Evidence for Persistent Low-level Viremia in Individuals Who Control HIV in the Absence of Antiretroviral Therapy

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ABSTRACT

A subset of antiretroviral-untreated, HIV-infected individuals are able to maintain undetectable plasma HIV RNA levels in the absence of antiretroviral therapy. These “elite” controllers are of high interest as they may provide novel insights regarding host mechanisms of virus control. The degree to which these individuals have residual plasma viremia has not been well-defined. We performed a longitudinal study of 46 elite controllers, defined as HIV-seropositive, antiretroviral-untreated individuals with plasma HIV RNA levels <50-75 copies/mL. The median duration of HIV diagnosis was 13 years, median baseline CD4+ T cell count was 753 cells/mm$^3$, and median duration of follow-up was 16 months. Plasma and cellular HIV RNA levels were measured using the Transcription Mediated Amplification (TMA) assay (estimated limit of detection <3.5 copies RNA/mL). A total of 1117 TMA assays were performed (median 5 time-points/subject and 4 replicates/time-point). All but one subject had detectable plasma HIV RNA on at least one time-point, and 15 (33%) subjects had detectable RNA at all time-points. The majority of controllers also had detectable cell-associated RNA and proviral DNA. A mixed effect linear model showed no strong evidence of change in plasma RNA levels over time. In conclusion, the vast majority (98%) of elite controllers had measurable plasma HIV RNA, often at levels higher than that observed in antiretroviral-treated patients. This confirms the failure to eradicate the virus, even in these unique individuals who are able to reduce plasma viremia to very low levels without antiretroviral therapy.
INTRODUCTION

The vast majority of HIV-infected individuals have readily detectable levels of plasma HIV RNA in the absence of highly active antiretroviral therapy (HAART). There exists, however, a rare subset of individuals who have undetectable plasma HIV RNA using conventional assays. These “elite controllers” are exceedingly rare, comprising less than 1% of the HIV-infected population (23, 31, 36). They are distinct from long-term non-progressors (LTNP), who have been classically defined as maintaining a CD4+ T cell > 500 cells/mm$^3$ over a period of several years; many (although not all) elite controllers maintain stable CD4+ T cells, while only a small subset of LTNP have undetectable HIV RNA levels (11). Elite controllers are now being recognized as a potentially informative model for vaccine research in which the goal is to decrease the level of viral replication in individuals who have already become infected (52). In addition, characterization of immunological mechanisms responsible for viral suppression in elite controllers may yield valuable insights for the development of novel immune-based treatment strategies for HIV-infected individuals.

The mechanisms by which elite controllers are able to maintain durable control of HIV is the focus of intensive investigation by our group and others. HIV controllers appear to be enriched for certain HLA alleles (15, 43) and often have high levels of HIV-specific T cells (4, 6, 14, 19, 42, 46, 47). Many controllers have favorable CCR5 genotypes (10, 40, 50) and/or high copy numbers of CCL3L1 (18), the natural ligand for CCR5 (13). More recently, it was shown that controllers are highly enriched for specific NK cell receptor genotypes (particularly when present with HLA-Bw4 alleles), arguing for a presently undefined role of NK cells in virologic control (39). Finally, it has been suggested that viral factors (such as nef deletions) may play a role (1, 9,
21, 25, 27, 41, 53, 55), although replication-competent virus has been recovered from a small number of elite controllers (5, 32) and gross genetic defects were not observed in viral sequences obtained from a large cohort of controllers (44). Comparable findings are also emerging from the SIV infected macaque model (17, 54).

Our group has developed a large cohort of well-characterized elite controllers in order to provide more clarity regarding the mechanisms of virologic control in these individuals. The primary objective of the current study was to systematically characterize longitudinal levels of plasma viremia and viral persistence in peripheral blood mononuclear cells (PBMCs) in a representative number of controllers. Several assays were performed, including quantification of very low level plasma HIV RNA, cell-based HIV RNA, and proviral DNA. We also measured HIV antibody levels over time as the dynamics of such responses may provide indirect insights into the degree of low-level HIV replication and ongoing antigenic stimulation (2, 8).

MATERIALS AND METHODS

Study Participants

Blood was obtained from individuals enrolled in SCOPE, an ongoing prospective cohort study based at the University of California, San Francisco. All subjects in SCOPE are seen every four months, at which time they are interviewed. Plasma and peripheral blood mononuclear cells are stored at each visit. Elite controllers were defined based on the following criteria: (1) HIV seropositive, (2) antiretroviral untreated, and (3) plasma HIV RNA levels below the level of detection using conventional assays (< 50 copies/mL by PCR, or < 75 copies/mL by bDNA). We also studied a group of long-term HAART-suppressed patients, who were defined based on
the following criteria: (1) HIV seropositive, (2) treated with antiretroviral therapy for at least five continuous years, and (3) plasma HIV RNA levels below the level of detection using conventional assays for at least five years (isolated viremic episodes < 1000 copies/mL were allowed). All subjects provided written informed consent. This study was approved by the University of California San Francisco Committee on Human Research.

Ultra-sensitive Plasma HIV RNA Levels

Longitudinal plasma HIV RNA levels were measured using the isothermal Transcription Mediated Amplification (TMA) assay (Aptima®, Gen-Probe, San Diego, CA). This is a nucleic acid-amplification test that has been FDA-approved for the early detection of HIV infection in blood donors and validated for clinical use (49, 51). It is a highly specific and sensitive assay, with a 50% detection limit of 3.6-14 copies RNA/mL when performed in singlicate (7, 37). The assay was performed in quadruplicate for each time-point (2 cc plasma total), improving the overall limit of detection to <3.5 copies RNA/mL. The output is a signal:cutoff (S/Co) ratio (range 0-30), with S/Co < 1.0 considered HIV RNA “negative” and S/Co ≥ 1.0 considered HIV RNA “positive.”

In vitro spiking experiments were conducted to validate the specificity of the TMA assay and its use for ultrasensitive, semiquantitative measurement of HIV RNA levels (Figure 1). Samples of known HIV viral load copy number (0, 1, 3, 10, 30, 100, and 300 copies/mL) were tested with the TMA assay 20 times each by 4 different laboratory technicians (Jeff Linnen, personal communication). A random selection of 4 replicates (the same number of replicates performed on the elite controllers) for each of the technicians showed excellent correlation
between HIV viral load copy number and S/Co ratio. Importantly, there were no false positive test results using the TMA assay.

HIV Antibody Levels

In addition, a “de-tuned” or less-sensitive enzyme immunoassay (Organon Tecnika Vironostika [OTV], BioMerieux) was used to obtain longitudinal, semiquantitative HIV antibody levels on the same 46 controllers. The OTV is a second-generation ELISA that detects both IgG and IgM antibodies to HIV-1 and is FDA-approved for diagnostic testing. The less-sensitive modification involves testing 1:20,000 dilutions of plasma under abbreviated incubation conditions and calculating a standardized optical density (SOD) for each sample. Previously, the less sensitive-EIA has been used to identify patients with early HIV infection, with such patients exhibiting a positive result on the standard EIA and a negative result on the less sensitive-EIA (26). In this study, we used the less sensitive-EIA to evaluate the natural history of the HIV antibody response in controllers and to screen for any individuals who had potentially seroreverted. All specimens with a “negative” less sensitive-EIA (SOD<0.2) underwent antibody testing using a standard third-generation EIA to assess for seronegativity. The less sensitive-EIA was also used to compare HIV antibody levels in elite controllers and in untreated, chronically-infected, first-time blood donors; all blood donors were HIV seropositive by standard third-generation EIA and had positive plasma RNA levels by the TMA assay.

Cell-associated RNA and Proviral DNA
The TMA assay was also used to measure cell-associated RNA in a subset of elite controllers (n=29) who exhibited consistently positive or negative plasma HIV RNA levels by the TMA assay. A modified approach of previously published methods for PBMC extraction and TMA amplification of HCV was used (3). All signal:cutoff (S/Co) ratios were normalized to the input number of PBMCs.

Proviral DNA levels were also measured in the same subset of controllers (n=29) with consistently positive or negative plasma HIV RNA levels. Total proviral HIV DNA was extracted from PBMCs using modifications of previously-described methods (34). This assay has an overall sensitivity of 1 copy/3 µg of input DNA, equivalent to approximately 450,000 PBMCs (33, 35). All proviral DNA levels were normalized to the input number of PBMCs.

Statistical Methods

Mixed effect linear models with random slopes and intercepts were assessed to examine change in plasma HIV RNA and antibody levels over time. All statistical analyses were conducted with the Stata version 9.0 software program (Stata Corp, College Station, TX).

RESULTS

Baseline characteristics

Forty-six controllers were followed for a median 16 months; 39% were female and 37% were African-American (Table 1). The median self-reported duration of HIV diagnosis at study entry was 13 years, and the median baseline CD4+ T cell count was 753 cells/mm³.
As a comparison group, we measured plasma HIV RNA levels in 37 long-term HAART-treated subjects who had undetectable plasma levels (using conventional assays) for at least five years. The median duration of viral load suppression was 7 years (IQR 6-7 years) and the median baseline CD4+ T cell count was 485 cells/mm$^3$. The self-reported CD4 nadir of this group was 85 (IQR 37 to 172).

Ultra-sensitive Plasma HIV RNA Levels

A total of 1117 TMA assays were performed (median 5 time-points per subject, 4 replicates per time-point) (Figure 2). Fifteen (33%) subjects had all replicates from all time-points positive, while only one subject (described in detail below) had all TMA assays negative across all available time-points. The level of viremia—as estimated by the signal:cutoff ratio—varied over time within any given subject (Figure 2), with many subjects having intermittent time-points at which no virus could be detected. For the entire cohort, however, a mixed effect linear model showed no strong evidence of change in plasma HIV RNA over time (average of 0.08 increase in S/Co per month, $p=0.08$).

As a comparison group, plasma HIV RNA levels were also measured in a cohort of 37 subjects who had been virologically suppressed with HAART for a median 7 years. A total of 180 TMA assays were performed (median 2 time-points per subject, 3 replicates per time-point). Nine (24%) subjects had all replicates from all time-points positive, while five (14%) subjects had all replicates from all time-points negative. The median S/Co for elite controllers was higher than in the HAART-suppressed subjects, although this difference did not reach statistical significance (9.3 and 6.3, respectively, Mann-Whitney test, $p=0.127$; Figure 3).
Cell-associated RNA and Proviral DNA

Longitudinal cell-associated RNA (CA-RNA) levels were measured using the TMA assay in 29 controllers (median 2 time-points per subject, 2 replicates per time-point). CA-RNA was detectable in the majority (25/29) of controllers. A mixed effect linear model showed no evidence of change in CA-RNA over time ($p=0.91$).

Longitudinal proviral DNA levels were measured in the same 29 controllers (median 2 time-points per subject, 4 replicates per time-point). Proviral DNA was detectable in most (21/29) individuals (median 16 copies DNA/10$^6$ PBMCs, IQR 6-48). A mixed effect linear model showed no evidence of change in proviral DNA levels over time ($p=0.45$).

Persistent lack of detectable viremia in one elite controller

Only one elite controller had all plasma HIV RNA assays negative across all available time-points (5 time-points, 16 total replicates), spanning a period of one year. Interestingly, this subject also had negative less sensitive-EIA HIV antibody levels across all four time-points that were tested, although all of these were positive by standard EIA testing. HIV infection was further confirmed by longitudinal HIV western blot testing (Bio-Rad Laboratories). The same four time-points that yielded negative plasma viremia and less sensitive-EIA results were tested and showed intermediate-intensity and generally stable band patterns (gp160 1+ to 2+, gp120 +/-, p65 negative, p55/p51 2+, gp41 1+, p40 2+, p31 negative, p24 2+, p18 +/- to 1+). It is notable, however, that there was no p31 band across all time-points; such a pattern has been
associated with false positive HIV test results in blood donors, as well as with early HIV infection (16, 28, 48).

Despite the lack of detectable plasma HIV RNA and low antibody levels in this subject, both cell-associated HIV RNA and proviral DNA assays were positive from the one time-point that was tested. However, levels of T cell activation were remarkably low in this individual (only 1.7% and 5.9% of CD4+ and CD8+ T cells expressed HLA-DR and CD38, respectively; this was the lowest levels of any in our cohort) (24). Gag-specific and Pol-specific T cell responses were also much lower than those observed in the rest of the cohort (data not shown) (14).

**HIV Antibody Levels**

A total of 249 less sensitive-EIAs were performed on the 46 elite controllers (median 4 time-points per subject). The median SOD was 4.5, which was higher than that observed in untreated, chronically HIV-infected individuals (n=543, median SOD=3.7, p=0.03). Although uncommon for the less sensitive-EIA to be negative in chronically HIV-infected individuals (30), 4/46 (9%) of our elite controllers were less sensitive-EIA negative at baseline. Only two subjects had all less sensitive-EIAs negative (SOD<0.2); however, all less sensitive-EIA negative specimens were positive by standard EIA. A mixed effect linear model showed no evidence of change in HIV antibody levels over time (average of 0.01 increase in SOD per month, p=0.40). There was a positive relationship between baseline HIV antibody level and each of the following: baseline plasma HIV RNA (rho=0.43, p<0.01; Figure 4A), baseline CA-RNA (rho=0.50, p=0.01; Figure 4B), and baseline proviral DNA (rho=0.40, p=0.06; Figure 4C) levels.
DISCUSSION

The majority (98%) of “elite” controllers have measurable plasma HIV RNA, albeit at very low levels. This is in contrast to long-term HAART-suppressed subjects, where a more sizable minority (14%) had undetectable plasma RNA levels. The persistent viremia in the elite controllers may reflect ongoing viral replication or release of RNA from a long-lived latent reservoir (which can theoretically persist indefinitely in the absence of active viral turnover). Determining the role of viral replication versus latency as the source of virus in elite controllers will be challenging, just as it has proven difficult to rule in or rule out active replication in long-term HAART treated patients. We believe that the totality of our findings (persistent viremia and detection of cell-associated RNA and proviral DNA in the majority of controllers, some of whom have been infected for decades) argues for the presence of ongoing viral replication; however, further studies will be needed. Regardless of the source of viremia, our data confirm a failure to eradicate the virus even in these unique individuals who appear to be able to control the virus without antiretroviral therapy for many years.

Across our entire elite controller cohort, only one subject had consistently undetectable plasma HIV RNA levels; however, both cell-associated RNA and proviral DNA levels were detected in this study participant. Interestingly, this subject also had repeatedly negative HIV antibody levels using a less-sensitive “detuned” assay (although all samples were positive by standard EIA) and had multiple HIV western blots that were consistent with a potentially false positive or early seroconversion serological reactivity (i.e., there was no p31 band across multiple time-points) (16, 28, 48). This particular subject first tested HIV-seropositive in 1989 and had a
strong exposure history (sexual and intravenous drug use). Given the positive cell-associated RNA and proviral DNA results, HIV-seropositivity by standard EIA testing, as well as low but detectable Gag-specific CD4+ T cell responses (data not shown), the subject is clearly HIV-infected. However, it is intriguing that this subject could have been considered to have a false positive or indeterminate HIV antibody test with conventional HIV testing, given negative plasma RNA levels and potentially false positive western blot pattern. Moreover, this one interesting case raises the possibility that there may be a “spectrum of HIV infection,” and that elite controllers, like highly-exposed HIV-seronegative individuals (29, 56), are on one extreme end of the spectrum.

Although as a cohort there was no strong evidence of change in plasma HIV RNA levels over time, there appeared to be significant individual variability over time (Figure 2). This lack of a clear and stable “steady-state” is consistent with recent data reported from two elite controllers followed over time (12), and is distinct from the trends observed in long-term HAART suppressed patients (38). Although the lack of a steady-state in our elite controllers may represent assay variability, this seems unlikely given that the within time-point TMA data were generally more consistent with each other. Alternatively, the variations in plasma RNA may reflect fluctuations in virus production by long-lived, chronically-infected cells. We propose that the dynamics of viremia in these controllers may reflect an ongoing host/virus interaction, with each waxing and waning over time (“predator-prey dynamics”) (2). In other words, the host response may increase as viremia increases, which results in improved control and a decline in viremia. This dynamic host/virus response with one driving the other is further supported by the observed positive relationship between plasma HIV RNA levels and HIV antibody levels (Figure
Moreover, the subset of elite controllers with a negative less sensitive-EIA (approximately 10% of our cohort) may be of particular interest as it pertains to the role of the virus and the host in a state of near-compete viral suppression. This group tended to have the lowest levels of viremia and presumably had insufficient antigen to generate a potent and sustained antibody response.

What are the consequences of very low-level viremia in elite controllers and in HAART-suppressed patients? Low-level viremia (below the conventional level of detection) has been associated with increased immune activation in HAART-suppressed subjects (45). Controllers have higher levels of immune activation compared to HAART-suppressed and HIV-negative subjects (24), and it appears that a small proportion of elite controllers with high levels of T cell activation even progress immunologically to AIDS despite maintenance of virologic control (24). Preliminary data from our group suggest that controllers have higher levels of atherosclerosis (as measured by intima-media thickness) compared to HIV-negative controls, even after adjustment for traditional cardiovascular risk factors (22). Thus, it is possible that oscillating, very low levels of viremia lead to high levels of immune activation in some controllers, which may lead to AIDS- and non-AIDS defining events.

This is the first such study to report longitudinal measurements of plasma HIV RNA in a large group of elite controllers. Several limitations of our study deserve comment, however. Although there was no strong evidence of change in plasma HIV RNA levels over time, there was a trend towards a slow increase in plasma HIV RNA levels in our cohort. It is possible that with longer periods of follow-up or a greater number of subjects, significant changes in plasma viremia.
and cell-associated viremia would have been documented. Moreover, the TMA assay provides a 
semiquantitative (although highly specific) measurement of ultrasensitive plasma HIV RNA and 
confirmatory studies utilizing a more quantitative measurement of very low levels of viremia are 
warranted. Similarly, the less sensitive-EIA is a semiquantitative measure of total antibody 
reactivity, although it has been studied in patients treated with HAART during acute (20) and 
chronic (8) infection and appears to correlate directly with level of plasma viremia. Finally, 
additional virologic studies (sequencing of virus, co-culture of virus) need to be systematically 
performed in a substantial number of elite controllers in order to confirm infection with 
replication-competent virus in these individuals.

Further study of elite controllers is necessary. Elite controllers provide a unique opportunity to 
examine the relationship between viral characteristics and host genetics/immune responses in a 
successful model of durable HIV control without the use of antiretroviral therapy. Insight gained 
from these individuals could be used as a platform for studies aimed at therapeutic vaccines and 
eradication of HIV. Virtually all of our elite controllers had evidence of viremia for a significant 
period of time. Thus, rather than a phenotype of an abortive attempt at infection (detectable 
proviral DNA with undetectable plasma RNA, seen in only one subject), the great majority of 
subjects had evidence of persistent viremia, implying a long-term capacity to control virus at 
very low levels. This would suggest that perhaps the host can be armed to durably control the 
virus, and that a therapeutic vaccine may be a future possibility.
ACKNOWLEDGMENTS

Potential conflicts of interest. JML is an employee of Gen-Probe, Inc. HH, ELD, PJN, THL, JDW, SLS, PWH, RH, JMM, JNM, MPB, SGD: No conflicts. Reagents provided by Gen-Probe and Novartis at no cost to this study.

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sensitivities of licensed nucleic acid amplification tests for detection of viremia in early human immunodeficiency virus and hepatitis C virus infection. Transfusion 45:1853-63.


Natural history of serum HIV-1 RNA levels in 330 patients with a known date of infection.


FIGURE LEGENDS

Figure 1. In vitro spiking experiments showing relationship between HIV RNA level and TMA assay. Spiking experiments showing relationship between HIV RNA level and signal:cutoff (S/Co) ratio using the Transcription Mediated Amplification (TMA) assay. Each dot represents the mean of four replicates (randomly selected from 20 replicates) performed by 4 different laboratory technicians; the lines represent the mean S/Co for each viral load copy number (0, 1, 3, 10, 30, 100, and 300 copies/mL).

Figure 2. Plasma HIV RNA levels in elite controllers. Plasma HIV RNA levels (signal:cutoff [S/Co] ratio) using the Transcription Mediated Amplification assay in a subset (n=16) of elite controllers with ≥ 20 months of follow-up. Each panel represents longitudinal plasma HIV RNA levels for one subject.

Figure 3. Baseline plasma HIV RNA level for 37 HAART-suppressed subjects and 46 EC (elite controllers). S/Co=signal:cutoff ratio using the Transcription Mediated Amplification assay. The lines represent the median S/Co for each group.

Figure 4A. Association between baseline plasma HIV RNA and HIV antibody level. Association between baseline plasma HIV RNA and HIV antibody level (n=46, Spearman’s rho=0.43, p<0.01). S/Co=signal:cutoff ratio using the Transcription Mediated Amplification assay. SOD=standardized optical density using the less-sensitive EIA.
Figure 4B. Association between baseline cell-associated RNA and HIV antibody level.

Association between baseline cell-associated RNA (CA-RNA) and HIV antibody level (n=25, Spearman’s rho=0.50, p=0.01). SOD=standardized optical density using the less-sensitive EIA.

Figure 4C. Association between baseline proviral HIV DNA and HIV antibody level.

Association between baseline proviral HIV DNA and HIV antibody level (n=24, Spearman’s rho=0.40, p=0.06). SOD=standardized optical density using the less-sensitive EIA. Plot excludes one outlier.
FIGURE 1

![Graph showing the relationship between S/Co (TMA) and HIV RNA (copies/mL).]
FIGURE 2

Plasma HIV RNA (S/Co)

Time (months)
Figure 3

Plasma HIV-1 RNA (S/Co)

HAART-suppressed (n=37)

EC (n=46)
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<th><strong>TABLE 1.</strong> Baseline characteristics (n=46)</th>
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<td>Age (years)</td>
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<td>5</td>
<td>Self-reported duration of HIV diagnosis (years)</td>
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<td>CD4+ T cell count (cells/mm&lt;sup&gt;3&lt;/sup&gt;)</td>
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<td>7</td>
<td>Self-reported nadir CD4+ T cell count (cells/mm&lt;sup&gt;3&lt;/sup&gt;)</td>
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<td>8</td>
<td>Plasma HIV RNA (copies/mL) (bDNA assay)</td>
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<td>9</td>
<td>Duration of follow-up (months)</td>
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Data are medians with interquartile ranges (IQR). <sup>a</sup>The cohort was 37% African-American (Af-Am), 35% Caucasian, 11% Hispanic, 4% Asian/Pacific Islander, 7% Mixed, and 7% Other.