

Relationship Between Residual Plasma Viremia and the Size of HIV Proviral DNA Reservoirs in Infected Individuals Receiving Effective Antiretroviral Therapy

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Residual plasma viremia (<50 copies/mL) persists in certain human immunodeficiency virus (HIV)-infected individuals receiving antiretroviral therapy (ART); however, the relationship between the degree of residual plasma viremia, the size of HIV reservoirs, and the level of immune activation has not been delineated. Here, we demonstrate that residual plasma viremia correlates with the size of the CD4⁺ T cell viral reservoir, but not with markers of immune activation, suggesting that reactivation of the latent viral reservoir may not be the sole source of residual plasma viremia. Novel therapeutic strategies aimed at targeting the source of residual viremia may be necessary to achieve viral eradication.

Prolonged suppression of plasma viremia is now achievable in most human immunodeficiency virus (HIV)-infected individuals receiving antiretroviral therapy (ART) [1]. Nonetheless, it has not been possible to eradicate HIV by ART alone, likely due in part to the persistence of various viral reservoirs [2–6]. A number of previous studies have demonstrated that HIV persists in latently and productively infected CD4⁺ T cells

in peripheral blood [2–4] as well as in gut-associated lymphoid tissues (GALT) [6, 7] of infected individuals receiving ART who have maintained undetectable plasma viremia for prolonged periods of time, as measured by clinically relevant assays (with a typical limit of detection of 50 HIV RNA copies/mL of plasma). With the advent of a laboratory-based real-time polymerase chain reaction (PCR) assay capable of detecting single copies of HIV RNA in plasma [8], several studies have recently demonstrated the presence of residual plasma viremia ranging from 1 to 49 copies/mL in some infected individuals receiving ART [8–10]. One such study observed multiphasic decay of residual plasma viremia and speculated that latently infected, resting CD4⁺ T cells and/or unidentified viral reservoirs, which are capable of producing low levels of genetically identical virions for prolonged periods of time without cellular turn-over, may be responsible for the persistence of residual plasma viremia in infected individuals receiving ART for extended periods of time [10, 11]. However, the relationship between residual plasma viremia and the frequency of CD4⁺ T cells carrying HIV proviral DNA and/or markers of immune activation has not been fully delineated. We conducted the present study to address this issue.

METHODS

Patient Population

Initially, 189 HIV-infected individuals who were receiving ART were screened. Of those, 127 individuals who had received ART for a median of 6.5 years (range, 1.3–15.8 years) and who had achieved suppression of plasma viremia were included in this study (Table 1). All patients were receiving various antiretroviral regimens containing at least 1 protease inhibitor and/or 1 nonnucleoside reverse-transcriptase inhibitor, in addition to 2 reverse-transcriptase inhibitors. The median CD4⁺ and CD8⁺ T-cell counts at the time of study were 580 cells/mm³ of blood (range, 100–1770 cells/mm³) and 760 cells/mm³ of blood (range, 200–2470 cells/mm³), respectively. All participants included in this study maintained undetectable levels of plasma viremia (<50 copies/mL) at the time of study and had fewer than 3 viral “blips” (defined as <100 HIV RNA copies/mL) after initiation of ART, as determined by frequent blood sampling (at least 3 times per year) (Table 1). Blood was collected from the study participants in accordance with protocols approved by the Institutional Review Boards of the University of Toronto, Toronto, Canada, and by the Office of Human Subjects Research at the National Institutes of Health.

Received 11 November 2010; accepted 11 February 2011.

Potential conflicts of interest: none reported.

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The Journal of Infectious Diseases 2011;204:135–8

Published by Oxford University Press on behalf of the Infectious Diseases Society of America 2011.

0022-1899 (print)/1537-6613 (online)/2011/2041-0018\$14.00

DOI: 10.1093/infdis/jir208

Table 1. Profiles of Study Participants With Human Immunodeficiency Virus Infection

Characteristic	Values
Sex, % of participants	
Male	96.9
Female	3.1
Age, median years (range)	48 (30–73)
Duration of antiretroviral therapy at time of study, median years (range)	6.5 (1.3–15.8)
Plasma viremia at time of study	<50 copies/mL ^a
No. of viral blips (<100 copies/mL), % of participants	
0	49.6
1	28.4
2	22.0
CD4 ⁺ T cell count at time of study, median cells/mm ³ (range)	580 (100–1770)
CD8 ⁺ T cell count at time of study, median cells/mm ³ (range)	760 (200–2470)
CD4 ⁺ /CD8 ⁺ cell ratio, median value (range)	0.8 (0.1–3.5)

NOTE. ^a Measured by a branched DNA assay with a detection limit of 50 copies/mL of plasma.

Determination of Residual Plasma Viremia in Infected Individuals Receiving Antiretroviral Therapy

Residual plasma viral loads were determined using Cobas Ampliprep/Cobas Taqman HIV-1 Test, version 2.0 (Roche Diagnostics), in quadruplicate. The published limit of detection for this system is 20 copies/mL of plasma. The actual copy number of <20 HIV RNA copies/mL was determined by averaging Ct values obtained from quadruplicates of plasma specimens per individual.

Isolation of Peripheral Blood CD4⁺ T Cells and Quantitative Real-time PCR for Measurements of HIV Proviral DNA

Peripheral blood mononuclear cells (PBMCs) were obtained from blood draw, and CD4⁺ T cells were isolated from PBMCs of HIV-infected individuals using an automated cell separation system (StemCell Technologies).

To determine the frequency of CD4⁺ T cells carrying HIV proviral DNA in infected individuals, real-time PCR was performed on genomic DNA isolated from 2×10^6 purified CD4⁺ T cells (Qiagen). Then 1 μ g of DNA was used as a template for real-time PCR in a 7500 Real-Time PCR System (Applied Biosystems). The amplification reaction was performed in triplicate using HIV-specific primers and probe (FAM), RNaseP-specific primers and probe (VIC) (Applied Biosystems), and Taqman Gene Expression Master Mix (Applied Biosystems) in 50 μ L total volume. The following primers were used for amplification of HIV LTR: 5'-GGTCTCTCTGGTTAGACCAGAT-3' (5' primer) and 5'-CTGCTAGAGATTTTCCACACTG-3' (3' primer), along with the fluorescent probe 5'-6FAM-AGTAGTGTGTGCCCGTC-TGTT-TAMRA-3'. PCR conditions consisted of a step at 95°C

for 10 min followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C. Serially diluted ACH-2 DNA was also subjected to the PCR described above to obtain standard curves. The detection limit of the assay was 2.6 HIV DNA copies/mL.

Detection of Activation Markers in Blood

The plasma levels of C-reactive protein (R&D Systems), D-dimer (BioMérieux), interleukin (IL)-6 (R&D Systems), and soluble tumor necrosis factor (TNF) receptor I (R&D Systems) were determined by enzyme-linked immunosorbent assay. The levels of CD38 expression on CD4⁺ and CD8⁺ T cells were determined by flow cytometry.

Statistical Analyses

Comparisons of independent groups were made by the Wilcoxon 2-sample test. Correlation was determined by the Spearman rank method. The Bonferroni method was used to adjust *P* values for multiple testing.

RESULTS

To determine the level of residual plasma viremia <50 HIV RNA copies/mL in 127 infected individuals receiving ART, we performed an automated viral load assay (Cobas Ampliprep/Cobas Taqman HIV-1 Test, version 2.0; Roche Diagnostics) in quadruplicate for each study participant. As shown in Figure 1A, residual plasma viremia ranged from 0 to 49 HIV RNA copies/mL (median HIV RNA level, 2.6 copies/mL). The plasma from the majority of the study participants (63.0%) contained detectable plasma viremia, whereas there was no measurable HIV RNA in the plasma of 37.0% of the individuals in our study.

To examine the correlation between residual plasma viremia (at the level of <50 HIV RNA copies/mL) and the frequency of CD4⁺ T cells carrying HIV proviral DNA, genomic DNA was prepared from highly purified CD4⁺ T cells from peripheral blood of study participants and subjected to real-time PCR specific for HIV proviral DNA. The median copy number of HIV proviral DNA for all study participants examined was 775.1 copies (range, <2.6–6890.6 copies) per 10^6 CD4⁺ T cells. There was a direct correlation between the level of residual plasma viremia and the frequency of CD4⁺ T cells carrying HIV proviral DNA ($r = .294$; $P = .001$) (Figure 1B). When the data on HIV proviral DNA burden were stratified on the basis of residual plasma viremia, there was a statistically significant difference between the study subjects with undetectable (0 copies) versus detectable (1–49 copies) plasma viremia ($P = .01$) (Figure 1C), indicating that the frequency of CD4⁺ T cells carrying HIV proviral DNA in infected individuals with undetectable plasma viremia (median, 448.5 copies per 10^6 CD4⁺ T cells; interquartile range, 166.7–1467.2 copies per 10^6 CD4⁺ T cells) as a group is lower than that of individuals with detectable plasma viremia (median, 1027.2 copies per 10^6 CD4⁺ T cells; interquartile range, 292.2–3040.7 copies per 10^6 CD4⁺ T cells). Of

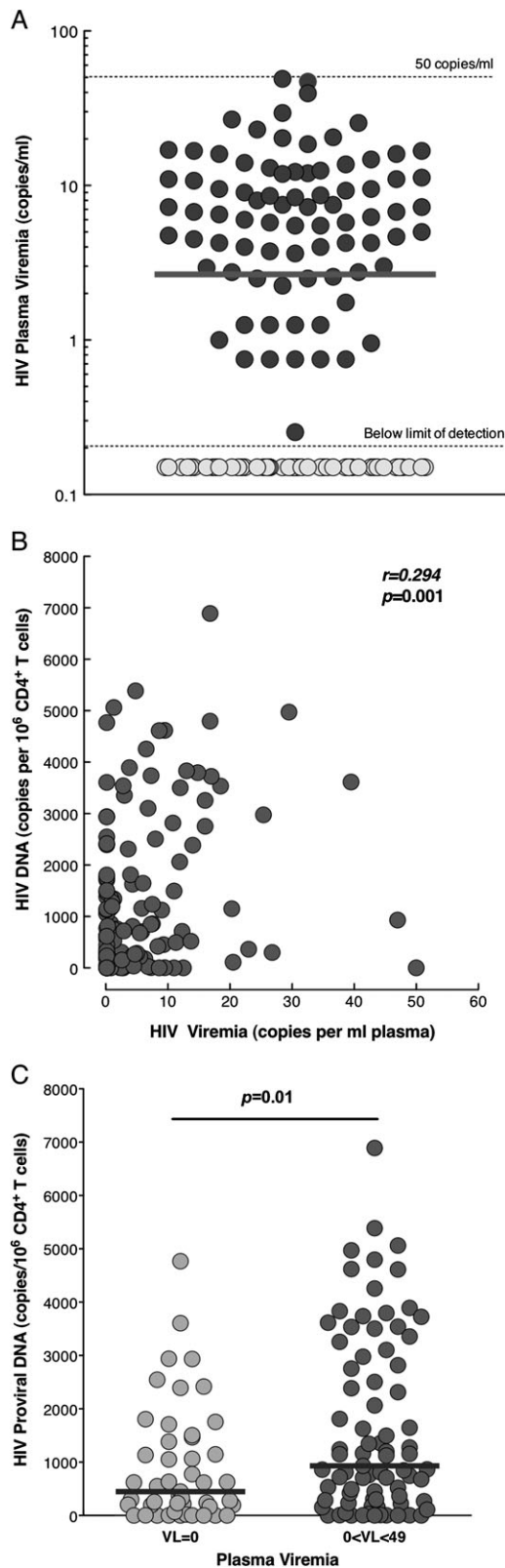


Figure 1. Relationship between residual plasma viremia and human immunodeficiency virus (HIV) proviral DNA in CD4⁺ T cells of HIV-infected individuals receiving antiretroviral therapy (ART) for prolonged periods of time. *A*, Distribution of residual plasma viremia in individuals with <50 HIV RNA copies/mL plasma. *B*, relationship between residual plasma

viremia and cell-associated HIV DNA. *C*, frequencies of CD4⁺ T cells carrying HIV proviral DNA based on plasma viremia. The open and closed circles represent undetectable and detectable values, respectively, determined by quadruplicate measurements. When quadruplicates of plasma samples were negative for HIV RNA, they were scored as undetectable (ie, below the limit of detection). The median value is shown as gray bars.

note, 38% of the study participants with undetectable plasma viremia carried ≥ 775 copies per 10^6 CD4⁺ T cells (median value of all data points), suggesting that, at least in some individuals, the size of the HIV reservoir in the CD4⁺ T-cell compartment in the peripheral blood may not necessarily equate to the level of residual plasma HIV.

To determine the effect of immune activation on residual plasma viremia and the size of the viral reservoir carrying HIV proviral DNA in infected individuals receiving clinically effective ART, we sought to evaluate possible correlations among immune markers (C-reactive protein, D-dimer, IL-6, soluble TNF receptor I, and CD38 expression on CD4⁺ and CD8⁺ T cells), residual plasma viremia, and the frequency of CD4⁺ T cells carrying HIV proviral DNA. There was no correlation among the above parameters ($P > .5$), with the exception of a direct correlation between CD4⁺/CD8⁺ T-cell count ratio and the level of CD4⁺ T cells carrying HIV proviral DNA ($P = .005$). However, there was no correlation between markers of immune activation and residual plasma viremia ($P > .5$).

DISCUSSION

The persistence of HIV proviral DNA and infectious virus in CD4⁺ T cells has long been considered as one of the major impediments to eradicating virus in infected individuals receiving ART for extended periods of time and in whom plasma viremia was suppressed below the level of detection, as measured by a number of US Food and Drug Administration–approved clinical assays (which have a limit of detection of 50 copies/mL) [2–4]. In recent years, studies using a laboratory-based real-time PCR assay that is capable of detecting single HIV RNA copies in plasma have shown that residual plasma viremia 50 copies/mL can be observed in certain infected individuals who are receiving effective ART [9, 10]. Given that delineating the mechanism by which residual plasma viremia persists and identifying the source of the <50 HIV RNA copies/mL in plasma may have profound implications for eradicating HIV in infected individuals receiving ART, it is important to understand the relationship between residual plasma viremia and various immunological and virological parameters, such as the level of cell-associated HIV proviral DNA and markers of immune activation.

In the present study, we set out to determine the degrees of residual plasma viremia in a large number of infected

viremia and cell-associated HIV DNA. *C*, frequencies of CD4⁺ T cells carrying HIV proviral DNA based on plasma viremia. The open and closed circles represent undetectable and detectable values, respectively, determined by quadruplicate measurements. When quadruplicates of plasma samples were negative for HIV RNA, they were scored as undetectable (ie, below the limit of detection). The median value is shown as gray bars.

individuals receiving ART and to determine immunological or virological parameters that may correlate with residual plasma viremia. Residual plasma viremia was determined in quadruplicate using an automated system to minimize quantitative errors often associated with the detection of extremely low levels of viral RNA. We have demonstrated detectable levels of residual plasma viremia (1–49 copies/mL) in the majority of study participants receiving ART in whom plasma viremia had been suppressed for extended periods of time, as measured by a standard clinical assay (with a limit of detection of 50 copies/mL). Furthermore, we found a correlation between the level of residual plasma viremia and the frequency of CD4⁺ T cells carrying HIV proviral DNA. Of note, due to the limited amounts of blood obtained from the study subjects, we could not conduct the quantitative coculture assays that are required for determining the frequency of cells carrying replication-competent HIV. It has been speculated that residual plasma viremia originates from the cellular activation of latently infected, resting CD4⁺ T cells [10]. In this regard, we have previously shown that activated CD4⁺ T cells in the peripheral blood of HIV-infected individuals receiving ART spontaneously released virions in the absence of activating stimuli [5]. In addition, we have reported higher frequencies of HIV infection in CD4⁺ T cells in the GALT and evidence for cross-infection between the blood and tissue compartments of infected individuals receiving ART [6]. The data presented in the present study do not rule out the possibility of reactivation of latently infected, resting CD4⁺ T cells contributing to residual plasma viremia. However, considering the relatively high frequency of HIV infection in the CD4⁺ T-cell compartment of nearly 40% of the study participants exhibiting undetectable plasma viremia (0 copy) and the lack of any correlation between residual plasma viremia and various markers of immune activation in blood, there is a possibility that residual viral replication, with or without the detection of plasma viremia, may also originate from productively infected CD4⁺ T cells in various lymphoid tissues. In this regard, recent studies investigating the effect of intensification of conventional ART have demonstrated evidence for the existence of ongoing viral replication, as measured by levels of cell-associated viral DNA in blood [12] and RNA in the GALT [13]; however, in other studies with similar settings, but in which plasma viremia was the sole virological indicator, there was no effect of drug intensification [14, 15]. With regard to these apparent discrepancies, it is important to point out that it is possible to have low levels of viral replication and cell-to-cell spread of virus, particularly in lymphoid organs, without such replication being reflected in the levels of plasma viremia, even as measured by the most sensitive assays.

Achieving eradication of HIV in infected individuals receiving ART remains a daunting challenge for the scientific community. To achieve a functional cure, as defined by the absence of detectable HIV for extended periods of time in the absence of ART,

therapeutic strategies aimed at eliminating cellular reservoirs in various tissue compartments must be accompanied by comprehensive virological assays that monitor infected CD4⁺ T cells that may or may not contribute to residual plasma viremia.

Funding

The Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Acknowledgments

We thank the study volunteers for their participation in this study, and Adam Ruppert, Julie Metcalf, Erika Benco, and Max Ridell, for their invaluable assistance in the execution of this study.

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