

# Increases in T Cell Telomere Length in HIV Infection after Antiretroviral Combination Therapy for HIV-1 Infection Implicate Distinct Population Dynamics in CD4<sup>+</sup> and CD8<sup>+</sup> T Cells

Sumesh Kaushal,\* Alan L. Landay,† Michael M. Lederman,‡ Elizabeth Connick,§ John Spritzler,<sup>¶</sup> Daniel R. Kuritzkes,§ Harold Kessler,† Bruce L. Levine,\*<sup>1</sup> Daniel C. St. Louis,\* and Carl H. June\*<sup>1</sup>

\*Henry M. Jackson Foundation for the Advancement of Military Medicine, U.S. Military HIV Research Program, Bethesda, Maryland 20889; †Departments of Immunology/Microbiology and Medicine, Rush-Presbyterian–St. Luke's Medical Center, Chicago, Illinois 60637;

‡Division of Infectious Diseases, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106;

§Division of Infectious Diseases, University of Colorado Health Sciences Center, Denver, Colorado 80262;

and <sup>¶</sup>Statistical and Data Analysis Center, Harvard School of Public Health, Boston, Massachusetts 02115

**Changes in mean telomeric terminal restriction fragment (TRF) length were examined as a marker for cellular replicative history in HIV-1-infected individuals after institution of anti-retroviral therapy (ART). Increases in mean T cell TRF lengths were observed in most patients following therapy; however, the contribution of individual T cell subsets was complex. An elongation of CD8<sup>+</sup> T cell TRF was nearly uniformly observed while changes in mean TRF length in CD4<sup>+</sup> T cells were heterogeneous as, despite potent suppression of viral replication, CD4 cell telomeres recovered in some patients, yet continued to decline in others. Increases in CD8 cell TRF correlated with decreased memory cells, suggesting a negative selection in the periphery for CD8 cells with extensive replicative history. In contrast, increases in CD4<sup>+</sup> T cell TRF length correlated with increases in naive cell subsets, suggesting that the CD4<sup>+</sup> T cell TRF increase may reflect a thymic contribution in some patients. These are the first increases in somatic cell telomere length in a population of cells observed *in vivo*, and the findings are compatible with therapy-induced reconstitution of the lymphoid compartment with cells having a more extensive replicative potential. These findings further distinguish lymphocytes from other somatic cell populations where only decreases in TRF over time have been noted. Thus, institution of ART in persons with moderately advanced HIV-1 disease reveals distinct population dynamics of CD4 and CD8 T cell subsets and also shows that the lymphocyte replicative history is dynamic.** © 1999 Academic Press

**Key Words:** HIV-1; telomere; telomerase; antiretroviral therapy; immunodeficiency.

## INTRODUCTION

Telomeres are specific structures found at the ends of eukaryotic chromosomes. In somatic cells, telomere length is shortened with each cell division due to the inability of DNA polymerase to replicate the extreme 5' end of the lagging strand of DNA (1). Substantial evidence supports the hypothesis that telomeres function in part as a mitotic clock for the cell. *In vivo*, lymphocytes have age-related reduction in telomere length (2), losing an average of 31 base pairs/year. *In vitro*, the rate of telomere loss from lymphocytes from normal individuals is approximately 50 to 120 bp/cell doubling, comparable to that seen in other somatic cells (2, 3). Thus, measurement of the telomeric terminal restriction fragment (TRF) length by Southern blot analysis is thought to serve as an index of replicative history *in vivo* and *in vitro*.

It has been suggested that replicative senescence (4) might contribute to the immunodeficiency of HIV infection (5). A related hypothesis is that increased clonal expansion and potential clonal exhaustion of T cells occur in response to HIV infection (6–8). Consistent with these models of immunosenescence, the mean TRF length was shown to decrease in CD8<sup>+</sup> T cells in most adult patients with HIV infection (9–11). However, telomere measurements of CD4 cells from HIV-infected individuals do not shorten excessively, suggesting that proliferative exhaustion is not a major component of CD4 cell decline in patients with HIV infection (9, 11–13). Recent measurements of T cell turnover during SIV infection in macaques using BrdU labeling document moderately elevated turnover of both CD4 and CD8 lymphocyte subsets (14, 15).

Treatment of HIV-1-infected individuals with potent antiretroviral drug combinations results in a dramatic decline in viral load and a partial restoration of the circulating T cell compartment. Soon after initiation of

<sup>1</sup> Present address: Department of Molecular and Cellular Engineering, University of Pennsylvania.

combination anti-retroviral therapy an initial rapid increase in CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts in the blood occurs, and this is followed by a more gradual increase in CD4<sup>+</sup> cells and a decline to below baseline in CD8<sup>+</sup> T cell counts (16–18). Further evidence for partial T cell reconstitution in patients treated with potent ART regimens includes the functional recovery of some CD4 T cell proliferative responses (17) accompanied by a late increase in proliferating CD4<sup>+</sup> T cells (19, 20). It is controversial as to whether the diversity of the T cell receptor repertoire recovers, and it is likely that this is a delayed and incomplete process (20, 21).

The mechanisms leading to incomplete T cell regeneration are still a matter of debate (22). It is likely that impaired thymic function contributes to the failure to completely restore T cells (23). In an effort to assess possible mechanisms for T cell reconstitution after profound inhibition of virus replication, we examined the changes in TRF as an indicator of T cell replicative history and correlated these to longitudinal changes in lymphocyte subpopulations following institution of potent ART.

#### METHODS

**Human subjects.** Two cohorts of patients were studied. Cryopreserved blood samples from patients with 350–500 CD4 cells were obtained from the National Naval Medical Center. The second set of samples was obtained from cryopreserved blood samples from patients participating in AIDS Clinical Trials Group Protocol (ACTG) 315. Protocol eligibility requirements included a history of prior zidovudine therapy, 100 to 300 CD4 cells, and no prior lamivudine or protease inhibitor therapy. Before initiating the ART protocol, patients discontinued all antiretroviral treatment for 5 weeks, then on day 0 ritonavir was started and increased as tolerated to 600 mg twice daily. On day 10, 150 mg of lamivudine twice daily and 300 mg of zidovudine twice daily were started. All patients provided written informed consent.

**T cell isolation.** PBMC were isolated from whole blood by density sedimentation, and CD3<sup>+</sup>, CD4<sup>+</sup>, or CD8<sup>+</sup> T cells were purified from PBMC using magnetic beads (Dyna). Briefly, to isolate T cells, PBMC were incubated with anti-CD3-specific beads for an hour at 4°C after first blocking nonspecific binding with human IgG. CD3<sup>+</sup> T cells were then obtained using positive selection with a magnetic gradient. For isolation of T cell subsets, PBMC were first incubated with anti-CD8-specific beads, and CD8<sup>+</sup> cells were obtained by flushing the retained cells using a magnetic bead separator. After washing thrice in cold PBS the CD8-depleted fraction was subjected to CD4 isolation using anti-CD4-specific magnetic beads. Fractions obtained using this method yielded a purity of >98% for posi-

tively selected cells.

**Analysis of terminal telomeric restriction fragment length.** DNA was isolated from purified T cells, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells according to a protocol recommended by the manufacturer (Puregene). Genomic DNA (2 µg) was digested with *Hinf*I and *Rsa*I (10 U each) overnight at 37°C. Digested DNA was then electrophoresed on a 0.6% agarose (Gibco BRL) gel at 4°C (30 V, 24 h), transblotted on a nylon membrane (Oncor) overnight, and UV-crosslinked (Stratagene). Hybridization was performed at 42°C with <sup>32</sup>P-labeled (TTAGGG)<sub>3</sub> probe in Hybrisol-1 (Oncor). Blots were washed twice in 3× SSC, 0.5% SDS, and finally in 1× SSC for 15 min each at 42°C, and hybridized blots were exposed to PhosphorImager screens (Kodak) for 4 h or overnight. Telomere lengths were analyzed by IPLab Gel software (Signal Analytics). The point of highest intensity was considered the peak TRF length and the mean TRF was calculated by obtaining the Gaussian distribution above the background from 50 segments drawn across the length of the band according to methods described elsewhere (24). See Oexle for a discussion of the theoretic aspects of telomere length measurement by Southern blot analysis (25). Mean TRF values were calculated as  $\Sigma(\text{OD}_i)/\Sigma(\text{OD}_i/L_i)$ , where OD<sub>i</sub> is the phosphorimager output (arbitrary units) and L<sub>i</sub> is the molecular weight of segment at position (i) relative to DNA standards. Sums were calculated over the range of 3–17 kb to obtain the mean TRF length. To minimize interassay variability, equal amounts of DNA were used and samples from the same patient were run on the same gel.

Assaying 5 independent samples assessed reproducibility of TRF measurement. The standard deviation of the intraassay variation was ±0.09 kb, and the coefficient of variation was 1.3%. Interassay variation was assessed by analysis of 14 samples. Samples were processed once, and the DNA was subjected to Southern blot analysis twice over a 3-month period of time. The interassay standard deviation was ±0.333 kb, and the coefficient of variation was 4.9%.

**Telomerase assay.** The telomeric repeat amplification protocol (TRAP) used is the modified version of the original method described by Kim *et al.* (26). Briefly, 2 × 10<sup>6</sup> cells were lysed in 200 µl of Chaps lysis buffer and cell extract representing 2 × 10<sup>4</sup> cells was used in a two-step, single-tube protocol (Oncor). In the first step, telomerase from the cell extract generated telomeric repeats by incubating at 30°C for 30 min, and the extended products were then amplified by PCR reaction at 94°C/30 s and 60°C/30 s for 28 cycles using <sup>32</sup>P-labeled TS primer. Generated PCR products were then analyzed on 12% PAGE followed by quantitative analysis on a PhosphorImager (Molecular Dynamics). The signal from each TRAP product ladder was calcu-

lated above the background of the corresponding heat-inactivated lane. To estimate the activity of telomerase, an internal 36-bp standard was included to permit quantification of PCR by competition, the signal from the 36-bp internal control was used to normalize inter-assay variation, and telomerase activity was calculated as the percentage of TRAP product generated by a TSR8 control template. To normalize interassay variation, telomerase activity was expressed as a percentage value of extracts from  $1 \times 10^4$  telomerase-positive 293 cells. Specificity of telomerase activity was demonstrated by heat inactivation of samples at 85°C for 10 min prior to TRAP assay. Additional controls included use of water as a template for PCR. The presence of telomerase inhibitors in the samples was also excluded by mixing telomerase-positive control extract from 293 cells with study samples.

**FACS analysis.** Two- and three-color flow cytometry was used to evaluate the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> peripheral blood T cells expressing HLA-DR, CD38, CD45RA, CD45RO, CD25, CD95, and CD28. The whole blood lysis technique was used utilizing appropriately labeled murine monoclonal antibodies (Pharmingen).

**Statistical analysis.** The two-tailed Student *t* test or the Wilcoxon signed rank test was used to interpret the significance of differences in the number of lymphocyte subsets or TRF lengths between patients before and after treatment. The global test of correlations adjusting for repeated measures on the same subject was done to assess changes in telomere length and CD4 and CD8 T cell subpopulations. For the global *P* value testing zero versus a positive or negative correlation between telomere length and cell type percentage, the interpretation of a small *P* value is that it indicates that when a subject's cell type percentage is greater than the mean of all people at that time point (i.e., week 0 or week 12, 24, or 48), then that person's telomere length tends to be higher (lower in the case of a negative correlation) than the mean of all people at that same time point. *P* values were adjusted for repeated measures on the same subject.

## RESULTS

### *Increase in Mean TRF Length in Lymphocytes Following Initiation of a Potent Anti-Retroviral Therapy (ART) Regimen*

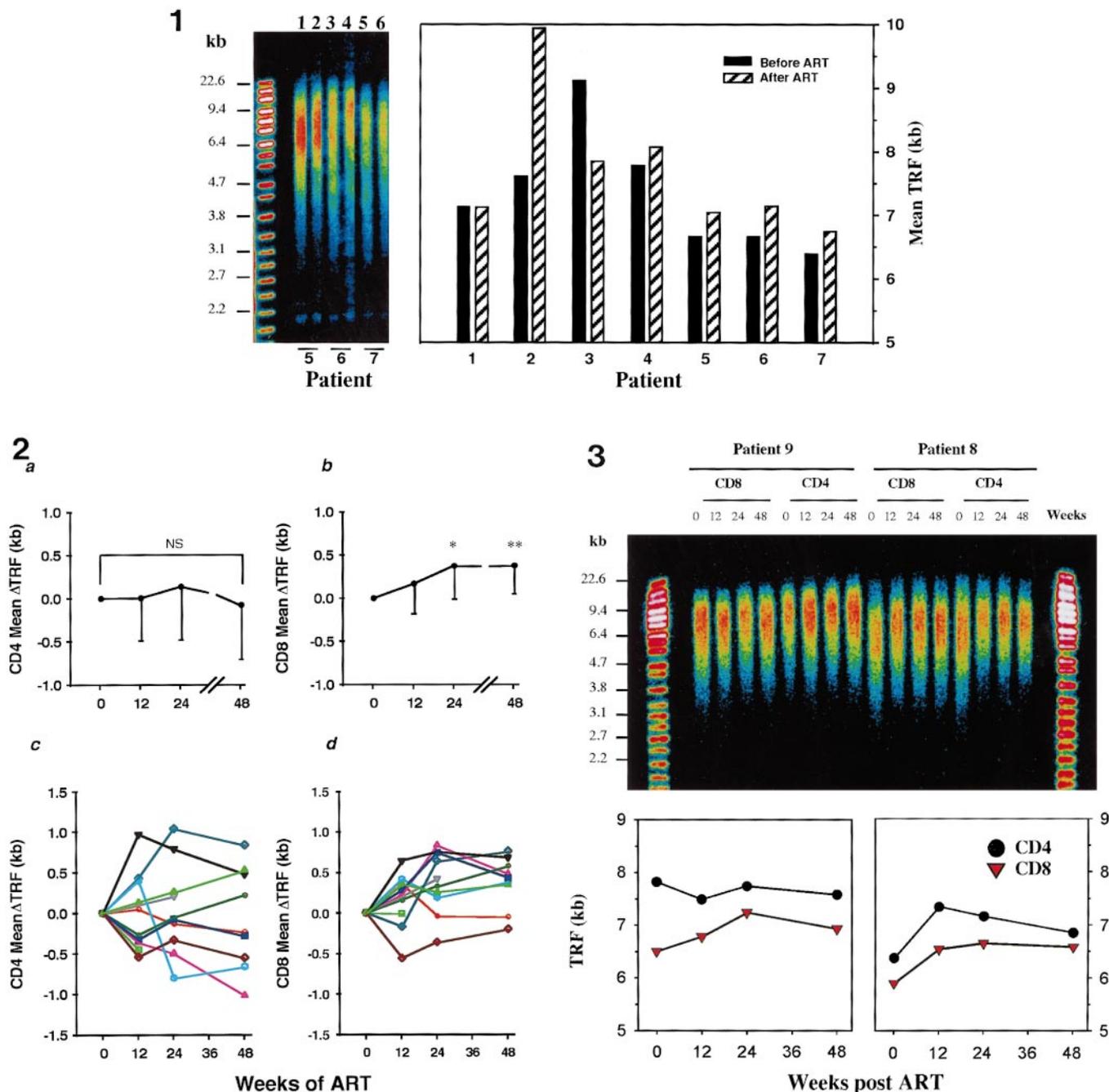
A previous study has shown an accelerated rate of telomere shortening in the leukocytes of patients during the natural progression of HIV infection, with a mean loss of 114 base pairs/year in PBMC in asymptomatic patients and 175 bp/year in progressors (9). To assess the effect of potent anti-retroviral therapy on

telomeres, TRF length was analyzed longitudinally in the T cells of seven men who had not had previous therapy with protease inhibitors. Four of these men had moderately advanced HIV infection (CD4 count 100–300), and three were asymptomatic individuals with baseline CD4 counts of 350 to 500 cells per cubic millimeter of blood. Five of the seven patients had increased T cell mean TRF after 6 to 12 months of potent anti-retroviral therapy (Fig. 1). The expected wide individual variations in baseline mean telomere length were noted. Thus, while the previously documented rate of telomere loss is 100 to 175 bp/year (9), we observed an average gain of 350 base pair T cells from patients treated for 6 to 12 months with protease inhibitor therapy.

### *Distinct Changes in CD4 and CD8 Telomeres after Institution of ART*

Although the number of patients tested was small, the above results suggest that inhibition of viral replication might slow the accelerated rate of loss of telomere length and may actually result in restoration of T cell telomere length in most HIV-infected individuals. To more formally test this possibility and to assess the changes in TRF length in T cell subpopulations, we performed a longitudinal study of 11 individuals enrolled in AIDS Clinical Trials Group Protocol 315. Patient recruitment for ACTG 315 and the study design was previously described (27). Briefly, all patients had documented HIV-1 infection and CD4 T lymphocyte counts of 100–300 cells/ $\mu$ l and all had received pre-treatment with AZT; none had received 3TC or a protease inhibitor. Eleven patients receiving triple combination drug therapy with lamivudine, zidovudine, and zalcitabine were selected based on sample availability for further analysis. The baseline immunologic and virologic indices of these patients are shown in Table 1 and are representative of the entire ACTG 315 cohort (27).

Nearly all patients had an increase in CD8<sup>+</sup> T cell mean TRF after institution of combination ART (Fig. 2b). The median TRF length of CD8<sup>+</sup> T cells increased 430 bp over the 48-week study period, and this increase was significant compared to baseline (*P* = 0.02 by Wilcoxon signed rank test; *P* = 0.007 by Student's *t* test. The univariate 95% confidence interval on the mean change in CD8 telomere length from baseline to week 48 of the study was 132 to 619 bp). The increase in CD8<sup>+</sup> T cell TRF was evident within 12 weeks after initiation of therapy and then stabilized after 24 to 48 weeks of therapy. In contrast, the mean TRF length in CD4<sup>+</sup> T cells did not increase (Fig. 2a). Confirming previous reports (9–11), we found that the baseline mean CD8<sup>+</sup> T cell TRF length was 714 bp shorter than the mean CD4<sup>+</sup> T cell TRF length in this cohort of patients (Fig. 3 and data not shown).



**FIG. 1.** Effect of combination ART therapy on T cell mean telomere terminal restriction fragment length (TRF) in seven HIV-infected patients. T cells were isolated from seven protease naive patients using positive selection with anti-CD3 and magnetic immunobeads (Dyna) on PBMC cryopreserved at baseline and after 6 to 12 months of potent anti-retroviral therapy (ART). (Left) Representative Southern blot from patients 5, 6, and 7 showing 0.34- to 0.47-kb increases in T cell mean TRF after ART therapy. Lanes 1, 3, and 5, baseline, and lanes 2, 4, and 6, after ART. (Right) T cell mean telomere length in the seven patients. Mean TRF of purified T cells was determined by Southern blot analysis and plotted as a bar graph.

**FIG. 2.** Distinct changes in mean TRF length in CD4 and CD8 T cells in HIV-infected patients after therapy with combination ART. The mean TRF was determined in CD4 and CD8 cryopreserved T cells from 11 patients in ACTG protocol 315. Samples were analyzed at baseline and longitudinally to 48 weeks. Telomere length at day = 0 was considered baseline and changes in telomere length were plotted. (a, b) Mean changes in 11 patients of the CD4 and CD8 mean TRF ( $-1$  SD). For CD8 cells, Student's *t* test was used to assess for significance of changes versus baseline: \* $P = 0.01$ ,  $n = 10$ ; \*\* $P = 0.007$ ,  $n = 9$ , normality  $\geq 0.2$ . In addition, a global one-sided test of no change versus an increase in telomere length over time at 12, 24, and 48 weeks yields  $P = 0.002$ , indicating that CD8 TRF length increased with time after ART. A statistically significant increase in CD8 TRF at 24 ( $P = 0.03$ ) and 48 ( $P = 0.02$ ) weeks was also indicated by the Wilcoxon signed rank test. For CD4 cells, the TRF changes were not significant, as analysis by *t* test ( $P = NS$ ) and by global analysis of the CD4 mean TRF changes

**TABLE 1**  
Virologic and Immunologic Parameters for the 11 HIV-Infected Subjects in ACTG Protocol 315

Patient	Age (years)	Plasma viral load (copies/ml)		CD4 <sup>+</sup> T cells (μl)	CD8 <sup>+</sup> T cells (μl)
		Baseline	Week 48	Baseline	Baseline
1	41	$3.61 \times 10^3$	$<10^2$	242	615
2	35	$7.26 \times 10^3$	$<10^2$	155	1240
3	47	$2.84 \times 10^5$	$<10^2$	182	604
4	64	$1.49 \times 10^5$	$<10^2$	257	1465
5	37	$2.82 \times 10^4$	$<10^2$	181	1406
6	36	$6.27 \times 10^4$	$1.40 \times 10^3$	167	1032
7	45	$6.54 \times 10^4$	$<10^2$	282	1290
8	36	$1.05 \times 10^5$	$<10^2$	132	1782
9	33	$2.65 \times 10^5$	$<10^2$	81	282
10	55	$2.14 \times 10^4$	$<10^2$	205	876
11	39	$9.23 \times 10^4$	$2.0 \times 10^4$	156	405
Mean ± SD	42 ± 10	$5.17 \times 10^{4a}$		186 ± 58	1000 ± 482

<sup>a</sup> Geometric mean.

Significant interpatient and inpatient heterogeneity was observed in the longitudinal analysis of the patterns of changes in telomere length in the T cell subsets from the 11 patients. Analysis of the mean TRF length changes in these treated patients revealed two primary patterns of CD4<sup>+</sup> T cell recovery. Mean TRF length in CD4<sup>+</sup> T cells modestly increased in 5 individuals, but remained unchanged or were slowly lost in the 6 other patients (Fig. 2c). The pattern was complex, as 2 of the patients had transient increases in CD4<sup>+</sup> T cell telomere length that were not sustained and were followed by subsequent decreases in length. In contrast, the pattern was more uniform in the CD8<sup>+</sup> T cell compartment, with 8 of 10 patients having increases in mean telomere length after 24 to 48 weeks of therapy (Fig. 2d). (One patient had no samples available after week 12). Two examples of inpatient heterogeneity of the changes in telomere length are shown in Fig. 3. In patient No. 8, there were increases in both the CD4 and the CD8 T cell mean TRF after institution of ART, while in patient No. 9 a “split response” was noted, with a decrease in the CD4 telomere length observed while the CD8 telomere length was increasing. Of the 10 patients assayed longitudinally for 24 weeks or more of therapy, 5 patients had increases in both CD4<sup>+</sup>

and CD8<sup>+</sup> T cell telomere length while 3 of the patients had decreases in CD4 and increases in CD8 TRF and 2 patients had TRF decreases in both T cell subsets.

The above results establish that the institution of ART is associated with changes in telomere length, and these changes differ strikingly from the expected monotonic decline observed in the PBMC from normal adults of about 30 to 50 base pairs per year (2), or from the accelerated decline of 100 to 300 base pairs per year in the CD8 subset that is reported in HIV-infected individuals (9). Perhaps most notable from these results is that some patients continue to have a decline in CD4 T cell TRF length while others have increases in CD4 cell TRF length after starting ART. This does not appear to be correlated with failure of control of HIV-1 replication as only two patients had detectable plasma HIV-1 after 48 weeks of therapy. Moreover, both of these patients had increased CD8 cell TRF while one had increased CD4 cell telomere length and the other decreased CD4 cell telomere length.

We have considered a number of other potential explanations for the changes in telomere length observed after ART. The initial response to potent ART is an increase in CD4 and CD8 cells that may be due primarily to the release of cells trapped in lymph nodes.

( $P = 0.31$ ) indicated that the mean TRF did not change with time after ART. (c, d) Heterogeneity of changes in individual m TRF length in the CD4 and CD8 subsets in the 11 patients. The mean of these data is depicted in (a) and (b) above. Patients as numbered in Table 1: ○ 1, ▽ 2, □ 3, ◇ 4, △ 5, ⊙ 6, ⊖ 7, ▽ 8, ⊞ 9, ⊕ 10, ▲ 11.

**FIG. 3.** Temporal patterns and heterogeneity of changes in CD4 and CD8 T cell mean TRF. (Top) Longitudinal analysis of two patients by Southern blot of CD4 and CD8 T cell TRF at baseline and after 48 weeks of ART. (Bottom) Plots of the Southern blot data for patients 8 and 9 of the changes in the mean TRF of the CD4 (●) and CD8 (▼) subsets; these patients are also shown in Fig. 2. Patient 9 (left) had a “split” response, with stable CD4 TRF and an increase in CD8 mean TRF while patient 8 (right) had increases in mean TRF in both T cell subsets. Inspection of the data in the Fig. 2 indicates that five patients had increases in mean TRF in both CD4 and CD8 cells, three patients had a split response with decreased mean TRF in CD4 cells and increased mean TRF in CD8 cells, and two patients had decreased mean TRF in both subsets.

Preferential mobilization of cells with longer telomeres such as naive T cells could contribute to the changes that we have observed. The major contributions to the increase in CD4 and CD8 cells from rebound occurs early, in the first several weeks of therapy (6, 7), and we did not observe the major increase in CD8 TRF until 24 weeks or more of therapy.

Activated T cells express telomerase (28), and hence it is possible that induction of telomerase activity could contribute to the increased telomere length during the T cell repopulation that follows institution of ART. To investigate this possibility we measured telomerase activity in the T cells from 11 patients in AIDS Clinical Trials Group protocol 315. Low levels of telomerase activity were detectable in the lymphocytes from patients before therapy, confirming previous studies (9). In 10 of the patients there was no change in the telomerase activity in relation to commencement of combination ART, while in 1 patient there was a temporally related increase in activity (Fig. 4). In this individual (patient 10), during the first 2 weeks after starting ART there was a substantial increase in the telomerase activity from 2% to more than 50% of the activity detected in the telomerase-positive tumor control cell line. This appears to be an uncommon occurrence, as we have since assayed multiple other samples from a total of 22 individuals and have not observed a similar temporal induction of telomerase activity (data not shown). Together, these results suggest that it is unlikely that increased telomerase activity could account for the nearly uniform increase in mean telomere TRF length in the CD8 compartment that we have noted. However, our results do not exclude the possibility that induction of telomerase activity limited to a compartment such as lymph nodes could contribute to the increased telomere lengths observed during cellular restoration following ART.

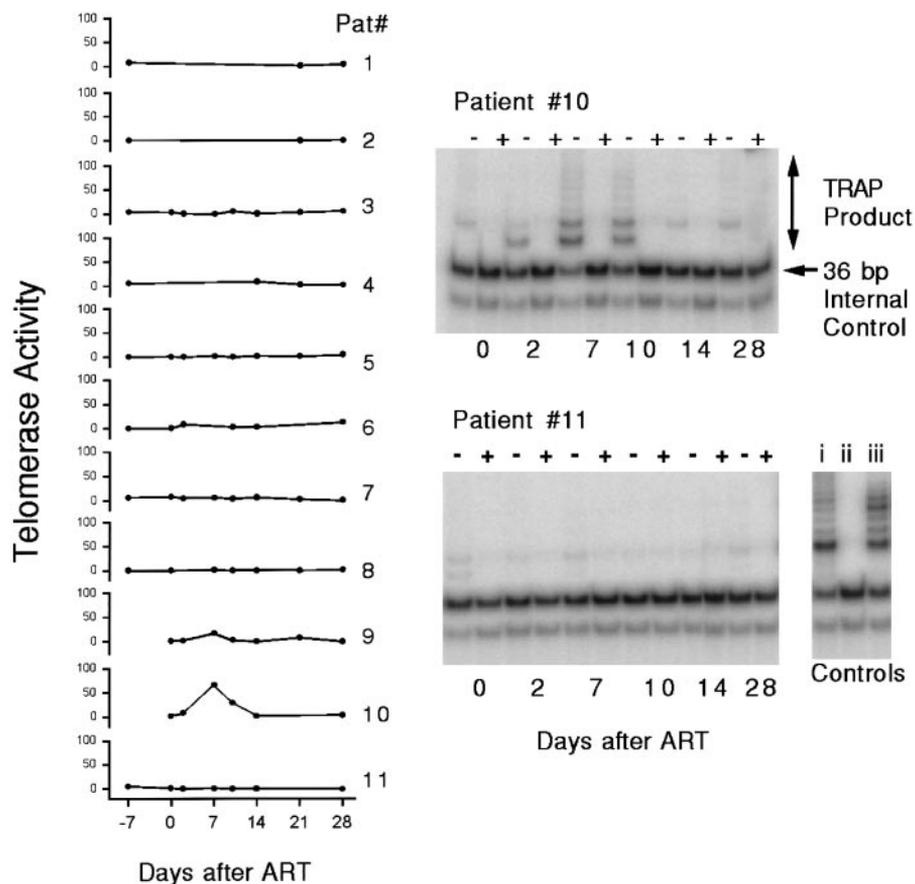
Striking changes in the numbers and immunophenotypes of the circulating lymphocytes occurred in the 11 patients after starting ART (Table 2). There was a significant increase in CD4<sup>+</sup> T cells at all time points. In contrast, while CD8<sup>+</sup> T cells increased early after starting therapy confirming previous reports (16), after 36 to 48 weeks of therapy the total CD8<sup>+</sup> T cell number was not different from the baseline number. There were, however, marked changes in the composition of the CD8<sup>+</sup> T cell subset, with a significant decrease in activated cells bearing the CD95 antigen. In contrast, naive CD4 and CD8 cells increased with therapy. Similarly, there was an increase in T cells that express CD28, and this was significant for CD4<sup>+</sup>28<sup>+</sup> ( $P = 0.002$ , Wilcoxon signed rank test) cells. There was, however, a significant decrease in the number of memory CD8<sup>+</sup>RO<sup>+</sup> cells. Recent studies have documented shorter telomeres in CD28<sup>-</sup> T cells compared to their CD28<sup>+</sup> counterparts (10, 29). Similarly, since memory CD45RO<sup>+</sup> T

cells have shorter telomeres than CD45RA<sup>+</sup> naive T cells, it is likely that the increase in TRF length in the CD8 cells of patients after starting potent ART is due to a selective persistence and/or renewal of cells with longer telomeres, such as naive and/or CD28<sup>+</sup> T cells. Furthermore, the selective depletion of activated CD8<sup>+</sup>RO<sup>+</sup>28<sup>-</sup> T cells with short telomeres following HAART is also likely to contribute to these findings. Thus, our results are most consistent with a mechanism for recovery of the CD8<sup>+</sup> T cell compartment that involves the replacement of CD8<sup>+</sup> T cell cells with high replicative history and shorter TRF length with cells having higher replicative potentials and longer TRF lengths.

In contrast to the marked decrease in CD8 cells that expressed the CD45RO or CD95 antigens, there was an increase in the absolute number of CD4 cells expressing CD45RO ( $P = 0.004$ ) or CD95 ( $P = 0.006$ ) (Table 2). The increase of CD4 cells with a memory phenotype likely contributes to the decline in CD4 cell telomere lengths noted in some patients.

To further clarify the distinct changes in telomeres induced by ART, the CD4 and CD8 T cell telomere lengths were correlated with the number and frequency of T cell subsets after 12 to 48 weeks of therapy (Table 3). It would be expected that changes in mean TRF length, an assay done on bulk populations of CD4 and CD8 cells, would correlate best with changes in the frequency of cell subsets rather than the absolute numbers of cell subsets. Changes in the mean TRF length in the CD4<sup>+</sup> T cell compartment correlated with changes in the frequency of CD45RA<sup>+</sup>62L<sup>+</sup> naive cells ( $P = 0.003$ ) and CD28<sup>+</sup> cells ( $P < 0.001$ ), suggesting that the mean TRF length observed in the CD4 compartment may relate to new production of CD4 T cells following combination ART (Table 3). Telomere lengths of CD4 cells also correlated with the absolute number of naive cells ( $P = 0.01$ ) but not with the absolute number of CD28<sup>+</sup> cells ( $P = 0.14$ ). Furthermore, elongation of CD4 T cell TRF also correlated with the frequency of CD25<sup>+</sup>CD4<sup>+</sup> T cells ( $P = 0.003$ ). It is known that naive cells have long telomeres, and it is likely that recent thymic emigrants have long telomeres (30). In contrast, while the increase in TRF lengths of CD8<sup>+</sup> T cells did not correlate with changes in naive cells, there was an inverse correlation of CD8 memory cells with telomere length that did not quite reach statistical significance ( $P = 0.06$ ).

Previous studies have shown that HIV infection induces increased proliferation of CD4<sup>+</sup> T cells (31), and recent studies have shown that there is a further increase in CD4<sup>+</sup> T cell proliferation induced by potent ART (19). We found that for both CD4 and CD8 T cells, the percentage and frequency of CD25<sup>+</sup> T cells correlated with increased telomere length. Together these results are consistent with the notion that the increase



**FIG. 4.** Longitudinal analysis of telomerase activity in PBMC from patients after initiation of combination ART. Lymphocytes from 11 patients from ACTG protocol 315 were assayed for telomerase activity before and at the indicated intervals following initiation of anti-retroviral therapy. Three of the patients in this cohort were also included in the data shown in Figs. 2 and 3. The telomeric repeat amplification protocol (TRAP) assay was used to assess telomerase activity in the extracts from PBMC. Extracts from  $2 \times 10^4$  cell equivalents per lane were assayed. The longitudinal results from the 11 patients are depicted graphically as a percentage of activity of telomerase-positive cell extracts from  $1 \times 10^4$  293 cells. The results from patients 10 and 11 are shown on the right. Non-heat-exposed extracts (-) were assayed while heat-inactivated extracts (+) were used as negative controls. (i) 293 cell extracts ( $1 \times 10^4$ ); (ii) water PCR template negative control; (iii) TSR8 template control.

in the telomere length in the  $CD8^+$  T cell compartment was related to the selective persistence/survival of  $CD8^+$  T cells that have longer telomeres, as well as the renewal/replacement with naive cells that have longer telomeres. Finally, the a positive correlation of CD25 expression with longer telomeres in both  $CD4$  and  $CD8$  T cells is consistent with these cells being a marker for recently produced cells that are derived from cells with a more extensive replicative capacity.

#### DISCUSSION

In this report we have extended our previous studies regarding telomere lengths in HIV-1 infection. Potent ART results in T cells with longer average terminal telomeric restriction fragment lengths. Longitudinal analysis of the replicative history of T cell subsets

shows that ART therapy has marked effects on the composition of the  $CD4$  and  $CD8$  T cell compartments which results in distinct changes in telomere length in  $CD4$  and  $CD8$  T cells. We found that the effect of ART in patients with moderately advanced HIV infection was to increase the median  $CD8^+$  TRF by about 430 base pairs while there was no change in the  $CD4^+$  cell TRF after 48 weeks of therapy. Thus, these are the first results to demonstrate the reconstitution of T cell compartment with T cells having a more extensive replicative capacity, as previous studies have only demonstrated age-related declines in lymphocyte telomere lengths during steady state hematopoiesis.

There are several limitations to the use of telomere length as a marker of T cell turnover and thymic renewal in HIV infection. First, McCune and Hellerstein have pointed out that since HIV-1 preferentially in-

TABLE 2

Alterations in T Lymphocyte Subsets after Combination Anti-retroviral Therapy

Subset	Baseline	Weeks 36–48	<i>P</i> value <sup>a</sup>
	cells/ $\mu$ l (mean $\pm$ SD)		
CD4	185 $\pm$ 58	389 $\pm$ 96	0.002
CD8	1000 $\pm$ 482	1126 $\pm$ 464	0.28
CD4 <sup>+</sup> 28 <sup>+</sup>	129 $\pm$ 31	320 $\pm$ 97	0.002
CD4 <sup>+</sup> 45RA <sup>+</sup> 62L <sup>+</sup>	49 $\pm$ 22	145 $\pm$ 50	<0.001
CD4 <sup>+</sup> RO <sup>+</sup>	155 $\pm$ 65	269 $\pm$ 98	0.004
CD4 <sup>+</sup> 95 <sup>+</sup>	121 $\pm$ 41	213 $\pm$ 85	0.006
CD8 <sup>+</sup> 28 <sup>+</sup>	230 $\pm$ 98	312 $\pm$ 140	0.10
CD8 <sup>+</sup> 45RA <sup>+</sup> 62L <sup>+</sup>	120 $\pm$ 105	224 $\pm$ 112	0.002
CD8 <sup>+</sup> RO <sup>+</sup>	847 $\pm$ 446	528 $\pm$ 211	0.01
CD8 <sup>+</sup> 95 <sup>+</sup>	785 $\pm$ 399	208 $\pm$ 248	0.002

Note. Lymphocyte subpopulations were enumerated by two- and three-color flow cytometry for the 11 patients shown in Table 1 and Figs. 2 and 3.

<sup>a</sup> *P* value, Wilcoxon signed rank test.

fects dividing CD4<sup>+</sup> T cells that are about to shorten their telomeres, the T cells surviving HIV infection should have longer telomeres (22). However, this is unlikely to be a significant limitation in the present study due to the effective interruption of viral replication consequent to potent anti-retroviral therapy. Second, telomerase is a ribonucleoprotein complex that can add hexameric repeats to telomeres. Previous studies have shown that telomerase activity is strongly upregulated after T cell activation (28, 32–34). Furthermore, HIV-1-infected individuals have normal telomerase activity in peripheral blood T cells (9, 11). We found that most patients do not have detectable increases in telomerase activity during the first month of HAART, and thus it is unlikely that telomerase activity accounts for the increase in telomere lengths that we have observed. However, we did find that telomere lengths were correlated with CD25 expression, and this would be consistent with telomerase-mediated lengthening of telomeres.

The central issue raised by our study concerns the nature of the differences between CD4<sup>+</sup> and CD8<sup>+</sup> T cell reconstitution. BrdU labeling studies in normal and SIV-infected macaques have shown similar CD4<sup>+</sup> and CD8<sup>+</sup> T cell turnover rates, and have also shown that infection with SIV induced comparable increases in the turnover rates of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (14, 15). In studies quantifying expression of the nuclear Ki-67 antigen as a surrogate marker for cell division, CD4<sup>+</sup> T cell proliferation was found to be increased two- to threefold in HIV-infected individuals with moderately advanced HIV infection (35, 36) but not in patients with early stage HIV infection (19). In contrast, the fraction of proliferating CD8<sup>+</sup> T cells in HIV infection in both early and later stage HIV infection was elevated approximately sixfold compared to control donors (19, 35, 36). Together, these studies are compatible with the previous studies showing that HIV infection was associated with decreased telomere length in CD8<sup>+</sup> T cells but not in CD4<sup>+</sup> T cells (9–11). Thus, on the one hand, patients begin ART with more extensive depletion of telomeres in the CD8 compartment, and simply the ART-mediated relief of the homeostatic stress would be predicted to lead to a more rapid normalization of the CD8<sup>+</sup> T cell telomere compartment. In addition, the rise in mean TRF length in the CD8<sup>+</sup> T cell compartment correlated inversely with the number of circulating CD8 memory cells, consistent with a negative selection in the periphery for CD8<sup>+</sup> T cells with a less extensive replicative history.

In contrast to the relative homogeneity of the response in the CD8 compartment, the telomere response of the CD4 compartment was characterized by wide individual heterogeneity as was shown in Fig. 2. Other studies have also noted high interindividual variability in CD4 cell growth and turnover rates compared to a uniformly high CD8 turnover rate in HIV-infected individuals as assessed by Ki-67 antigen expression (19, 35). There are other indications that the population dynamics of the CD4 and CD8 compartments differ.

TABLE 3

Correlations of CD4 and CD8 Cell Telomere Length and T Cell Subsets

	CD4 T cells				CD8 T cells			
	CD62L <sup>+</sup> RA <sup>+</sup>	CD28 <sup>+</sup>	CD45 RO <sup>+</sup> RA <sup>-</sup>	CD25 <sup>+</sup>	CD62L <sup>+</sup> RA <sup>+</sup>	CD28 <sup>+</sup>	CD45 RO <sup>+</sup> RA <sup>-</sup>	CD25 <sup>+</sup>
	<i>P</i> value <sup>a</sup>							
Percent	0.003	<0.001	-0.037	0.003	0.41	-0.39	-0.20	0.01
Absolute number	0.01	0.14	-0.16	0.07	0.14	-0.29	-0.06	0.05

<sup>a</sup> *P* value for global one-sided test of zero versus a positive or negative correlation of CD4 or CD8 T cell telomere length and the indicated CD4 or CD8 T cell subset for all weeks. Correlations are shown for both absolute number and for percentage of cells with a given immunophenotype. Correlations were tested at baseline and at weeks 12, 24, and 48 after starting combination therapy. For positive correlations a small *P* value indicates that when a person's T cell phenotype percentage is greater than the mean of all subjects at that time point, then that subject's telomere length tends to be longer than the mean of all patients at that same time point.

For example, the regenerative capacity of the adult CD4<sup>+</sup> T cell repertoire is limited (37, 38). Furthermore, following chemotherapy or marrow transplantation, CD8<sup>+</sup> T cell regeneration occurs much earlier than CD4 recovery (39). Mackall and co-workers have summarized evidence that the regeneration of CD4<sup>+</sup> and CD8<sup>+</sup> T cell compartments is distinct (40). Most notable is the observation that CD4 cell recovery is associated with thymic enlargement while CD8 T cell recovery is not (41). McCune and co-workers found that the number of circulating naive CD4<sup>+</sup> T cells correlates with patients who have more abundant thymic mass (42). Thus, it is possible that the large individual variations in CD4 T cell telomere length changes might be relevant to individual differences in disease progression and the response to ART.

Reverse transcriptase inhibitors have been shown to have effects on telomeres and telomerase. For example, zidovudine has been shown to incorporate into telomeric DNA and to cause telomere shortening in HeLa cells at high concentrations (43). In human leukemic cell lines, zidovudine caused progressive telomere shortening in some cultures and inhibited telomerase activity (44). In cultures of primary human T cells, we have been unable to detect inhibition of telomerase activity or induction of telomere shortening by zidovudine (SK, unpublished). Thus, reverse transcriptase inhibitors are unlikely to have caused the increase in telomere length that we observed following the start of potent ART and it remains unclear if they might contribute to the telomere shortening observed in HIV-infected patients.

Uncovering of the mechanism underlying the wide disparity in CD4 cell telomere length changes following potent ART will require further study. Given that we found that increases in TRF length correlated with increases in naive (CD4<sup>+</sup>45RA<sup>+</sup>62L<sup>+</sup>) cells, a marker of thymic-derived T cells in CD4 cells but not CD8 T cells, it is possible that thymic regenerative capacity will correlate with recovery of naive CD4 T cells having a more extensive replicative capacity. Recent studies of T cells containing excisional DNA products of TCR-gene rearrangement provide a measurement of thymic output, and these studies suggest substantial heterogeneity of thymic function in patients with HIV infection (45). Autopsy studies also indicate substantial thymic pathology in patients with HIV-1 infection (46). In addition we found that CD4 telomere lengths correlated inversely with CD4 memory cells, similar to CD8 cells. Together, our data are in broad agreement with the Red Queen Model recently put forward by Haase and co-workers (36), which states that T cells, and particularly CD4 T cell replacement mechanisms, normally operate in adults at close to maximum capacity just to maintain steady state. Our results add to this model by suggesting that CD8 T cells are able to more

rapidly catch up after perturbation of the steady state by HIV and that a thymic component to CD4 cell renewal is more active in some individuals than in others. In summary, the present results show that potent ART therapy has marked effects on composition of the T cell compartment. However, the differential recovery rates of T cell compartments suggest that CD4 and CD8 T cell reconstitution are regulated by distinct mechanisms.

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