Evidence of Thymic Function in Heavily Antiretroviral-Treated Human Immunodeficiency Virus Type 1–Infected Adults with Long-Term Virologic Treatment Failure

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Thymic function was evaluated in 32 heavily antiretroviral-treated human immunodeficiency virus type 1 (HIV-1)–infected adults with long-term virologic treatment failure by measuring thymic volume, by determining the absolute number of naive T cell phenotypes, and by determining the number of cells carrying T cell receptor excision circles (TRECs). There was a significant inverse correlation between age and thymic volume (r = −0.415; P = .018), and there was a significant direct correlation between thymic volume and total naive T cell counts (r = 0.529; P = .002), naive CD4+ cell counts (r = 0.437; P = .012), naive CD8+ cell counts (r = 0.467; P = .007), and TRECs (r = 0.391; P = .027). In conclusion, this study found clear evidence that the thymus of heavily antiretroviral-treated HIV-1–infected adults with long-term virologic treatment failure is actively engaged in thymopoiesis, which generates new naive T cells for the peripheral lymphocyte pool.

Although highly active antiretroviral therapy (HAART) in most human immunodeficiency virus type 1 (HIV-1)–infected patients leads to a rapid decrease in virus load and a rise in T cells [1], a proportion of subjects have high virus loads, despite consecutive changes in antiretroviral regimens. Failure of a regimen may occur for many reasons, including initial viral resistance to ≥1 agents, altered absorption or metabolism of the drug, multidrug pharmacokinetics that adversely affect therapeutic drug levels, and poor patient adherence to a regimen [2]. In addition to virologic treatment failure, some of these patients have stable or slow increases in CD4+ T cell counts [3].

The mechanisms responsible for this so-called discordant treatment response are not well known. Several hypotheses have been proposed to explain this seemingly contradictory phenomenon.

First, HAART could maintain partial efficacy in persons with this discordant response to treatment. Hawley-Foss et al. [4], studying the effect of therapy cessation on 8 patients with discordant immunologic responses to therapy, found that improved CD4+ T cell responses are dependent on ongoing drug pressure. Second, viruses that accumulate several resistance mutations because of selective pressure of antiretroviral treatment have significant impairment in replicative fitness (fitness) and are less pathogenic [5]. Last, thymic output could contribute to the restoration of circulating CD4+ T cells [6].

Moreover, while studying 18 patients with discordant treatment responses, Lecossier et al. [7] found a significant correlation between the improvement in CD4+ T cell counts and thymic output as assessed by measurement of T cell receptor excision circles (TRECs). In regard to this finding, it has been speculated that patients with discordant treatment responses could have an active thymic function. In this way, the thymus might play a role in the restoration of CD4+ T cells destroyed by HIV. However, little is known about the role that the thymus might play in heavily antiretroviral-treated HIV–infected adults with long-term virologic treatment failure. The objective of this work was to further study thymus function in this subpopulation of patients by 3 different approaches: measuring thymic volume and determining the naive phenotypes and the number of cells carrying TRECs.

Patients and Methods

Patients. Study subjects were recruited from a cohort of 239 HIV-infected patients receiving HAART between March 2000 and
January 2001 at University Hospital Virgen del Rocío (Seville, Spain). Forty-six of these patients (19%) were heavily antiretroviral treated, had long-term virologic treatment failure, and met the following study inclusion criteria: (1) previous virologic treatment failure of several regimens, including almost all nucleoside reverse-transcriptase inhibitors, at least 2 protease inhibitors (PIs), and 1 nonnucleoside reverse transcriptase inhibitor, and (2) HIV loads >1000 copies/mL in 2 consecutive determinations (separated by 12 weeks) prior to enrolling in the study. Patients identified as meeting the inclusion criteria were asked to undergo thoracic computer tomography (CT) for the measurement of thymic volume. A total of 32 patients agreed to participate in this cross-sectional study.

Lymphocyte subpopulations. Absolute numbers of CD3+, CD4+, and CD8+ T cells were determined in fresh peripheral blood mononuclear cell (PBMC) samples obtained at the time thoracic CT was done. A frozen aliquot was used to determine percentages of T cell subsets, as described elsewhere [8]. In brief, naive and memory T cell subsets were defined as CD4+CD45RA+CD45RO− (naive CD4+ T cells), CD4+CD45RA−CD45RO+ (memory CD4+ T cells), CD8+CD45RA−CD11a− (naive CD8+ T cells), and CD8+CD45RA−CD11a+ (memory CD8+ T cells). Absolute counts of naive and memory subsets of CD4+ or CD8+ T cells were calculated by multiplying the counts in frozen samples by the absolute CD4+ and CD8+ T cell subset counts obtained from fresh blood samples.

Quantification of HIV-1 RNA. Plasma HIV-1 RNA was measured by quantitative polymerase chain reaction (PCR), using the Amplicor HIV-1 Monitor Test (Roche Molecular Systems), according to the manufacturer’s instructions. This assay has a detection limit of 50 RNA copies/mL.

Quantification of TREC’s. To determine thymic function, a proposed molecular marker for recent thymic emigrants was quantified. This marker, TREC, is a DNA circle generated during the rearrangement of T cell receptor genes. TREC concentration was measured in PBMC samples from HIV-infected patients by using a real-time quantitative PCR according to a modification of a previously described method [9]. PCR was adapted to a real-time PCR setting, using a LightCycler (Roche Molecular Biochemicals) to measure the characteristic signal-joint sequences harbored in the generated TREC.

The level of TREC in each PBMC sample was determined by using an external standard curve made with DNA from a unique pediatric thymic sample that was used for the entire study. The number of molecules of TREC’s/10⁶ thymocytes of the reference thymic sample was then calculated using known concentrations of a 375-bp amplification product of the signal-joint sequence that was cloned into the pGEM T-EASY vector (Promega). To determine the proportion of TREC-positive T cells in HIV-positive PBMC samples, the TREC level in each sample was compared with the level in the reference thymic sample. The absolute count of TREC-positive T cells per microliter was also calculated. However, since only CD3+ T cells can harbor TREC’s, the percentage of CD3+ cells present in PBMC was determined by flow cytometry, and the proportion of TREC-positive cells/10⁶ PBMC was adjusted accordingly to yield the proportion of TREC-positive cells in CD3+ cells. This fraction was then multiplied by absolute CD3 counts (determined by flow cytometry) in fresh blood.

PCR reactions were done in a 20-µL volume. For TREC amplification, 400 nmol/L of each primer (TCRαe, 5′-AAA GAG GGC AGC CCT CTC CAA GGC, and TCRαr, 5′-AGG CTG ATC TTT TCT GAC ATT TGC TCC G), 100 nmol/L of each detection probe (TCR FL, 5′-AGG GAT GTG GCA TCA CCT TTG ACA, and TCR LC, 5′-GGC ACC CCT CTG TCT CCC ACA GGA), and 100–400 ng of DNA were used. Hybridization buffer (LightCycler DNA Master Hybridization Probes buffer; Roche Molecular Biochemicals) with a final Mg²⁺ concentration of 3.5 mM was used. PCR conditions were as follows: 120 min at 95°C followed by 45 cycles at 95°C, 40 min at 63°C, and 40 min at 72°C. The TREC concentration in each sample was normalized for its DNA concentration by measuring the β-globin gene concentration.

Determination of thymic volume. Thoracic CT was done by a modification of the method of Choyke et al. [10]. Scans were obtained using a Sytec 3000 scanner (GE Medical Systems), taking 5 mm–thick sections at 5-mm intervals in the thymus fossa. The “slice volume” of each section was then calculated and added to obtain measurements in cubic centimeters. The first and last slices were halved to account for partial volume averaging (CT Sytec software, 2.02 version; GE Medical Systems). A wide density range (between −900 and +900 Hounsfield units) was used to avoid incorrect measurements due to hardening beam artifacts. Next, the margin of the gland was delimited by the cursor in each section, and then the computer integrated all analyzed sections, supplying a volumetric measurement in cubic centimeters. It is important to underscore that thymic volume measured by CT was due to thymic tissue, not to mediastinal fat; this determination was made on the basis of density differences (i.e., a higher density for soft tissue and lower density for surrounding fat). Thymic index was scored on a 0–5 scale to distinguish mediastinal fat infiltration within the parenchyma: a score of 0 indicates full fat infiltration, and a score of 5 indicates thymic tissue with no fat infiltration.

Statistical analysis. Spearman’s correlation coefficient (r) analysis was used to assess the correlation between variables. The Wilcoxon rank sum test was used to analyze paired values. For all analyses, P < .05 was considered significant. Statistical analyses were performed using the SPSS software package (version 9.0; SPSS).

Results

Characteristics of the 32 subjects at the time of CT scan are shown in table 1. Thymic volume was quite variable among the 32 patients. The median volume was 3.95 cm³ (range, 0–14.07). The median age of the subjects was 38 years (range, 30–57). There was a significant inverse correlation between age and thymic volume (r = −0.415; P = .012), with younger subjects demonstrating larger thymic volume by chest CT (figure 1A). There was, however, a significant direct correlation between thymic volume and total naive cell counts (r = 0.529; P = .002), naive CD4+ cell counts (r = 0.437; P = .012; figure 1B), and naive CD8+ cell counts (r = 0.467; P = .007; figure 1C). We also observed a direct correlation between the molecular marker of thymic function, the TREC levels, and the thymus volume measured by CT (r = 0.391; P = .027; figure 1D).

In addition, there was a nearly significant direct correlation...
between thymic volume and total CD4$^{+}$ cell count ($r = 0.320; P = .074$). We did not find a significant correlation between thymic volume and counts for total memory cells and memory CD4$^{+}$ and CD8$^{+}$ T cells or HIV virus load. In addition, we retrospectively analyzed CD4$^{+}$ cell counts and plasma virus loads for the 32 subjects 1 year before entry into this cross-sectional study (data not shown). The median change in CD4$^{+}$ cell counts during that year was $-23.5$ cells/µL (range, $-210$ to $99$; $P = .02$), and the median change in plasma virus load was $-0.16 \log_{10}$ copies/mL (range, $-3.05$ to $3.46$; $P = .80$). A direct correlation was observed between the change in $\log_{10}$ CD4$^{+}$ cell counts and TREC levels ($r = 0.375; P = .035$), and a near significant direct correlation was observed with naive CD4$^{+}$ cell counts ($r = 0.326; P = .068$). In contrast, the increment in $\log_{10}$ plasma virus load did not correlate with TREC levels or naive CD4$^{+}$ cell counts.

**Discussion**

In this cross-sectional study, we found clear evidence that the thymus of heavily antiretroviral-treated HIV-1–infected adults with long-term virologic treatment failure is actively engaged in thymopoiesis, generating new naïve T cells to the peripheral lymphocyte pool. We have reported a relationship between the amount of thymic tissue (as estimated by thoracic CT) and thymic output by determining the number of cells carrying TREC and the number of circulating naïve CD4$^{+}$ and CD8$^{+}$ T cells. In this way, sustained thymic output is maintained in adults with long-term virologic treatment failure, although we, like others [9, 11], have observed age involution in the thymus.

We retrospectively analyzed CD4$^{+}$ T cell counts and plasma virus load for the 32 HIV-1–infected patients 1 year before study entry. We found a direct correlation between thymic function as determined by measurement of TREC and the changes in CD4$^{+}$ T cell counts during the previous year. We did not find a correlation with changes in plasma virus load during that period. In this way, we found that those patients who had the highest TREC levels were those who had the greatest increases in CD4$^{+}$ T cell counts, and this correlation was not due to residual antiviral activity from HAART. These results are in agreement with those of Lecossier et al. [7], who found a significant correlation between the improvement in CD4$^{+}$ T cell counts and quantification of TREC cells among 18 patients with discordant immunologic responses to therapy. However, we did not study only patients with discordant responses, but we included all of the heavily antiretroviral–treated patients with long-term virologic failure. Moreover, we used different but complementary methods for studying thymic function among these subjects.

We used 3 different tools to study the role of the thymus in these patients: measurement of thymic volume by use of CT, determination of the number of naïve T cell phenotypes, and determination of the number of cells carrying TREC. Each of these methods has been used extensively alone in previous work; however, each one of them taken separately has some limitations. First, the images visualized by CT may not represent true thymic tissue; they may instead represent a pathologic accumulation of inflammatory or malignant cells [12]. However, the evaluation of thymus size as proposed in this work is a precise method that has been improved from that of others [10], and the finding of a significant inverse correlation of thymic volume and patient age, as described elsewhere [11], supports the reliability of the technique for measuring thymic tissue. Second, not all naïve human T cells are generated directly from the thymus. Other possibilities include peripheral expansion of existing subpopulations [13], release of lymphocytes sequestered in lymphoid tissues [14], or reversion of memory-phenotype T cells to naïve-phenotype T cells [13]. Last, TREC are markers of naïve T cells that either have just left the thymus or have not divided since thymic emigration. Previous studies have expressed TREC values as a proportion of naïve T cells carrying these DNA circles [7]. In this way, increases in the total number of naïve T cells can decrease the TREC/naïve T cell ratio independently of thymic output. We used the absolute count of TREC-positive T cells per microliter; thus, results are independent of changes in cell number and phenotype. However, redistribution of sequestered TREC and naïve T cells from lymph nodes might be an additional mechanism. We found a high correlation among these 3 methods. Thus, despite the limita-
Figure 1. Correlations between thymic volume and age (Spearman’s test, $r = -0.415; P = .018$) (A); naive CD4$^+$ cell count (Spearman’s test, $r = 0.437; P = .012$) (B); naive CD8$^+$ cell count (Spearman’s test, $r = 0.467; P = .007$) (C); and concentration of T cell receptor excision circles (TRECs) (Spearman’s test, $r = 0.391; P = .027$) (D) in a study of thymic function in heavily antiretroviral-treated HIV-1–infected adults with long-term virologic treatment failure.

The mechanisms through which thymic output may be maintained or restored in HIV-1–infected adults with long-term virologic treatment failure are still unknown. Recently, Stoddart et al. [15] found that the replication of PI-resistant, but not wild-type, HIV-1 isolates was highly impaired in thymocytes. They proposed that the inability of PI-resistant HIV-1 to replicate efficiently in the thymus contributes to the preservation of CD4$^+$ T cell counts in patients showing virologic rebound while receiving PI therapy. Our patients have been exposed extensively to antiretroviral therapy (they received a median of 4 HAART regimens before entering this study). Most of them probably carry mutations in the reverse transcriptase and/or in the viral protease that could reduce their overall replicative capacity. However, this hypothesis could not explain why some patients with long-term virologic treatment failure after several treatments containing PIs have low levels of TRECs and/or low thymic volume. Future studies are necessary to better understand the thymic function in this subpopulation of HIV-1–infected patients. In conclusion, our study suggests a role of thymic tissue in the maintenance of circulating T cells in heavily antiretroviral-treated HIV-1–infected adults with long-term virologic treatment failure.

References


