Association between Larger Thymic Size and Higher Thymic Output in Human Immunodeficiency Virus–Infected Patients Receiving Highly Active Antiretroviral Therapy

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To examine the impact of thymic size on immune recovery in patients with human immunodeficiency virus (HIV) infection, the thymus was visualized, using computed tomographic scans, in 25 HIV-infected patients who had received highly active antiretroviral therapy (HAART) for 6–18 months and had levels of viremia <500 copies/mL. For comparison, 10 control subjects were included in the study. Total and naive CD4+ cell counts were determined by flow cytometry. To determine thymic output, the number of CD4+ cells containing T cell receptor excision circles (TRECs) was measured. Qualitative immune recovery was evaluated by determination of CD4+ T cell receptor repertoire in 19 of the HIV-infected patients. Larger thymic size was associated with higher CD4+ cell counts (r = 0.498; P = .011) and higher CD4+ TREC frequency (r = 0.652; P < .001). Furthermore, patients with abundant thymic tissue seemed to have broader immunologic repertoires, compared with patients with minimal thymic tissue (P = .054). These findings suggest that thymopoiesis is ongoing in the adult thymus and contributes to immune reconstitution in HIV-infected patients receiving HAART.

Human immunodeficiency virus (HIV) infection is characterized by a progressive loss of circulating CD4+ T lymphocytes (CD4+ cells). Treatment with highly active antiretroviral therapy (HAART) leads to some immunologic reconstitution, specifically, an increase in the number of CD4+ cells of both memory (CD45RO+) and naive (CD45RA−CD62L+) phenotypes [1–3]. The origin of the CD4+ cells that appear in the blood after initiation of treatment is still uncertain. Peripheral blood CD4+ cells can be replaced either by peripheral expansion of existing T cell clones or by production of new naive T cells from the thymus. However, it is known that the human thymus involutes with age, and its function as a T cell generator in adults has been assumed, therefore, to be limited [2, 4–7].

 Recently, thymic tissue has been detected in adult HIV-infected patients on computed tomographic (CT) scans, and it has been found that the size of this thymic tissue correlates with increases in naive CD4+ cells during the early phase of treatment with HAART [8, 9]. Assuming that the naive CD4+ cells are newly produced cells, this suggests that some degree of thymopoiesis is still occurring in the adult thymus. However, it has been shown that memory cells are able to convert from a memory to a naive phenotype, and the naive phenotype may, therefore, not be a reliable marker of recent thymic emigrants [10, 11]. Determination of the number of T cell receptor excision circles (TRECs) as a method of measuring thymic output more directly has been suggested [12]. Because TRECs are stable and are not copied during mitosis, they are diluted out with each mitosis of the cell and can be used as a marker of the cell’s proximity to the thymus and of recent thymic emigrants. It has been proposed that the presence of TRECs containing cells in the peripheral CD4+ cells in a 70-year-old patient is evidence that the adult thymus is not completely quiescent [12].

Previous studies have shown that the depletions within the CD4+ cell pool that occur during HIV infection can result in severe disruptions of the CD4+ T cell receptor (TCR) repertoire (immunologic repertoire), disruptions that are not or are only partly restored during antiretroviral treatment [4, 13]. A disrupted immunologic repertoire may reflect all deletions of T cells of a particular clonal type and could explain the vulnerability to opportunistic pathogens that is seen in HIV-infected patients. In theory, presence of a functional thymus that is capable of generating new naive T cells to the circulating CD4+ cell pool would result in a higher degree of normalization of the skewed repertoire than restoration of the CD4+ cell pool by expansion of existing CD4+ cells [4, 13–15].

Thus, a large thymus does seem to be associated with the early increase in CD4+ cell counts that is seen during initial HAART.
However, the importance of a functional thymus for long-term immunologic reconstitution, with regard to increased CD4+ cell counts, increased number of cells containing TREC, and improvement of immunologic repertoire, in adult HIV-infected patients has yet to be clearly determined.

Patients and Methods

HIV-infected patients and healthy blood donors. A total of 25 HIV-infected patients and 10 healthy control subjects were included in this study at some point in the period November 2000–March 2001. All participants were men aged 24–47 years. HIV-infected patients were enrolled after 6–18 months of treatment with HAART. All patients were naive to any antiretroviral treatment before HAART. The clinical characteristics of the 25 HIV-infected patients are presented in table 1. HIV-negative control subjects were recruited from among hospital personnel. All tests were performed at study entry. Blood collected in tubes containing EDTA was used to obtain a full blood cell count and for flow cytometry. To determine virus load, plasma HIV RNA levels were measured with a polymerase chain reaction (PCR) quantitative kit (Amplicor HIV-1 Monitor; Roche) according to the manufacturer’s instructions. The detection threshold was 20 copies/mL. Additional blood samples were drawn into tubes containing heparin to obtain peripheral blood mononuclear cells (PBMC) by means of density gradient centrifugation. PBMC were used for determination of numbers of TREC and for analysis of TCR repertoire.

Visualization of the thymus. To visualize the thymus, CT scans of the chest were performed. With the patient in a supine position, a noncontrast chest CT was performed using a CT scanner (Somatom Plus 4; Siemens), with a single-slice technique (slice, 3 mm; feed/rotation, 4.5 mm), from the sternal notch and ~6 cm caudally. The images were read by a single radiologist who was blinded to patients’ clinical data. The scans were scored on a scale from 0 to 5, as described elsewhere [8]: 0, no visible thymic tissue; 1, minimal thymic tissue, barely recognizable; 2, minimal, but more obvious, thymic tissue; 3, moderate amount of thymic tissue; 4, moderate but greater amount of thymic tissue; 5, thymic mass large enough to raise concern about thymoma. Each scan was scored twice, at an interval of 2 weeks. The 2 scores were compared for each subject, and, when a discrepancy was found, the scan was scored a third

Table 1. Clinical characteristics of 25 human immunodeficiency virus (HIV)–infected patients receiving steady-state highly active antiretroviral therapy (HAART) who were included in a study of the relationship between thymic size and immune reconstitution.

<table>
<thead>
<tr>
<th>Group, patient</th>
<th>Age, years</th>
<th>CD4+ cell count at initiation of HAART, cells/μL</th>
<th>CD4+ cell count at study entry, cells/μL</th>
<th>Virus load at study entry, copies/mL</th>
<th>HIV-related illness</th>
<th>HAART regimen at study entry</th>
<th>Duration of HAART at study entry, months</th>
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NOTE. Group mean values are given as estimates from analysis of variance. Age was included as a covariate in the analysis. ABC, abacavir; d4T, stavudine; Efv, efavirenz; Idv, indinavir; NfV, nelfinavir; Nvp, nevirapine; PCP, Pneumocystis carinii pneumonia; Rtv, ritonavir; Sqv, saquinavir; TB, tuberculosis; Zdv, zidovudine; 3TC, lamivudine.
time, and the most frequently occurring score of the 3 designated the “thymic index.” All scores from each subject were within 1 grade of each other.

**Flow cytometry.** Measurement of lymphocyte subsets was done essentially as described in [16]. In brief, 100 mL of blood was incubated with 10 mL of fluorescent dye–conjugated monoclonal antibodies at room temperature for 15 min. Erythrocytes were lysed with 2 mL of NH4Cl buffer at room temperature for 10 min, and the samples were washed and resuspended in PBS supplemented with 10% CellFIX (Becton Dickinson). All samples were analyzed using a FACScan (Becton Dickinson) equipped with a 488-nm argon-ion laser. Data were processed using CELLQuest software (Becton Dickinson).

The number of CD4+ cells and the number of CD4+ cells with the naive phenotype (i.e., CD4+CD45RA−CD62L+) were determined. These measurements were made at the Department of Clinical Biochemistry (Hvidovre Hospital, Hvidovre, Denmark). In some patients, naive CD4+ cell count measurements taken before our investigation were obtained from the hospital records. These measurements also had been made at the Department of Clinical Biochemistry. The fluorescence of 5000 cells was measured. To obtain the absolute number for a lymphocyte population, the fraction of cells in a lymphocyte gate that expressed lymphocyte markers was multiplied by the lymphocyte count. Monoclonal antibodies used to define a lymphocyte gate that expressed lymphocyte markers was multiplied by the lymphocyte count. Monoclonal antibodies used to determine lymphocyte subsets were isotype control antibodies, and the absolute number for a lymphocyte population, the fraction of cells expressing lymphocyte markers was multiplied by the lymphocyte count.

**Enrichment of CD4+ cells.** CD4+ cells were enriched from freshly collected PBMC, using a magnetic cell separator (MACS; Miltenyi Biotech), 1.25 mM magnesium chloride (Life Technologies), 1 mM each dNTP (Amersham Pharmacia Biotech), 1.25 μM random hexanucleotide primers (DNA Technology), 2.5 U/μL reverse transcriptase, and 1 U/μL RNase inhibitor (Applied Biosystems). The conditions were 42°C for 30 min followed by 94°C for 5 min. It was possible to obtain cDNA from 19 HIV-infected patients and 5 control subjects.

**TCR spectratyping.** Analysis of complementary determining region 3 (CDR3) length within the TCR β chain variable region (Vβ) was performed by multiplex PCR for the detection of the 23 functional TCR Vβ families. Each of the 23 Vβ gene subfamilies was amplified across constant-variable junctions, using the 23 Vβ subfamily specific primers, as described elsewhere [20], as well as a fluorescent dye–conjugated constant β region–specific primer (DNA Technology). Each of the 5 multiplex PCRs contained TCR Vβ primers specific to 4 or 5 different families and selected to allow size discrimination of the outcome PCR products. The total PCR mixture volume (20 μL) contained 10× PCR buffer (Life Technologies), 0.2 mM magnesium chloride (Life Technologies), 0.2 mM each dNTP (Amersham Pharmacia Biotech), 0.25 U/μL Platinum Taq DNA polymerase (Life Technologies), 2 μL of cDNA (equivalent to ~20,000 cells), and primer mix (DNA Technology), as described elsewhere [20].

The initial denaturation was done at 94°C for 2 min. Cycling conditions were 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 5 min.

Fluorescent PCR products (2 μL) and a size standard (1 μL) (TAMRA 500; Applied Biosystems) were mixed with 13 μL of formamide (Amresco) and denatured at 100°C for 2 min for 2 samples. The electrophoreses were through Performance Optimized Polymer 6 (Applied Biosystems) on an ABI 310 automated sequencer (Applied Biosystems). GeneScan software (Applied Biosystems) was used to analyze the data.

**Statistical analysis.** Data are given as mean ± SEM or estimated mean ± SEM. Differences between results for HIV-infected patients and control subjects and between results for different subgroups of patients were evaluated using analysis of variance. Age was included as a covariate in the analysis. The correlation between measurements was calculated using Pearson’s correlation coefficient. A 5% significance level was used.

The TCR repertoire was analyzed as described elsewhere [13]. Each CDR3 profile was translated into a probability distribution, p(i) = Ai/[∑j(Aj)], using the fraction of the area (Aj) under the profile for each CDR3 length, i, from minimum to maximum length in steps of 3 nt. A control profile was established for each Vβ family, using the average probability distribution of the 5 control subjects. The extent of perturbation in a sample was calculated for each Vβ family, using the distance (D) between the probability distributions of the sample, j, and the control, c: D(i) = p(i) − p(i). The sum of the absolute values of the distances, Dj = 100 ∑i(D(i))/2, in each
$\text{V} \beta$ family over all TCR lengths yields the perturbation of that TCR profile as a percentage. The overall repertoire perturbation of a sample was calculated by the average perturbation of all 23 $\text{V} \beta$ families.

**Results**

*Thymic tissue in HIV-infected patients receiving HAART.* To determine the impact of thymic size on immune recovery in HIV-infected patients receiving steady-state HAART (patients who had received HAART for 6–18 months and had levels of viremia <500 copies/mL), thymic size was measured in 25 patients and 10 control subjects. To visualize the thymus, CT scans of the chest were performed. Thymic tissue was detectable in all HIV-infected patients and control subjects (figure 1). All thymic indices fell within the range of 1–4, with a mean value of 2.52 in the group of HIV-infected patients and 2.50 in the group of control subjects ($P = .964$). The thymic index of the HIV-infected patients was distributed as follows: thymic index of 1, 7 patients; thymic index of 2, 4 patients; thymic index of 3, 8 pa-

![Figure 1](https://academic.oup.com/jid/article-abstract/185/11/1578/835309)  
Images from chest computed tomographic scans of patients with thymic indices of 1 (A), 2 (B), 3 (C), or 4 (D). The thymus is the triangular mass above the heart, at the top center of each image.
patients; and thymic index of 4, 6 patients. As would be expected (because thymic volume diminishes with age), older age was associated with smaller thymic size ($r = -0.593$ and $P = .002$, for HIV-infected patients; $r = -0.761$ and $P = .011$, for HIV-negative control subjects). No correlation was detected between thymic size and virus load ($r = 0.197$; $P = .346$). Likewise, no correlation was detected between thymic size and duration of HAART ($r = 0.176$; $P = .400$).

HIV-infected patients were divided into 2 groups on the basis of thymic size: group A included patients with minimal thymic tissue (thymic index $\leq 2$; $n = 11$), and group B included patients with abundant thymic tissue (thymic index $> 2$; $n = 14$) [8]. A statistically significant difference in mean age was seen between the 2 groups (41.1 vs. 35.1 years for group A and group B, respectively; $P = .010$). No difference in virus load was seen between the 2 groups. However, a difference in estimated mean duration of HAART was seen (10.6 vs. 13.9 months for group A and group B, respectively; $P = .035$).

**Effect of thymic size on total and naive CD4+ cell counts.** To investigate whether thymic size is associated with immunologic reconstitution, as demonstrated by increases in CD4+ cell counts, the number of circulating CD4+ cells was measured in 25 HIV-infected patients and 10 control subjects. Among the HIV-infected patients, thymic size was associated with the absolute number of CD4+ cells ($r = 0.498$; $P = .011$) (figure 2A). In contrast, a correlation between thymic size and the number of naive CD4+ cells was not found ($r = 0.312$; $P = .129$). However, because the patients included in the present study had received HAART for a duration of 6–18 months, naive CD4+ cell counts after 6 months of treatment were obtained from the hospital records. A nearly significant correlation was found between thymic size and the naive CD4+ cell count after 6 months of treatment ($r = 0.478; P = .052$). There was no difference in the absolute number of CD4+ cells ($P = .404$) (figure 3A) or in the number of naive CD4+ cells ($P = .806$) (estimated means) between patients with minimal (group A) and abundant (group B) thymic tissue.

**Correlation between thymic size and the number of CD4+ cells containing TRECs.** The number of CD4+ cells containing TRECs is considered to be a more reliable marker of recent thymic emigrants, and the TREC frequency was, therefore, measured in 25 HIV-infected patients and 10 control subjects. An age-dependent decrease in thymic output has been observed elsewhere [12], and we found that older age was associated with a lower percentage of cells containing TRECs ($r = -0.426$ and $P = .034$, for HIV-infected patients, and $r = -0.901$ and $P < .001$, for control subjects). Among HIV-infected patients, thymic size correlated with the percentage of CD4+ cells containing TRECs ($r = 0.652$; $P < .001$) (figure 2B). When the group of patients with minimal thymic tissue (group A) was compared with the group of patients with abundant thymic tissue (group B), a statistically significant difference in the estimated mean number of CD4+ cells containing TRECs was found ($0.56\% \pm 0.14\%$ in group A vs. $1.07\% \pm 0.15\%$ in group B; $P = .027$) (figure 3B). This difference was independent of age (figure 4). There was no correlation between the number of cells containing TRECs and duration of HAART ($r = -0.057; P = .788$).

**Possible association between larger thymic size and broader immunologic repertoire.** To determine whether thymic size is associated with less perturbation of the immunologic repertoire, the CD4+ TCR repertoire was measured in 19 HIV-infected patients and 5 control subjects. The mean perturbation of the immunologic repertoire in HIV-infected patients was $15.5\% \pm 4.1\%$, versus $11.5\% \pm 2.9\%$ in control subjects ($P = .024$). Among the

![Figure 2](https://academic.oup.com/jid/article-abstract/185/11/1578/835309)

**Figure 2.** Associations between thymic index and CD4+ cell count (A), CD4+ T cell receptor excision circle (TREC) frequency (B), and degree of perturbation in the T cell receptor (TCR) repertoire of CD4+ cells (C) at study entry. A, Thymic indices are correlated to absolute nos. of circulating CD4+ cells in 25 human immunodeficiency virus (HIV)–infected patients ($P = .011$). B, Thymic indices are correlated to nos. of newly produced CD4+ cells, expressed as the percentage of CD4+ cells containing TRECs, in 25 HIV-infected patients ($P < .001$). C, A tendency toward a negative correlation between degree of perturbation in the CD4+ TCR repertoire and thymic index is seen in 19 HIV-infected patients ($P = .064$). Correlations between measurements were calculated using Pearson’s correlation coefficient.
HIV-infected patients, there seemed to be a negative correlation between thymic size and the degree of perturbation of the immunologic repertoire ($r = -0.434; P = .064$) (figure 2C). Likewise, when patients with minimal (group A) and abundant (group B) thymic tissue were compared, a trend toward a significant difference in the estimated mean degree of perturbation of the immunologic repertoire was found (17.7% ± 1.4% vs. 13.9% ± 1.1%, respectively; $P = .054$) (figure 3C). This difference was independent of age. A difference in the degree of perturbation was seen between the control group and group A ($P = .008$) but not between the control group and group B ($P = .229$). No correlation was found among the HIV-infected patients between the degree of perturbation of the immunologic repertoire and the duration of HAART ($r = 0.007; P = .977$).

**Discussion**

The present study was designed to investigate the role of the adult thymus in the reconstitution of immune function in HIV-infected patients receiving steady-state HAART. Thymic size was measured, using CT scans, in 25 HIV-infected patients and 10 control subjects. The study demonstrated a relationship between thymic size and absolute number of circulating CD4+ cells. Furthermore, an association was found between thymic size and the percentage of CD4+ cells containing TRECs. The immunologic repertoire of CD4+ cells showed a tendency to be less perturbed in patients with large thymuses. The data presented suggest that the adult thymus is involved in reconstituting the immune function in HIV-infected patients receiving steady-state HAART.

That it is possible to visualize the adult thymus on CT scans and to grade these images according to a thymic tissue scale has been demonstrated by others [8, 9] and was confirmed in the present study. However, because the adult thymus is somewhat infiltrated with adipose tissue, compared with the thymus during early life, the CT appearance of the thymic tissue may not be completely unambiguous. If CT appearance of thymic tissue is a reliable measure of thymic function, a relationship between thymic index and levels of newly produced CD4+ cells would be expected. Estimating the number of newly produced CD4+ cells often relies on measurements of the number of CD4+ cells expressing the naive phenotype CD45RA+CD62L+ [3] or containing TRECs [12].

In the present study, a positive correlation between thymic size and the CD4+ cell count was found. In contrast, no association was found between thymic size and the number of naive CD4+ cells. In previous studies, a correlation between thymic size and both the number of circulating naive CD4+ cells and the increase in naive CD4+ cell counts during the early phase of HAART has
been demonstrated [8, 9]. A possible explanation for these conflicting results may be found in the heterogeneity of the group of patients included in this study with regard to the duration of HAART. The patients included in the present study had all gone through the early phase of HAART treatment, but the duration of HAART on study entry ranged from 6 to 18 months. However, a recent study examining the increase in naive CD4+ cell counts in a comparable population of HIV-infected patients during 3 years of treatment with HAART demonstrated only a modest increase in median naive CD4+ cell count, of 25 cells/μL, from months 6 through 18 [21]. This indicates that the period from 6 through 18 months of HAART is a stable period for reconstitution of the naive pool. Yet, when the number of naive CD4+ cells after 6 months of treatment was determined from the hospital record, to investigate further the modest increase in the number of naive CD4+ cells between months 6 and 18 of HAART treatment, a nearly significant correlation with thymic size was found.

The pool of naive CD4+ cells has been shown to reflect not only newly produced cells from the thymus but also cells converted from a memory to a naive phenotype [10, 11]. Naive CD4+ cells also can have a long quiescent life span [22]. Furthermore, it has been shown that the number of naive CD4+ cells can increase during HAART in previously thymectomized HIV-infected patients [23], and it has been suggested that extrathymic expansion of naive cells occurs [24]. Thus, the naive phenotype may not be a measure of recent thymic emigrants. Estimating the naive T cell pool by using other markers of naive phenotype, such as CD27+ and CD45RO−, may also contribute to a purer definition of the population of naive cells [19]. However, use of quantification of TREC as a more reliable marker of thymic output has been suggested [12]. Previous studies have shown that the TREC frequencies in HIV-infected patients are low, compared with those in control subjects [12, 25, 26]. This may result from HIV-induced inhibition of thymopoiesis [12, 23] or from HIV-induced impairment of progenitor cell function [16, 27]. It has also been proposed that decreases in TREC levels are due to increases in cell division [28]. During HAART, the number of CD4+ T cells containing TREC has been shown to increase, and this increase has been explained by de novo lymphopoiesis resulting from suppression of virus load [12]. Furthermore, hematopoietic progenitor cells regain function during HAART and seed the thymus with functional T cell progenitors [16, 27]. One study has reported a relationship between the increase in TREC levels during HAART and development of response to neoantigens, which further supports the suggestion that TREC levels increase as a result of de novo lymphopoiesis [29]. Furthermore, in a population of untreated HIV-infected patients, lower TREC levels have been shown to be associated with faster progression to AIDS. Patients with long-term nonprogression were found to have significantly higher levels of TREC, and these values remained relatively stable over time, whereas the concentrations of TREC in patients with rapid progression decreased over time [30]. Thus, the TREC value may be a good prognostic marker of progression of HIV disease. In the present study, a positive correlation between thymic size and the percentage of CD4+ cells containing TREC was demonstrated. This finding indicates that a larger thymus represents a higher degree of thymopoiesis and may be associated with a better prognosis for the HIV-infected patient.

Although estimation of the quantitative T cell recovery is important for evaluating immune reconstitution in patients receiving HAART, the qualitative restoration of the immunologic repertoire must also be considered. The consequences of depletions within the CD4+ cell pool for the CD4+ TCR repertoire have been investigated eagerly. Perturbations of the immunologic repertoire with both clonal expansions and entire deletions of T cell clones in HIV-infected patients have been reported in several studies that included control groups [4, 14, 15, 31], and a relationship has been reported between a greater degree of perturbation of the immunologic repertoire, low CD4+ cell counts [4, 14], and high viremia [13]. After initiation of HAART, both improvements in and persistent perturbations of the immunologic repertoire have been found [4, 13]. Improvement in the immunologic repertoire is believed to be dependent on production of new CD4+ cells by the thymus. However, changes in TCR diversity following therapy that are due to the loss of antigen-responsive effector cells after the removal of viral antigen or to increased numbers of CD4+ cells (because fewer cells are killed by infection) cannot be ruled out at present. An association between restoration of TCR diversity and the number of naive CD4+ cells appearing in the blood after T cell–depleted bone marrow transplantation has been demonstrated [32], which supports the hypothesis that recovery of TCR diversity is dependent on production of new cells from the thymus. Moreover, increased TREC frequency in adults after hematopoietic stem cell transplantation has been shown to be associated with broadening of the TCR repertoire [33]. Furthermore, thymocytes from the adult thymus seem capable of broadening the repertoire; it has been shown that aging does not lead to major gaps in the TCR repertoire at the level of the thymus [34]. The present study demonstrates a trend toward a significant association between a broader immunologic repertoire and a large thymus, which suggests that thymopoiesis occurs in the adult thymus and contributes to immune recovery, with new CD4+ cells expressing numerous TCR Vβ gene families and broadening the immunologic repertoire. Because the immunologic repertoire has been shown to be associated with clinical status, as classified by the CDC [15], this implies that a large thymus is beneficial to the patient.

In conclusion, we have demonstrated that, in adult HIV-infected patients receiving steady-state HAART, a relationship exists between thymic size (as measured by CT scan) and CD4+ cell counts and between thymic size and the percentage of newly produced CD4+ cells (expressed as the percentage of CD4+ cells containing TREC). Furthermore, a large thymus seems to be associated with a less perturbed CD4+ TCR repertoire. Together,
these findings suggest that the adult thymus is functional to a certain degree and is involved in reconstituting the immune function in HIV-infected patients receiving steady-state HAART, generating new CD4+ cells that contain TREC, and contributing to a broader immunologic repertoire. Developing strategies to increase residual thymic function may be beneficial for HIV-infected patients whose immune systems are not fully reconstituted during HAART.

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References