Original Article

Thymic Function during 12 Months of Highly Active Antiretroviral Therapy in Thai HIV-Infected Patients with Normal and Slow Immune Recovery

Doungnapa Kingkeow¹, Sarinee Srithep¹, Jutarat Praparattanapan², Khuanchai Supparatpinyo³, and Sakorn Pornprasert³*

¹Research Institute for Health Sciences; ²Division of Infectious Disease, Faculty of Medicine; and ³Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand

SUMMARY: The aim of this study was to determine and compare thymic output during 12 months of highly active antiretroviral therapy (HAART) in HIV-infected patients with different types of immune recovery. In total, 18 Thai HIV-infected patients with normal immune recovery (NR) and 13 Thai HIV-infected patients with slow immune recovery (SR) were enrolled. T-cell receptor rearrangement excision circle (TREC) levels in peripheral blood mononuclear cells (PBMCs) and CD4⁺ T cells were quantified at baseline, and after 6 and 12 months of HAART. CD4⁺ T-cell counts in NR patients were significantly higher than those in SR patients after 6 and 12 months of HAART. However, the median TREC levels in PBMCs and CD4⁺ T cells in both groups were comparable. Moreover, TREC levels showed similar trends in PBMCs and CD4⁺ T cells in both groups during 12 months of HAART. Only patients with SR had significant increases in median TREC levels in PBMCs and CD4⁺ T-cells during the first 6 months of HAART. No correlations were found between CD4⁺ T-cell count and TREC levels in PBMCs and CD4⁺ T cells. These results imply that the increase in CD4⁺ T-cell count in SR patients after 12 months of HAART is likely attributable to thymic output and other sources.

INTRODUCTION

The morbidity and mortality of HIV-infected patients have been effectively decreased by highly active antiretroviral therapy (HAART) through control of viral replication and immune restoration (1). However, up to 30% of patients achieve inadequate CD4⁺ T-cell count recovery despite years of suppressive HAART (2–4). As a primary source of naïve T cells, the thymus plays a key role in establishing and maintaining the peripheral T-cell pool (5). In humans, the thymus reaches its maximum volume by 1 year of age; thereafter, its size and function decrease (6). Thymic production of naïve T cells can be quantified by measuring the T-cell receptor rearrangement excision circles (TRECs) formed during T-cell receptor gene rearrangement. These thymic outputs are episomal and are diluted during cell division because they do not replicate during mitosis (7). Low TREC content in T cells and peripheral blood mononuclear cells (PBMCs) have been observed in a large percentage of untreated HIV-infected individuals (8,9). The rapid decrease in TREC levels during HIV infection may result from the expansion of memory T cells after chronic activation by pathogens and thymic impairment (10). Effective suppression of viral replication in HIV-infected patients receiving HAART has been associated with immune reconstitution and an increase in TREC levels, which suggest improving thymic function (8). However, no studies have examined for thymic function in Thai HIV-infected patients with normal immune recovery (NR) and slow immune recovery (SR) after HAART. Therefore, the aim of this study was to analyze thymic output during 1 year of HAART by quantifying and comparing TREC levels in PBMCs and CD4⁺ T cells in between these patients.

MATERIALS AND METHODS

Study population: Thirty-one Thai HIV-infected patients were enrolled at Chiang Mai University Hospital (Chiang Mai, Thailand) before the initiation of HAART. The inclusion criteria were as follows: (i) age >18 years; (ii) antiretroviral treatment naïve; (iii) no evidence of active or treated opportunistic infection at the time of HAART initiation; and (iv) no history of alcoholism or intravenous drug use. The treatment was initiated with a first-line standard HAART regimen composed of two nucleoside reverse transcriptase inhibitors (lamivudine plus stavudine, zidovudine, or tenofovir) and one non-nucleoside reverse transcriptase inhibitor (nevirapine or efavirenz).

This study was approved by the Ethics Committee of the Faculty of Medicine and Research Institute for Health Sciences of Chiang Mai University (Chiang Mai, Thailand). All participating patients provided written informed consent before enrolment. Demographic data, historic treatment of opportunistic infection, and HAART regimens were recorded before treatment be-
gan (baseline). CD4+ T-cell counts and HIV RNA loads were measured by using BD Tritek (Becton-Dickinson, San Jose, CA, USA) and Abbott Real Time HIV-1 (Abbott Molecular, Des Plaines, IL, USA), respectively, at baseline and at 6- and 12-month follow-ups. All patients were virologic responders with HIV RNA loads of <50 copies/mL after 6 months of treatment. Immune recovery was assessed by measuring changes in CD4+ T-cell counts between the baseline and 6 months of HAART. NR was defined as a CD4+ T-cell counts increase of ≥100 cells/µL from base line (11), whereas SR was defined as a CD4+ T-cell count increase of <100 cells/µL from baseline.

Blood cell preparation: Blood samples were collected in EDTA anticoagulation tubes (BD Vacutainer™, Franklin Lakes, NJ, USA) before HAART began and after 6 and 12 months of HAART. Within 3 h of collection, the samples were shipped to the Research Institute for Health Sciences at Chiang Mai University, where they were separated into PBMCs and CD4+ T cells. PBMCs were stored in liquid nitrogen until use. CD4+ T-cell counts were thawed and washed twice in cold phosphate-buffered saline solution. The viability of the thawed PBMCs, determined with 0.4% trypan blue exclusion assay, was ≥90%. A magnetic cell separator was used to separate CD4+ T cells (EasySep, STEMCELL Technologies, Vancouver, Canada) according to the manufacturers’ instructions. The PBMCs and CD4+ T cells were counted and adjusted to 10⁶ cells/mL.

DNA preparation and quantification of TRECs: DNA was extracted from 10⁶ cells/mL PBMCs and separated CD4+ T cells with a NucleoSpin kit (Machery-Nagel, Düren, Germany), according to the manufacturers’ instructions and kept at −20°C until use. TRECs were quantified with quantitative real-time polymerase chain reaction (PCR) as previously described (12) with slight modification. DNA amplification was performed in a 25-µL volume of reaction mixture. The reaction mixture included 1x real-time PCR Master Mix (ABsolute Fast QPCR ROX Mix [Thermo Scientific, Surrey, UK]), 400 nM each primer (forward, 5' - CACATCCCTTTCAACCATGCT -3' , and reverse, 5' - GCCAGCTGAGGTTTAGG -3' : GenBank accession number DQ858179.1), 200 nM fluorogenic probe (5' - ACACCTCTGGTTTTTGTAAAGGTGCCCACT -3') conjugated with 6-carboxyfluorescin at the 5'-end and 6-carboxytetramethylrhodamine at the 3'-end, and 5 µL DNA or sterile distilled water as a no-template control. The primers and probe were specifically designed for the detection of human TRECs. The amplification was performed with a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: preheating at 95°C for 15 min, then 50 cycles of 95°C for 15 sec followed by 60°C for 1 min. A cycle threshold (Ct) is the PCR cycle at which the detected fluorescence exceeds the baseline signal. The Ct value is inversely correlated with the copy number of TRECs. TREC concentrations were determined with a standard curve of a plasmid clone containing TRECs that was run in parallel with the DNA samples. TRECs in both the DNA and the plasmid clone were quantified in duplicate. TREC levels in PBMCs and CD4+ T cells were presented as copy number of TRECs per 10⁶ cells.

Statistical analysis: The data are presented as mean and standard deviation (SD) or median and interquartile range (IQR). Quantitative and qualitative variables between groups of patients were compared with the independent samples t-test, Mann-Whitney U test, or Fisher’s exact test. The paired t test or Wilcoxon matched pair test was used to compare follow-up values. Furthermore, the correlation was analyzed with Pearson’s correlation coefficient and linear regression. Statistical analyses were carried out with the SPSS software package ver. 11.0 (SPSS, Chicago, IL, USA). A two-sided P-value (P)<0.05 indicated statistical significance.

RESULTS

Patient characteristics: Based on their immune recovery after 6 months of HAART, 18 patients with CD4+ T-cell count increases of ≥100 cells/µL from baseline and HIV RNA loads of <50 copies/mL were classified as NR, and 13 patients with CD4+ T-cell count increases of <100 cells/µL from baseline and HIV RNA loads of <50 copies/mL were classified as SR. The baseline characteristics of all patients are shown in Table 1. No statistical differences in mean age, sex, CD4+ T-cell count, or median HIV RNA load were found between NR and SR patients.

Virologic and immunologic responses to HAART: Undetectable plasma HIV RNA loads (<20 copies/mL) were found in 11 of 18 (61%) NR patients and 10 of 13 (77%) SR patients after 6 months of HAART and in 12 of 18 (67%) NR patients and 11 of 13 (85%) SR patients after 12 months of HAART. CD4+ T-cell counts in NR patients were significantly higher than those in SR patients after 6 and 12 months of HAART (303.83 ver-

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal immune recovery (NR) (n = 18)</th>
<th>Slow immune recovery (SR) (n = 13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years (SD)</td>
<td>38.26 (9.40)</td>
<td>38.14 (8.99)</td>
<td>0.97¹</td>
</tr>
<tr>
<td>Sex: Male/Female</td>
<td>16/2</td>
<td>8/5</td>
<td>0.10²</td>
</tr>
<tr>
<td>Baseline CD4+ T-cell counts, mean cells/µL (SD)</td>
<td>114.28 (77.27)</td>
<td>117.31 (110.34)</td>
<td>0.93¹</td>
</tr>
<tr>
<td>Baseline HIV load, median log_{10} HIV RNA copies/mL (IQR)</td>
<td>5.4 (5.04-5.82)</td>
<td>4.86 (4.29-5.72)</td>
<td>0.21³</td>
</tr>
</tbody>
</table>

¹: Independent samples t test.
²: Fisher’s exact test.
³: Mann-Whitney U test.
sus 174.85 cells/µL and 363.89 versus 248.92 cells/µL, \( P = 0.003 \) and 0.04, respectively; Fig. 1A). The CD4+ T cells in NR patients rapidly increased from baseline during the first 6 months of HAART and then slightly increased between 6 and 12 months of HAART. In SR patients, a slight increase in CD4+ T cells was observed through 12 months of HAART. Moreover, in both groups, CD4+ T-cell counts measured after 6 months of HAART were significantly higher than those at baseline (303.83 versus 114.28 cells/µL and 174.85 versus 117.31 cells/µL, \( P < 0.001 \) and 0.01, respectively) and those after 12 months of HAART (363.89 versus 303.83 cells/µL and 248.92 versus 174.85 cells/µL, \( P = 0.02 \) and <0.001, respectively; Fig. 1A).

**Changes in TREC levels during 12 months of HAART:** TREC levels in PBMC and CD4+ T cells were not significantly different between NR and SR patients at baseline and after 6 and 12 months of HAART (Fig. 1B and 1C, respectively), and the changes in TREC levels in PBMCs and CD4+ T cells in patients with NR and SR showed similar trends. TREC levels in PBMCs and CD4+ T cells rapidly increased during the first 6 months of HAART and then declined from 6 to 12 months of HAART. In the SR group, levels of TREC in PBMCs and CD4+ T cells at 6 months of HAART were significantly higher than those at baseline (10.59 versus 3.72 copies/10^6 cells and 35.43 versus 2.68 copies/10^6 cells, \( P = 0.03 \) and <0.01, respectively; Fig. 1B and 1C).

**Correlation between TREC levels and CD4+ T-cell counts:** Correlation between TREC levels and CD4+ T-cell counts were analyzed at baseline and after 6 and 12 months of HAART in NR and SR patients. The levels of TREC in PBMCs and CD4+ T cells at every time point were not correlated with CD4+ T-cell count in either groups (data not shown).

**DISCUSSION**

Compared with SR patients, NR patients had higher CD4+ T-cell counts during 12 months of HAART. However, the slow immune reconstitution in SR patients was probably not due to lower baseline CD4+ T-cell counts as previously described in other studies (2,3) because the baseline CD4+ T-cell counts in NR and SR patients were not significantly different. Other factors such as a highly activated apoptotic T-cell compartments, residual viremia, and elevated levels of interleukin-7, a key cytokine necessary to regulate the proliferation and survival of circulating naïve and memory T cells, likely influenced the lower CD4+ T-cell counts in SR patients after HAART, as proposed previously (4,13–15).

The thymus plays a key role in the immune recovery of HIV-infected patients receiving HAART (8,16,17). In this study, we investigated whether thymic function differed between NR and SR patients during 12 months of HAART. The median TREC levels in PBMCs and CD4+ T cells in SR patients were not significantly different from those in NR patients at every time point. TREC levels in PBMCs and CD4+ T cells in patients with SR significantly and rapidly increased during the first 6 months of HAART. These results implied that a slow immune reconstitution in the SR group was unlikely related to impaired thymic function.

Our results disagreed with those of previous studies by Benveniste et al. (18) and Teixeira et al. (19), which suggested that the failure of immune reconstitution after HAART was caused by impaired thymic output. The inconsistency in results is likely due to difference in study design. Our study was a cohort study with a 12-month follow-up, whereas the previous studies were cross-sectional studies in which patients received HAART for more than 12 months. Therefore, compared with our study, these 2 previous studies evaluated thymic function during different periods after HAART. In addition, the patient characteristics were different:
the poor immune responders in the study by Benveniste et al. (18) were patients with CD4+ T-cell counts of <250 cells/µL after 12 months of HAART, whereas those in the study by Teixeira et al. (19) and in the present study were patients with CD4+ T-cell count increases of <100 cells/µL after 12 months and 6 months of HAART, respectively. Although the criterion for poor immune response in our study and the study by Teixeira et al. (19) were similar, the poor immune responders in the latter were older than those in our study (38 versus 46 years). Thymic size and function reportedly decrease with age (6); therefore, older age may have contributed, in part, to the impaired thymic output found in poor immune responders in the study by Teixeira et al. (19).

In the present study, both NR and SR patients showed similar changes in TREC levels in PBMCs and CD4+ T cells during 12 months of HAART. These levels rapidly increased during the first 6 months of HAART and then declined. These results suggest that thymic function was similar in NR and SR patients. TREC levels in PBMCs and CD4+ T cells increased non-significantly in NR patients but significantly in SR patients during the first 6 months of treatment. This result in SR patients corresponds with the results of a study by Franco et al. (20), which showed a significant increase in TREC levels during 6 months of HAART. Thus, the increase of CD4+ T-cell counts in SR patients likely resulted from de novo production of naive T cell in the thymus. However, TREC levels in PBMCs and CD4+ T cells did not correlate with CD4+ T-cell counts. Therefore, the increase of CD4+ T-cell count in SR patients might be attributable not only to thymic output but also to the redistribution of CD4+ T cells from lymphoid tissues as described in previous studies (21,22).

A limitation of our study is small sample size (18 NR and 13 SR patients) which may introduce bias, deviate the results, and reduce statistical power to detect real difference. However, other studies measuring TREC levels and immune recovery after HAART have used small sample sizes of good and poor immune recovery, 19 NR and 19 SR patients in the study by Benveniste et al. (18) and 10 NR and 12 SR patients in the study by Teixeira et al. (19).

In summary, the results of our study suggest that the thymic function contributes to early immune recovery in HIV-infected patients particularly SR patients receiving HAART. A long-term prospective study with a larger cohort may be needed to verify these results and further elucidate the kinetics of thymic function after HAART in patients with different types of immune recovery.

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Conflict of interest None to declare.

REFERENCES