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QUANTIFYING RESIDUAL HIV-1 REPLICATION IN PATIENTS RECEIVING COMBINATION ANTIRETROVIRAL THERAPY

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ABSTRACT

Background In patients infected with human immunodeficiency virus type 1 (HIV-1), combination antiretroviral therapy can result in sustained suppression of plasma levels of the virus. However, replication-competent virus can still be recovered from latently infected resting memory CD4 lymphocytes; this finding raises serious doubts about whether antiviral treatment can eradicate HIV-1.

Methods We looked for evidence of residual HIV-1 replication in eight patients who began treatment soon after infection and in whom plasma levels of HIV-1 RNA were undetectable after two to three years of antiretroviral therapy. We examined whether there had been changes over time in HIV-1 proviral sequences in peripheral-blood mononuclear cells, which would indicate residual viral replication. We also performed in situ hybridization studies on tissues from one patient to identify cells actively expressing HIV-1 RNA. We estimated the rate of decrease of latent, replication-competent HIV-1 in resting CD4 lymphocytes on the basis of the decrease in the numbers of proviral sequences identified during primary infection and direct sequential measurements of the size of the latent reservoir.

Results Six of the eight patients had no significant variations in proviral sequences during treatment. However, in two patients there was sequence evolution but no evidence of drug-resistant viral genotypes. In one patient, extensive in situ studies provided additional evidence of persistent viral replication in lymphoid tissues. Using two independent approaches, we estimated that the half-life of the latent, replication-competent virus in resting CD4 lymphocytes was approximately six months.

Conclusions These findings suggest that combination antiretroviral regimens suppress HIV-1 replication in some but not all patients. Given the half-life of latently infected CD4 lymphocytes of about six months, it may require many years of effective antiretroviral treatment to eliminate this reservoir of HIV-1. (N Engl J Med 1999;340:1605-13.)

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BETTER understanding of the dynamics of the replication of human immunodeficiency virus type 1 (HIV-1) in vivo¹⁻⁴ has provided an important rationale for early and aggressive treatment of this infection. The advent of combination antiretroviral therapy has made it possible to suppress the replication of HIV-1 in infected persons to such an extent that the virus becomes undetectable in the plasma for more than two years.⁴⁻⁶ For the first time in the history of this epidemic, the eradication of HIV-1 from an infected person is a real scientific objective.^{4,7} However, a major obstacle to the elimination of HIV-1 became apparent when latent, replication-competent virus was found within resting memory CD4 lymphocytes,⁸ which have a long life span.⁹⁻¹¹

In addition, this latent reservoir of HIV-1 is established early in infection and persists after two years of seemingly effective combination antiretroviral therapy,^{12,13} even when treatment is initiated during the primary phase of infection.¹⁴ But the true size of the latent reservoir cannot be properly assessed unless it is known whether viral replication has been completely stopped by the drug regimen. In other words, unrecognized residual replication of HIV-1 would result in an overestimation of the size of the latent reservoir. We therefore undertook a study to look for evidence of ongoing HIV-1 replication as well as a

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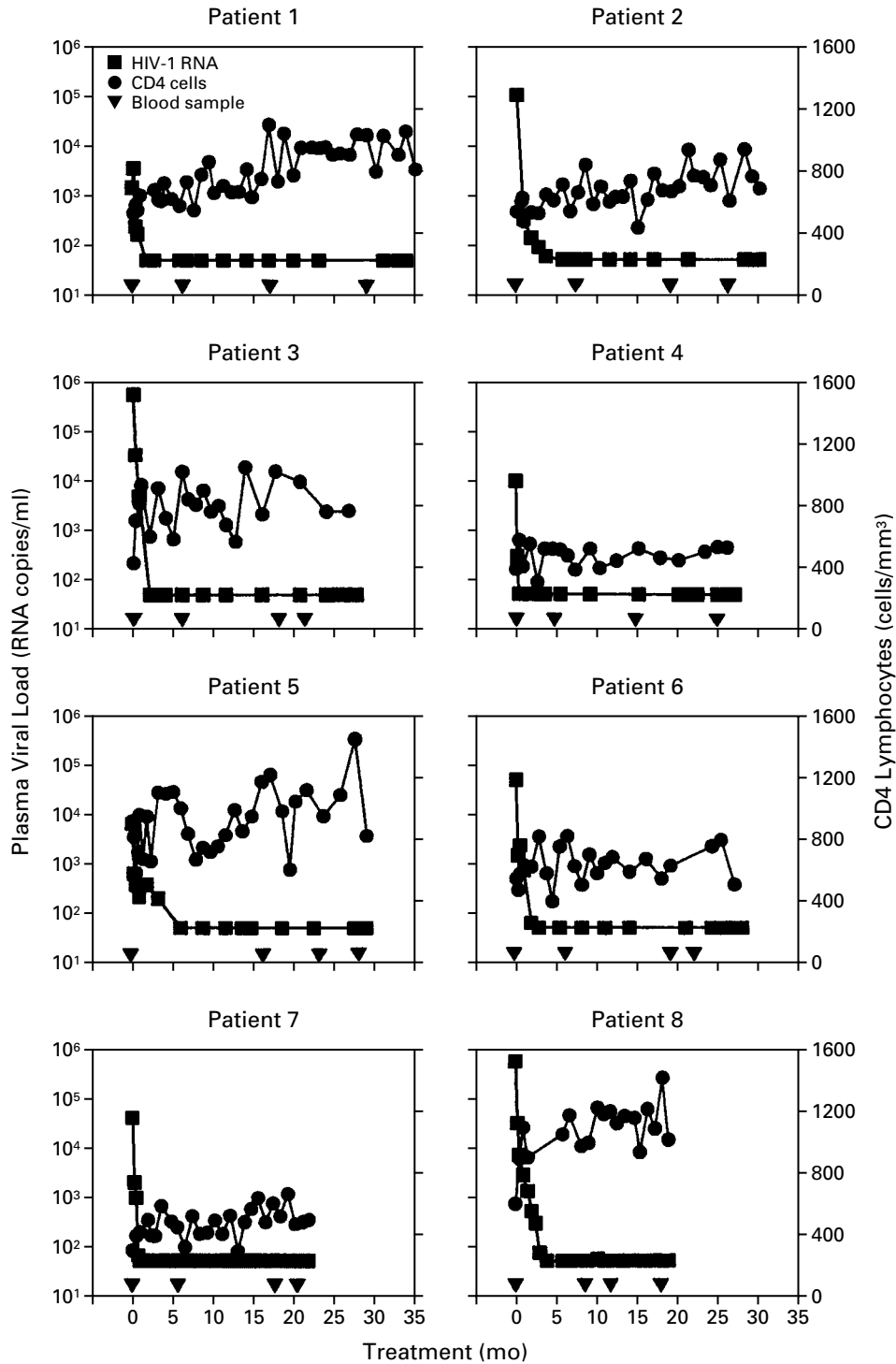


Figure 1. Changes in Plasma Viral Load and CD4 Lymphocyte Count during Combination Antiretroviral Therapy in the Eight Men.

Patients 1, 2, and 5 were taking zidovudine, lamivudine, and ritonavir; Patients 3, 4, and 6 were taking zidovudine, lamivudine, and indinavir; and Patients 7 and 8 were taking zidovudine, lamivudine, ritonavir, and saquinavir. Patients 3 and 6 were 30 years old, Patients 1 and 5 were 32 years old, Patient 4 was 34 years old, Patient 7 was 35 years old, Patient 2 was 36 years old, and Patient 8 was 37 years old. The arrowheads indicate the days on which peripheral-blood mononuclear cells were obtained for DNA-sequence analysis.

decrease in the size of the latent reservoir in a select group of patients who began treatment early and in whom the HIV-1 RNA had been completely undetectable in plasma for approximately two to three years during combination therapy with three or four drugs.

METHODS

Patients

Eight men ranging in age from 30 to 37 years were chosen for the study (Fig. 1) from over 100 subjects enrolled in our clinical trials, because they had had complete suppression of HIV-1 in plasma while being fully compliant with the prescribed antiretroviral therapy. At base line they had a mean CD4 lymphocyte count of 533 per cubic millimeter and an average plasma viral load of 174,000 RNA copies per milliliter. The level of HIV-1 in plasma was measured by a commercial reverse-transcriptase–polymerase-chain-reaction (PCR) assay (Amplicor Ultrasensitive HIV-1 Monitor assay, Roche Molecular Diagnostic Systems, Branchburg, N.J.), which has a limit of detection of 50 HIV-1 RNA copies per milliliter of plasma. All patients were enrolled in treatment protocols within the first 90 days after acute infection. The antiviral regimens consisted of zidovudine (600 mg per day) and lamivudine (300 mg per day), along with either ritonavir (1200 mg per day) or indinavir (2400 mg per day) or with both ritonavir (800 or 1200 mg per day) and saquinavir (1200 mg per day). As shown in Figure 1, each patient had a steep decline in plasma HIV-1 RNA levels during treatment; levels below 50 RNA copies per milliliter were quickly reached and then sustained for 20 to 35 months of therapy. After month 5, plasma viremia was not detected in any of the patients. As compared with base-line values, there was an average increase in the mean CD4 lymphocyte count of approximately 300 cells per cubic millimeter for the last four measurements.

Amplification, Sequencing, and Sequence Analysis

DNA was extracted from peripheral-blood mononuclear cells as previously described.¹⁵ Single molecules of the provirus were amplified after limiting-dilution analysis and sequenced directly with an automated sequencer (Prism 377, Applied Biosystems, Foster City, Calif.) to avoid errors introduced by the amplification of DNA *in vitro*.¹⁵ The region amplified in gp120 spanned the V3, V4, and V5 hypervariable domains. The outer *env* primer sequences, with their positions in HIV-1 clone NL4-3 indicated in parentheses, were V3a 5'CCAATTCCCACATACATTATTG3' (nucleotide 6848) for the forward primer and V3i 5'GCGTTATTGACGCTGCG-CCCAT3' (nucleotide 7823) for the reverse primer, and the respective inner primers were V3e 5'GTACAATGTACACATGGAAT3' (nucleotide 6947) and V3h 5'AATTCACCTTCTCCAATTGTC3' (nucleotide 7662). The outer primers for protease and reverse transcriptase were PRouter 5'GAGCAGACCAGGCCAACAGC-CCA3' (nucleotide 2139) for the forward primer and RTouter 5'GCCCTGCTTCTGTATTCTGC3' (nucleotide 3549) for the reverse primer, respectively, and the inner primers were PRinner 5'GAAGCAGGAGCCGATAGACAAGG3' (nucleotide 2211) and RTinner 5'GTGGTACTACTTCTGTTAGTGC3' (nucleotide 3432), respectively. Each round of PCR consisted of 30 cycles, with the first 5 cycles at 94°C for one minute, 52°C for one minute, and 72°C for one minute, followed by 25 cycles at 94°C for one minute, 55°C for one minute, and 72°C for one minute.

All DNA extractions and amplification reactions were carried out with appropriate negative controls in parallel to detect contamination at each step of the procedure. Nucleotide sequences were aligned with use of the Clustal V program.¹⁶ Pairwise distances among sequences were estimated by the DNADIST program in the PHYLIP package.¹⁷ Phylogenetic analysis of the nucleotide sequences was carried out with use of the neighbor-joining method.¹⁸ Bootstrap analysis was also performed on 1000 replicates to evaluate the reliability of the neighbor-joining results (PHYLIP programs SEQBOOT and CONSENSE).

Quantitative Viral Culture

The virus was cultured after quantitative limiting-dilution analysis according to the method of Finzi et al.¹³ in CD4-enriched peripheral-blood mononuclear cells that had been depleted of CD8 lymphocytes with the use of immunomagnetic beads (DynaL, Lake Success, N.Y.). Cells were serially diluted (up to six replicates for each inoculum size) and plated in medium containing phytohemagglutinin (2 µg per milliliter) and interleukin-2 (10 U per milliliter) with gamma-irradiated allogeneic (donor) CD4 peripheral-blood mononuclear cells in a concentration that was 2 to 10 times the concentration of CD4 cells in the patient. The culture supernatant was replaced the next day by medium containing only interleukin-2. On day 2, CD8-depleted, phytohemagglutinin-stimulated blasts from an HIV-1–seronegative donor were added. Thereafter, half of the medium was changed every three to four days and fresh CD8-depleted, stimulated blasts were added weekly until day 28. The serial-culture supernatant was then examined for HIV-1 by measuring the p24 antigen concentration with use of an enzyme immunoassay (Abbott Laboratories, Abbott Park, Ill.). The infectious titer of latent, replication-competent virus was then determined according to the maximum-likelihood method.⁴ The sensitivity of the assay was limited by the number of cells available for multiple replicate cultures.

Tissue Collection and Processing and *In Situ* Hybridization

We obtained multiple biopsy samples from Patient 5 to look for cells actively expressing HIV-1 RNA. The tissue samples were fixed in 10 percent buffered formalin for approximately 24 hours and then embedded in paraffin with use of an automated tissue processor under heat and vacuum pressure. Tissue sections were processed and examined by *in situ* hybridization, as described previously.¹⁹

Statistical Analysis

We used least-squares analysis to estimate the best-fit slope for each patient so as to determine the exponential rate of decrease in the numbers of replication-competent HIV-1 within resting memory CD4 lymphocytes and of parental proviral sequences identified during the primary infection. We calculated the average and standard error of the slopes, using data points from month 8 to month 28 of therapy. For the estimates of the half-lives of the virus, we used the 95 percent confidence intervals, which we calculated as the mean values ± 2 SE for the average slopes.

RESULTS

Residual HIV-1 Replication

Four sequential samples of peripheral-blood mononuclear cells were obtained from each patient and examined to determine whether there were changes in the DNA sequences, which would indicate ongoing replication of HIV-1. A total of 414 amplicons (10 to 21 fragments per sample of peripheral-blood mononuclear cells; 42 to 64 fragments per patient) that ranged from 612 to 669 bp in length were obtained at the limits of the serial-dilution PCR. These nucleotide sequences, deposited in GenBank (accession numbers AF093912 to AF094325), were matched with available sequences in the Los Alamos Database with use of the Clustal V program.¹⁶ When analyzed phylogenetically by the neighbor-joining method,¹⁸ sequences from each patient formed a tight cluster within clade B (Fig. 2) and were distinct from those of control subjects or other patients (data not shown). Thus, there is no indication that the sequences ex-

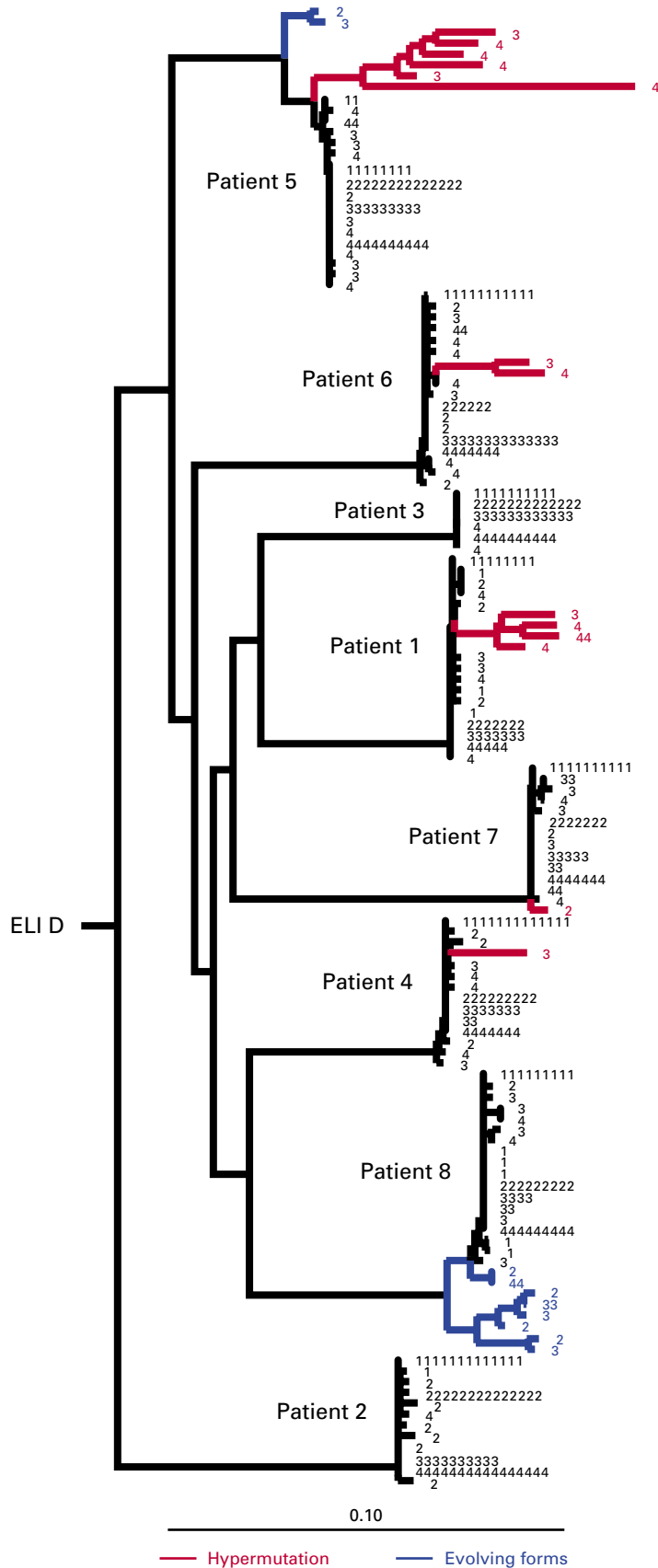


Figure 2. Phylogenetic Tree Depicting HIV-1 *env* Sequences Found in the Eight Patients.

All sequences fall within HIV-1 clade B and are shown with ELI subtype D (GenBank accession no. K03454) as the outgroup. Sequences from each patient clustered together, as expected for a population of viruses that are highly related. The genetic relatedness of two different sequences in the phylogenetic tree is represented only by the horizontal distance that separates them, with the length of the bar at the bottom denoting a sequence divergence of 0.10 (or 10 percent). Vertical distances in the tree are not counted since they are drawn arbitrarily for the sake of clarity. Sequences containing hypermutations are shown in red, whereas evolving sequences are shown in blue. All other sequences are within three mutations from the dominant form at point 1 (the time at which the initial sample of peripheral-blood mononuclear cells was obtained). The numbers 1, 2, 3, and 4 denote the times at which the sequences were obtained and match the times indicated by the arrowheads in Figure 1. Each number represents one sequence.

amined were the result of contamination during PCR amplification.

As described previously,^{20,21} viral sequences obtained during or shortly after primary HIV-1 infection (time 1) were extremely homogeneous (Fig. 2). Four patients (Patients 3, 4, 6, and 7) had no sequence variation at the initial time point; in no patient did the sequence differ by more than two mutations from the predominant form. The mean variation within samples for the eight patients at time 1 was 0.03 percent, with the highest degree of variation (0.14 percent) found in Patient 8. In three patients (Patients 2, 3, and 7), there were no appreciable increases in the degree of sequence variation (differing by no more than two mutations from the parental sequence). Greater divergence was found in samples obtained at subsequent points from the other patients. However, in Patients 1, 4, and 6, the more divergent sequences were the results of hypermutations, typically a cluster of mutations in which A had been substituted for G (Fig. 2). Since hypermutations are believed to be the product of a single replication cycle,^{22,23} they cannot be regarded as evidence of gradual sequence evolution. Thus, there was no genetic evidence to indicate continued replication of HIV-1 in these three patients.

On the other hand, substantial sequence divergence, exclusive of hypermutated forms, was found in Patients 5 and 8 (Fig. 2). Up to 16 and 25 nucleotide substitutions were found in Patients 5 and 8, respectively. Such diversity is possible only through continued viral replication. However, most of the variation had occurred by the time of the second analysis (at months 16 and 9, respectively), suggesting that much of the replication of residual virus may have occurred during the early phase of treatment. We also extensively sequenced proviruses from various tissues from Patient 5. Divergent proviral sequences similar to those found in peripheral-blood mononuclear cells were identified in the lymph node and tonsil but not in the sigmoid colon or rectum 15 months after treatment began (data not shown).

To determine whether the residual replication in Patients 5 and 8 was due to the emergence of drug-resistant HIV-1, we looked for genotypic evidence of drug-resistant virus in the DNA from peripheral-blood mononuclear cells obtained at the fourth measurement in each patient (months 29 and 18, respectively). Again, we used limiting-dilution PCR to amplify the relevant regions of the protease and reverse transcriptase genes. Twelve of 16 protease clones and 11 to 12 reverse transcriptase clones were obtained from each patient for nucleotide sequencing. All sequences encode for wild-type amino acids at protease or reverse transcriptase residues that are involved in conferring resistance to ritonavir, indinavir, saquinavir, zidovudine, and lamivudine (GenBank accession numbers AF097943 to AF097992). Thus, the residual replication of HIV-1 does not appear to

be under any appreciable selective pressure exerted by the antiretroviral agents. This conclusion, in turn, implies that the virus might be replicating within a small compartment free from the influence of the antiretroviral agents. This compartment could be a specific anatomical location or diffusely scattered cell populations that are not reached or affected by the antiretroviral agents for pharmacokinetic reasons or because of aberrant drug metabolism by the cells.

Next, we studied tissue samples and body fluids from Patient 5 to confirm the presence of residual HIV-1 replication as well as to search for anatomical sites where the virus continued to be expressed. Biopsies of the rectum, sigmoid colon, and descending colon were performed at months 12 and 15 of treatment. In addition, semen samples were obtained during months 13 and 15, and a sample of cerebrospinal fluid was obtained during month 15. A cervical-lymph-node biopsy was also performed during month 15, and tonsillar biopsies during months 15 and 20. HIV-1 RNA was undetectable (<50 RNA copies per milliliter) in the seminal or cerebrospinal fluid. A total of 175 tissue sections from the lymph node, tonsil, and gastrointestinal tract were processed and examined by *in situ* hybridization as described previously.¹⁹

In contrast to the findings in lymphoid tissues from an untreated patient with HIV infection (Fig. 3A, 3B, and 3C), in 162 tissue sections from Patient 5, there was a complete absence of cells expressing viral RNA in the network of follicular dendritic cells in germinal centers (Fig. 3D, 3E, and 3F). The lymphoid architecture was also largely intact. However, in 13 tissue sections (6 lymph-node sections, 5 tonsillar sections, and 2 gastrointestinal sections), 19 cells expressing viral RNA were identified (Fig. 3G, 3H, and 3I). Morphologically, every RNA-positive cell resembled a lymphocyte; the grain count generated from the isotope-labeled probes ranged from 15 to 60 per cell (insets in Fig. 3G and 3H), which is markedly lower than typical grain counts in untreated patients.¹⁹ Interestingly, many of the RNA-positive lymphocytes were found in lymphoid sinuses, suggesting that they might be in transit.

No evidence of viral trapping in follicular dendritic cells was found, even in the sections containing RNA-positive cells. Taken together, these results not only confirm the presence of ongoing HIV-1 replication in Patient 5, but also demonstrate that this residual activity was occurring in the lymphocyte population usually affected and in the expected anatomical sites.

Changes in the Latent Virus

The genetic data also allowed us to measure the decrease in the level of the parental virus (the dominant form at time 1, or form 1) in peripheral-blood mononuclear cells during treatment. At each of the three subsequent times the sequences were analyzed,

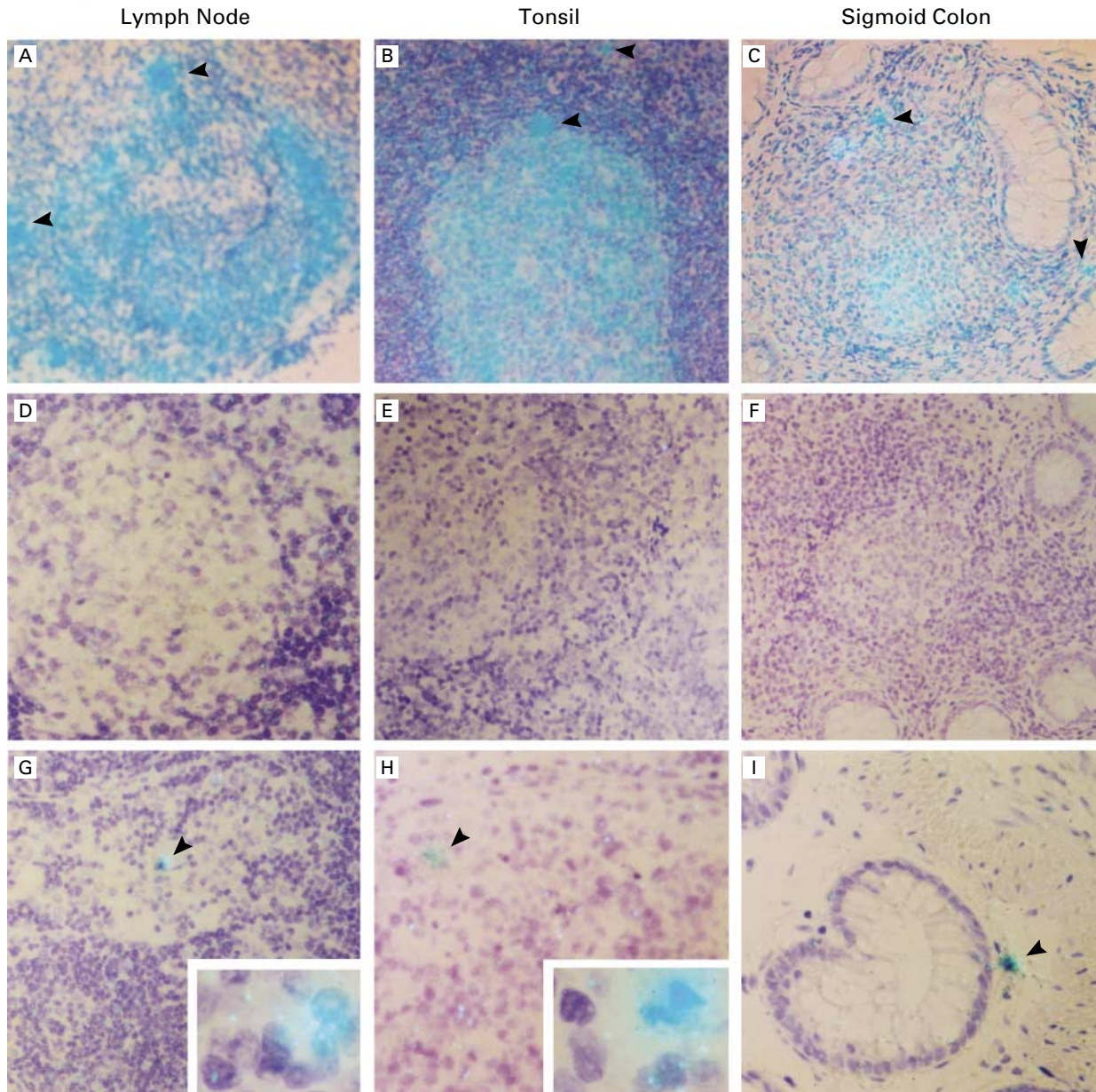


Figure 3. In Situ Hybridization Studies of Tissue from an Untreated Patient with HIV Infection (Panels A, B, and C) and Patient 5 (Panels D through I).

There is extensive expression of HIV-1 (blue staining) in the network of follicular dendritic cells in germinal centers found in the lymph node (Panel A), tonsil (Panel B), and sigmoid colon (Panel C) from the untreated patient. Cells expressing viral RNA are indicated by arrowheads. Most tissue sections from Patient 5 had no RNA-positive cells (Panels D, E, and F), but some sections had a few RNA-positive cells (arrowheads in Panels G, H, and I). The RNA-positive cells had the morphologic appearance of lymphocytes (insets in Panels G and H). (Panels A, B, C, E, F, and G, $\times 200$; Panels D, H, and I, $\times 800$; insets, $\times 1260$.)

the fraction of sequences identical to form I was determined for each patient. In addition, at each time, the number of copies of proviral DNA in peripheral-blood mononuclear cells was determined by quantitative PCR-based assays.²⁴ These two sets of results allowed us to calculate the absolute number of form

I at each point in time. Figure 4A shows that in every patient there was a gradual loss of the parental sequence, with a mean half-life of 6.4 months (95 percent confidence interval, 4.9 to 8.9 months). Why is this half-life important? Although much of the proviral DNA does not yield infectious HIV-1, it is pri-

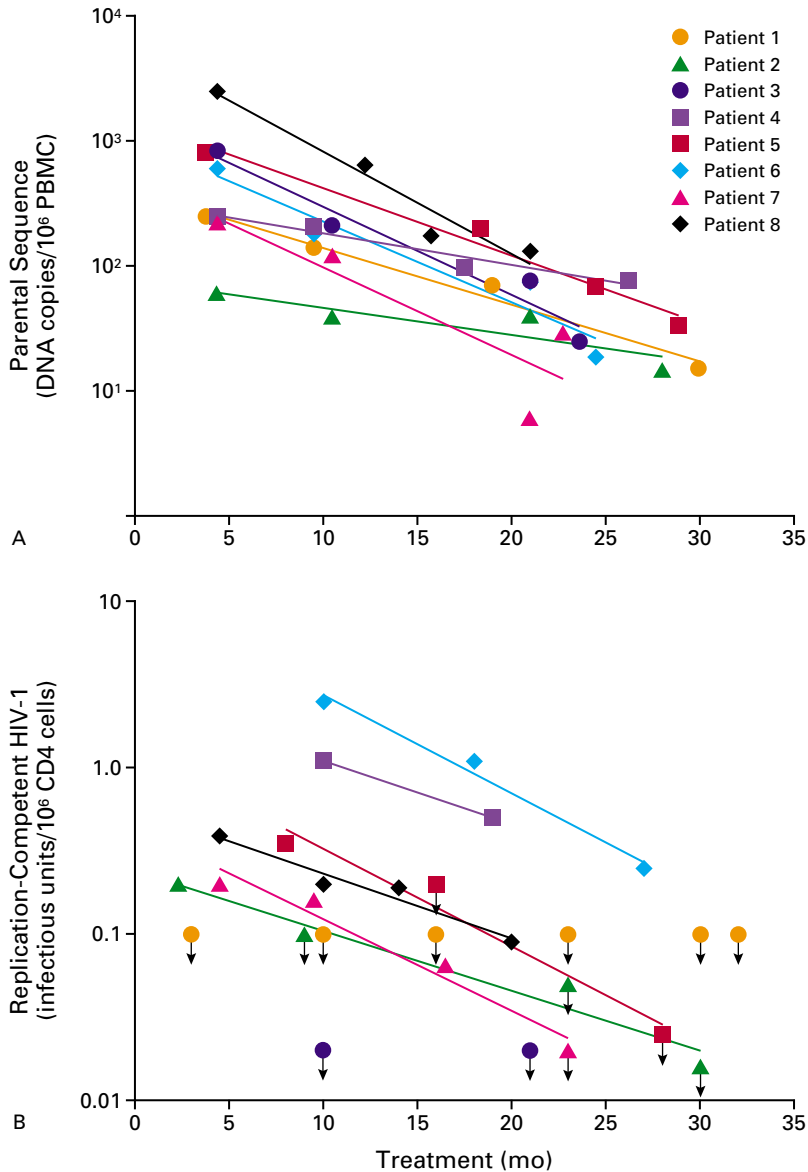


Figure 4. Changes in the Levels of the Parental Proviral Sequence in Peripheral-Blood Mononuclear Cells (Panel A) and in the Numbers of Latent, Replication-Competent HIV-1 (Panel B) during Antiretroviral Therapy in the Eight Patients.

The linear regression line for each data set is shown. In Panel B, the arrows indicate values below the limit of detection. No regression lines are shown for Patients 1 and 3, who had no detectable reservoir of latent virus. PBMC denotes peripheral-blood mononuclear cells.

marily harbored within resting memory CD4 lymphocytes, the same cell population in which latent, replication-competent HIV-1 is found.⁸ Thus, the decrease in levels of the parental proviral DNA provides an indirect assessment of the turnover rate of the latent virus.

The decrease in the numbers of latent, replication-competent HIV-1 was also measured directly with use of a limiting-dilution culture technique based on the method initially described by Finzi et al.¹³ As

shown in Figure 4B, Patients 1 and 3 had no detectable replication-competent virus in cultures of up to 10 million to 50 million CD4 lymphocytes. In the other six patients, there was a gradual decrease in the levels of latent, replication-competent HIV-1, with a mean half-life of 6.2 months (95 percent confidence interval, 5.3 to 7.5 months). This turnover rate is remarkably close to that determined by the indirect method and to the average life span of resting memory CD4 lymphocytes.⁹⁻¹¹

DISCUSSION

We studied eight patients who had had undetectable levels of HIV-1 RNA in plasma for up to 35 months during potent combination antiretroviral therapy. There was no evolution of the viral sequence during treatment in six of these patients. This finding suggests that treatment decreased the rate of replication of HIV-1 to an undetectable level. Two patients, however, did have sequence changes. It could be argued that the new sequences resulted from the selection of variant viruses not found initially. But such a possibility is unlikely for two reasons. First, the variant forms were not detected among the large number of independent HIV-1 clones examined at the beginning of treatment. Second, the extent of the sequence differences (up to 16 and 25 substitutions in fragments of approximately 650 bp) would be unusual during primary infection.^{20,21} Thus, we believe that the sequence changes are more likely due to ongoing residual replication, albeit at an exceedingly low level. This conclusion is supported by evidence of continued expression of viral RNA in lymphoid tissues, detected by *in situ* hybridization, in one patient. Almost certainly, most patients treated in the usual clinical setting would have greater degrees of residual viral activity, since they typically do not have the sustained suppression of plasma viremia seen in our highly selected study subjects. The continued replication of HIV-1 in two patients seems to be due to the presence of drug-sensitive viruses within lymphoid tissues. We are unable, however, to explain why drug-sensitive HIV-1 is capable of replicating at low levels during treatment with three or four drugs. But it is essential to the therapeutic effort that the answer, be it pharmacokinetic or cellular in nature, be obtained promptly.

That there is unrecognized residual replication of HIV-1 in some patients despite apparently effective combination therapy is both good and bad news. On the one hand, this finding tells us that the extent of the persistence of a latent reservoir of virus^{8,12-14} may be overestimated, because there is unrecognized replenishment of the pool by active viral replication. But, on the other hand, it is sobering to realize that the so-called highly active antiretroviral therapy is actually not always active enough. As we strive to eradicate HIV-1 infection or induce a remission,⁷ we must focus on the possibility of further intensifying antiretroviral treatment, even though current therapies are already toxic, costly, and complex.

What does our estimate of the half-life of latent, replication-competent HIV-1 imply about the duration of effective treatment that is required to eliminate this pool? The size of the latent reservoir ranges from 10,000 to 1 million cells^{7,8} (Fig. 4B). Therefore, as a crude estimate, it will take 14 to 20 half-lives for the pool to decrease to a size of less than 1. If the half-life is 6 months, roughly 7 to 10 years of con-

tinuous, truly effective therapy will be necessary to eliminate this reservoir. It will be difficult to maintain treatment for such a long time; thus, we must find ways to facilitate a decrease in the size of the pool of latent virus.

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