Progressive Telomere Shortening of Epstein-Barr Virus–Specific Memory T Cells during HIV Infection: Contributor to Exhaustion?

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Individuals infected with human immunodeficiency virus (HIV) have low numbers of functional Epstein-Barr virus (EBV)–specific CD8$^+$ T cells in the face of a high EBV load, suggesting that these cells have become exhausted. We investigated whether the observed chronic EBV loads during HIV infection could cause exhaustion of EBV-specific T cells by using flow-FISH (flow cytometry in combination with fluorescence in situ hybridization) to analyze the telomere length of EBV-specific CD8$^+$ T cells. Enhanced telomere shortening of EBV-specific T cells was observed during HIV infection, compared with the decline in telomere length observed in the CD8$^+$ T cells of healthy subjects. Thus, chronic exposure to high antigen levels may lead to the progressive shortening of telomeres of antigen-specific T cells, which may impair viral control.

Adequate numbers of antiviral T cells are essential to maintain immunological memory. However, excessive numbers of cell divisions may result in the generalized age-related decline in T cell responses [1]. Phenotypic changes have been shown to occur within the memory T cell pool during aging. Furthermore, memory CD8$^+$ T cells may lose their replicative capacity as a result of telomere loss, which may restrict their function or persistence during aging [2]. Although telomeres do not shorten during excessive proliferation in acute infections [3, 4] because of up-regulation of the enzyme telomerase, the ability of T cells to up-regulate telomerase activity progressively decreases on repeated stimulation [1], resulting in telomere shortening in virus-specific CD8$^+$ T cells after acute infection. Telomere erosion of memory CD8$^+$ T cells, especially those specific for persistent antigens, may eventually restrict the capacity of these cells to expand.

Telomere erosion may occur in several clinical situations. Patients with X-linked lymphoproliferative syndrome (XLP) experience uncontrolled polyclonal expansion of both T and B cells, usually in response to acute Epstein-Barr virus (EBV) infection. The excessive CD8$^+$ T cell proliferation was shown to lead to end-stage differentiation and loss of functional EBV-specific CD8$^+$ T cells through replicative senescence [5], which may contribute to defective immunity in patients with XLP. Another situation in which EBV infection is not controlled optimally is during HIV infection. During the course of HIV infection, CD8$^+$ T cells increasingly become end-stage effector cells, similar to T cells in elderly persons [6, 7]. Interestingly, HIV-infected individuals have low numbers of functional EBV-specific CD8$^+$ T cells [8] in the face of a high EBV load [9], suggesting that these T cells have become exhausted due to chronic exposure to EBV. Therefore, we investigated whether continuous exposure to high levels of EBV during HIV infection may cause increased telomere shortening of EBV-specific T cells.

Methods. To study the telomere length of EBV-specific T cells, we selected 4 of 7 HIV-negative blood bank donors (age range, 38–53 years) and 5 of 13 HIV-positive individuals (age range, 36–44 years) from the Amsterdam Cohort Studies of HIV infection, all of whom carried the HLA-B8 allele and had substantial numbers of EBV-specific T cells. Procedures followed were in accordance with the institutional medical ethical committee and with the Helsinki Declaration of 1975, as revised in 1983. Written informed consent was obtained from the subjects at inclusion in the Amsterdam Cohort Studies. The HIV-positive individuals were compared 1 and 8 years (median follow-up, 7.3 years) after HIV seroconversion. After this period, 4 of the individuals progressed to AIDS. Data from the HIV-positive individuals over time were related to data from an additional group of healthy control subjects ($n = 10$; age range, 0–65 years).

For determination of EBV load, genomic DNA from 2×10^5 cells was amplified in duplicate using polymerase chain reaction (PCR) primers specific for the nonglycosylated membrane protein BNRF1 p143. Real-time PCR amplification was performed

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using a fluorogenic probe to detect the 74-bp product. As a control for the input DNA, the amount of β-albumin DNA was also determined [9].

Major histocompatibility complex (MHC) class I tetramers complexed with EBV peptides were produced. The peptides used (synthesized by solid-phase methods using an automated multiple peptide synthesizer and 9-fluorenylmethoxycarbonyl chemistry) were 2 immunodominant epitopes restricted through HLA-B8: RAKFKQLL (B8-RAK) from the lytic cycle antigen BZLF-1 and FLRGRAYGL (B8-FLR) from the EBV latent antigen EBNA-3A [10]. We included both of these epitopes because they are presented during different phases of the EBV life cycle. Biotinylated class I peptide complexes were tetramerized by addition of cyanine 5 (Cy5)–conjugated streptavidin, in addition to the "conventional" allophycocyanin (APC)– or phycoerythrin (PE)–conjugated streptavidin. Staining using Cy5-labeled tetramers was compared with that using the APC- or PE-labeled tetramers and was found to give similar results (data not shown).

The telomere length of specific $CD8^+$ T cells was measured using a 2-color flow-FISH (flow cytometry in combination with fluorescence in situ hybridization) assay, as described elsewhere [4]. Briefly, peripheral blood mononuclear cells (PBMCs) were thawed, and 1.5×10^6 cells were stained with MHC class I tetramers containing a Cy5 label, after which cells were washed in PBS and fixed with bis(sulfosuccinimidyl)suberate (BS^3). After being washed in hybridization buffer, cells were incubated with the peptide nucleic acid telomeric $(C_3TA_2)_3$ probe conjugated to fluorescein isothiocyanate. Samples were heated for 10 min at 82°C, rapidly cooled on ice, and hybridized for 1 h at room temperature in the dark. Cells were washed, and at least 250,000 events were acquired using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences). Cy5-tetramer staining of $CD8^+$ T cells was confirmed using parallel staining with the APC- or PE-labeled tetramers in conjunction with CD8 (BD Biosciences).

In vitro T cell proliferation was measured using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes), in accordance with the manufacturer's protocol. Briefly, PBMCs were thawed, cells were labeled with CFSE for 8 min, and labeling was stopped using human pool serum. Cells were washed, and 2×10^6 cells in 0.5 mL of RPMI 1640 medium were aliquoted in round-bottom tubes. Cells were then stimulated with either RAK or FLR peptide (concentration, 1 μg/mL). As a positive control, 0.02 μg of anti-CD3 (Sanquin Reagents) and 2 μg of anti-CD28 (Sanquin Reagents) was used. After 6 days of incubation at 37°C and 5% CO_2, cells were stained using anti–CD8-APC and anti–CD3-PerCP (BD Biosciences). The percentage of $CD3^+CD8^+$ T cells that underwent at least 1 division was calculated by subtracting the proliferation in the absence of stimulus. Stimulation indices (SIs) were calculated by dividing the percentage of proliferation for $CD3^+CD8^+$ T cells after stimulation by the percentage of proliferation for unstimulated cells. In EBV-positive healthy control individuals, $CD8^+$ T cells showed proliferation on EBV peptide stimulation, with a median SI of 4 (data not shown).

Results and discussion. To investigate whether the observed chronic exposure to high levels of EBV during HIV infection may cause increased telomere shortening of EBV-specific T cells, we analyzed telomere lengths of both EBV lytic (peptide **RAK**FKQLL) and EBV latent (peptide **FLR**GRAYGL) antigen–specific $CD8^+$ T cells in HLA-B8–positive individuals. To this end, we performed staining of specific T cells with HLA-B8 tetrameric complexes containing the EBV peptides in combination with flow cytometry analysis of telomere length using in situ hybridization with fluorescent telomere probes (flow-FISH) [4]. Telomere lengths of EBV-specific T cells in HIV-negative ($n = 4$) and HIV-positive individuals ($n = 5$) were compared 1 and 8 years (median follow-up, 7.3 years) after HIV infection. EBV loads were elevated at these time points during HIV infection, varying from a median of 15,648 copies/1×10^6 PBMCs at the early time point to 5000 EBV copies/1×10^6 PBMCs at the late time point (data not shown).

T cells specific for lytic antigen RAK, as identified by HLA-B8 tetrameric complexes labeled with Cy5, were always more abundantly present (range, 0.11%–1.24% tetramer-positive T cells) than those specific for latent antigen FLR (range, 0.15%–0.57% tetramer-positive T cells), as analyzed in a larger group of HIV-negative ($n = 7$) and HIV-positive ($n = 13$) individuals (figure 1*A*). Subsequently, telomeric probe fluorescence was analyzed within the RAK- and FLR-specific T cell populations in 4 HIV-negative and 5 HIV-positive individuals with substantial numbers of both RAK- and FLR-specific T cells (>0.4%). Figure 1*B* shows telomeric probe fluorescence of EBV-specific $CD8^+$ T cells in a representative HIV-infected patient at early and late time points during HIV infection. Although $CD8^+$ T cells specific for the RAK peptide increased from 0.48% to 0.68% (figure 1*B*) and those for the FLR peptide increased from 0.40% to 0.63% (figure 1*B*), telomeric probe median fluorescence intensity (MFI) decreased both for RAK-specific $CD8^+$ T cells, from 119.71 to 93.08 (figure 1*B*), and for FLR-specific $CD8^+$ T cells, from 115.48 to 100 (figure 1*B*). Overall, RAK-specific T cells had shorter telomeres than did FLR-specific T cells (figure 1*C*), in both HIV-negative (MFI, 89.85 vs. 104.64) and HIV-positive individuals (MFI, 88.19 vs. 101.83 early), a difference that was significant late during HIV infection (MFI, 77.74 vs. 93.06; $P = .042$, Mann-Whitney *U* test) (figure 1*C*). This finding suggests that lytic antigen–specific T cells underwent more proliferation cycles, and it fits with the described phenotype of EBV-specific T cells showing lytic antigen–specific T cells to be more differentiated than latent antigen–specific T cells in healthy carriers [10].

Interestingly, further telomere shortening of both lytic and latent antigen–specific T cells was observed during HIV infec-

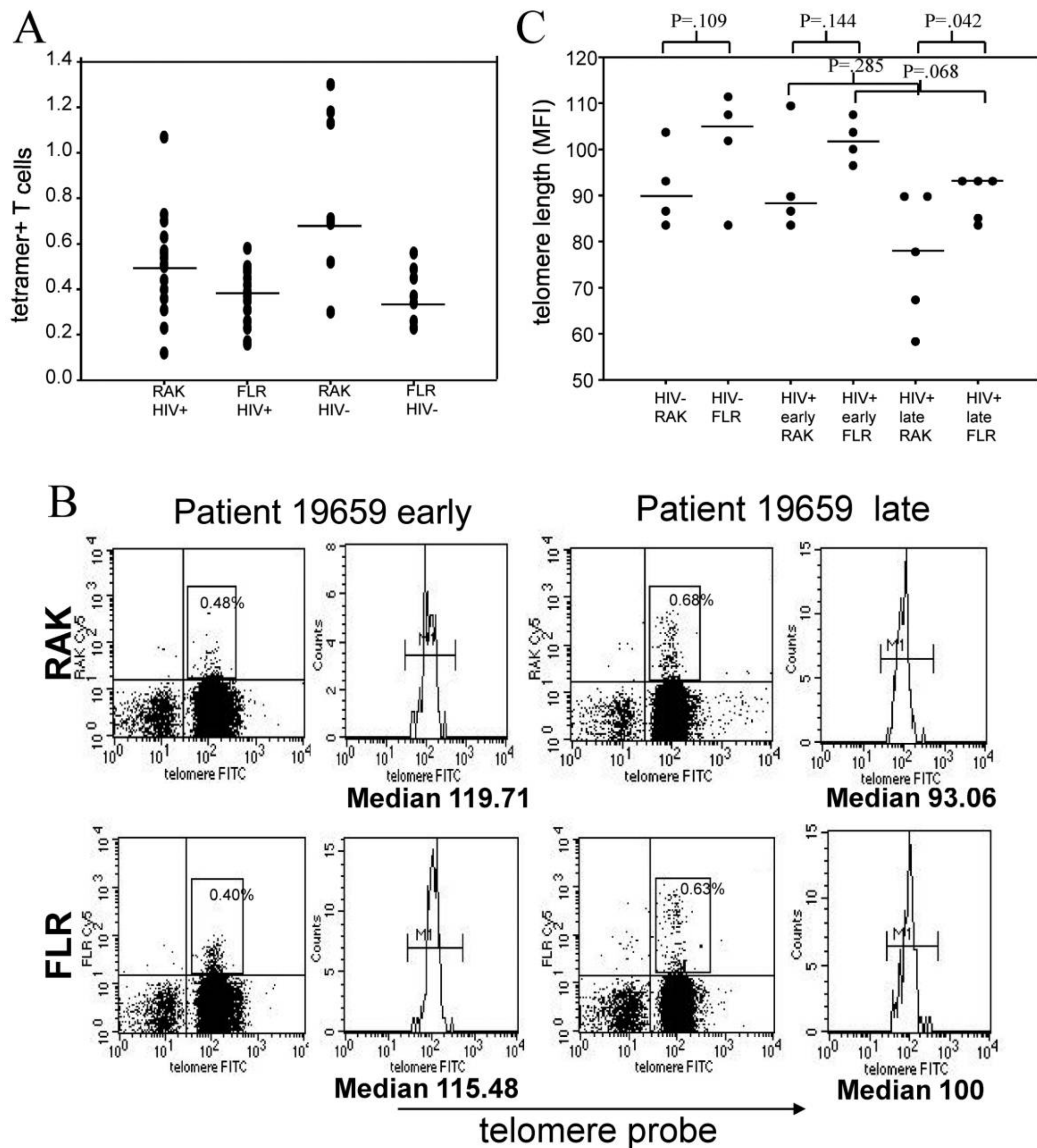

Figure 1. Analysis of telomeric probe fluorescence of Epstein-Barr virus (EBV)–specific T cells in HIV-negative and HIV-positive individuals. *A,* Percentages of lytic antigen RAK– and latent antigen FLR–specific $CD8^+$ T cells in 7 HIV-negative and 13 HIV-positive HLA-B8–positive individuals, as measured by flow cytometry analysis of staining with cyanine 5 (Cy5)–labeled HLA-B8 tetrameric complexes containing either the RAK or FLR peptide. *B,* Flow cytometry analysis of telomere probe fluorescence in tetramer-positive T cells for 1 HIV-infected individual early *(left panels)* and late *(right panels)* during HIV infection. RAK-specific *(upper panels)* and FLR-specific *(lower panels)* T cells are shown in dot plots, and median fluorescence intensity (MFI) is given as histograms of the tetramer-positive/telomere-positive population *(upper right quadrants of dot plots). C,* Telomere probe MFI after telomere probe hybridization as a measure of the telomere lengths of lytic antigen (RAK)–specific and latent antigen (FLR)–specific $CD8^+$ T cells in HIV-negative healthy control subjects ($n = 4$) and HIV-positive individuals early ($n = 4$; 1 year after seroconversion) and late ($n = 5$; median follow-up, 7.3 years) during HIV infection. Cells were stained with Cy5-labeled HLA-B8 tetrameric complexes containing 1 of the 2 specific peptides (RAK and FLR) in combination with flow cytometry analysis of telomere length using in situ hybridization with fluorescent (fluorescein isothiocyanate [FITC]–labeled) telomere probes (flow-FISH), and the MFI of the telomere probes within the HLA-B8-RAK– or HLA-B8-FLR–specific T cells was determined. *P* values are indicated.

tion. The telomeric probe MFI of RAK-specific T cells decreased from 88.19 to 77.74 (figure 1*C*). In the case of FLR-specific $CD8^+$ T cells, the tendency to have shorter telomeres in late HIV infection (telomere probe MFI, 93.06) than in early infection (MFI, 101.83) was most striking ($P = .068$, Wilcoxon test), suggesting that telomere shortening of EBV-specific T cells can occur

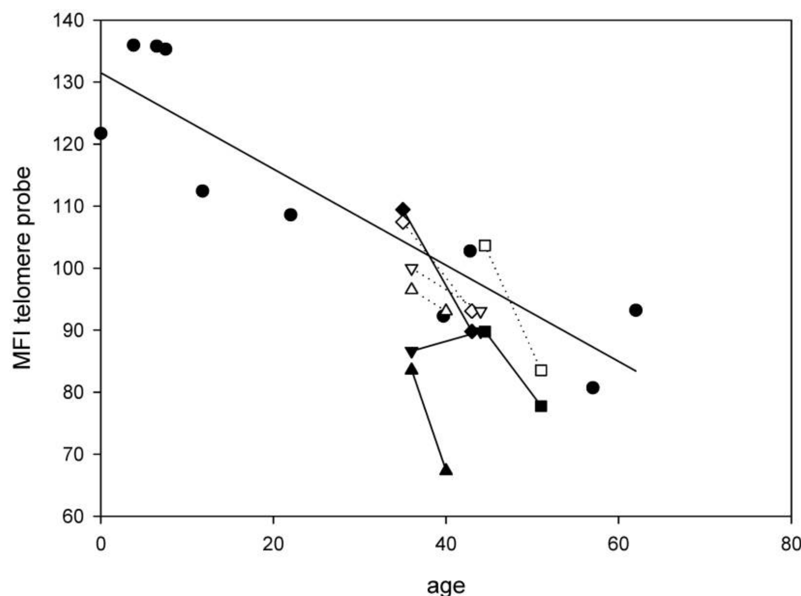

Figure 2. Telomeric probe fluorescence of Epstein-Barr virus–specific T cells in HIV-negative vs. HIV-positive individuals over time. The median fluorescence intensity (MFI) related to age (in years) is shown for $CD8^+$ T cells from a group of healthy control subjects *(black circles)* and, longitudinally, for lytic antigen RAK–specific T cells *(other black symbols)* and latent antigen FLR–specific T cells *(white symbols)* from 4 HIV-infected subjects. Three of 4 HIV-infected individuals showed a steeper decline in telomere fluorescence of RAK-specific T cells *(upright triangles)* or both RAK- and FLR-specific T cells *(squares and diamonds)*.

within 7 years of HIV infection (figure 1*C*). In line with this finding, we did not observe any proliferative capacity of EBV-specific T cells late in HIV infection, as measured using a CFSE dye dilution assay, and we observed it in only 1 of the HIV-infected individuals at the early time point during HIV infection (SI, 6), whereas T cells did proliferate on anti-CD3 stimulation (SI, 5) (data not shown); this individual did not progress to AIDS.

To relate these data on telomere length to the normal rate of decline in telomere length with age, we analyzed the telomeric probe fluorescence of $CD8^+$ T cells in a group of healthy donors ($n = 10$) with ages ranging from 0 (cord blood) to 80 years. Figure 2 shows that telomeric probe MFI decreases by 7.86 per 10 years. From this curve, the "normal" decline in MFI was calculated to be 5.7 over a period of 7.3 years (the median follow-up for our HIV-positive individuals) (figure 2). The decline in MFI observed for FLR- and RAK-specific T cells was ~2-fold greater in 3 of 4 HIV-infected individuals (figure 2), with median declines of 8.77 and 10.45, respectively. This indicates that telomere shortening of EBV-specific T cells occurs at a faster rate than expected in HIV-infected individuals and may explain the observed functional and phenotypic features of $CD8^+$ T cells during HIV infection.

HIV-infected individuals (30–40 years of age) resemble elderly individuals (>80 years of age) with respect to a lack of CD28 and CD27 expression on $CD8^+$ T cells, and $CD28^-$ T cells from these HIV-positive persons have telomere lengths identical to those of centenarians [1]. This suggests that $CD8^+$ T cells during HIV infection have undergone considerable proliferation, comparable to those in elderly persons. Moreover, a longitudinal study in young HIV-infected individuals showed a decrease in telomere length in $CD8^+$ T cells over time [11].

Functional defects that have been described in HIV-positive individuals include reduced numbers of cytokine-producing T cells upon antigen stimulation late in infection. Furthermore, only T cells from nonprogressors were shown to maintain high proliferative capacity, which was shown to be coupled with increases in perforin expression [12], suggesting that proliferation is required for obtaining efficient effector T cells. Thus, diminished proliferative capacity leads to a memory T cell population that is less able to control infections because of lower effector functions.

EBV-specific T cells have been shown to be important for control of latent EBV infection and to protect against uncontrolled lymphoproliferation of infected B cells and lymphoma development [8, 13]. In both elderly individuals and patients suffering from XLP, EBV-specific T cells show reduced functionality associated with enhanced telomere erosion in the latter group [5]. We now show that EBV-specific T cells during HIV infection also show progressive telomere shortening. Decreased numbers of interferon (IFN)–γ–producing EBV-specific T cells upon antigen stimulation in these HIV-infected individuals [8] resemble lower EBV-specific responses (IFN-γ) in elderly people. This suggests that, with respect to EBV-specific T cell responses, HIV-positive individuals seem to display a more "se-

nescent" T cell phenotype, which may be explained by the persistently high EBV DNA levels found in these individuals [9].

In conclusion, during chronic antigen stimulation, such as during HIV infection, antigen-specific T cells may progressively shorten their telomeres due to frequent activation through increased rates of virus exposure. Although the role played by lytic antigen–specific T cells is still unclear, latent antigen–specific T cells have been shown to be important for control of EBV infection [14, 15]. This suggests that loss of proliferative potential due to telomere shortening, potentially leading to exhaustion of these cells, may impair EBV control and may predispose these individuals to EBV-related lymphomas.

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