Discordant Increases in CD4+ T Cells in Human Immunodeficiency Virus–Infected Patients Experiencing Virologic Treatment Failure: Role of Changes in Thymic Output and T Cell Death

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Some patients infected with human immunodeficiency virus (HIV) who are experiencing antiretroviral treatment failure have persistent improvement in CD4+ T cell counts despite high plasma viremia. To explore the mechanisms responsible for this phenomenon, 2 parameters influencing the dynamics of CD4+ T cells were evaluated: death of mature CD4+ T cells and replenishment of the CD4+ T cell pool by the thymus. The improvement in CD4+ T cells observed in patients with treatment failure was not correlated with spontaneous, Fas ligand–induced, or activation-induced T cell death. In contrast, a significant correlation between the improvement in CD4+ T cell counts and thymic output, as assessed by measurement of T cell receptor excision circles, was observed. These observations suggest that increased thymic output contributes to the dissociation between CD4+ T cell counts and viremia in patients failing antiretroviral therapy and support a model in which drug-resistant HIV strains may have reduced replication rates and pathogenicity in the thymus.

Treatment of patients infected with human immunodeficiency virus type 1 (HIV-1), using a combination of antiretroviral drugs that includes a protease inhibitor, leads to a rapid decrease in virus load and an improvement in the absolute number of circulating CD4+ T cells. In a proportion of individuals, however, virus load either fails to decline or rapidly rebounds after treatment with combined antiretroviral agents. Despite this apparent treatment failure, some, but not all, of the patients have substantial and persistent improvement in their CD4+ T cell counts, so-called discordant responses [1–3].

The mechanisms responsible for discordant treatment responses are not well understood. Two recent studies evaluating patients with persistently detectable plasma viral mRNA after treatment have found that the reduction in virus load from pretreatment levels correlated with the improvement in CD4+ T cell counts [4, 5]. A decrease in virus load, however, is not the only factor explaining discordant responses, because, for any given reduction in virus load, a wide range of improvement in CD4+ T cell counts can be observed, and some patients develop discordant responses despite no reduction or actual increases in plasma virus load.

A variety of processes have been identified that may contribute to T cell depletion in HIV infection, including the direct cytopathic effect of the virus [6], the elimination of infected cells by the immune response [7], virus-induced apoptosis of uninfected cells [8, 9], induction of abnormalities in cell trafficking [10], and impairment of the generation of T cells within the thymus [11–13]. Demonstrating the involvement of ⩾1 of these pathways in the paradoxical increase in circulating CD4+ T cells in patients with virologic treatment failure would directly support the conclusion that the given mechanism has a real impact on T cell depletion in the course of HIV infection. At present, however, no information is available concerning the contribution of changes in these pathways to the improvement in CD4+ T cell counts in patients with discordant responses. To explore this question, we identified a cohort of patients who, after being treated for the first time with a protease inhibitor in conjunction with 2 reverse-transcriptase inhibitors, experienced confirmed virologic failure. In these patients we evaluated (1) spontaneous and activation-induced death of cultured peripheral blood lymphocytes and (2) thymic output, as assessed by quantification of T cell receptor excision circles.

Patients and Methods

Study participants. The group of patients with virologic treatment failure consisted of 18 adult patients followed up at Hôpital Bichat-Claude Bernard who met the following criteria: (1) the pa-

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Informed consent was obtained from all patients; the studies were performed in accordance with the human experimentation guidelines of our institution.

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tient had been treated with a protease inhibitor for the first time in combination with ⩾2 reverse-transcriptase inhibitors; (2) plasma HIV RNA levels had been measured before initiating therapy with the protease inhibitor; (3) treatment with protease inhibitors had been continued for ⩾1 year after virologic treatment failure had become evident; and (4) on the day of evaluation, plasma HIV RNA had returned to levels of $\geqslant 10^4$ copies/mL and had rebounded to within 1 $\log_{10}$ of the pretreatment levels or was higher than pretreatment levels. All patients in our cohort who were identified as meeting the inclusion criteria were evaluated. Initial CD4 T cell counts or changes in CD4 T cell counts were not used as criteria for inclusion. These patients (all men) had a mean ± SD age of 41 ± 9 years.

Twelve HIV-1–infected patients who had never received antiretroviral therapy were evaluated. These patients (8 men and 4 women) had an average age of 41 ± 13 years. On the day of evaluation, $CD4^+$ T cell counts were $305 \pm 214 \times 10^6$ cells/L (range, $17–619 \times 10^6$ cells/L), and virus load was 4.5 ± 1.1 $\log_{10}$ copies/mL (range, <2.3–6.4 $\log_{10}$ copies/mL). Twelve healthy volunteers not infected by HIV served as controls. These individuals (4 men and 8 women) had an average age of 36 ± 9 years. Informed consent was obtained from all participants.

Evaluation of cell death. Mononuclear cells were isolated from peripheral blood mononuclear cells (PBMC) by centrifugation on Ficoll-Paque (Pharmacia). Cells were resuspended at 1×10^6 cells/mL in complete medium (RPMI-1640 containing 25 m*M* HEPES, 10% fetal calf serum, 2 m*M* glutamine, 200 U/mL penicillin G, and 250 μg/mL streptomycin) and 0.5-mL aliquots cultured in 5-mL polypropylene culture tubes (Falcon 2005, Becton Dickinson). To test the sensitivity of T cells to death induced by cross-linking receptors of the tumor necrosis factor (TNF) receptor family, cells were cultured with 100 ng/mL (final concentration) of fusion proteins containing the extracellular domain of the corresponding cognate ligand linked to a Flag epitope (FasL-Flag, TRAIL-Flag TNF-α–Flag, or TWEAK-Flag) in the presence of 2 μg/mL anti-Flag monoclonal antibody (M2, Sigma) [14]. To evaluate activation-induced cell death, 4 μg/mL activating anti-CD3 monoclonal antibody (Beckman-Coulter) was added. Cultures were maintained at 37°C in 95% air/5% CO_2. Samples were evaluated either before or after 48 h of culture. The cells were resuspended, and 100-μL aliquots were removed and were incubated at 4°C for 45 min with fluorescein isothiocyanate (FITC)–conjugated anti-CD4, phycoerythrin-conjugated anti-CD8, and PC5-conjugated anti-CD14 monoclonal antibodies, using the concentrations suggested by the manufacturer (Beckman Coulter). Fifty microliters of Flow-Count Fluorospheres (Coulter) was added, and the samples were analyzed by cytometry (FACScan, Becton-Dickinson). Debris was excluded during acquisition, and 50,000 total events were evaluated. The number of events corresponding to viable $CD4^+$ T cells, viable $CD8^+$ T cells, and fluorospheres was determined by setting appropriate gates, and, by use of this information, the total number of viable $CD4^+$ and $CD8^+$ T cells remaining in the culture at the time of analysis was calculated.

Cells in gates used to identify viable $CD4^+$ and $CD8^+$ T cells contained <1% positive cells after incubation with FITC-conjugated Annexin V (Beckman Coulter). Incubation of Jurkat cells with the fusion proteins containing the extracellular domain of Fas-ligand or TRAIL under the conditions used in these experiments led to massive apoptosis after 24 h of culture (data not shown). The fusion proteins containing the extracellular domains of TNF-α and TWEAK have been shown elsewhere to induce apoptosis in WEHI-164 fibrosarcoma cells and interferon-γ–treated HT29 colon carcinoma cells, respectively, in the presence or absence of anti-FLAG antibody [14].

Evaluation of thymic output. To evaluate thymic output, T cell receptor excision circles (TRECs) were quantified, using a modification of the approach described by Douek et al. [15]. TRECs are episomal DNA circles formed as by-products of the rearrangement of the T cell receptor locus during T cell development. In this study, the "signal-joint" TREC, a TREC formed in 70% of all T cells expressing αβTCR, which results from recombination between the δRec and ψJα loci, was evaluated. The percentage of T cells expressing the $CD4^+$ $CD45RA^+$ $CD14^-$ "naive" phenotype was determined by flow cytometry. To measure TRECs, $CD4^+$ T cells were purified from PBMC by using magnetic beads coated with anti-CD4 monoclonal antibodies (CD4 Positive Isolation Kit, Dynal), following the manufacturer's instructions (purity >95%), and were stored at −80°C. DNA was extracted (QIAamp DNA Blood Mini Kit, Qiagen), and the number of TRECs was determined by real-time polymerase chain reaction (PCR). Each reaction contained 1× *Taq*Man Universal PCR Master Mix, 5 m*M* $MgCl_2$, and 200 n*M* of each primer and probe (50 μL final volume): TREC-F2, 5′-GCAACTCGTGAGAACGGTGA; TREC-R1, 5′-CTTTC-AACCATGCTGACACCTC; TREC probe, 5′-(6-FAM)-CCGTG-CCAGCTGCAGGGTTTAGG(Tamra)(phosphate). As an index of total cells, the number of copies of the albumin gene (*ALB;* present in only 2 copies per cell and without pseudogenes) [16] was determined in parallel reactions, as described above, by use of 200 nM each of the following primers and probe: ALB-F1, 5′-GTGAA-CAGGCGACCATGCT; ALB-R1, 5′-GCATGGAAGGTGAAT-GTTTCAG; ALB probe, 5′-(VIC)-TCAGCTCTGGAAGTCGA-TGAAACATACGTTC(Tamra)(phosphate). For both systems, cycling parameters were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 58°C for 1 min. In each case, the number of cycles required to reach threshold fluorescence (C_t) was determined. For quantification of both TREC and ALB, serial dilutions of DNA extracted from thymic tissue obtained from a 4-month-old infant were used. The difference in C_t (ΔC_t) for the amplification of albumin and TREC in this standard was constant over a 100-fold range of dilution of the template, which indicates that the 2 amplicons had the same efficiency of amplification [17]. Thus, by comparing the Y intercept of the standard curves, the preparation was found to have 37.5 albumin sequences per 1 TREC sequence. To calculate TREC/10^3 $CD4^+$ $CD45RA^+$ T cells, the following formula was used: TREC/10^3 $CD4^+$ $CD45RA^+$ T cells = [(1000 × number of TREC sequences)/(number of albumin sequences/2)]/[% $CD45RA^+$ $CD4^+$ T cells/100]. Each sample was evaluated in duplicate at 3 dilutions of template, and the values were averaged. To assess the reproducibility of the technique, the same preparation of DNA from normal PBMC was evaluated in 15 consecutive experiments and gave values of 234 ± 70 TREC/10^6 cells (mean ± SD). TRECs were undetectable in T cell lines when this technique was used.

Statistical methods. All results are reported as mean ± SD, unless otherwise indicated. Spontaneous cell death was determined by comparing the number of viable cells present before and after

Table 1. Plasma human immunodeficiency virus (type I) RNA levels and circulating $CD4^+$ T cell counts in patients with virologic treatment failure before treatment with a protease inhibitor and on the day of evaluation.

Patient	Length of treatment failure, mo[a]	Viral RNA, $\log_{10}$ copies/mL			$CD4^+$ T cells, cells $\times 10^6$/L		
		Before protease inhibitor treatment	Day of evaluation	Δ[b]	Before protease inhibitor treatment	Day of evaluation	Δ[b]
1	32	5.61	5.01	−0.60	45	713	668
2	27	3.14	5.44	2.30	72	462	390
3	27	4.89	4.88	−0.01	32	301	269
4	31	5.09	4.64	−0.45	107	370	263
5	25	5.21	4.29	−0.92	68	301	233
6	31	4.87	4.25	−0.62	32	254	222
7	33	5.08	4.33	−0.75	5	195	190
8	8	5.05	5.07	0.02	172	350	178
9	27	5.37	4.38	−0.99	59	230	171
10	21	3.31	4.13	0.82	50	209	159
11	32	4.96	5.05	0.09	140	271	131
12	34	5.28	5.60	0.32	18	146	128
13	22	5.44	5.63	0.19	34	133	99
14	32	5.19	4.76	−0.43	15	96	81
15	32	4.26	5.74	1.48	80	146	66
16	32	5.18	5.43	0.25	110	107	−3
17	31	5.79	5.78	−0.01	27	19	−8
18	29	5.72	5.50	−0.22	22	6	−16

[a] Number of months that viral RNA was continuously detectable before evaluation.
[b] Change between the intiation of therapy and the day of evaluation.

48 h of culture. Cell loss induced by ligands of the TNF family and activation-induced cell death were determined by comparing the number of T cells remaining at 48 h in cultures performed without or with the corresponding ligands or the activating anti-CD3 monoclonal antibody. Both cell viability and thymic output were evaluated for 13 patients; for the remaining patients only cell viability ($n = 3$) or thymic output ($n = 2$) was studied. Comparisons between groups were made by analysis of variance; posttest comparisons (performed only if $P < .05$) were made using the Dunnett multiple comparisons test. Results for HIV-infected and -uninfected individuals were compared using the Mann-Whitney U test. Correlations were evaluated by linear regression. For all analyses, $P = .05$ was considered significant. To confirm the robustness of conclusions drawn from regression analysis, all analyses were repeated using the nonparametric Spearman correlation and after excluding individual outlier points. In no case did these procedures change the interpretation of the findings.

Results

Clinical characteristics of patients with virologic treatment failure. We sought to identify factors influencing the extent of improvement in circulating $CD4^+$ T cell counts in HIV-infected patients with persistently elevated virus loads after antiretroviral therapy. To minimize the impact of partial treatment responses on our results, the study was restricted to patients whose plasma HIV RNA was $\geq 10^4$ copies/mL and had returned to levels that were within 1 $\log_{10}$ of pretreatment levels or higher than pretreatment levels.

All patients had been treated with ≥1 nucleoside analogue reverse-transcriptase inhibitors before receiving protease inhibitors, and all had detectable plasma HIV-1 RNA when therapy with a protease inhibitor was initiated (ritonavir [$n = 10$], indinavir [$n = 5$], saquinavir [$n = 2$], or nelfinavir [$n = 1$]). Thirteen patients had an initial decrease of ≥1 $\log_{10}$ in plasma HIV-1 RNA after treatment with protease inhibitors, and virus became undetectable (<200 copies/mL) in 6 patients. Evidence of virologic treatment failure was apparent within 6 months of treatment in 17 of 18 patients, and, at the time of evaluation, plasma HIV-1 RNA had been continuously detectable for >2 years in 16 of 18 patients (table 1; mean, 28 ± 6 months). During the year before evaluation, virus load was fairly stable in these individuals, even in patients for whom therapeutic changes were made (mean change in virus load in the 12 months before evaluation: -0.2 ± 0.6 $\log_{10}$ copies/mL).

The evolution of virus load and absolute $CD4^+$ T cell counts between the initiation of therapy and the time of evaluation are shown in table 1. On the day of evaluation, mean virus load was 5.0 ± 0.6 $\log_{10}$ copies/mL; the average change since initiation of therapy was $+0.03 \pm 0.8$ $\log_{10}$ copies/mL (range, 0.99 to +2.3 $\log_{10}$ copies/mL). Before treatment with protease inhibitors, $CD4^+$ T cell counts were generally low ($60 \pm 46 \times 10^6$ $CD4^+$ T cells/L) and were $<100 \times 10^6$ cells/L in 14 of 18 patients. After therapy with protease inhibitors, $CD4^+$ T cell counts increased in most patients despite virologic treatment failure. At the time of evaluation, the average increase in $CD4^+$ T cells was $+179 \pm 162 \times 10^6$ cells/L, and $CD4^+$ T cell counts had remained higher than pretreatment values for 15 of 18 patients. Nevertheless, considerable variability in the increase in $CD4^+$ T cell counts was observed ($\Delta CD4^+$ T cells $\times 10^6$/L: −100 to 0, $n = 3$; 1 to 100, $n = 3$; 101 to 200, $n = 6$; 201 to 300, $n = 4$; >300, $n = 2$.

Loss of cultured T cells. The spontaneous loss of both

$CD4^+$ and $CD8^+$ T cells during the 48-h culture period was variable for HIV-infected individuals but, when considered for the patients as a group, was not significantly different from that observed for uninfected control subjects ($P = 0.5$ for both comparisons). For patients with virologic treatment failure, the spontaneous death during culture of $CD8^+$ T cells was significantly greater than that of $CD4^+$ T cells (26% ± 27% and 9% ± 14%, respectively; $P < .002$ by paired analysis). In these patients, the spontaneous loss during culture of both $CD4^+$ and $CD8^+$ T cells was correlated significantly with virus load (figures 1*A,* 1*B*). Most T cells with light scatter properties of dead cells were stained with annexin V, which suggests that T cell loss was occurring, at least in part, through apoptotic mechanisms (data not shown). It is important to note, however, that no correlation was observed between the spontaneous death during culture of either $CD4^+$ or $CD8^+$ T cells and the improvement in $CD4^+$ T cell counts observed in patients with virologic treatment failure (figures 1*C,* 1*D;* $P > .2$ for both comparisons).

Fas ligand–induced death of both cultured $CD4^+$ and $CD8^+$ T cells from HIV-infected individuals was significantly increased, compared with that of the corresponding T cell populations from uninfected controls (figure 2*A*). For patients with virologic treatment failure, the increase in death induced by culture with Fas ligand over spontaneous cell death was of similar magnitude for $CD4^+$ and $CD8^+$ T cells (cell loss induced by Fas ligand for $CD4^+$ cells, 21% ± 19%; for $CD8^+$ cells, 17% ± 14%; $P > .2$). As expected, annexin V staining confirmed that many of these T cells were dying by apoptosis. There was no difference, however, in Fas-mediated cell death during culture between untreated HIV-infected patients and patients with virologic treatment failure, and the extent of Fas-mediated death did not correlate with the improvement in $CD4^+$ T cell counts observed in patients with virologic treatment failure (figure 2*B;* $P > .3$ for both comparisons). Culture of T cells in the presence of the death-inducing ligands TRAIL, TNF, and TWEAK did not significantly increase the death of either $CD4^+$ or $CD8^+$ T cells from HIV-infected or -uninfected individuals (figure 2*A*).

For patients with virologic treatment failure, the loss of cultured T cells stimulated through CD3 was increased, compared with that of unstimulated cells; the magnitude of this effect was comparable for both $CD4^+$ and $CD8^+$ T cells (8% ± 16% and 16% ± 15% decreases in cell number, respectively, compared with those in unstimulated cultures). Activation-induced cell death of cultured $CD8^+$ T cells from patients with virologic treatment failure was increased, compared with that of $CD8^+$ T cells from uninfected controls ($P < .05$). Again, no correlation was observed between activation-induced cell death during culture of either $CD4^+$ or $CD8^+$ T cells and the extent of improvement in $CD4^+$ T cell counts occurring in patients with

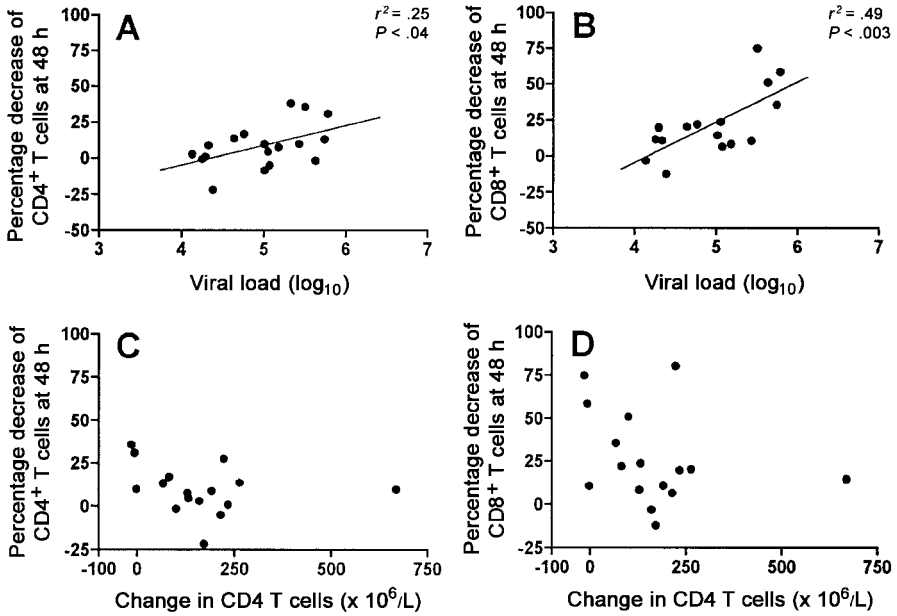

Figure 1. Loss of $CD4^+$ and $CD8^+$ T cells in culture correlates with virus load but not with improvement in $CD4^+$ T cell counts. Peripheral blood mononuclear cells from patients with virologic treatment failure were cultured for 48 h, and the percentages of $CD4^+$ *(A* and *C)* and $CD8^+$ *(B* and *D)* T cells lost during culture were determined. These results were correlated with plasma virus load on the day of evaluation *(A* and *B)* and with the improvement in $CD4^+$ T cell counts observed since the initiation of treatment with a protease inhibitor *(C* and *D)*. No correlation was observed between the loss of $CD4^+$ T cells in culture and the change in CD4 T cells ($r^2 = .06$; $P > .3$). No correlation was found between the loss of $CD8^+$ T cells in culture and the change in $CD4^+$ T cells by linear regression ($r^2 = .08$; $P > 0.2$), when the nonparametric Spearman correlation was used ($r = -.32$; $P > .2$) or after individual outlier points were excluded.

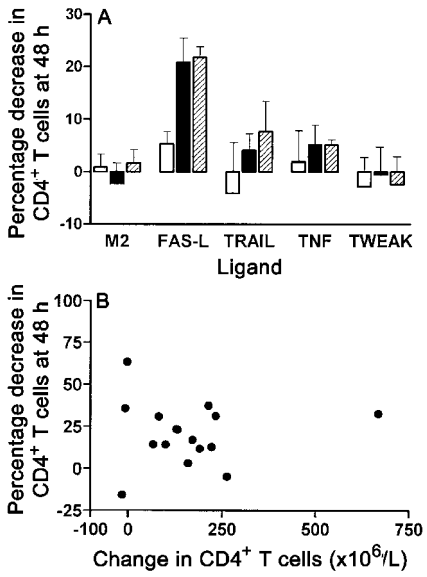

Figure 2. Effect of stimulation of receptors of the tumor necrosis factor (TNF) receptor family on survival of $CD4^+$ T cells in vitro. Peripheral blood mononuclear cells from uninfected controls *(open bars),* patients with virologic treatment failure *(solid bars),* and human immunodeficiency virus (HIV)–infected untreated patients *(hatched bars)* were cultured for 48 h with fusion proteins containing the extracellular domain of the indicated ligands linked to a Flag epitope in the presence of the M2 anti-Flag monoclonal antibody *(A)*. The number of viable $CD4^+$ T cells was determined, and the percentage of decrease in cell numbers was determined relative to that of parallel cultures not containing ligands or anti-Flag antibody. M2 indicates cultures containing the anti-Flag antibody in the absence of ligand. Results are presented as mean ± SE. For patients with virologic treatment failure, no correlation was observed by linear regression between the percentage of decrease in $CD4^+$ T cells resulting from culture with Fas-ligand fusion protein and the change in $CD4^+$ T cell counts after beginning treatment with a protease inhibitor ($r^2 = .01$; $P > .7$), when the nonparametric Spearman correlation ($r = -.05$; $P > .8$) was used or after individual outlier points were excluded *(B)*.

virologic treatment failure ($P > .2$ for both comparisons; data not shown).

Evaluation of TRECs. In vitro studies have shown that treatment with antiproteases leads to the emergence of viruses with impaired replicative capacity in the thymus [18], suggesting that increased regeneration of T cells may contribute to the increase in $CD4^+$ T cell counts occurring in some patients with virologic treatment failure. To test this hypothesis, $\delta Rec/\psi J\alpha$ "signal-joint" TRECs were evaluated in patients with treatment failure, an approach developed by Douek et al. [15]. As reported elsewhere, TREC/10^3 $CD4^+$ $CD45RA^+$ T cell counts were lower in untreated HIV-infected individuals than in uninfected control subjects, although considerable overlap was observed between the 2 groups ($U = 23$ [Mann-Whitney U test]; $P < .05$). Considerable variability in TREC levels was seen for patients with virologic treatment failure, but, when the patients were considered as a group, they were not significantly different from TREC levels of uninfected control subjects. As shown in figure 3*A,* however, a significant correlation was observed between TREC levels and the improvement in $CD4^+$ T cell counts ($r^2 = .48$; $P < .005$). In contrast, TREC levels did not correlate with plasma virus load (figure 3*B*) or with the change in virus load after antiprotease therapy (data not shown).

Several studies have shown that TREC levels decrease with age, an observation that we confirmed both for patients with

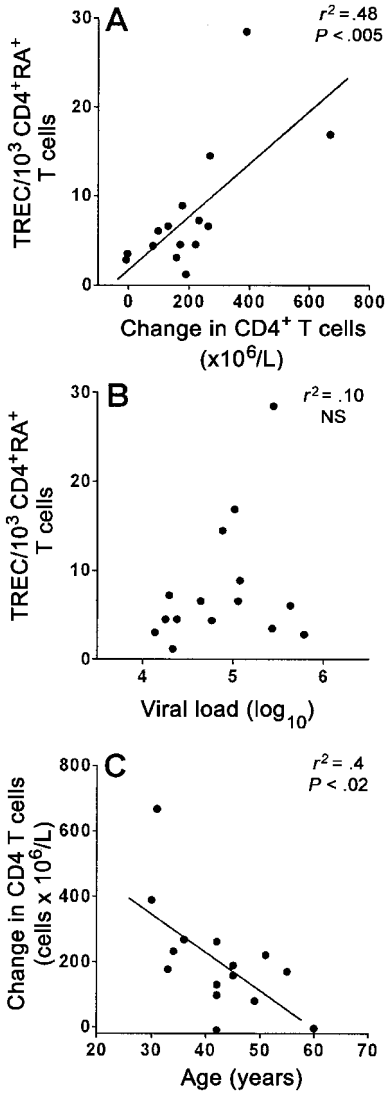

Figure 3. Relationships between the increase in $CD4^+$ T cell counts and T cell receptor excision circle (TREC) levels, virus load, and age. $CD4^+$ T cells were purified from peripheral blood of patients with virologic treatment failure, DNA was extracted, and TREC/10^3 $CD4^+$ $CD45RA^+$ T cell counts were determined by real-time polymerase chain reaction. These results were correlated with *(A)* the improvement in $CD4^+$ T cell counts after beginning treatment with a protease inhibitor and *(B)* plasma virus load at the time of evaluation. The relationship between the improvement in $CD4^+$ T cell counts and patient age is shown in *C*. NS, not significant.

virologic treatment failure and for uninfected control subjects ($P < .01$ and $P < .03$, respectively). If TREC levels are directly linked to increased T cell counts in patients with virologic treatment failure, as suggested by the strong correlation, one would predict a greater increase in $CD4^+$ T cell counts in the younger individuals. Consistent with this hypothesis, we found that the increase in $CD4^+$ T cell counts in patients with virologic treatment failure was inversely correlated with age (figure 3*C*).

Discussion

When combined antiretroviral therapy fails to control viral replication in treated patients, peripheral $CD4^+$ T cell counts can remain paradoxically high, clearly above those recorded before the initiation of therapy. Although higher CD4 counts are more common in patients whose plasma viremia remains below pretherapy levels [4, 5], such "dissociated" response profiles are observed in a significant proportion of patients experiencing virologic therapy failure, including individuals whose virus load returns to pretreatment levels. In this study, we have examined 2 possible mechanisms for this phenomenon: (1) regeneration of $CD4^+$ T cells by the thymus in spite of high levels of virus replication and (2) a decrease in spontaneous or induced peripheral $CD4^+$ T cell death.

Several authors have suggested that an impairment of thymic function could contribute to the pathogenesis of HIV-1 infection [11, 12, 15, 19–21]. Recently, several authors have used the quantification of TREC in circulating T cells as a surrogate marker for thymic output, to further evaluate this hypothesis [15, 22, 23]. Both Douek et al. and Zhang et al. demonstrated that TREC levels are lower in HIV-infected individuals than in age-matched uninfected control subjects [15, 22], and Hatzakis et al. showed that a progressive decline in the concentration of TREC is characteristic of patients with progressive disease [23]. After successful antiretroviral therapy, TREC levels have been shown to rise in conjunction with $CD4^+$ T cells [15], compatible with the possibility that thymic output can contribute to the restoration of circulating T cells, at least in some individuals [21, 24]. In our study, we found that TREC levels were lower in untreated HIV-infected patients than in uninfected controls, although considerable overlap in the 2 groups was observed, as previously described. In patients in whom the improvement in $CD4^+$ T cells occurred despite the persistence of high levels of circulating virus, we observed a significant correlation between the improvement in $CD4^+$ T cell counts after treatment and TREC levels.

It is recognized that factors other than thymic output can affect the TREC:"naive" T cell ratio. In particular, increases in the proliferation of naive T cells and the reconversion of "memory" T cells to a naive phenotype [24–27], by increasing the total number of naive cells, can decrease the TREC:naive T cell ratio independent of thymic output, and the flux through these pathways can be increased by stimulation of the immune system [26, 27]. Thus, in the context of HIV-1 infection, changes in the extent of virus-induced immune stimulation must be taken into consideration. For example, when virus load is dramatically reduced by effective antiretroviral therapy, the increased proliferation of naive T cells returns toward normal levels [26] and can produce an increase in the TREC:naive T cell ratio [27]. Several findings suggest that this was not a confounding variable in our study. First, all of our patients had a persistently high plasma virus load that, on average, was similar before and after treatment, which suggests that these individuals were subject to intense, persistent, and relatively constant immune stimulation. The increased sensitivity of T cells from these patients to Fas ligand–induced cell death is compatible with the persistence of strong virus-induced immune response (see below). Second, we observed a fairly strong correlation between age and both the improvement in $CD4^+$ T cell counts and TREC/10^3 $CD4^+CD45RA^+$ T cells in patients with virologic treatment failure. Thymic output is known to be age dependent [21, 28], whereas no evidence for age-dependent changes in the other factors influencing the total size of the naive T cell pool has been presented. Together, these results suggest that differences in thymic output explain the variation in the TREC:naive T cell ratio seen in our patients.

Although the mechanisms through which thymic output may have been restored in these patients were not directly addressed by our studies, recent experimental evidence suggests that a decrease in HIV replicative capacity may be involved. It is now well established that viruses escaping antiretroviral therapy accumulate mutations in the reverse transcriptase and/or in the viral protease that often reduce their overall replicative capacity [29, 30]. It is interesting to note that the extent of impairment of viral replication is highly dependent on the nature of the target cells. In particular, Stoddart et al. have recently shown that viruses carrying resistant protease sequences from treated patients replicated almost normally in phytohemagglutinin-stimulated human PBMC but failed to replicate and to deplete thymic T cells in thymic implants in SCID-hu Thy/Liv mice [18]. According to this model, reduced HIV replicative capacity in thymocytes would improve thymic function, whereas near-normal viral replication in mature peripheral T cells would explain the high plasma virus load seen in our patients. Other, more indirect mechanisms could also contribute to the restoration of thymic function. Cytokines, including interleukin (IL)–7, are known to be required for thymopoiesis but are also required for high-level HIV-1 replication in thymocytes [31, 32]. IL-7 levels have recently been found to be negatively correlated with peripheral $CD4^+$ T cell counts in HIV-infected individuals [33, 34]. Thus, improved thymic output resulting from reduced HIV-1 fitness in the thymus might further reduce local virus replication by feedback inhibition of IL-7 production.

Further studies will be required to better define the overall contribution of improved thymic output to the restoration of $CD4^+$ T cell counts in these patients with discordant responses

and the mechanisms responsible for this effect. Nevertheless, we propose that decreased viral replicative capacity due to accumulated drug resistance mutations, together with adequate residual thymic function (e.g., younger patients without irreversible thymic damage), both contribute to improved $CD4^+$ T cell counts through the restoration of thymic output.

In contrast to the findings concerning thymic output, we found no evidence that a reduction in the death of T cells contributes to discordant immunovirologic responses. Several earlier studies quantifying apoptotic cells have shown that the spontaneous apoptosis of cultured T cells from HIV-infected individuals is increased compared with that of T cells from uninfected control subjects, although the effect is generally smalI (~2-fold increase), and considerable overlap between HIV-infected patients and uninfected controls has been found [9, 35–40]. In this study, the number of viable cells present before and after 48 h of culture was determined, an approach that permits the quantification of total cell loss—regardless of the mechanism—in contrast to earlier studies in which only relatively intact cells in an early stage of apoptosis were evaluated. Using this approach, we did not observe a significant difference in spontaneous cell death between HIV-infected patients and uninfected controls. The differences in the techniques used, the relatively small number of patients evaluated in our study, and the small number of HIV-infected individuals with very low $CD4^+$ T cell counts—patients who have the highest levels of spontaneous apoptosis—are possible explanations for this discrepancy. Consistent with earlier studies, we found that both $CD4^+$ and $CD8^+$ T cells from HIV-infected individuals were considerably more sensitive to Fas ligand–induced apoptosis than were T cells from uninfected controls and that activation-induced cell death of $CD8^+$ T cells from HIV-infected patients was greater than that of T cells from uninfected control subjects.

Of particular importance for this study were the observations that the extent of spontaneous cell death, Fas ligand–induced cell death, and activation-induced cell death showed no correlation with the improvement in $CD4^+$ T cell counts in patients experiencing virologic treatment failure. Thus, our findings do not support the idea that the selection of drug-resistant viral variants changes their propensity to activate pathways, resulting in the accelerated elimination of T cells. This is interesting, because all of our patients were being treated with HIV protease inhibitors at the time of their evaluation, so our observations suggest that these antiviral compounds do not by themselves significantly affect the levels of T cell death, for example, through a nonspecific inhibitory action on cellular proteases involved in apoptosis [41].

The mechanisms responsible for accelerated T cell death in HIV-infected individuals remain controversial. The direct killing of infected cells by HIV-1 may play a role [6]. In this regard, we observed a fairly strong correlation between virus load and spontaneous death of cultured $CD4^+$ T cells in the HIV-infected patients and patients with virologic treatment failure. Against this possibility, however, we also observed that virus load correlated with the spontaneous death of $CD8^+$ T cells, that spontaneous death was greater for $CD8^+$ than for $CD4^+$ T cells in patients with virologic treatment failure, and that activation-induced apoptosis of $CD8^+$, but not $CD4^+$, T cells was increased in these patients. These findings better fit the idea that virus-induced activation of the immune system increases the number of T cells susceptible to death through apoptotic or other mechanisms [40]. Our observation that Fas-mediated and activation-induced cell death remains elevated in patients with virologic treatment failure offers a possible explanation for the observation that T cell counts do not return to normal levels in patients with discordant responses, despite improved thymic output.

Together, our findings suggest that an improved thymic output may contribute to the restoration of circulating $CD4^+$ T cells seen in some patients with persistently elevated virus loads after antiretroviral therapy. These results further support the potential usefulness of therapeutic strategies designed to restore thymic function in HIV-infected individuals.

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