Clonal Sequences Recovered from Plasma from Patients with Residual HIV-1 Viremia and on Intensified Antiretroviral Therapy Are Identical to Replicating Viral RNAs Recovered from Circulating Resting CD4$^+$ T Cells$^\nabla$

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Despite successful antiretroviral therapy (ART), low-level viremia (LLV) may be intermittently detected in most HIV-infected patients. Longitudinal blood plasma and resting CD4$^+$ T cells were obtained from two patients on suppressive ART to investigate the source of LLV. Single-genome sequencing of HIV-1 *env* from LLV plasma was performed, and the sequences were compared to sequences recovered from limiting-dilution outgrowth assays of resting CD4$^+$ T cells. The circulating LLV virus clone was identical to virus recovered from outgrowth assays from pools of millions of resting CD4$^+$ T cells. Understanding the sources of LLV requires evaluation of all possible reservoirs of persistent HIV infection.

Antiretroviral therapy (ART) reduces the level of plasma HIV-1 RNA to below the detection limit of clinical assays (<50 copies/ml). However, approximately 75% of patients are found to have stable, persistent low-level viremia (LLV) when tested with more sensitive methods (12, 14, 18, 21, 23). Although such patients remain clinically stable on ART, persistent virus expression may contribute to long-term complications (11), and the cellular source of LLV poses a significant challenge to future attempts to induce a drug-free remission of HIV disease and eradication of infection (25).

The source(s) of LLV is unclear and controversial (10, 19). Latent, persistent infection of resting CD4$^+$ T cells is well described (5), and induction of viral replication in this substantial reservoir of infection might produce LLV in some or all patients. However, cellular sources outside the resting CD4$^+$ T cell pool have been postulated. Macrophages can survive and produce virus for long periods of time in cell culture (20). Hematopoietic progenitor cells may be infected by HIV (7), although this is also disputed (33). Other persistent, durable cellular reservoirs may exist.

Bailey et al. carried out an extensive study of sequences found in patients with LLV (4). In five of nine patients, LLV was populated with a predominant viral clone. This viral clone was found to be underrepresented in DNA recovered from circulating resting CD4$^+$ T cells and in some cases was also underrepresented in replication-competent virus recovered from these cells. The researchers concluded that LLV represented the production of a small number of viral clones without evident evolution, possibly by cells other than circulating

CD4$^+$ T cells. Herein, we report two cases in which the circulating LLV virus clone, as identified by single-genome sequencing, was similarly monomorphic but where an identical replicating viral clone was discovered only after analysis of the replication-competent virus recovered from millions of resting CD4$^+$ T cells cultured in a limiting dilution. While our findings are complementary to those of Bailey and colleagues, they highlight the possibility that LLV is derived from virus that persists and may expand by homeostatic proliferation (8) within resting CD4$^+$ T cells but may be difficult to uncover without a detailed analysis of the replication-competent subpopulation of provirus harbored by resting CD4$^+$ T cells.

Per study protocol, lymphocytes from two patients on suppressive ART were obtained by continuous-flow leukapheresis. Resting CD4$^+$ T cells were isolated, and the frequency of replication-competent virus was assessed by limiting-dilution culture (2). Both patients provided written informed consent, and the study was approved by the University of North Carolina Office of Human Research Ethics Institutional Review Board. Outgrowth assays found the frequencies of resting cell infection to be 0.41 to 0.60 per million resting CD4$^+$ T cells for patient 15 and 3.15 to 6.00 per million resting CD4$^+$ T cells for patient 41.

HIV-1 RNA from 7 ml of stored plasma from patient 15 at a single time point, and 14 ml of plasma from patient 41 at two time points was isolated as previously described (1, 16, 22, 28). In parallel, to obtain virus sequences from resting CD4$^+$ T cells, cell-free viral RNA from cell culture supernatants was extracted. HIV-1 RNA was reverse transcribed to cDNA, and the *env* gene was amplified from plasma by using a limiting-dilution approach and sequenced as previously described (1, 16, 22, 28). If full-length *env* could not be amplified from plasma, then nested PCR was performed using V1 (5′-TTAT GGGATCAAAGCCTAAAGCCATGTGTA-3′) and V2 (5′-
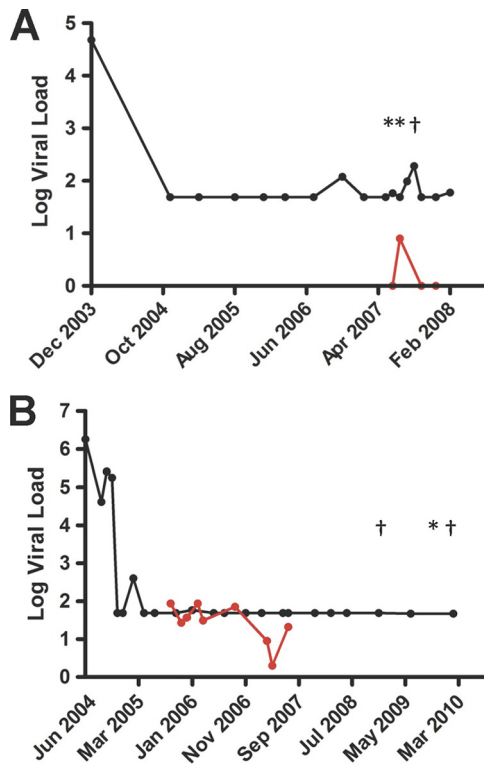

FIG. 1. Longitudinal viral load analyses of patient 15 (A) and patient 41 (B). Patient 15 was diagnosed with established HIV-1 infection in December 2003, and ART was promptly initiated. Patient 41 was diagnosed with acute HIV-1 infection in June 2004 and initiated ART in December 2004. HIV-1 RNA viral loads determined by Roche Amplicor assay are shown in black, and those determined by single-copy assay (SCA) are shown in red (24). Asterisks represent time points when leukophoresis was performed to obtain virus for resting cell outgrowth. Daggers represent time points when blood plasma was assayed by single genome amplification.

CTTAATTCCATGTGTACATTGTACTGTGCT-3′) primers. For a subset of the cell culture supernatant-derived full-length *env* amplicons for patient 15, a bulk PCR approach of oligo(dT)-primed cDNA was utilized. A bulk PCR approach was used to generate all of the cell culture supernatant-derived amplicons for patient 41.

Longitudinal viral loads for patients 15 and 41 are shown in Fig. 1. Patient 15 was diagnosed with late-stage HIV-1 infection with a CD4+ T cell count of 38 cells/µl. ART was initiated at that time, resulting in suppression of viremia and maintenance of <50 copies/ml for 40 months prior to enrollment in an ART intensification clinical trial (2). In contrast to patient 15, patient 41 was diagnosed with acute HIV-1 infection (Fiebig stage IV) (13) with an indeterminate HIV-1 Western blot and a CD4+ T cell count of 448 cells/µl. ART was initiated approximately 6 months postinfection, resulting in suppression of viremia and maintenance of <50 copies/ml for several months prior to enrollment and after completion of an ART intensification clinical trial (2, 3).

To infer viruses present in resting CD4+ T cells, we initially used single-genome amplification (SGA) to generate 37 full-length *env* amplicons from a single cell culture supernatant derived from a culture of 2.5 million resting CD4+ T cells (Fig.

2A). Within the major clade, the 36 sequences were highly homogeneous (with 19 of 36 sequences completely identical). Subsequently, a bulk PCR approach was utilized for additional limiting-dilution culture supernatants, as SGA was not necessary to analyze diversity within the resting cells.

Bulk PCR was used to generate 18 additional *env* amplicons (Fig. 2B). For these amplicons, the hypervariable regions V1 to V5 were sequenced. Thus, a total of 20 *env* sequences (including the SGA-derived major and minor variants identified in Fig. 2A) were used to measure the diversity within the resting CD4+ T lymphocytes, and these sequences have a maximum pairwise distance of 5.5%, consistent with a patient who has chronic HIV-1 infection and has been infected for several years (29).

Sixteen amplicons were isolated from resting CD4+ T lymphocytes of patient 41 nearly 5 years after ART initiation. The resting cell population was extremely homogeneous, with a mean pairwise genetic distance of 0.22% (Fig. 2C and D), and the case is analogous to a subject infected with a single variant sampled during acute infection (1, 16, 28). In addition, four amplicons were generated by SGA from longitudinal blood plasma samples separated by a 14-month interval (Fig. 2C and D) and differ from the resting CD4+ T cell population by 2 mutations at most. Thus, a minimal amount of viral diversification occurred since initiation of therapy, and this suggests that viral populations do not diversify in the setting of effective ART.

To examine the viral diversity of the LLV present within the blood plasma of patient 15, eight full-length *env* amplicons and seven V1-V2 amplicons were generated (Fig. 2B). Two clades are identified within the phylogenetic tree. The major clade was highly homogeneous, with 13 of 15 identical sequences and with one sequence 0.3% divergent from the major clade. In addition, one sequence was 4.2% divergent from the major clade. The sequences present in the blood plasma had identical/nearly identical sequences present in the resting CD4+ T cells. Similarly, identical/nearly identical sequences were obtained from the LLV in the blood plasma and the resting CD4+ T cells from patient 41 (Fig. 2C and D). These results confirm that the source of ongoing virus production detected in these patients is the latently infected resting CD4+ T cell population or another cell type infected with a genetically identical virus and that this virus is primarily clonal in origin.

The twin problems of the persistence of integrated HIV provirus and chronic low-level viral expression despite effective ART must be addressed if eradication of HIV infection is to be achieved. Some mechanisms that contribute to the persistence of replication-competent HIV DNA genomes within cells are understood, but the predominant sources of persistent, low-level expression of virions have not been fully defined.

Some investigators reported that when ART is interrupted, rebound viremia appears to originate from the CD4+ T cell reservoir (15), but others have found that the source of viral rebound cannot always be identified (9). Similarly, Bailey et al. (4) found that the predominant circulating viral species isolated from patients with LLV could not be identified within the proviral DNA recovered from the resting CD4+ T cells in 5 of 9 patients (4).

This study specifically compared the LLV virus to virus capable of replication that is released after *ex vivo* activation of
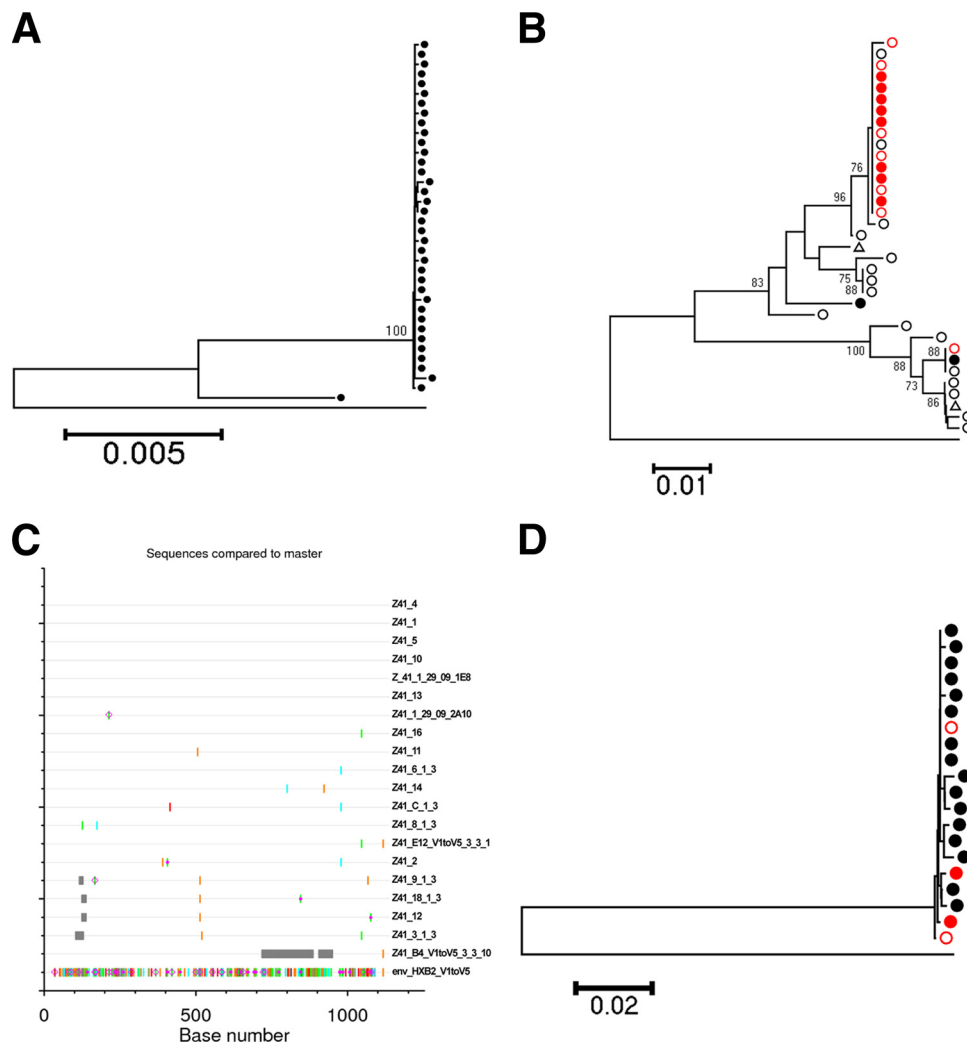
FIG. 2. Phylogenetic analyses of patients 15 and 41. DNA sequences were aligned using CLUSTAL W (32). Phylogenetic trees were constructed using the neighbor-joining method (27) implemented in CLUSTAL W with Kimura's correction (17) by using Mega 4.0 (30). Evolutionary distances were computed using the maximum-composite-likelihood method (31). (A) Neighbor-joining tree of 37 SGA-derived full-length *env* sequences from resting CD4+ T lymphocytes in patient 15. Black circles represent SGA-derived *env* sequences. (B) Neighbor-joining tree of viruses derived from resting CD4+ T cells and blood plasma in patient 15. Closed red circles, full-length SGA-derived *env* sequences from plasma; open red circles, hypervariable V1-V2 SGA-derived sequences from plasma; closed black circles, full-length SGA-derived *env* sequences derived from cell supernatants from first leukophoresis; open black circles, hypervariable V1 to V5 bulk PCR sequences from cell supernatants from first leukophoresis; open black triangles, hypervariable V1-V5 bulk PCR sequences from all supernatants at second leukophoresis. (C) Highlighter analysis of viruses derived from resting CD4+ T cells and blood plasma from patient 41. The consensus sequence is included as the master. Sequence names are indicated on the right. Each vertical tick represents a mismatch from the master as indicated in the figure. (D) Neighbor-joining tree of V1 to V5 amplicons derived from resting CD4+ T cells (black circles) in August 2009, blood plasma in January 2009 (open red circles), and blood plasma in March 2010 (filled red circles). In each neighbor-joining tree, the percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (values of >60% with 500 replicates) are shown next to the branches. The trees are drawn to scale, and the horizontal scale bar represents genetic distance (nucleotide subsititutions per site). All positions containing gaps and missing data were eliminated from the data set. Strain HXB2 is included in the trees as an outgroup.

resting CD4+ T cells. Two levels of homogeneity were seen. First, the LLV virus from plasma was predominately a single variant. Second, approximately 50% of the induced virus was in clusters, suggesting that half of the virus in resting CD4+ T cells is clonally expanded from a small number of cells.

The predominant LLV plasma viral sequence in patient 15 was found in only 2 of 21 outgrowth assays performed using resting CD4+ T cells. In order to detect the two infected resting CD4+ T cells containing the virus also found in LLV plasma, approximately 50 million resting CD4+ T cells were

cultured in 30 individual cultures. Therefore, in some patients, it is possible that LLV viral species may originate from a minor population of circulating CD4+ T cells. Obviously, our finding does not eliminate the possibility that replication-competent HIV persists in cell populations other than resting CD4+ T cells for long periods of time despite ART, as suggested in recent reports (6, 26). However, these reports similarly cannot rule out the possibility that a minor population of resting CD4+ T cells containing virus identical to the LLV virus was not assayed. Given the variability of the immune response and

differences in disease progression, persistent viremia could originate from different sources in different patients. It will be necessary to examine large numbers of CD4$^+$ T cells and other potential viral reservoirs to characterize persistent reservoirs of HIV.

Finally, our data indicate that viral diversification is effectively halted after initiation of ART, given the identical/nearly identical sequences observed from patient 41 (Fig. 2C and D). An alternate scenario is that there is differential decay of several populations of latently infected resting cells, and as some populations express virus and are cleared, the remaining population becomes more homogeneous over time. This population of "deeply latent" proviruses may also increase in predominance if indeed it is able to undergo homeostatic proliferation (8). Analyses of longitudinal samples of resting cells capable of releasing replication-competent virus from additional patients are needed to address this question further.

Nucleotide sequence accession numbers. JF421359 through JF421448.

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