Origin of Rebound Plasma HIV Includes Cells with Identical Proviruses That Are Transcriptionally Active before Stopping of Antiretroviral Therapy

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ABSTRACT
Understanding the origin of HIV variants during viral rebound may provide insight into the composition of the HIV reservoir and has implications for the design of curative interventions. HIV single-genome sequences were obtained from 10 AIDS Clinical Trials Group participants who underwent analytic antiretroviral therapy (ART) interruption (ATI). Rebounding variants were compared with those in pre-ART plasma in all 10 participants and with on-ART peripheral blood mononuclear cell (PBMC)-associated DNA and RNA (CA-RNA) in 7/10 participants. The highest viral diversities were found in the DNA and CA-RNA populations. In 3 of 7 participants, we detected multiple, identical DNA and CA-RNA sequences during suppression on ART that exactly matched plasma HIV sequences. Hypermutated DNA and CA-RNA were detected in four participants, contributing to diversities in these compartments that were higher than in the pre-ART and post-ATI plasma. Shifts in the viral rebound populations could be detected in some participants over the 2- to 3-month observation period. These findings suggest that a source of initial rebound viremia could be populations of infected cells that clonally expanded prior to and/or during ART, some of which were already expressing HIV RNA before treatment was interrupted. These clonally expanding populations of HIV-infected cells may represent an important target for strategies aimed at achieving reservoir reduction and sustained virologic remission.

IMPORTANCE
Antiretroviral therapy alone cannot eradicate the HIV reservoir, and viral rebound is generally rapid after treatment interruption. It has been suggested that clonal expansion of HIV-infected cells is an important mechanism of HIV reservoir persistence, but the contribution of these clonally proliferating cells to the rebounding virus is unknown. We report a study of AIDS Clinical Trials Group participants who underwent treatment interruption and compared rebounding plasma virus with that found within cells prior to treatment interruption. We found several incidences in which plasma HIV variants exactly matched that of multiple proviral DNA copies from infected blood cells sampled before treatment interruption. In addition, we found that these cells were not dormant but were generating unspliced RNA transcripts before treatment was interrupted. Identification of the HIV reservoir and determining its mechanisms for persistence may aid in the development of strategies toward a cure for HIV.

The discovery of strategies that target the HIV reservoir and induce sustained antiretroviral therapy (ART)-free remission is one of the highest priorities of the HIV research field. The evaluation of such therapeutic strategies will require demonstration of effectiveness in analytic treatment interruption (ATI) studies, either through a significant delay in the timing of viral rebound or through a reduction in the viral load set point. Our knowledge of the source and diversity of rebounding HIV after ATI is still limited, but such information will be crucial to design and evaluate interventions aimed at eliminating the HIV reservoir.

During suppressive antiretroviral therapy, there is little to no evidence of active HIV evolution in plasma viremia, suggesting an absence of ongoing viral replication in peripheral blood mononuclear cells (PBMCs) during optimal ART (1). However, ART alone is unable to eradicate infected cells, and without lifelong treatment, HIV plasma viremia almost invariably rebounds (2, 3). One study of individuals undergoing multiple short ATIs detected generally homogeneous populations of rebounding virus with different lineages present at different ATI cycles, suggesting stochastic reactivation of monoclonal or small oligoclonal populations of latently infected cells (4). However, other studies have shown a

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relatively large number of rebounding founder variants, suggesting
that viral reactivation occurs from many latently infected cells,
possibly from multiple anatomic sites (5). There is evidence that
virus after treatment interruption is similar to that found in the
pre-ART plasma quasispecies (1, 6), but the exact cellular origin of
these rebounding variants is still unclear, especially after pro-
longed suppressive ART (7, 8).

ART results in rapid 3- to 4-log_{10} declines in levels of HIV
plasma RNA but only ~10-fold declines in the number of infected
PBMCs (9). Clonal proliferation of HIV-infected cells may be a
key contributor to the persistence of infected cells on ART (10,
11); however, it is not known if these cells are a source of viral
rebound after stopping ART. This uncertainty has been fueled by
a study suggesting that clonally expanded PBMCs contain only
replication-incompetent proviruses (12). The evaluation of HIV
proviral DNA and actively transcribed intracellular HIV RNA
may provide a better predictor of the rebounding HIV variants,
especially as the number of actively expressing HIV-infected cells
has been associated with the timing of viral rebound (13). Under-
standing the origin of HIV variants during early and late rebound
would provide insight into the composition of the HIV reservoir
and has implications for the design of curative interventions. In
this study, we evaluated a cohort of 10 participants who initiated
ART a median of 4 years earlier as part of an AIDS Clinical Trials
Group (ACTG) study and then enrolled in a subsequent ACTG
study with an ATI component. These participants all had plasma
samples available both before ART initiation and shortly after the
ATI. In addition, seven of these participants had available PBMC
samples shortly before the ATI. Using single-genome sequencing
(SGS), we compared the post-ATI rebounding virus to the plasma
virus from pre-ART and to the on-ART PBMC-associated DNA
and RNA in an attempt to link infected cells and their transcrip-
tional activity to rebound viremia after stopping ART.

**MATERIALS AND METHODS**

**Study population.** Ten participants of prior ACTG ATI studies with avail-
able pre-ART and post-ATI plasma samples were included. Seven of these
participants had available on-ART PBMC samples taken shortly prior to
ATI and while virologically suppressed (<50 HIV RNA copies/ml). These
participants initiated first-line ART as part of either ACTG trial 384 (14,
15) or ACTG trial A5095 (16) and underwent an ATI in one of the follow-

**TABLE 1 Characteristics of study participants**

<table>
<thead>
<tr>
<th>PID</th>
<th>Gender</th>
<th>Race</th>
<th>No. of CD4 cells/mm³</th>
<th>No. of yrs on ART</th>
<th>No. of wks following ATI:</th>
<th>Pre-ART</th>
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<td>Pre-ART</td>
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<td></td>
<td></td>
<td>Median (Q1–Q3)</td>
<td>474 (424–543)</td>
<td>742 (619–1,006)</td>
<td>4.0 (3.9–4.5)</td>
<td>4.5 (4–8)</td>
</tr>
</tbody>
</table>

† The on-ART (pre-ATI) PBMC samples were collected immediately prior to the ATI with the exception of one participant (PID 7), whose sample was collected 7 months prior to the ATI.

‡ TP, time point.
events, Taq polymerase errors, or multiple templates. Sequences with evidence of mixed bases were excluded from analysis. We sequenced ~20 to 50 SGS from each plasma and PBMC sample, which provided the sensitivity to detect variants present in 10% of the population with ≥90% certainty.

The resulting single-genome sequences were aligned using ClustalW. To ensure that there was no cross-contamination of patient samples, we generated a neighbor-joining (NJ) tree including all the sequences obtained for the study and confirmed that all SGS clustered appropriately (Fig. 1). Population genetic diversity was calculated as the average pairwise difference (APD) using an in-house program (1, 22). In brief, the in-house program tabulates the number of nucleotide differences between all possible pairs of aligned sequences, divides the number of differences between each pair by the sequence length, and sums the total. The total is then divided by the number of sequence pairs, \( N(N - 1)/2 \), where \( N \) is the number of sequences in the population. Indels were not counted as differences between pairs of sequences. This program is available by request. Shifts in population structure were calculated using a subdivision test for panmixia with a significance cutoff level at \( P \) values of \( 10^{-3} \) (23).

The probability of \( 10^{-3} \) for assigning a significant change in viral populations obtained from SGS was derived statistically, taking into consideration the very large numbers of comparisons across sequences (1, 23). Neighbor-joining phylogenetic analyses and divergence calculations were done using MEGA6. Trees were rooted on the subtype B consensus sequence (www.HIV-1.lanl.gov).

**Statistical analysis.** HIV-1 sequence diversity and divergence between time points were assessed by Spearman correlation and the nonparametric Friedman test. Paired sample comparisons were performed by the Wilcoxon signed-rank test. The Friedman test excluded the post-ATI plasma #2 time point, as one of the participants did not have a sample available.

**RESULTS**

**Patient and study characteristics.** Participants had a median pre-ART CD4 count of 474 cells/mm\(^3\) and a median on-ART CD4 count of 742 cells/mm\(^3\) at the time of ATI (Table 1). The median (Q1, Q3) plasma HIV RNA counts at the first and second post-ATI time points were 6,386 (2,050, 33,165) and 18,128 (6,022, 22,616) HIV RNA copies/ml, respectively. A median of 27 (24, 31) SGS was obtained from each sample type and time point (Table 2). There was no evidence of cross-participant sequence contamination in the phylogenetic analysis (Fig. 1).

**Intracellular and plasma HIV diversity before, during, and after ART.** Viral diversity was evaluated within each sample by average pairwise distance (APD). The highest levels of viral diver-
sity were observed in the PBMC DNA populations (Fig. 2). The levels of DNA diversity (APD, 1.5%) were significantly higher than those of the CA-RNA (1.3%; Wilcoxon signed-rank test, \( P < 0.03 \)), pre-ART plasma (1.1%; \( P < 0.02 \)), and post-ATI plasma (0.5%; \( P < 0.02 \)) populations. CA-RNA had the second highest median levels of diversity, and it was lowest in the post-ATI plasma (Fig. 2). Plasma HIV RNA diversity did not significantly change over the 1 to 2 months between the first and second post-ATI time points (APD post-ATI time points #1 and #2: 0.5% versus 0.5%; Wilcoxon signed-rank test, \( P = 1.0 \)) but was significantly higher in pre-ART than in post-ATI (1.1% versus 0.5%, \( P < 0.04 \)). The lower diversity of the rebound virus than in the pre-ART virus indicates that the initial rebound virus represents only a subset of the virus population pre-ART.

**Divergence between HIV populations before, during, and following ART.** We measured the divergence of the initial post-ATI plasma HIV sequences compared to viral sequences from pre-ART, on-ART DNA and CA-RNA, and the second post-ATI plasma time point. The rebounding virus was overall more similar to pre-ART plasma HIV than to on-ART DNA or CA-RNA populations (Fig. 3). This is also supported by the finding that early post-ATI plasma HIV diversity was closely associated with pre-ART plasma virus diversity (Spearman \( r = 0.72, P = 0.02 \)) but not significantly correlated with either on-ART DNA or CA-RNA viral diversity. These results are consistent with a minor subset of HIV-infected cells being responsible for HIV viremia before ART, persisting despite ART, and subsequently contributing to viral variants detected after ATI.

**Probable expanded clonal populations may be a source of rebound HIV.** We used phylogenetics to investigate the relationship between sequences detected in pre-ART, on-ART, and post-ATI (Fig. 4) samples. In two of seven participants (PID 1 and 7; Fig. 4A and G), we detected multiple, identical DNA and CA-RNA gag-pol sequences that exactly matched sequences from rebound plasma HIV (indicated with red arrows), suggesting that this expanded population could be a source of rebound viremia. These clonal plasma sequences represented 15% and 21%, respectively, of all plasma sequences identified at the first post-ATI viremic time point. The pre-ART plasma virus populations in both cases were relatively diverse (1.16% and 0.74% for PID 1 and 7, respectively); the typical pol APD in chronically infected individuals is ~1.0% and consists of all unique variants when sampled by SGS), indicating that these individuals likely initiated treatment in

<table>
<thead>
<tr>
<th>PID</th>
<th>Pre-ART plasma</th>
<th>On-ART DNA</th>
<th>On-ART CA-RNA</th>
<th>Post-ATI plasma #1</th>
<th>Post-ATI plasma #2</th>
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<td>29</td>
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**FIG 2** HIV sequence diversity measured by average pairwise distance in the pre-ART plasma, on-ART PBMC, and post-ATI plasma. Post-ATI plasma #1 and #2 refer to the initial and subsequent sampling of plasma HIV, respectively. Each symbol represents a different patient. The nonparametric Friedman test excluded the post-ATI plasma #2 time point, as one of the participants did not have a sample available at that time point. Paired sample comparisons were performed with the Wilcoxon signed-rank test.

**FIG 3** Sequence divergence compared to the initial post-ATI plasma time point. Each symbol represents a different patient. Paired sample comparisons were performed by Wilcoxon signed-rank test.

**TABLE 2** Number of single-genome sequences obtained for each participant sample
FIG 4 Neighbor-joining trees from the 10 participants. The date for each participant time point is labeled. For the post-ART plasma time points, the number of weeks after treatment discontinuation is also indicated. The red arrows highlight the multiple identical on-ART CA-RNA and DNA sequences that exactly matched post-ART plasma HIV sequences. Black arrows indicate exact matches between pre-ART plasma sequences and either on-ART CA-RNA and DNA sequences or post-ART plasma sequences. PID 3 (C) illustrates the shifts in divergent viral populations after HIV rebound and the possible evolution of variants from a later rebound time point (purple arrows) from an early rebound viral population (green arrows). The presence of G-to-A hypermutations in both the DNA and CA-RNA sequences was detected and is labeled.
chronic infection (22). This result suggests that the identical proviral sequences in the PBMCs may have resulted from clonal expansion and not from a single HIV variant infecting multiple cells prior to initiating ART. Importantly, in both cases, matching CA-RNA sequences were found in the on-ART (pre-ATI) PBMC populations, suggesting that expanded cell populations with identical proviruses may be the source of rebound viremia. PID 7 is the best example of rebound from a presumably expanded, transcriptionally active cell population persisting during ART (Fig. 4G, boxed sequences). However, most rebounding variants detected did not match DNA or CA-RNA from pre-ART, suggesting that these arose from different reservoirs or tissues or that their presence in blood was below the level of detection by SGS.

Rebound viremia can result from cells that were infected prior to initiating ART. In three cases (PID 2, 6, 9; Fig. 4B, F, and I), rebound sequences were found to exactly match a pre-ART plasma HIV variant (indicated with black arrows), and in one of these cases (PID 2), PBMCs were available for analyses and matching DNA/RNA were also detected (Fig. 4B, black arrows). Two of these participants had low levels of viral diversity prior to ART (PID 2 = 0.24%, PID 6 = 0.16%), suggesting that ART was initiated during early infection. Although it has been shown previously that viral evolution is inhibited by ART, halting viral diversification when ART is initiated early (1, 24), this study is the first to report that at least some of these infected cells or their descendants can continue to express HIV RNA during treatment for at least 4 years. This finding provides evidence that some PBMCs infected prior to initiating ART can express unspliced HIV RNA during ART, which can result in rebound viremia when ART is interrupted.

Hypermutated proviruses are expressed during ART. We detected both hypermutated PBMC DNA and CA-RNA sequences in three of seven participants for which sequences were obtained from PBMCs during ART (Fig. 4B, E, and G). This result confirms findings by others that cells infected with hypermutated proviruses persist for years on ART (25) but also shows that at least some proviruses can be expressed in cells during treatment despite being hypermutated. This finding is important because it explains, at least in part, the weakness of the association between levels of HIV CA-RNA and residual plasma viremia during ART (26). Since G-to-A hypermutants are defective (27), this finding also suggests that HIV proviruses that encode nonfunctional viruses due to other mutations, insertions, or deletions may be expressed as well.

HIV populations sometimes shift after treatment interruption. Nine of the 10 participants had plasma HIV sampled at a second post-ATI time point 1 to 2 months after the first post-ATI time point. Although there were no significant differences in viral diversity between the first and second post-ATI time points (Fig. 2) and the overall levels of divergence were also low (Fig. 3), in 3 individuals (PID 1, 3, and 4), there were significant population shifts between the rebounding viral populations across the two time points that were detected by a test for panmixia (P < 0.001) and by phylogenetic analyses (Fig. 4A, C, and D, indicated with blue arrows). Rebounding populations from the temporally spaced plasma samples both contained highly homogenous, but distinct, subpopulations. In all cases, the two sets of rebounding variants appear to have originated from different cellular sources that may have been consecutively activated over a 4-week period (PID 1 and 3 are shown as examples of this in Fig. 4A and C). This observation suggests that latently infected cells persisting during ART could be activated sequentially during ART or after stopping ART, leading to the rebound of multiple HIV variants that continues for at least several weeks. However, one shifting rebound virus populations in PID 3 (Fig. 4C) may have occurred by a different mechanism. In this case, one population of variants detected in the second rebound time point (variants near the purple arrow) are present on the same main node but on longer branches on the phylogenetic tree than variants from the first rebounding time point (green arrow), making it a possibility that the variants in the later rebound sample evolved from those in the initial rebounding population.

DISCUSSION

Although the HIV reservoir is often referred to as the pool of infected cells that persist in patients during ART, it is now known that the vast majority of these cells carry defective proviruses (28) and, hence, cannot be responsible for rebound viremia. As such, these cells are not a “true” reservoir for HIV. The true reservoir is more accurately defined as those cells that carry replication-competent proviruses and have the potential to result in rebound of HIV plasma RNA when treatment is interrupted. Such cells are clearly only a tiny fraction of all of the infected cells that persist during therapy (12, 28). Clonal expansion of HIV-infected cells have been identified as a potential key mechanism in the maintenance and persistence of the HIV reservoir (10, 11). While there is evidence that clonally expanded populations of HIV-infected cells may contribute to residual viremia during ART (29), it has also been claimed that that many of the clonally expanded cellular populations harbor replication-defective variants (12). It is not known if clonally expanded populations within PBMCs that persist during ART are the source of rebound viremia when treatment is interrupted and, therefore, represent one of the true reservoirs of HIV. As the HIV field turns toward the search for successful curative strategies, it will be essential that we identify this replication-competent reservoir of HIV and elucidate its mechanisms for renewal and persistence.

To identify the sources of rebound viremia, we performed single-genome sequencing on samples from 10 participants, including plasma from pre- and post-ART time points as well as cells obtained immediately prior to rebound. We identified examples of identical gag-pol proviruses that matched both intracellular HIV RNA during ART and plasma HIV RNA during rebound, suggesting that clonally expanded, infected cells may be a source of rebound viremia and hence a true reservoir for HIV. The gag-pol region that we selected is one that has previously been shown (30) to result in virtually all unique sequences when sampled by SGS during chronic HIV infection, and therefore, identical sequences in this region (when detected during chronic infection) likely result from the proliferation of infected cells rather than from viral replication. However, it is important to note that while we show that some HIV DNA and RNA sequences from PBMCs match variants that rebound after treatment interruption, most of the viral rebound sequences did not have exact matches to the PBMC DNA or RNA. This discrepancy could reflect limited sampling of infected PBMCs, viral evolution after treatment interruption, or the presence of anatomic reservoirs of HIV that were not sampled but may also contribute to rebound viremia like those described by Rothenberger et al. (5). Despite this limited sampling, the fact
that we found gag-pol matches between multiple proviral variants and rebound viremia suggests that these apparently proliferating cell populations are present in the blood at high levels, consistent with a report from Wagner et al. (31). While the detection of identical HIV gag-pol proviruses in this study suggests the presence of clonally expanded cellular populations, we cannot definitively rule out the alternative explanation that such populations were created by the infection of multiple cells with a homogeneous population of viruses, perhaps during acute infection. The fact that the majority of participants were in the chronic phase of HIV infection pre-ART, as reflected by their relatively high levels of viral diversity, would argue against the latter hypothesis. However, integration site analyses that include their corresponding HIV gag-pol sequences need to be developed in order to definitively show that these identical HIV sequences are derived from clonally expanded cellular populations, and full-length HIV sequencing is needed to verify that these variants are not defective elsewhere in the genome.

We also found examples of rebound virus identical to proviral sequences in cells that appeared to express HIV RNA prior to interrupting treatment and possibly to proviruses that may have become activated after treatment was interrupted, resulting in a shift in the plasma virus population over the course of treatment interruption. Furthermore, we found that the intracellular HIV RNA populations during ART were diverse and not structurally different from the proviral populations (sequences intermingled in phylogenetic trees) or the pretherapy virus, and most did not result in rebound viremia. This finding, along with the detection of hypermutated HIV RNA sequences, implies that defective HIV proviruses are expressed during ART and may explain previous reports on the weak association between levels of HIV cellular and plasma RNA compartments (26), as most defective HIV proviruses would not lead to successful viral protein production (28). This finding also highlights the uncertainty surrounding the use of CA-RNA as the main efficacy outcome in the evaluation of latency-reversing agents (32, 33), since a subset of the induced CA-RNA may be replication incompetent and subject to considerable stochastic fluctuation from one patient to the next. These considerations highlight the limits of PCR-based assays for HIV reservoir assessment. It is interesting that despite this finding, CA-RNA was set as the main efficacy outcome in the evaluation of latent infection pre-ART, as reflected by their relatively high levels of viral diversity, would argue against the latter hypothesis. How-

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REFERENCES


