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Evidence for Persistent Low-Level Viremia in Individuals Who Control Human Immunodeficiency Virus in the Absence of Antiretroviral Therapy▽

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A subset of antiretroviral-untreated, human immunodeficiency virus (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 of <50 to 75 copies/ml. The median duration of HIV diagnosis was 13 years, the median baseline CD4$^+$ T-cell count was 753 cells/mm^3, and the 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 of <3.5 copies RNA/ml). A total of 1,117 TMA assays were performed (median of five time points/subject and four 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.

The vast majority of human immunodeficiency virus (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 when tested 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 nonprogressors, who have been classically defined as maintaining a CD4$^+$ T-cell count of >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 long-term nonprogressors 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 are 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 simian immunodeficiency virus-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 quantifications 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).

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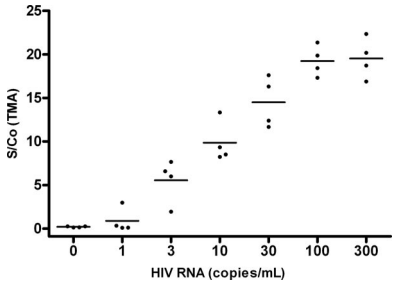

FIG. 1. In vitro spiking experiments showing the relationship between HIV RNA levels and S/Co ratio using the TMA assay. Each dot represents the mean of the results for four replicates (randomly selected from 20 replicates) performed by four different laboratory technicians; the lines represent the mean S/Co ratio for each viral load copy number (0, 1, 3, 10, 30, 100, and 300 copies/ml).

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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, CA. All subjects in SCOPE are seen every 4 months, at which time they are interviewed. Plasma samples and PBMCs are stored at each visit. Elite controllers were defined based on (i) being HIV seropositive, (ii) being antiretroviral untreated, and (iii) having plasma HIV RNA levels below the level of detection by using conventional assays (<50 copies/ml by PCR or <75 copies/ml by branched-DNA assay). We also studied a group of long-term HAART-suppressed patients, who were defined based on (i) being HIV seropositive, (ii) being treated with antiretroviral therapy for at least five continuous years, and (iii) having plasma HIV RNA levels below the level of detection by using conventional assays for at least 5 years (isolated viremic episodes of <1,000 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 has been validated for clinical use (49, 51). It is a highly specific and sensitive assay, with a 50% detection limit of 3.6 to 14 copies of RNA/ml when performed in singlicate (7, 37). The assay was performed in quadruplicate for each time point (2 ml plasma total), improving the overall limit of detection to <3.5 copies of RNA/ml. The output is a signal-to-cutoff (S/Co) ratio (range, 0 to 30), with S/Co ratios of <1.0 considered HIV RNA "negative" and S/Co ratios of ≥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 (Fig. 1). Samples of known HIV viral load copy numbers (0, 1, 3, 10, 30, 100, and 300 copies/ml) were tested with the TMA assay 20 times each by four different laboratory technicians (J. Linnen, personal communication). Results from a random selection of four replicate experiments (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 "detuned" or less-sensitive enzyme immunoassay (Organon Tecnika Vironostika; bioMerieux) was used to obtain longitudinal, semiquantitative HIV antibody levels on the same 46 controllers. The Organon Tecnika Vironostika is a second-generation enzyme-linked immunosorbent assay that detects both immunoglobulin G and immunoglobulin M 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 enzyme immunoassay (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 result (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 (CA-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 of hepatitis C virus was used (3). All 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 by 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 changes 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 of 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/mm^3.

As a comparison group, we measured plasma HIV RNA levels in 37 long-term HAART-treated subjects who had undetectable plasma levels (when using conventional assays) for at least 5 years. The median duration of viral load suppression was 7 years (interquartile range [IQR], 6 to 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).

Ultrasensitive plasma HIV RNA levels. A total of 1,117 TMA assays were performed (median of five time points per subject and four replicates per time point) (Fig. 2). Fifteen (33%) subjects had all replicates from all time points test positive, while only one subject (described in detail below) had all negative TMA assay results across all available time points. The level of viremia—as estimated by the S/Co ratio—varied over time within any given subject (Fig. 2), with many subjects

TABLE 1. Baseline characteristics ($n = 46$)

Characteristic	Value[a]
Male/female (%)	61/39
Af-Am/Caucasian (%)[b]	37/35
Median age (yr)	46 (43–49)
Median self-reported duration of HIV diagnosis (yr)	13 (8–17)
Median $CD4^+$ T-cell count (cells/mm^3)	753 (537–1039)
Median self-reported nadir $CD4^+$ T-cell count (cells/mm^3)	571 (400–694)
Median plasma HIV RNA (copies/ml) (branched-DNA assay)	75 (75–75)
Median duration of follow-up (mo)	16 (7–25)

[a] Data in parentheses are IQRs.
[b] The cohort was 37% African-American (Af-Am), 35% Caucasian, 11% Hispanic, 4% Asian/Pacific Islander, 7% mixed, and 7% other.

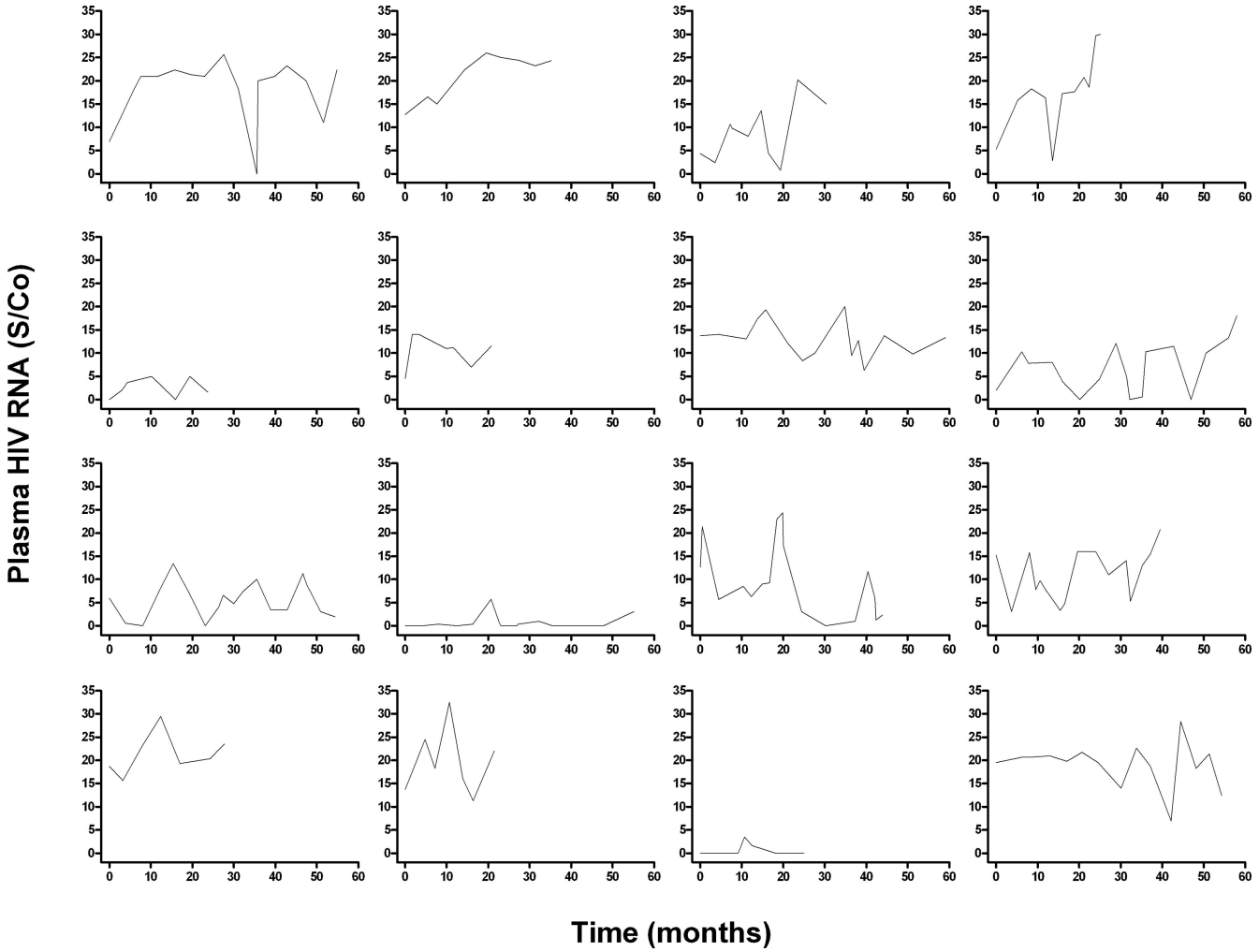

FIG. 2. Plasma HIV RNA levels in elite controllers. Plasma HIV RNA levels (S/Co ratio) using the TMA assay in a subset ($n = 16$) of elite controllers with ≥20 months of follow-up. Each panel represents longitudinal plasma HIV RNA levels for one subject.

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 changes in plasma HIV RNA levels over time (average of 0.08 increase in S/Co ratio 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 of 7 years. A total of 180 TMA assays were performed (median of two time points per subject and three replicates per time point). Nine (24%) subjects had all replicates from all time points test positive, while five (14%) subjects had all replicates from all time points test negative. The median S/Co ratio for elite controllers was higher than that for the HAART-suppressed subjects, although this difference did not reach statistical significance (9.3 and 6.3, respectively, by Mann-Whitney test; $P = 0.127$) (Fig. 3).

CA-RNA and proviral DNA. Longitudinal CA-RNA levels were measured using the TMA assay for 29 controllers (median of two time points per subject and two 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 levels over time ($P = 0.91$).

Longitudinal proviral DNA levels were measured in the same 29 controllers (median of two time points per subject and four replicates per time point). Proviral DNA was detectable in most (21/29) individuals (median, 16 copies DNA/10^6 PBMCs;

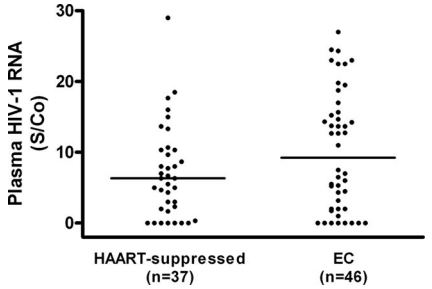

FIG. 3. Baseline plasma HIV RNA level for 37 HAART-suppressed subjects and 46 elite controllers (EC). The lines represent the median S/Co ratio for each group.

IQR, 6 to 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 test negative across all available time points (five time points, 16 total replicates), spanning a period of 1 year. Interestingly, this subject also had negative less-sensitive EIA results for 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, −; p55/p51, 2+; gp41, 1+; p40, 2+; p31, −; p24, 2+; p18, ± to 1+ [+ and − signs refer to degrees of intensity of the bands]). 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; these were 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 of four 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 result to be negative in chronically HIV-infected individuals (30), 4/46 (9%) of our elite controllers tested negative by less-sensitive EIA at baseline. Only two subjects had all negative less-sensitive EIA results (SOD, <0.2); however, all specimens with negative less-sensitive EIA results tested 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 level (rho = 0.43; $P < 0.01$) (Fig. 4A), baseline CA-RNA level (rho = 0.50; $P = 0.01$) (Fig. 4B), and baseline proviral DNA level (rho = 0.40; $P = 0.06$) (Fig. 4C).

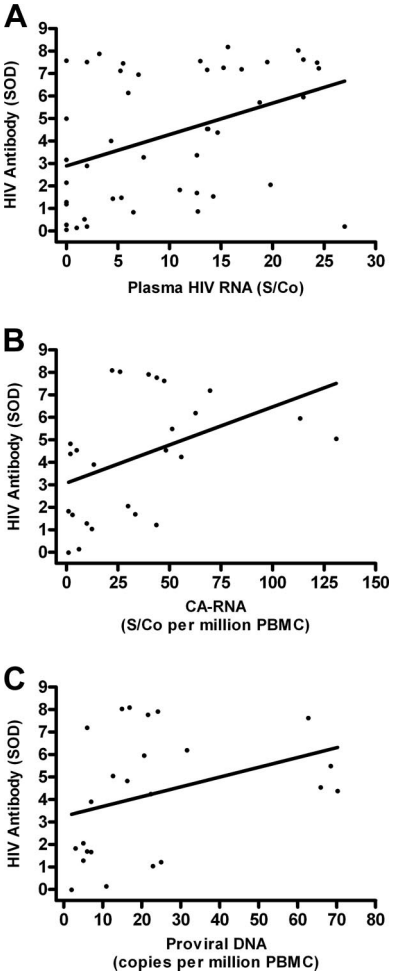

FIG. 4. (A) Association between baseline plasma HIV RNA and HIV antibody levels ($n = 46$; Spearman's rho = 0.43; $P < 0.01$). (B) Association between baseline CA-RNA and HIV antibody levels ($n = 25$; Spearman's rho = 0.50; $P = 0.01$). (C) Association between baseline proviral HIV DNA and HIV antibody levels ($n = 24$; Spearman's rho = 0.40; $P = 0.06$). Plot excludes one outlier.

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, of which 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 that of 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 CA-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 CA-RNA and proviral DNA levels were detected in this study participant. Interestingly, this subject also had repeatedly negative HIV antibody levels when tested 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 CA-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 a 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 (Fig. 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 (Fig. 4A). Moreover, the subset of elite controllers with negative less-sensitive EIA results (approximately 10% of our cohort) may be of particular interest, as it pertains to the roles 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 than do 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) than do 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 toward 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- 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) infections and appears to correlate directly with level of plasma viremia. Finally, additional virologic studies (sequencing of virus, coculture of virus) need to be systematically performed on 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 the 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.

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