 20.

The notion of genetic disparity between plasma and tonsil biopsy specimens was substantiated by an analysis of combined data from these 4 patients (patients 4, 17, 20, and 21). Results of the analysis showed that the distribution of virus quasi species in both lymphoid virus DNA and lymphoid extracellular RNA was significantly different from that in plasma ($P = .004$ and
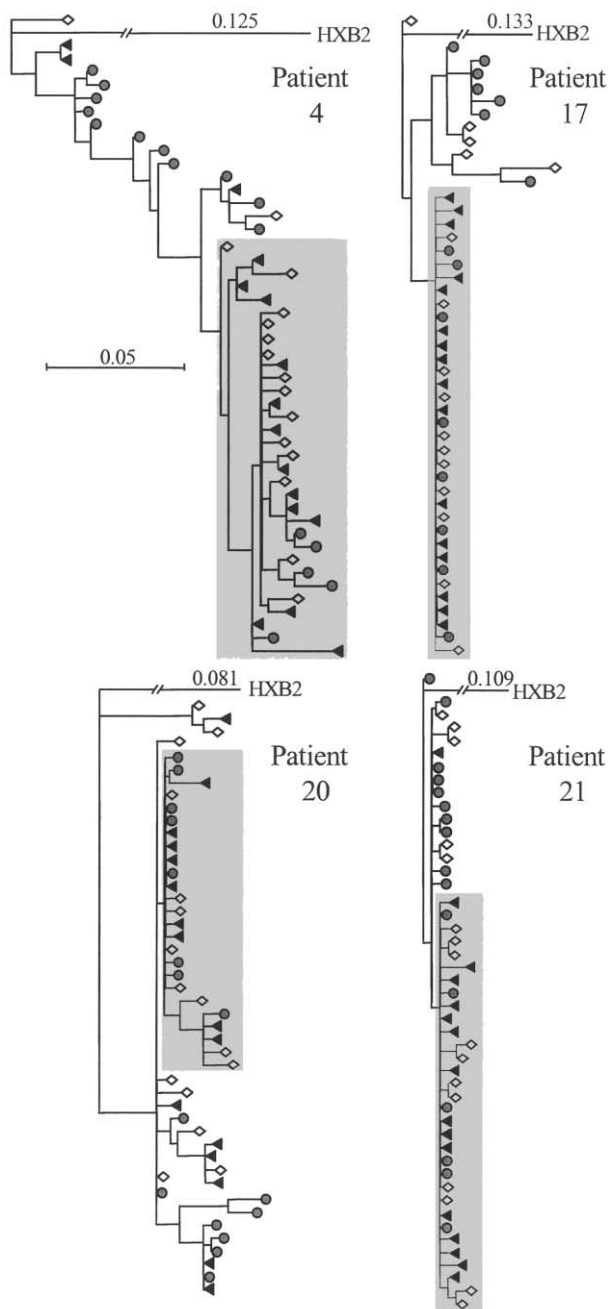
Figure 4. Phylogenetic analysis of virus quasi species in plasma and tonsils. Maximum-likelihood phylogenetic tree of HIV-1 *env* clones obtained from plasma *(shaded circles)*, tonsillar extracellular unspliced HIV RNA (HIV UsRNA; *closed triangles*), and tonsillar HIV DNA *(open diamonds)* of patients 4, 17, 20, and 21. Shaded areas denote monophyletic clusters of viral clones of predominantly tonsillar origin (i.e., "lymphoid clusters"). Individual clones were compared with the HXB2 reference sequence [42].

$P < .0001$, respectively) (table 3); however, the distribution of virus quasi species in lymphoid virus DNA was not significantly different from that in lymphoid extracellular RNA ($P = .22$).

Determination of the average viral productivity of lymphoid HIV-1–infected cells. We calculated the average content of

HIV MsRNA and intracellular HIV UsRNA per infected cell, to test whether the reduction in HIV-1 RNA levels in the lymphoid tissue of patients receiving HAART, compared with that in untreated patients, was the result of a reduction in HIV-1–infected cells or of differences in viral expression patterns in the persisting infected cells. HIV-infected cells of untreated patients contained a median of 1.63 HIV MsRNA copies/cell (range, 0.04–4.3 copies/cell) and a median of 113 intracellular HIV UsRNA copies/cell (range, 7–958 copies/cell). In the tonsil biopsy specimens of patients receiving HAART, highly significant reductions in average viral RNA productivity were observed ($\geqslant$48-fold reductions in HIV MsRNA [figure 5A] and $\geqslant$340-fold reductions in intracellular HIV UsRNA [figure 5B]). The cellular production of HIV-1 RNAs in lymphoid tissue samples obtained from patients undergoing STI was considered to be intermediate (figure 5A and 5B).

Because extracellular HIV UsRNA primarily reflected local production of HIV-1 virions, the average productivity of HIV-1 virions per infected cell could be calculated. In untreated patients, a median of 49 virions/infected cell (range, 1–352 virions/infected cell) was observed. The virion productivity of lymphoid tissue was reduced by $\geqslant$729-fold in patients receiving

Table 3. Differential clustering of virus quasi species in plasma and lymphoid tissue.

Patient, lymphoid cluster status[a]	Frequency in plasma HIV-1 RNA, %	Frequency in tonsil biopsy specimens, %			
		Extracellular HIV UsRNA	P[b]	HIV DNA	P[b]
4			.01		.003
Present	31	81		88	
Absent	69	19		13	
17			.002		.47
Present	53	100		69	
Absent	47	0		31	
20			1.0		1.0
Present	50	56		50	
Absent	50	44		50	
21			.002		.16
Present	38	94		69	
Absent	63	6		31	
All[c]			<.0001		.004
Present	43	80		69	
Absent	57	20		31	

NOTE. HIV UsRNA, unspliced HIV RNA.
[a] Defined by analysis of recent common ancestors, as shown in figure 4.
[b] Differences in the distribution of clones in lymphoid clustered or nonclustered sequences, compared with the distribution of clones derived from plasma RNA. By use of Fisher's exact test, 16 clones were analyzed for each nucleic acid pool, with the exception of the plasma RNA of patient 17, for whom 15 clones were analyzed for each nucleic acid pool.
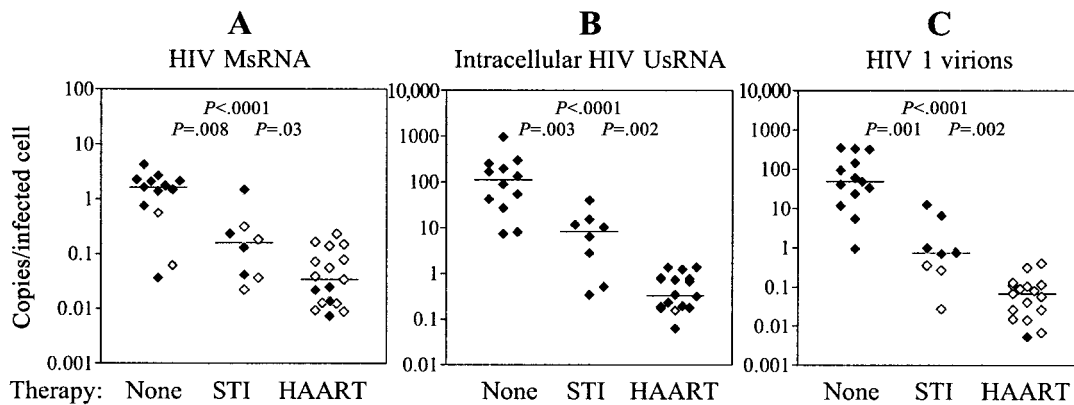[c] Combination of all data from patients 4, 17, 20, and 21.

Figure 5. Viral productivity of HIV-1–infected cells in tonsil biopsy specimens. *A*, Levels of expression of multiply spliced HIV-1 RNA encoding *tat* and *rev* (HIV MsRNA) normalized to HIV-1–infected cells. *B*, Expression levels of intracellular unspliced HIV RNA (HIV UsRNA) normalized to HIV-1–infected cells. *C*, Virion production of HIV-1–infected cells. Productivities were calculated by dividing the no. of copies of HIV RNA by the number of copies of HIV DNA. In panel *C*, the no. of copies of extracellular HIV UsRNA was divided by 2, to account for the fact that 1 virion contains 2 copies of genomic HIV-1 RNA. *Closed diamonds,* Values calculated from detectable polymerase chain reaction (PCR) measurements; *open diamonds,* values calculated using detection limits of PCR-negative specimens. *Horizontal lines* denote medians, and *P* values denoting the significance of differences between groups are shown within each panel. Note that only patients who had levels of plasma viremia >50 copies/mL were included in the group showing data from patients after 2 weeks of structured treatment interruption (STI). HAART, highly active antiretroviral therapy.

HAART (figure 5*C*), whereas, again, patients undergoing STIs showed intermediate productivity.

DISCUSSION

In the present study, analysis of viral transcription patterns in the lymphatic tissue of HIV-1–infected patients, by use of highly sensitive PCR-based assays, resulted in 3 major findings:

1.   The content of extracellular HIV-1 virions in lymphoid tissue was correlated with the number of infected cells and the viral transcriptional activity of infected cells in a given organ and, as verified by phylogenetic analysis, thus greatly depended on local productive infection.

2.   During STI, increasing transcription of multiply spliced HIV MsRNA encoding *tat* and *rev* was tightly linked to increases in lymphoid virion content. Thus, simultaneous expression of HIV MsRNA and virion-encapsidated HIV UsRNA represented a specific correlate of productively infected cells in lymphatic tissue.

3.   In patients receiving HAART, basal HIV-1 transcription persisted in lymphoid tissue, but productive infection was virtually abolished, as shown by the profound depletion of HIV MsRNA and virions.

To understand the interdependence of different viral nucleic acids in vivo, their correlations in the tonsil biopsy specimens of patients with viremia were studied. These analyses revealed high correlations among tonsillar cell-associated HIV-1 nucleic acids (figure 2*B* and figure 3*B*), findings that presumably reflect the interplay and linkage of virion production in infected cells

and reinfection of new target cells by FDC-entrapped virus [44]. On the other hand, linkage of lymphatic tissue and plasma, although significantly discernible, was limited to a single significant correlation. In particular, no correlation of plasma virus with extracellular tonsillar HIV UsRNA was observed. This lack of correlation implies either that the pool of lymphoid virions was saturated under conditions of ongoing viral replication, as was previously proposed by Haase et al. [12], or that it may not be replenished directly from plasma but, rather, primarily from virions originating in neighboring HIV-1–infected cells in the same organ. Supporting the latter hypothesis, a recent study showed locally confined spread of HIV-1 quasi species within single splenic germinal centers [45].

In agreement with these findings, the phylogenetic analysis performed, in the present study, on lymphoid DNA, lymphoid extracellular HIV UsRNA, and also the plasma HIV RNA of 1 untreated patient and 3 patients undergoing STI suggested that the pool of virus quasi species in the extracellular fraction of tonsillar HIV UsRNA closely overlapped with provirus quasi species in the same tissue, whereas the virus pool in plasma showed significant overall disparity from these lymphoid clusters ($P \leq .004$). Of note, our data complement the previous observation that rebounding virus in plasma during STI was different from the viral population present in the pool of persistently infected cells in peripheral blood [46]. The fact that the majority of rebounding virus quasi species present in lymphoid tissue after cessation of therapy, as represented by extracellular HIV UsRNA, was monophyletic, whereas the rebounding viruses in plasma were more heterogeneous, suggests that plasma HIV RNA represents a larger virus pool, including

the progeny of different lymphoid sites and, potentially, also of other viral reservoirs, such as the central nervous system [47] and the genital tract [48]. It is likely that these viruses may consist of a spectrum of virus quasi species derived from different bursts of replication triggered by activation of latently infected cells [49].

The concept of local and compartmentalized viral replication in lymphoid tissue was further supported by longitudinal correlation analysis of tonsil biopsy specimens obtained from patients before and after 2 weeks of STI. No correlation of elevations in lymphoid extracellular HIV UsRNA with changes in plasma viremia was observed. However, an outstanding linkage with increases in HIV MsRNA was observed (figure 3). Expression of HIV MsRNA is a prerequisite for virus production of HIV-1–infected cells [19] and may be viewed as a surrogate marker of productively infected cells [8], with some restrictions regarding its occurrence at low levels in different types of latently infected cells [8, 21, 26]. In tonsil biopsy specimens obtained from patients receiving HAART, expression of HIV-1 particles was almost completely absent. The number of virions per HIV-1–infected cell, which is an estimate of the average viral productivity of the infected cells in a given organ, was reduced by ⩾729-fold, compared with the number in specimens from patients with viremia.

That expression and trapping of HIV-1 virions were hardly discernible in patients receiving HAART, as measured by the highly sensitive RT-PCR assay [8] used in the present study, verifies and extends observations of previous studies that used in situ hybridization [5, 6, 50]. In addition, the data from the present study show that residual HIV UsRNA persisting in the lymphoid tissue of patients receiving HAART is almost exclusively located within cells and therefore cannot be attributed to the presence of FDC-trapped virions. These findings are in opposition to the hypothesis that FDC-trapped virions are a major viral reservoir during HAART [51]. Nevertheless, it cannot totally be excluded that extracellular particles were present, at frequencies below the limits of detection of the test used in the current study, in tonsil biopsy specimens obtained from patients receiving HAART. Considering that FDC-entrapped HIV-1 virions in mice have been shown to be highly infectious in vivo even months after deposition [52], it may be hypothesized that the incomplete viral replication observed in only 6 of 11 biopsy specimens tested may have been initiated by rare FDC-bound particles present in a minority of lymphoid locations. However, in our view, it is more plausible that this stochastic pattern of viral bursts after cessation of therapy was the result of random activation of latently infected cells [49], which may have been present in all tested tonsils, as indicated by persistence of HIV DNA.

Similar to extracellular HIV UsRNA, HIV MsRNA was significantly depleted in patients receiving HAART ($P = .01$), with occasional low-level expression being detectable in 26% of the biopsy specimens tested. The average HIV MsRNA productivity of HIV-1–infected cells was reduced >48-fold, compared with that noted in specimens from patients with viremia. In contrast, intracellular HIV UsRNA was detected in 86% of the biopsy specimens obtained from patients receiving HAART, while also showing a 340-fold decrease in the average productivity per infected cell.

Thus, the pattern of HIV-1 transcription and viral expression of HIV-1–infected cells observed in the lymphoid tissue of patients receiving HAART was profoundly different from that observed in patients with viremia. Findings of such a repressed pattern, persistence of intracellular HIV UsRNA at low levels, minute expression of HIV MsRNA, and almost-complete absence of extracellular HIV-1 particles were intriguingly reminiscent of the findings for the HIV-1–infected PBMCs of patients receiving HAART for the long term [8]; such PBMCs were shown to be depleted of productively infected cells, whereas 2 distinct classes of HIV-1–infected cells persisted (with 1 class expressing solely intracellular HIV UsRNA and a second, less frequently occurring class also expressing HIV MsRNA at minute levels). Analogous to these observations in PBMCs [8], it may be concluded that equivalent classes of HIV-1–infected cells persist in the lymphoid tissue of patients receiving HAART. Such cells expressing low levels of viral RNA can be viewed as latently infected, by use of a definition of viral latency that encompasses a lack of virion production [53] but, in accordance with the herpes simplex–paradigm of viral latency, not necessarily absolute viral transcriptional quiescence [54].

In support of this assumption, we observed that, during short interruptions of HAART, only incomplete and stochastic viral replication occurred in lymphoid tissue. This implies that during antiretroviral therapy, bursts of viral replication in lymphoid tissue can be expected to be even rarer and may only occur in a small fraction of the body's lymphoid organs.

Furthermore, previous reports that used ultrasensitive in situ hybridization protocols revealed persistence of rare HIV-1–infected cells bearing low levels of intracellular HIV-1 RNA and absence of cells expressing high levels of viral RNA in the lymphoid tissue of patients receiving HAART [5, 6, 50, 55]. However, in conflict with our view of residual HIV-1 infection in lymphoid tissue being dominated by latently infected cells, Hockett et al. [56] reported the viral RNA contents of HIV-1–infected cells to be high and uniform in lymphoid tissue, regardless of the levels of plasma viremia. The discrepancy between the findings of Hockett et al. [56] and those of the present study may be explained by incomplete suppression of viral replication during the short treatment time (⩽36 weeks) in the study by Hockett et al. [56], compared with the longer treatment period (>72 weeks) in the present study. Furthermore, the lower sensitivities of the PCR assays used by Hockett et al.

[56] (100 copies/PCR), compared with those of the assays used in the present study (<2 copies/PCR), likely explain the apparent absence of HIV-1–infected cells with low levels of viral expression in the study by Hockett and colleagues.

In summary, the present study demonstrated that a profound shift in the transcription pattern of HIV-1–infected cells in lymphoid tissue in vivo occurred in response to HAART. The average production of viral RNAs per infected cell was greatly reduced, and there was a vast depletion in the pool of locally produced extracellular virions. These observations suggest that the majority of residual viral RNA in the lymphoid tissue of patients receiving HAART can be attributed to basal viral transcription in latently infected cells and that it reflects neither persistence of productively infected cells nor persistence of virions entrapped in the FDC network. The findings of the present study emphasize the impact of latency on persistence of HIV-1 during HAART. Therefore, novel treatment strategies to purge the latent viral reservoirs are needed to revisit the aim of eradicating HIV-1.

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