Measuring Recent Thymic Emigrants in Blood of Normal and HIV-1–infected Individuals before and after Effective Therapy

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Summary

The role of the thymus in HIV-1 pathogenesis remains unclear. We developed an assay to quantify the number of recent thymic emigrants in blood based on the detection of a major excisional DNA byproduct (termed α1 circle) of T cell receptor rearrangement. By studying 532 normal individuals, we found that α1 circle numbers in blood remain high for the first 10–15 yr of life, a sharp drop is seen in the late teen years, and a gradual decline occurs thereafter. Compared with age-matched uninfected control individuals, α1 circle numbers in HIV-1–infected adults were significantly reduced; however, there were many individuals with normal α1 circle numbers. In 74 individuals receiving highly active antiretroviral therapy, we found no appreciable effect on α1 circle numbers in those whose baseline values were already within the normal range, but significant increases were observed in those with a preexisting impairment. The increases in α1 circle numbers were, however, numerically insufficient to account for the rise in levels of naive T lymphocytes. Overall, it is difficult to invoke thymic regenerative failure as a generalized mechanism for CD4 lymphocyte depletion in HIV-1 infection, as α1 circle numbers are normal in a substantial subset of HIV-1–infected individuals.

Key words: HIV • thymus gland • aging • pathogenesis • drug therapy

Multiple lines of evidence indicate that HIV-1 infects thymocytes, not only in vitro but also in a SCID mouse implanted with human fetal thymus and liver (SCID-hu mouse) in vivo (1–3). Autopsy studies of HIV-1–infected individuals (4) or SIV-infected macaques (5, 6) show both infection and altered histopathology of the thymus. Evaluation of thymic function in humans would be facilitated by noninvasive techniques to measure the output of thymocytes from this primary lymphoid organ. In mice given bromodeoxyuridine, cells that recently left the thymus could be detected by their low-level incorporation of this label (7); in chickens, such cells could be tracked by the expression of the chT1 antigen (8, 9). These techniques, however, are not applicable to human studies. Computer tomography scan of the chest has been used to correlate thymic sizes of HIV-1–infected individuals to their $CD4^+$ naive ($CD45RA^+CD62L^+$) lymphocyte counts (10), but such an imaging technique cannot reliably measure the true lymphoid mass of the thymus (4, 11).

As progenitor T cells undergo TCR rearrangement in the thymus, certain chromosomal sequences are excised to produce episomal DNA byproducts termed TCR excisional circles (TRECs)[1] (12, 13). Although TRECs are quite stable, they are diluted out by cell division or lost

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[1]*Abbreviations used in this paper:* HAART, highly active antiviral therapy; RTEs, recent thymic emigrants; SI, syncytium-inducing; TRECs, TCR excisional circles.

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with cell death (14). Thus, the detection of TRECs in T lymphocytes in blood could serve as a marker for recent thymic emigrants (RTEs) (8, 9). "Recent" denotes T lymphocytes that have undergone no more than a few cellular divisions since leaving the site of TCR rearrangement. It does not necessarily reflect the time elapsed. In fact, cells that are destined to become α/β T cells first delete the δ locus from the α locus during thymic maturation (15, 16). Furthermore, there is a preferred way of deleting the δ locus to give rise to $\sim$70% of α/β T cells (17). This rearrangement forms the δRec–ΨJα coding joint in the chromosomal DNA, and an 89-kb TREC (α1 circle) is generated as an episomal byproduct (see Fig. 1 A). Recently, Douek et al. used a quantitative competitive PCR assay to quantify this particular TREC (18). They found that the number of TRECs in $CD4^+$ and $CD8^+$ lymphocytes in the blood of 25 normal individuals decreased exponentially by 1–1.5 log from birth to age 73 and that the low TREC numbers in 10 HIV-1–infected individuals were restored upon treatment with highly active antiretroviral therapy (HAART). We now describe a comprehensive study using a real-time PCR/molecular beacon assay to quantify α1 circles as a measure of RTEs in the blood of 532 normal persons, 171 untreated HIV-1–infected individuals, and 74 HIV-1–infected individuals whose plasma viremia had been well suppressed by HAART.

Materials and Methods

Real-Time PCR Detection of α1 Circles. Genomic DNA was extracted using TriReagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. To detect α1 circles, a molecular beacon was used in combination with real-time PCR. The method of detection using molecular beacons (19, 20) and a fluorescence detector system (21–24) has been previously described. Each 50-μl reaction contained 5 μl of DNA, and the final concentration of each component was as follows: 1.0× Taqman buffer A (Perkin-Elmer Corp.), 3.5 mM $MgCl_2$, 0.4 pmol/μl of molecular beacon, 0.4 pmol/μl of each primer, and 1.25 U of AmpliTaq Gold DNA Polymerase (Perkin-Elmer Corp.). The primers (sense, 5′-GGATGGAAAACACAGTGTGACATGG-3′ and antisense, 5′-CTGTCAACAAAGGTGATGCCACATCC-3′) amplified a 208-bp product. The amplicon was sequenced and found to contain the appropriate motifs for a recombination signal sequence (12, 13).

A molecular beacon was included in the reaction mixture throughout PCR to serve as a real-time detector for the amplified product. The molecular beacon was designed to recognize a region upstream from the signal joint. This beacon was specifically designed to have a hairpin structure, with a 6-bp stem and a 26-nucleotide target recognition loop, plus a fluorophore (FAM [6-carboxyfluorescein]) and quencher (DABCYL [4-dimethylaminophenlazo benzoic acid]) in close proximity to the two ends of the oligonucleotide. The target recognition sequence for the molecular beacon was 5′-GAGAACGGTGAATGAAGAG-CAGACAG-3′.

One cycle of denaturation (95°C for 10 min) was performed, followed by 45 cycles of amplification (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s). PCR was carried out in a spectrofluorometric thermal cycler (ABI PRISM 7700; Applied Biosystems, Inc.) that monitors changes in the fluorescence spectrum of each reaction tube during the annealing phase while simultaneously carrying out programmed temperature cycles. The cycle number during PCR that yields a fluorescence intensity significantly above the background is designated as the threshold cycle (C_T). As shown here (see Fig. 1 A) and reported elsewhere (21–25), the C_T is directly proportional to the log of the copy number of the target sequence in the input DNA. We have found that this α1 circle assay is efficient (<4 h), specific (see Results and Dicussion), sensitive (10 copies), dynamic (7-log range), and accurate (10% coefficient of variance). To normalize for cell equivalents in the input DNA, we used a separate real-time PCR/molecular beacon assay to quantify the CCR5 coding sequence, as it is known that this gene is present at only two copies per cell, i.e., there are no pseudogenes (Kostrikis, L. and D.D. Ho, personal communication).

Patients. HIV-1–infected individuals were considered acutely infected if they were enrolled in treatment protocols within the first 90 d of symptoms of their acute HIV-1 infection. If enrolled after 90 d of symptoms, they were considered chronically infected. In the studies of the effect of treatment on α1 circle numbers, every patient was treated with a three- or four-drug combination. The regimens were standard dosages of zidovudine and lamivudine plus ritonavir, indinavir, nelfinavir, ritonavir/saquinavir, or abacavir/amprenavir. 13 of the chronically infected individuals were treated with the combination of stavudine, lamivudine, nelfinavir, saquinavir, and IL-2 (9 MU/day for 5 d in 6-week intervals for 8 cycles after day 28).

Immunological and Virological Monitoring. Quantitation of $CD3^+$, $CD4^+$, $CD8^+$, and $CD45RA^+62L^+$ subpopulations in peripheral blood samples from HIV-1–infected individuals was done by four-color flow cytometry using the following mAbs: anti-CD3–PerCp (Becton Dickinson), anti-CD4–APC (Exalpha), anti-CD8–APC (PharMingen), anti-CD45RA–FITC (Becton Dickinson), and anti-62L–PE (Becton Dickinson). In the lymphocyte subsets study, a minimum of 10^5 naive or memory cells were isolated using a fluorescence-activated cell sorter, MoFLO (Cytomation, Inc.). Using four-color flow cytometry, naive cells were defined as $CD45RA^+$ and $CD62L^+$, whereas memory cells were defined as $CD45RO^+$ for both $CD4^+$ and $CD8^+$ populations. The purity of the sorted cells was >99%, as analyzed by a FACSCalibur™ flow cytometer (Becton Dickinson). Quantitation of HIV-1 RNA in plasma was determined using the Roche Ultrasensitive Amplicor HIV-1 Monitor assay (lower limits of detection, 50 HIV-1 RNA copies/ml; Roche Molecular Systems).

Statistics. Comparisons between groups were done by nonparametric analysis using the Kolmogorov-Smirnov test (Statview, SAS Institute, Inc.). *P* values over 0.05 are regarded as not statistically significant.

Results and Discussion

Characteristics of the Real-Time PCR Assay for Measuring α1 Circles. A real-time PCR assay with a molecular beacon detection system (19, 26) was used to quantify α1 circles in DNA extracted from cells of interest. As shown in Fig. 1 B, α1 circles were detected in great abundance ($\sim 10^5$ copies/10^6 cells) in fetal thymic tissue but were not detected (<10 copies/10^6 cells) in nonlymphoid tissues or immortalized cell lines. In blood $CD4^+$ and $CD8^+$ lymphocytes, α1 circles were enriched in naive (defined as $CD45RA^+CD62L^+$ cells) subpopulations by 10–50-fold

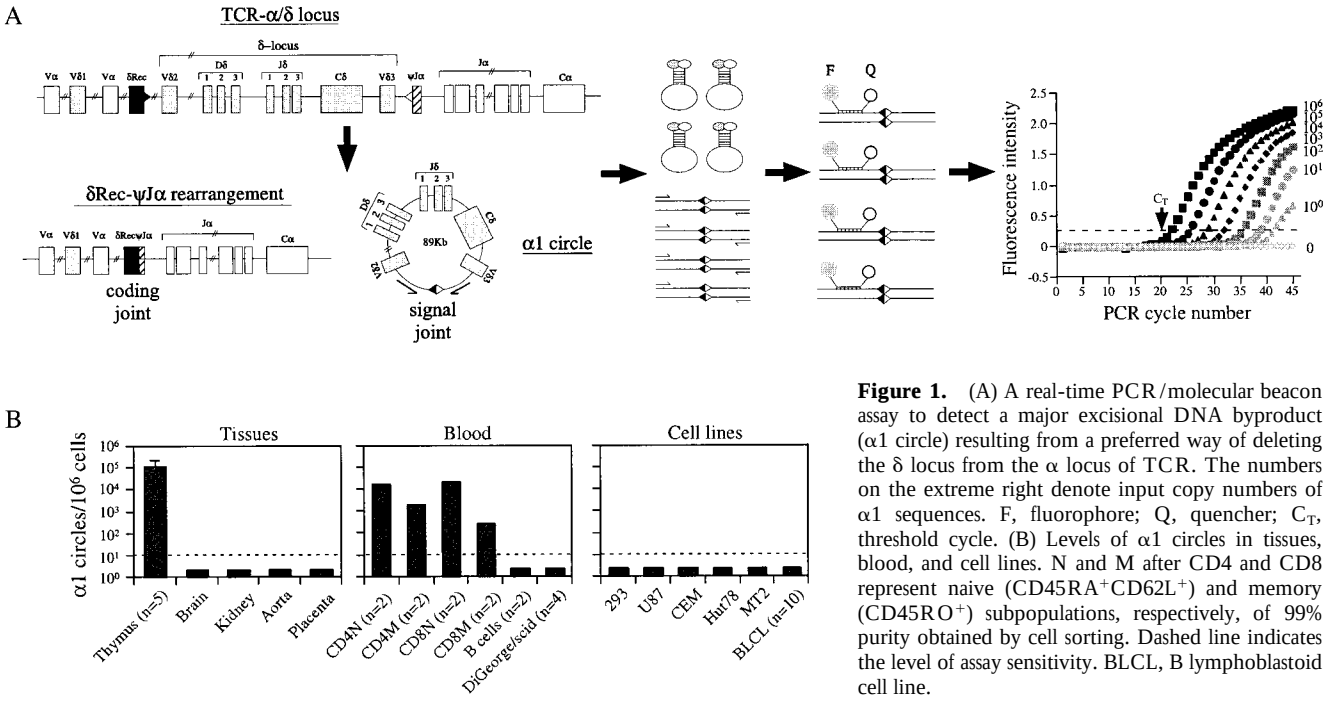

Figure 1. (A) A real-time PCR/molecular beacon assay to detect a major excisional DNA byproduct (α1 circle) resulting from a preferred way of deleting the δ locus from the α locus of TCR. The numbers on the extreme right denote input copy numbers of α1 sequences. F, fluorophore; Q, quencher; C_T, threshold cycle. (B) Levels of α1 circles in tissues, blood, and cell lines. N and M after CD4 and CD8 represent naive ($CD45RA^+CD62L^+$) and memory ($CD45RO^+$) subpopulations, respectively, of 99% purity obtained by cell sorting. Dashed line indicates the level of assay sensitivity. BLCL, B lymphoblastoid cell line.

compared with memory ($CD45RO^+$) subpopulations. The finding of low levels of α1 circles in memory cells was not unexpected, as these cells are derived from naive cells after antigen-induced proliferation during which TRECs are diluted. The specificity of the assay was further confirmed by the absence of α1 circles from sorted B cells and PBMCs of four individuals with severe DiGeorge syndrome or SCID (Fig. 1 B).

α1 Circle Numbers Decline with Age. The numbers of α1 circles in PBMCs were assessed in a cohort of 532 normal, HIV-1–seronegative persons who ranged in age from infants to 95-yr-olds. Fig. 2 A shows that the mean number of α1 circles remained relatively stable at a high level of $\sim 10^5/10^6$ PBMCs for the first 10–15 yr of life. Stability of α1 circle numbers during early childhood was confirmed by studying sequential PBMC samples from 12 subjects followed from birth to age 3 or 4. Not a single child showed any significant decrease in α1 circle numbers over this period of observation (data not shown). Given that the total lymphocyte concentration decreases in the first 5 yr of life, if we expressed RTE concentration as the concentration of α1 circles per microliter of blood, then a decline in concentration of α1 circles per microliter of blood would be observed with time in this age range (data not shown). Between the teen years and age 20–25, a sharp drop of ~1–1.5 logs was noted, followed by a gradual decline (downslope of ~0.03/yr) thereafter. No differences were found between males and females. A total decrease of ~2 logs over 10 decades was observed. It should be noted that even persons over the age of 70 have significant numbers of α1 circles in their PBMCs. In addition, when the results in Fig. 2 were converted to α1 circle numbers per microliter of blood, a drop in the concentration of RTEs was seen in the first 15 yr of life due to the known decrease in total lymphocyte numbers during this period. Overall, these results agree with the age-related decline in thymic lymphoid volume measured in an autopsy series (27). The multiphasic decrease in α1 circles over time shown in Fig. 2 A, however, differs from the monotonic exponential decline reported recently (18).

The same trend in age-related decline is seen when the results are analyzed by box plots (Fig. 2 B) denoting the 90th, 75th, 50th, 25th, and 10th percentiles for each age category. The prominent transition observed for age 16–20 is consistent with prior findings that $CD4^+$ lymphocyte regeneration after chemotherapy is severely impaired after the age of 20 (28, 29). Fig. 2 B also demonstrates the considerable normal variation (1–1.5 logs) in α1 circle numbers within any age category.

α1 Circle Numbers Are Reduced in a Subset of HIV-1–infected Adults and Children. In a cross-sectional study, we compared 126 HIV-1–infected adults who had never received antiretroviral therapy with 88 age-matched seronegative controls. Of the HIV-1–infected individuals, 65 were studied during the acute stage of HIV-1 infection, i.e., within the first 90 d of infection. As shown in Fig. 3 A, both acutely and chronically infected individuals have significantly lower α1 circle numbers than controls ($P < 0.05$). Many HIV-1–infected individuals, however, have α1 circle numbers in the normal range, in contrast to the previous suggestion that every HIV-1–infected adult has a low concentration of RTEs in blood (18). We performed our studies on PBMC samples, whereas the study by Douek et al. (18) measured the number of TRECs in separated $CD4^+$

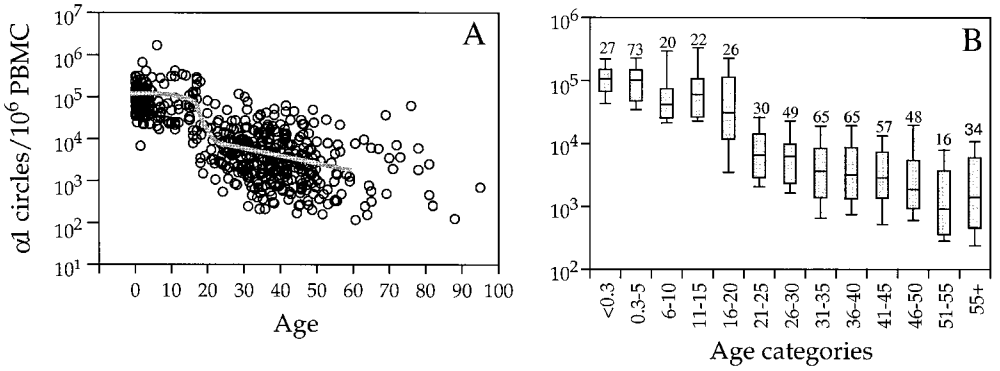

Figure 2. Changes in α1 circle numbers with age by (A) scattered plot or (B) box plot analysis. The gray line in A represents the best nonlinear fit to the data. The number of cases in each age category is shown at the top of each box plot in B. The top and bottom of each rectangular box denote the 75th and 25th percentiles, respectively, with the median shown inside the box. Horizontal bars extending from each box represent the 90th and 10th percentiles.

and $CD8^+$ T cell populations. Note as well the absence of supranormal α1 circle numbers from infected individuals to support the suggestion of a "thymic rebound" that had been raised by imaging studies on HIV-1–infected humans (10) or histopathologic examinations of SIV-infected macaques (6). Among HIV-1–infected individuals, no significant correlation was observed between α1 circle numbers and concurrent $CD4^+$ lymphocyte count or plasma viral load ($P > 0.05$; data not shown).

A more prominent impact of HIV-1 infection on the number of α1 circles was seen in a similar cross-sectional comparison of 42 HIV-1–infected children (ages 0–10 yr) with 124 age-matched seronegative controls ($P < 0.05$; Fig. 3 A). The HIV-1–infected children were either on no therapy or monotherapy with a nucleoside analogue, typically zidovudine. The median α1 circle value for HIV-1–infected children was 3.7-fold lower than that in normal subjects, with ~50% having values below the normal 10th percentile. A low α1 circle number in PBMCs of HIV-1–infected persons could be due to either decreased thymic output or to an increased rate of cell death or proliferation. No mechanistic discrimination can be made from these cross-sectional analyses.

The consequence of HIV-1 infection on the number of α1 circles was then assessed longitudinally in 16 homosexual men who were enrolled in an HIV-1 natural history cohort. They were all initially HIV-1 seronegative but became infected during the course of observation. Cryopreserved PBMC samples pre- and postseroconversion (up to ~7 yr of follow-up, and without the use of antiretroviral drugs except for zidovudine for four individuals in their last few time points) were assayed for α1 circles. A considerable person-to-person variation was again noted. As exemplified by the five cases shown in Fig. 3 B, some individuals demonstrated a progressive decline in α1 circle numbers, whereas others had sequential values that were essentially no different from their preinfection baselines. The data from each case were then analyzed by linear regression, and the slope of the change in α1 circle numbers over time was individually calculated (Fig. 3 C). Although the slope in half of these individuals did not show an appreciable decline, in the other half there was a downslope (>0.30/yr) that was >10 times steeper than the normal rate of decline. What might account for these dichotomous results was not immediately evident. However, the slope of the decline in α1 circle numbers correlated significantly with each patient's rate of decrease of CD4 lymphocytes ($R = 0.65$, $P < 0.05$; Fig. 3 D). The precise mechanistic implication of this correlation remains unclear, as it could be interpreted to mean that the rate of loss of $CD4^+$ T cells is linked either to a faster rate of drop in thymic output or to a faster rate of death or proliferation for RTEs.

The Impact of HAART on the Number of α1 Circles in HIV-1–infected Individuals. We studied the impact of HAART on the number of α1 circles in PBMCs of 74 previously drug-naive individuals (mean age, 36; ages ranged from 24 to 59). 27 patients began treatment within the first 90 d of their HIV-1 infection, whereas 47 were chronically infected for varying lengths of time. DNA extracted from sequential PBMC samples (generally ≥5) from each case were assayed for α1 circles. Variable patterns of response to HAART were observed, in contrast to the nearly complete response rate previously reported (18). As displayed in Fig. 4 A, those individuals with baseline values of >2,200 α1 circles/10^6 PBMCs (the median value for age-matched normal controls) in general did not have a rise in α1 circle numbers while receiving HAART. In contrast, individuals with baseline values ≤2,200 typically showed increases during treatment. These differences were quantified by calculating the slope of the regression line for all α1 circle data points from each individual. For example, in the cohort of chronically infected individuals, the mean slope of the change in α1 circles during HAART was +1.13/yr for those with a low baseline value and −0.51/year for those with a normal baseline value (Fig. 4 A). That the overall slope difference between individuals with low baselines and those with normal values was statistically significant ($P < 0.05$, t test) suggests that an increase in the number of RTEs during HAART is seen primarily in individuals with an existing impairment.

The thymus is known to express high levels of CXCR4 (30–32), the major entry coreceptor for syncytium-inducing (SI) strains of HIV-1 (33). Both X4 and R5 viral isolates can replicate in SCID-hu mice, although X4 viruses replicate with greater cytopathicity (30). Perhaps individuals with SI viruses have a lower concentration of RTEs due to increased thymic destruction by SI viruses, especially

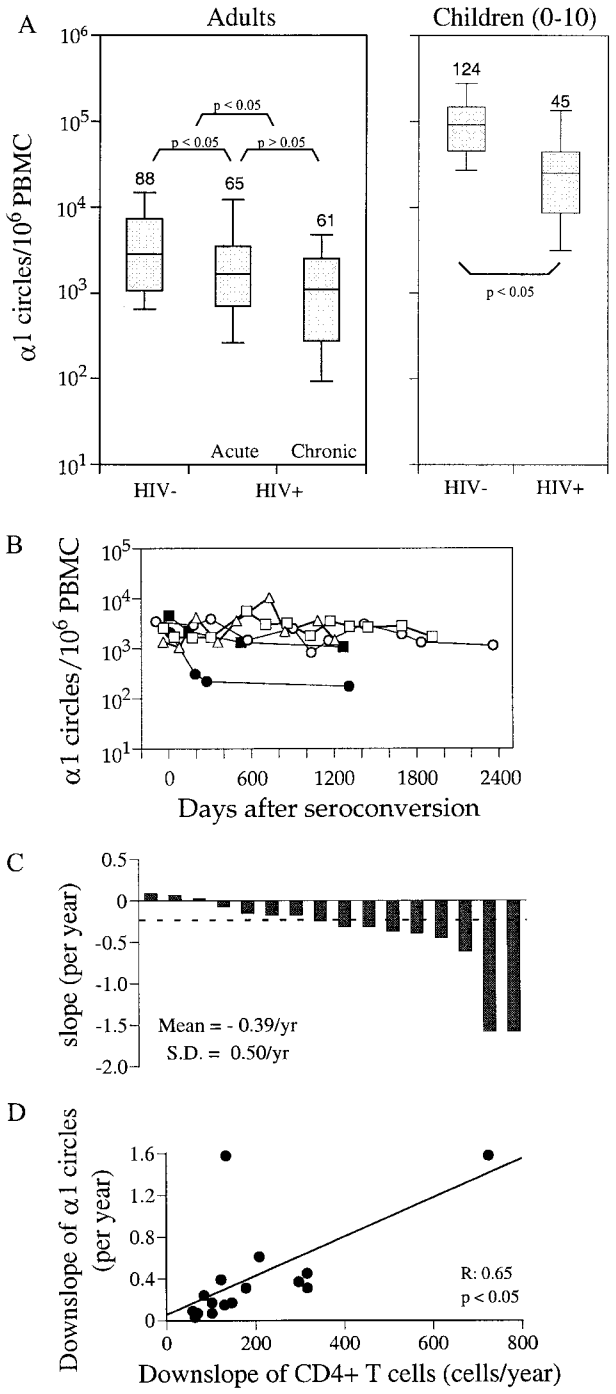

Figure 3. (A) Distribution of α1 circle numbers in uninfected and infected adults and children. The number of cases studied is shown on top of each box plot. The *P* values were determined by the Kolmogorov-Smirnov method. (B) Examples of seroconverters whose longitudinal α1 circle numbers did not change with time (open symbols) and whose numbers did (filled symbols). The slope of the change in α1 circle numbers with time in seroconverters (C) and its correlation with the slope of CD4 T cell loss (D). Dashed line in C indicates 10 times the normal rate of decline seen with aging alone.

in light of the observation that such individuals have a faster rate of $CD4^+$ T lymphocyte loss (34–36). However, in preliminary studies, we have noted that many individuals with low α1 circle numbers have no detectable SI viruses in their blood (data not shown). Additional work will be necessary to define the mechanistic pathways by which the concentration of RTEs in blood is affected in some and spared in others. Important clues might come from studies to understand why HIV-1–infected children are more likely than adults to have a lower number of RTEs. Some have suggested that certain infected infants have an immunophenotype resembling that seen in congenital athymic defects (37, 38). The application of new assays to measure TRECs to this clinical setting could be particularly revealing.

Increases in $CD4^+CD45RA^+62L^+$ and $CD8^+CD45RA^+62L^+$ Cells Do Not Correlate with Changes in α1 Circle Numbers. The sequential α1 circle numbers of eight individuals on an identical HAART regimen (standard dosages of zidovudine, lamivudine, abacavir, and amprenavir; median duration follow-up, 450 d) were converted to α1 circle copies per volume of blood based on concurrent total lymphocyte counts in blood. These results were then compared with the $CD4^+$ and $CD8^+$ naive ($CD45RA^+$ $CD62L^+$) T cell numbers (Fig. 4 B). As has been reported (39–42), mean $CD4^+$ T lymphocyte counts increased more significantly in the initial months of therapy, followed by a slower rise thereafter (mean total increase of 218 cells/μl). Mean $CD4^+$ naive cell numbers climbed steadily throughout the treatment period (mean total increase of 93 cells/μl), as did $CD8^+$ naive cells (mean total increase of 65 cells/μl), despite a clear drop in total $CD8^+$ T lymphocyte counts (mean total decrease of 250 cells/μl). Six of the eight individuals had low levels of α1 circles before therapy (i.e., <2,200 α1 circles/10^6 PBMCs). The kinetics of the changes in α1 circles per microliter of blood did not, however, temporally correspond to those of $CD4^+$ and $CD8^+$ naive cells. There was an initial rise in the concentration of α1 circles within the first 90 d of therapy followed by a subsequent decrease. More importantly, the total number of RTEs, as reflected by α1 circle measurements (<10 copies per microliter of blood), was numerically insufficient to account for the rise seen in $CD4^+$ and $CD8^+$ naive lymphocytes. This could be partly explained by proliferation of RTEs before or soon after emigration from the thymus, a phenomenon reported to occur in the mouse (43). Alternatively, this discrepancy could suggest that much of the observed rise in $CD45RA^+CD62L^+$ T cells after HAART is not due to direct outpouring from the thymus.

Conclusions. Using a simple and accurate real-time PCR/molecular beacon assay, we have shown that the number of RTEs in blood remains high for the first 10–15 yr of life, followed by a sharp drop in the late teenage years and a gradual decline thereafter. HIV-1 infection was found to lower the concentration of RTEs in a subset of but not all adult individuals. The rate of loss of RTEs correlated directly with the rate of $CD4^+$ T lymphocyte decline. However, it remains unclear whether the abnormality seen in some HIV-1–infected individuals is due to

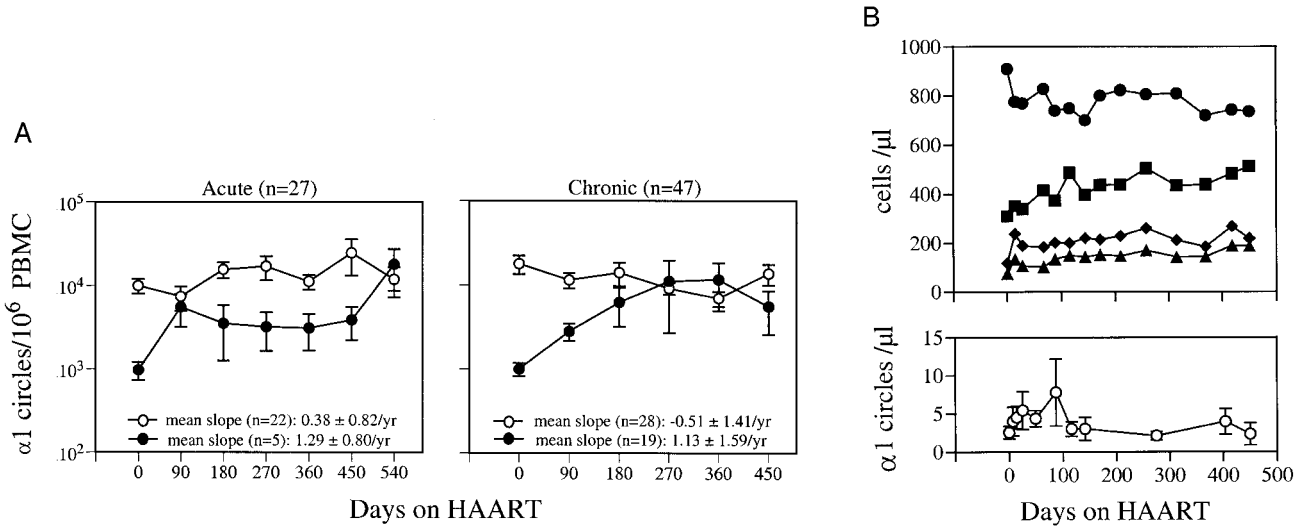

Figure 4. (A) Changes in α1 circle numbers in 74 individuals treated with combination antiretroviral therapy. Both acutely and chronically infected individuals were stratified into two different categories according to their pretreatment α1 circle values: ≤2,200 (●) or >2,200 (○) per 10^6 PBMCs. (B) HAART-associated changes in mean $CD4^+$ (■), $CD8^+$ (●), $CD4^+CD45RA^+CD62L^+$ (▲), and $CD8^+CD45RA^+CD62L^+$ (◆) T cell counts as well as changes in the mean α1 circle numbers (○).

decreased thymic output or to an increased death or proliferation rate of thymic emigrants. Because many HIV-1–infected persons had normal numbers of α1 circles, including some with low $CD4^+$ T cell counts, it is difficult to invoke thymic regenerative failure as a generalized mechanism for the depletion of $CD4^+$ lymphocytes.

HAART had no appreciable effect on the number of α1 circles in those individuals whose baseline levels were already within the normal range. On the other hand, significant increases were observed in those with a preexisting impairment. These increases, however, were numerically insufficient to account for the rise in naive ($CD45RA^+CD62L^+$) $CD4^+$ and $CD8^+$ T lymphocytes. This finding indicates that the rise in naive T lymphocytes during HAART may not be a direct measure of thymic output; instead, it may largely reflect either peripheral expansion of naive lymphocytes without a phenotypic switch (7) or the reversion of phenotype from memory cells (7, 44, 45). This conclusion is consistent with the observed increase in $CD45RA^+CD62L^+$ T lymphocytes after HAART in individuals who had been thymectomized (11). We therefore caution against the use of such phenotypic markers as a direct indicator of thymic output.

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