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

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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 replicating-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 outgrowth 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'-
Amplicon analysis of LLV tropism: tropism was determined by single genome amplification (SGA) of the envelope (env) gene. The env sequences of six amplicons from patients 15 and 41 were determined using the SGA approach. For patient 15, two amplicons were sequenced, and for patient 41, four amplicons were sequenced. The sequences were then compared and clustered to identify major and minor env variants. The env sequences of the LLV present in the blood plasma of patient 15 had a pairwise genetic distance of 1.4%, indicating a single variant. Similarly, the env sequences of the LLV present in the blood plasma of patient 41 had a pairwise genetic distance of 0.6%, also indicating a single variant. These results suggest that the LLV virus is highly homogeneous and clonal in origin.

To examine the viral diversity of the LLV present within the blood plasma of patients 15 and 41, eight full-length env amplicons from each patient were sequenced. The env sequences were then compared and clustered to identify major and minor env variants. The env sequences of the LLV present in the blood plasma of patient 15 had a pairwise genetic distance of 1.4%, indicating a single variant. Similarly, the env sequences of the LLV present in the blood plasma of patient 41 had a pairwise genetic distance of 0.6%, also indicating a single variant. These results suggest that the LLV virus is highly homogeneous and clonal in origin.
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

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 leukopheresis; open black circles, hypervariable V1 to V5 bulk PCR sequences from cell supernatants from first leukopheresis; open black triangles, hypervariable V1-V5 bulk PCR sequences from all supernatants at second leukopheresis. (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 substitutions 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.
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 and 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.


