Relationship between the Size of the Human Immunodeficiency Virus Type 1 (HIV-1) Reservoir in Peripheral Blood CD4⁺ T Cells and CD4⁺:CD8⁺ T Cell Ratios in Aviremic HIV-1–Infected Individuals Receiving Long-Term Highly Active Antiretroviral Therapy

Tae-Wook Chun, J. Shawn Justement, Punita Pandya, Claire W. Hallahan, Mary McLaughlin, Shuying Liu, Linda A. Ehler, Colin Kovacs, and Anthony S. Fauci

It has been demonstrated that human immunodeficiency virus type 1 (HIV-1) replication persists in most infected individuals receiving highly active antiretroviral therapy (HAART). However, studies addressing the relationship between low levels of ongoing viral replication and immunologic parameters, such as the CD4⁺:CD8⁺ T cell ratio, in such individuals have been lacking. Here, a statistically significant inverse correlation is shown between the frequency of CD4⁺ T cells carrying HIV-1 proviral DNA and the CD4⁺:CD8⁺ T cell ratio in infected individuals receiving HAART and in whom plasma viremia had been suppressed below the limit of detection for prolonged periods of time. No correlation was found between the frequency of HIV-1–specific cytotoxic CD8⁺ T lymphocytes (CTLs) and the CD4⁺:CD8⁺ T cell ratios in those individuals. These data suggest that persistent, low-level, ongoing viral replication, although not sufficient to maintain HIV-1–specific CTL responses, may explain, in part, why normalization of the CD4⁺:CD8⁺ T cell ratio is not achieved in some infected individuals successfully treated with HAART.

The use of highly active antiretroviral therapy (HAART) in the treatment of human immunodeficiency virus type 1 (HIV-1)–infected individuals has dramatically changed the clinical outcome for many infected persons and has led to a substantial decline in the incidence of AIDS and of mortality [1]. However, the presence of replication-competent virus [2–4], HIV-1 proviral DNA [2] (including 2 long terminal repeat circles [5]), spliced and unspliced HIV-1 RNA [6, 7] in CD4⁺ T cells, and unidentified virus reservoirs [8] has been demonstrated in most infected individuals in whom plasma viremia has fallen below the limit of detection, and these sources of ongoing replication have emerged as the major obstacle in preventing eradication of HIV-1.

After the initiation of HAART, plasma viremia rapidly declines to below the limit of detection in a high proportion of infected individuals and is followed by a gradual increase of CD4⁺ and decrease of CD8⁺ T cell counts. However, normalization of CD4⁺:CD8⁺ T cell ratios is not achieved in some infected individuals who have otherwise successfully responded to HAART [9, 10]. In this regard, it has been suggested that the chronically elevated levels of CD8⁺ T cells or abnormal CD4⁺:CD8⁺ T cell ratios in the peripheral blood are driven by active viral replication in infected individuals who are untreated or receiving suboptimal antiviral therapy [11]. In addition, the expression of the activation marker CD38 on CD8⁺ T cells has been shown to be a prognostic marker of disease progression [12]. Although it has been suggested that the failure to normalize the CD4⁺:CD8⁺ T cell ratio in some patients receiving HAART is attributed to incomplete suppression of viral replication [10], there are no reported data addressing this question directly, especially in patients who have been receiving HAART for prolonged periods of time and in whom plasma viremia has been well suppressed.

It has been established that the reservoir of infected CD4⁺ T cells in the peripheral blood, as determined by the number of cells carrying HIV-1 proviral DNA, is maintained by ongoing viral replication, even at very low levels [2]. In the present study, we evaluated the relationship between the ratios of CD4⁺:CD8⁺ T cell counts and the frequency of CD4⁺ T cells carrying HIV-1 proviral DNA in purified CD4⁺ T cells, as well as the frequency of HIV-specific cytotoxic CD8⁺ T lymphocytes (CTLs) in infected individuals who had been receiving HAART for prolonged periods of time and in whom plasma viremia has been suppressed to below the level of detection, as determined by standard assays.

The Journal of Infectious Diseases 2002;185:1672–6
© 2002 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2002/18511-0019$02.00

Received 30 November 2001; revised 21 January 2002; electronically published 8 May 2002.

The study protocol was approved by the institutional review board of the University of Toronto and by the National Institute of Allergy and Infectious Diseases, National Institutes of Health. All participants signed an informed consent form.

Reprints or correspondence: Dr. Tae-Wook Chun, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Laboratory of Immunoregulation, Bldg. 10, Rm. 6A32, Bethesda, MD 20892 (twchun@nih.gov).
Patients and Methods

Study patients. Thirty-two HIV-1–infected individuals receiving various HAART regimens containing at least 1 protease inhibitor and 2 reverse-transcriptase inhibitors of HIV-1 for >2.5 years (mean, 45.9 months; range, 30.5–54.0 months) were studied. The mean CD4+ and CD8+ T cell counts were 840 cells/mm³ (range, 170–1650 cells/mm³) and 1110 cells/mm³ (range, 380–1990 cells/mm³), respectively, and the CD4+:CD8+ T cell ratio was 0.90 (range, 0.18–2.79). At the time of study, all infected individuals had consistently maintained HIV-1 RNA plasma viremia levels of <50 copies/mL for >2.0 years (mean, 32.0 months; range, 24.0–46.0 months), as determined by reverse-transcription polymerase chain reaction (PCR; Roche).

Isolation of CD4+ T cells. Peripheral blood mononuclear cells (PBMC) were obtained by ficoll-hypaque density gradient centrifugation. CD4+ T cells were isolated from PBMC of HIV-1–infected individuals, using a column-based cell-separation technique (Stem-Cell Technologies), as described elsewhere [8].

Quantitative real-time HIV-1 DNA PCR. To determine the frequency of CD4+ T cells carrying HIV-1 provirus in infected individuals, a real-time PCR was carried out as described below. Genomic DNA was isolated from 1–2 x 10⁶ purified CD4+ T cells, using the Puregene DNA isolation kit (Gentra), according to the manufacturer’s specifications. DNA (1 µg) was then used as template for real-time PCR in an iCycler (Bio-Rad). The amplification reaction was done in triplicate using 0.5 µM primers, 0.2 µM fluorescent probe, 0.8 mM dNTPs, 5 mM MgCl₂, and 2.5 U Platinum Taq Polymerase (Life Technologies) in a 50 µL total volume. Primers 5’-GGTCTCTCTGGTTAGACCAGAT-3’ (5’ primer) and 5’-CTGCTAGAGATTTCACACTG-3’ (3’ primer) were used along with the fluorescent probe 5’-6FAM-AGTAGGTTGTGCACCTGT-3’ (Tamra). PCR conditions consisted of a denaturation step at 95°C for 3 min, followed by 45 cycles of 15 s at 95°C and 1 min at 58°C. Serially diluted ACH-2 DNA was also subjected to the above PCR to obtain standard curves.

Flow cytometric analysis of HIV-1–specific CD8+ T cells. The frequency of CD8+ T cells specific for HIV-1 was determined by analysis of intracellular interferon (IFN)-γ–positive cells after stimulation with HIV-1–specific peptides (20 mer overlapping 15 aa). Pools of overlapping peptides (1 µg each; National Institutes of Health AIDS Reagent Program) spanning the entire HIV-1 gag, pol, env, and nef sequences were initially incubated with 3 x 10⁶ PBMC for 10 min in a round-bottom 5-mL tube (Becton Dickinson) in 0.1 mL of complete medium in a 37°C CO₂ incubator. Next, 0.4 mL of complete medium was added to the tube, and, after a 2-h incubation, Brefeldin A (Sigma) was added to the medium at a final concentration of 10 µg/mL to inhibit secretion of IFN-γ. After 6 h of incubation, the cells were washed twice, fixed with 1X fixing solution (Becton Dickinson) for 10 min at room temperature, and washed again. Cells were permeabilized with 1X permeabilization solution 2 (Becton Dickinson) and were further incubated at room temperature for 10 min. After being washed again, the cells were stained with the following antibodies: fluorescein isothiocyanate–conjugated anti-CD8, phycoerythrin-conjugated anti–IFN-γ, peridinin-chlorophyll protein–conjugated anti-CD69, and allophycocyanine-conjugated anti-CD3 antibodies (Becton Dickinson). With a gate on CD3+CD8+ lymphocytes, ~100,000 events were collected, using a FACSCalibur (Becton Dickinson), and the frequency of IFN-γ+CD69+ cells was determined by use of Cellquest software (Becton Dickinson).

Statistical analysis. Spearman’s rank correlation was used as a measure of correlation between (1) the frequency of CD4+ T cells carrying HIV-1 proviral DNA and CD4+ and CD8+ T cell counts and the CD4+:CD8+ T cell ratio and (2) the frequency of HIV-1–specific CTLs and CD4+ and CD8+ T cell counts, the CD4+:CD8+ T cell ratio, and the numbers of CD4+ T cells carrying HIV-1 proviral DNA. Fisher’s exact test was used to determine the association of CD4+:CD8+ T cell ratios ≥ 1.0 and < 1.0 with proviral loads in the CD4+ T cell compartment. The Bonferroni method for the adjustment of P values for multiple testing was applied to the correlations of CD4+ and CD8+ T cell counts and CD4+:CD8+ T cell ratio with the frequency of CD4+ T cells carrying HIV-1 proviral DNA, and it was applied to the correlations of CD4+ and CD8+ T cell counts, CD4+:CD8+ T cell ratio, and the levels of proviral HIV-1 DNA in the CD4+ T cell compartment with the frequency of HIV-1–specific CTLs.

Results

Correlation between the size of the CD4+ T cell reservoir of HIV-1 and immunologic parameters. To investigate the relationship between the size of the peripheral blood CD4+ T cell reservoir for HIV-1 and abnormal peripheral immunologic parameters in infected individuals receiving successful HAART for prolonged periods of time, we sought to establish correlations between the frequency of CD4+ T cells carrying HIV-1 proviral DNA and the CD4+ and CD8+ T cell counts and CD4+:CD8+ T cell ratio. There was a statistically significant inverse correlation between the frequency of CD4+ T cells carrying HIV-1 proviral DNA and the CD4+ and CD8+ T cell counts and CD4+:CD8+ T cell ratio. When these 2 immunologic parameters were combined and expressed as CD4+:CD8+ T cell ratio, a strong inverse correlation was observed between these values and the frequency of CD4+ T cells carrying HIV-1 proviral DNA (P < .01; figure 1A). In contrast, no correlation was found between the HIV-1 proviral DNA load in CD4+ T cells and the CD8+ T cell counts (P = .34; figure 1B). When these 2 immunologic parameters were combined and expressed as CD4+:CD8+ T cell ratio, a strong inverse correlation was observed between these values and the frequency of CD4+ T cells carrying HIV-1 proviral DNA (P < .01; figure 1C). In addition, 14 (74%) of 19 patients with CD4+:CD8+ T cell ratios < 1.0 had HIV-1 DNA proviral loads of > 1000 copies/µg of genomic DNA derived from CD4+ T cells. On the contrary, 10 (77%) of 13 patients with CD4+:CD8+ T cell ratios ≥ 1.0 had HIV DNA proviral loads of < 1000 copies/µg of genomic DNA derived from CD4+ T cells (P = .01; figure 1C).

Correlation between the frequency of HIV-1–specific CTLs and immunologic and virologic parameters. It has been demonstrated that the frequency of HIV-1–specific CTLs declines gradually after the initiation of HAART in HIV-1–infected individuals because of the decreasing availability of HIV-1 antigens [13]. We investigated the relationship between the frequency of HIV-1–specific CTLs and CD4+:CD8+ T cell ratios.
in infected individuals who had responded successfully to HAART for prolonged periods of time. No statistically significant correlation was found between the frequencies of CTLs that responded to HIV-specific peptides derived from \textit{gag}, \textit{pol}, \textit{env}, and \textit{nef} genes and \textit{CD4}+/\textit{CD8}+ T cell ratios (figure 2). In addition, no statistically significant correlation was found between the number of HIV-1–specific CTLs and \textit{CD4}+ and \textit{CD8}+ T cell counts and the number of \textit{CD4}+ T cells carrying HIV-1 proviral DNA (data not shown). Of note, there was a trend toward higher numbers of CTLs in individuals with lower \textit{CD4}+/\textit{CD8}+ T cell ratios.

\textbf{Discussions}

The widespread use of HAART in the treatment of HIV-1 disease has led to a dramatic improvement in clinical outcome [1].

\textbf{Figure 1.} Relationship between the frequency of CD4+ T cells carrying human immunodeficiency virus type 1 (HIV-1) proviral DNA and various immunologic parameters. Genomic DNA isolated from highly enriched CD4+ T cells of HIV-1–infected patients receiving long-term highly active antiretroviral therapy was subjected to HIV-1 long terminal repeat–specific real-time polymerase chain reaction, as described in Patients and Methods. Correlations were assessed between the frequency of cells carrying HIV-1 proviral DNA and the numbers of CD4+ (A) and CD8+ (B) T cells and CD4+:CD8+ T cell ratios (C).

\textbf{Figure 2.} Relationship between the frequency of human immunodeficiency virus type 1 (HIV-1)–specific cytotoxic CD8+ T lymphocytes and \textit{CD4}+:\textit{CD8}+ T cell ratios. The frequency of \textit{CD8}+ T cells specific for HIV-1 was determined by analysis of intracellular interferon (IFN)–\gamma–positive cells after stimulation with HIV-1–specific peptides derived from \textit{gag}, \textit{pol}, \textit{env}, and \textit{nef} genes. Spearman’s rank correlation method was used to obtain \textit{P} values.
Although plasma viremia can be maintained below the limit of detection in many infected individuals receiving HAART, the persistence of low levels of ongoing viral replication in CD4+ T cells has posed a major obstacle in achieving eradication of HIV-1 [2–4]. Indeed, persistent low levels of viral replication are felt to be the major factor involved in the maintenance of the reservoir of HIV-1, as reflected by the frequency of CD4+ T cells carrying HIV-1 proviral DNA in patients whose plasma viremia has been suppressed by HAART to below the level of detection of standard assays [2]. In addition, the sustained levels of elevated CD8+ T cell counts have been observed in a high proportion of infected patients receiving HAART [9, 10]. In this regard, it has been suggested that active viral replication is responsible for the elevated levels of CD8+ T cells [11] and that the levels of expression of the activation marker CD38 on these cells has been shown to be a prognostic marker of disease progression in the absence of effective antiviral therapy [12].

Despite a lack of published data, it has been speculated that inverted CD4+:CD8+ T cell ratios in the peripheral blood of infected individuals who are treated successfully with HAART are due to low levels of ongoing viral replication [10]. In the present study, we examined whether the inverted CD4+:CD8+ T cell ratios in some infected individuals receiving HAART for prolonged periods of time were due to incomplete suppression of HIV-1 replication, as reflected by the size of the HIV-1 proviral DNA reservoir in CD4+ T cells. The persistent presence of HIV-1 proviral HIV DNA in the CD4+ T cell compartment in infected patients receiving HAART has been convincingly described elsewhere [2] and has been thought to reflect incomplete inhibition of residual on-going viral replication, despite suppression of plasma viremia to undetectable levels [2]. We demonstrated a strong inverse correlation between the frequency of infected CD4+ T cells containing HIV-1 proviral DNA and CD4+ and CD8+ T cell ratios, suggesting that low levels of residual viral replication may have been partly responsible for the abnormal CD4+:CD8+ T cell ratios in peripheral blood.

Although the frequency of CD4+ T cells carrying proviral HIV-1 DNA may be directly associated with CD4+:CD8+ T cell ratios, other factors may affect this relationship. Parameters such as the existence of unknown virus reservoirs that support active viral replication in the presence of HAART, abnormalities in the host immune system, the potency of given therapeutic regimens, or other confounding factors may have an effect on both CD4+:CD8+ T cell ratios and the proviral DNA loads in the CD4+ T cell compartment. Of note, our data suggest that on-going viral replication at levels that are not detectable by standard assays for plasma viremia but that maintain the CD4+ T cell reservoir of HIV-1, although sufficient to impede normalization of the CD4+:CD8+ T cell ratios in the patients examined in this study, are not sufficient to maintain HIV-1–specific CTL responses.

It might be argued that the sustained abnormal CD4+:CD8+ T cell ratios in infected individuals receiving HAART for prolonged periods could also be attributed to both immunologic and virologic factors. In this regard, it has been demonstrated that, in the absence of disease progression and sufficient recovery of CD4+ T cells, total T cell homeostasis is maintained in infected individuals by elevated levels of CD8+ T cells that may, in turn, interfere with regeneration of CD4+ T cells [14]. However, we did not find a correlation between levels of CD4+ T cells carrying HIV-1 proviral DNA and CD8+ T cell counts.

Finally, previous studies have shown that generation of CD4+ T cells in the thymus is severely impeded by either direct or indirect consequences of active viral replication [15]. In this regard, decreased levels of thymic output, as measured by the frequency of naive CD4+ T cells carrying T cell receptor–rearrangement excision circles, have been demonstrated in infected individuals [15]. Although it is not clear whether precursors of CD4+ T cells are infected by HIV-1 in the thymus, our data strongly suggest that incomplete suppression of viral replication in infected individuals receiving HAART continues to spread infection in the CD4+ T cell compartment in the peripheral blood, resulting in the maintenance of the CD4+ T cell reservoir for HIV-1.

Acknowledgments

We thank Paul Parks for scheduling patient visits, Susan Moir for reading this manuscript and for helpful discussions, and the patients for their participation in this study.

References


