MAJOR ARTICLE

Mitochondrial DNA Decline in T Cells of HIV-1 Seroconverters May Be Dependent on Immune Activation

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Background. Earlier reports have indicated that human immunodeficiency virus type 1 (HIV-1) infection itself might cause mitochondrial DNA (mtDNA) decline in peripheral blood mononuclear cells (PBMCs). However, the mtDNA dynamics within this heterogeneous cell population during HIV-1 infection are not fully understood.

Methods. mtDNA content was assessed longitudinally in PBMCs and in isolated $CD4^+$ and $CD8^+$ T cells from 16 documented HIV-1 seroconverters who were naive to antiretroviral therapy. The correlation between the mtDNA content of $CD4^+$ and $CD8^+$ T cells and their immunologically activated proportion was studied. Additionally, mtDNA content was measured within isolated activated and nonactivated $CD4^+$ and $CD8^+$ T cells obtained from 5 antiretroviral-naive men with chronic HIV-1 infection.

Results. In the seroconverter group, mtDNA content in $CD8^+$ T cells decreased 5 years after seroconversion ($P = .007$). mtDNA content in either $CD4^+$ or $CD8^+$ T cells did not correlate with the proportion of activated cells within either population. However, for the chronically infected men, mtDNA content in activated $CD8^+$ T cells was lower than that in nonactivated cells ($P = .043$). A similar trend was observed in the $CD4^+$ T cell fraction.

Conclusions. These findings indicate that HIV-1 infection affects mtDNA content, particularly in the most immunologically activated cells. Furthermore, the importance of measuring mtDNA in specific cell fractions rather than in the heterogeneous PBMC population is emphasized.

Highly active antiretroviral therapy (HAART) has altered the course of HIV-1 infection by significantly reducing its related morbidity and mortality. In spite of this, HAART and nucleoside reverse-transcriptase inhibitors (NRTIs) in particular have been associated with the occurrence of adverse effects, including peripheral neuropathy [1, 2], myopathy, lactic acidosis, hyperlactatemia, and lipoatrophy [3, 4]. Mitochondrial toxicity has been suggested as one of the possible causal mechanisms underlying the development of several of the above-mentioned complications, mainly because of the inhibitory effect that NRTIs have on mtDNA polymerase γ (Pol γ), the enzyme needed for mtDNA replication [5, 6]. According to the mitochondrial toxicity hypothesis, the inhibition of Pol γ may cause a decline in mtDNA content, leading to mitochondrial dysfunction [7]. For this reason, several research groups, including our own, have been investigating mtDNA as a possible marker for the assessment of mitochondrial toxicity and related adverse events caused by NRTIs. However, over the years it has become clear that mitochondria are affected not only by NRTIs but also by HIV-1 infection itself [8, 9].

We recently performed a longitudinal study that assessed mtDNA content in peripheral blood mononuclear cells (PBMCs) from 36 documented, antiretroviral-naive seroconverters of the Amsterdam Cohort Study in homosexual men [10]. We found that mtDNA content in PBMCs had declined 1 year after seroconversion and showed a nonsignificant decrease 5 years after seroconversion. These results, in addition to those of several cross-sectional studies, suggest that HIV-1

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infection itself is associated with a decline in mtDNA content in PBMCs [11–13].

Interesting results were obtained in a recent study by Mussini et al. [14], in which mtDNA was measured in highly purified $CD4^+$ and $CD8^+$ T cells from patients undergoing structured treatment interruption. These patients were followed up for 48 weeks. Results showed that mtDNA dynamics differed in the 2 cell fractions. mtDNA content in $CD8^+$ T cells was reported to increase 6 months after therapy discontinuation, whereas no change occurred in the $CD4^+$ T cell fraction [14]. These observations, together with the consideration that the effects of HIV-1 infection and immune activation on mtDNA dynamics are unknown, led us to perform the present study.

Our aim was to assess the effect of HIV-1 infection itself as well as the impact of HIV-1–induced immune activation on mtDNA content separately in both $CD4^+$ and $CD8^+$ T cells. To address this issue, we performed a longitudinal study whereby we measured mtDNA content in PBMCs and in isolated $CD4^+$ and $CD8^+$ T cell fractions obtained from a group of documented HIV-1 seroconverters. We then evaluated the possible correlations between immune activation markers and the mtDNA content in the different cell populations. These immune activation data regarding both activated and nonactivated $CD4^+$ and $CD8^+$ T cells had been previously generated from these same seroconverters by Hazenberg et al. [15]. Unfortunately, because of the insufficient availability of stored samples from these seroconverters at the time of the present study, we could not measure the mtDNA content separately in activated and nonactivated $CD4^+$ and $CD8^+$ T cells. It was for this reason that we decided to perform an additional cross-sectional study in 5 different patients who were chronically infected with HIV-1 and were naive to antiretroviral therapy. The specific aim of this additional study was to assess mtDNA content in separated activated and nonactivated $CD4^+$ and $CD8^+$ T cell fractions isolated from freshly obtained PBMCs from these 5 patients.

PATIENTS, MATERIALS, AND METHODS

Patient-sample collection. A serial collection of PBMC samples from 16 individuals with documented HIV-1 seroconversion who were naive to antiretroviral therapy was obtained from the central repository laboratory. Sample selection was based on the availability of cryopreserved PBMC samples at the repository laboratory. These patients were homosexual men who had been enrolled in the Amsterdam Cohort Study. A description of the Amsterdam Cohort Study has been given elsewhere [16]. Available cryopreserved samples were selected from time points at a median of 5 years (interquartile range [IQR], 3–8 years) before seroconversion and at ~1 and ~5 years after seroconversion. The time point of seroconversion was set to zero.

For the additional study, another set of fresh samples was obtained from 5 HIV-1–infected men consecutively attending the HIV outpatient clinic of the Academic Medical Center (AMC) in Amsterdam and fulfilling the following criteria: naive to antiretroviral therapy, clearly detectable plasma HIV-1 RNA, and a $CD4^+$ T cell count between 200 and 500 cells/mm^3 on multiple occasions. These clinical criteria were used to try to avoid selecting patients with long-term nonprogressive HIV-1 infection. All patients provided written informed consent.

Sample preparation and isolation of $CD4^+$ and $CD8^+$ T cells. All patient PBMC samples were isolated from heparinized blood by means of ficoll-hypaque density-gradient centrifugation, after which cells were viably frozen by means of an automated freezing program and were cryopreserved in liquid nitrogen until further processing. After thawing and then washing to avoid platelet contamination [17], an aliquot equal to 1×10^5 cells of the seroconverter PBMCs was put in 0.9 mL of L6 (a lysis buffer that contains chaotropic guanidium thiocyanate [18]) and was stored at −80°C until subsequent DNA extraction. $CD4^+$ and $CD8^+$ T cells were purified from the remaining seroconverter PBMCs by magnetic bead separation by means of the MiniMACS multisort kit (Miltenyi Biotec), in accordance with the manufacturer's instructions. This procedure allows the avoidance of platelet contamination [19]. An equivalent aliquot of 1×10^5 cells of both $CD4^+$ and $CD8^+$ T cell fractions was put in 0.9 mL of L6 and stored at −80°C for later DNA extraction. Of note, these $CD4^+$ and $CD8^+$ T cell fractions were not separated into activated and nonactivated fractions.

Immune activation marker ($CD38^+HLA\text{-}DR^+$) expression for both $CD8^+$and $CD4^+$ T cells from the 16 selected seroconverters at the same sampling dates as those reported here had previously been measured by means of a FACSCalibur instrument (Becton, Dickinson and Company) in an earlier study by Hazenberg et al. [15]. Here, this immune activation data set was retrospectively related to the mtDNA quantification results obtained in the present study.

To be able to measure mtDNA separately within the activated ($CD38^+HLA\text{-}DR^+$) and nonactivated ($CD38^-HLA\text{-}DR^-$) $CD4^+$ and $CD8^+$ T cell populations, the cryopreserved PBMCs recently obtained from the 5 HIV-1–infected patients from the AMC HIV outpatient clinic were incubated with anti-CD38–fluorescein isothiocyanate, anti–HLA-DR–phycoerythrin (Becton, Dickinson and Company), anti-CD4–peridinin chlorophyll protein, and anti-CD8–allophycocyanin (Becton, Dickinson and Company) monoclonal antibodies. The specified cell fractions were purified by cell sorting on a FACSAria machine (Becton, Dickinson and Company). The purity of the sorted cells was >95%.

Mitochondrial nucleic acid assessment. An equivalent of 1×10^5 PBMCs, $CD8^+$ T cells, and $CD4^+$ T cells was used for a silica-based DNA-extraction method [18]. The mtDNA content of all samples was measured by means of a real-time, duplex, nucleic acid sequence–based amplification assay (Retina

Table 1. CD4⁺ and CD8⁺ T cell count data for the HIV-1 seroconverter group.

Variable	Before seroconversion (n = 12)	After seroconversion	
		1 year (n = 16)	5 years (n = 9)
CD4⁺ T cell count	925 (685–1175)	565 (393–895)[a]	550 (320–705)[a]
CD8⁺ T cell count	550 (300–775)	810 (562–1007)[a]	830 (585–1370)

NOTE. Data are median (interquartile range) cells/mm^3.

[a] $P < .05$, compared with the preceding time point.

Mitox; Primagen) [17]. The nuclear gene measured by the assay is the small nuclear RNA U1A gene, whereas the mtDNA measured stretches from the 3′ end of the ribosomal RNA 16S gene into the tRNA, just downstream of the mitochondrial transcription terminator [17]. An equivalent of 3×10^3 cells was used as assay input for each sample, for which both mtDNA and nuclear DNA (nDNA) were amplified simultaneously in an 1-tube reaction. The samples were assayed in duplicate, and the relative mean of each mtDNA:nDNA ratio was subsequently extrapolated from the calibration curve, to determine the mtDNA copy number per cell. The calibration range included in each assay run consisted of a dilution series of plasmid mixes containing both mtDNA and nDNA. mtDNA content is reported as the $\log_{10}$ copy number per cell [17].

Statistical methods. Our primary objective was to assess the effect of HIV-1 infection on mtDNA content in PBMCs and in isolated CD4⁺ and CD8⁺ T cell populations from the seroconverters and to determine any relationship between mtDNA content and the immune activation data that were previously generated from these individuals in the study by Hazenberg et al. [15]. mtDNA changes relevant to the different cell types as well as changes in CD4⁺ and CD8⁺ T cell counts assessed at the 3 available time points were analyzed by means of the nonparametric Wilcoxon signed-rank test. Spearman's correlation analysis was performed for the investigation of the association between mtDNA content in CD4⁺ and CD8⁺ T cells and immune activation markers in these cell populations. Finally, mtDNA content in isolated populations of activated ($CD38^+HLA\text{-}DR^+$) and nonactivated ($CD38^-HLA\text{-}DR^-$) CD4⁺ and CD8⁺ T cells from the 5 HIV-1–infected antiretroviral-naive men was analyzed by means of the nonparametric Wilcoxon signed-rank test. A logarithmic transformation was applied to all mtDNA data to obtain a normal distribution. Analyses were performed by means of SPSS for Windows (version 12.0.1; SPSS). The level of significance was set at $P < .05$ for all analyses.

RESULTS

Seroconverters. All 16 patients from the Amsterdam Cohort Study in homosexual men were naive to antiretroviral therapy throughout the study's observation period. All had samples

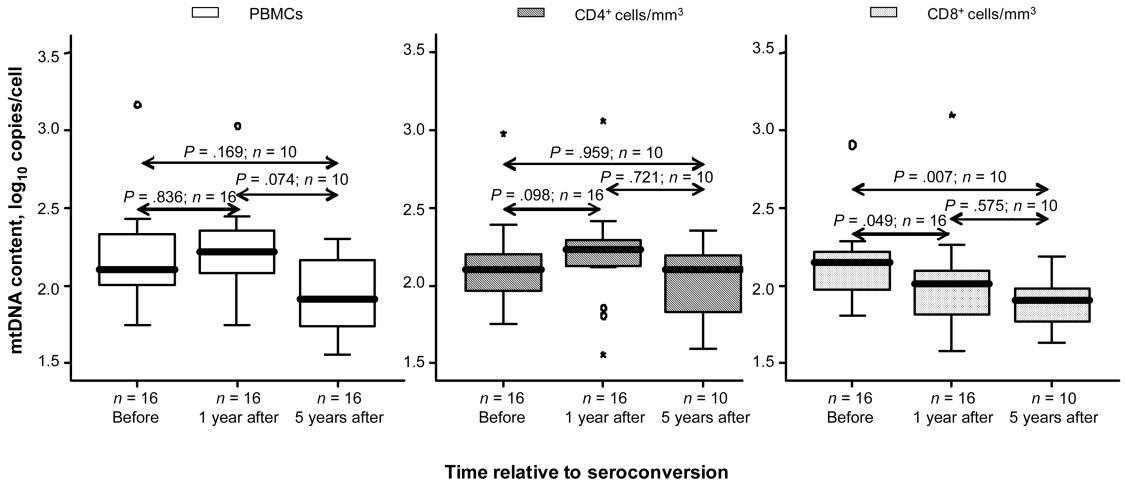

Figure 1. Box-whisker plots of logarithmically transformed mtDNA content values before seroconversion ($n = 16$ patients) and 1 and 5 years after seroconversion ($n = 16$ and $n = 10$ patients, respectively) in peripheral blood mononuclear cells (PBMCs), total CD4⁺ T cells, and total CD8⁺ T cells. The plots illustrate the median (middle line), the interquartile range (top and bottom of each box), and the largest and smallest nonoutlier observations (ends of the vertical lines). The small stars and circles represent the outliers.

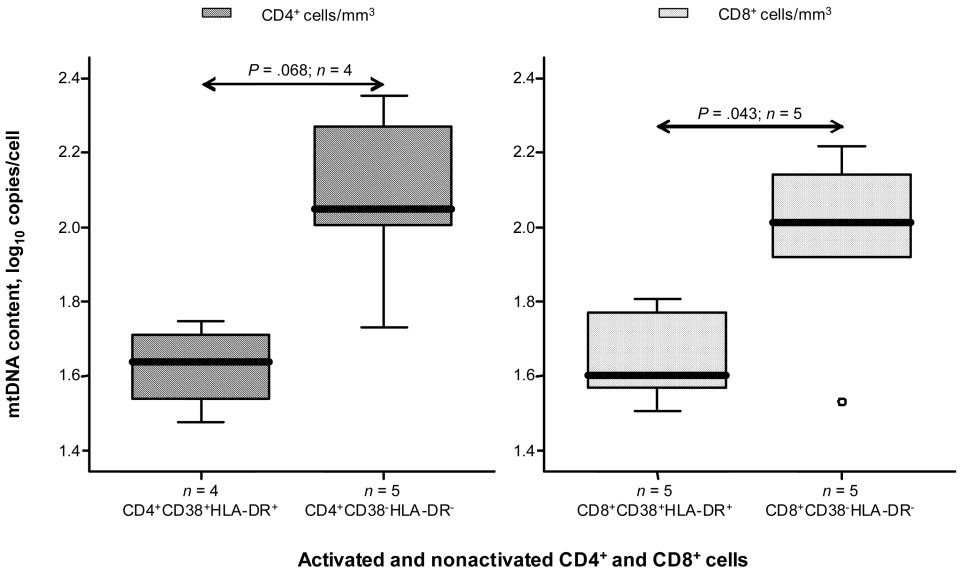

Figure 2. Box-whisker plots of logarithmically transformed mtDNA content values in activated ($CD38^+HLA\text{-}DR^+$) and nonactivated ($CD38^-HLA\text{-}DR^-$) $CD4^+$ and $CD8^+$ T cells from 5 antiretroviral-naive men with chronic HIV-1 infection. The plots illustrate the median (middle line), the interquartile range (top and bottom of each box), and the largest and smallest nonoutlier observations (ends of the vertical lines). The small circle represents an outlier.

available from both before and 1 year after seroconversion; however, at 5 years after seroconversion, samples were available from only 10 patients. Preseroconversion samples had been obtained at a median of 5 years (IQR, 3–8 years) before seroconversion. The median age at seroconversion was 41 years (IQR, 35–49 years).

$CD4^+$ and $CD8^+$ T cell counts from these individuals were retrospectively retrieved from the Amsterdam Cohort Study database. $CD4^+$ and $CD8^+$ T cell counts were not available for all patients before seroconversion and for 1 patient at 5 years after seroconversion. The median $CD4^+$ T cell count before seroconversion was 925 cells/mm^3 (IQR, 685–1175 cells/mm^3) ($n = 12$ patients). $CD4^+$ and $CD8^+$ T cell counts at the 3 time points analyzed are shown in table 1. From before seroconversion to 5 years after seroconversion, retrospective data analysis showed that the proportion of activated cells (i.e., $CD38^+$, $HLA\text{-}DR^+$, and $CD38^+HLA\text{-}DR^+$) had statistically significantly increased ($P < .05$) in both the $CD4^+$ and $CD8^+$ T cell populations, as has previously been reported for a larger group of seroconverters that included the individuals investigated in the present study [15].

mtDNA results for the seroconverters. Cross-sectional and longitudinal correlation analyses between mtDNA content in the $CD4^+$ and $CD8^+$ T cell populations and the corresponding cell counts did not yield any statistically significant results. The mtDNA content in PBMCs and in the $CD4^+$ T cell fraction was not statistically significantly different between before and after seroconversion (figure 1). In contrast, the mtDNA content decreased in the $CD8^+$ T cell fraction, from a median of 2.09 $\log_{10}$ copies/cell (IQR, 1.97–2.22 $\log_{10}$ copies/cell) before seroconversion to a median of 1.95 $\log_{10}$ copies/cell (IQR, 1.81–2.10 $\log_{10}$ copies/cell) 1 year after seroconversion ($P = .049$) and to a median of 1.85 $\log_{10}$ copies/cell (IQR, 1.77–2.00 $\log_{10}$ copies/cell) 5 years after seroconversion ($P = .007$) (figure 1). Correlation analysis between the proportion of immune-activated cells (i.e., $CD38^+$, $HLA\text{-}DR^+$, and $CD38^+HLA\text{-}DR^+$) in either $CD4^+$ or $CD8^+$ T cells (data retrospectively obtained from Hazenberg et al. [15]) and logarithmically transformed mtDNA content in the $CD4^+$ and $CD8^+$ T cell population did not show any correlation (data not shown).

HIV clinic patients. The median $CD4^+$ T cell count of the 5 HIV-1–infected patients from the AMC HIV outpatient clinic was 320 cells/mm^3 (IQR, 150–495 cells/mm^3), and the median $CD8^+$ T cell count was 730 cells/mm^3 (IQR, 595–1775 cells/mm^3). The median plasma HIV-1 load was 4.06 $\log_{10}$ copies/mL (IQR, 3.46–5.52 $\log_{10}$ copies/mL). The median age was 47 years (IQR, 35–56 years). The patients in this group had $CD4^+$ and $CD8^+$ T cell counts similar to those of the seroconverters.

mtDNA results for the HIV clinic patients. The mtDNA content in the activated $CD4^+$ T cell fraction from one of these patients could not be reliably assessed because of an insufficient cell yield after flow cytometry sorting. Cross-sectional analysis of the mtDNA content in the activated ($CD38^+HLA\text{-}DR^+$) and nonactivated ($CD38^-HLA\text{-}DR^-$) $CD4^+$ and $CD8^+$ T cells obtained from the 5 HIV-1-infected antiretroviral-naive men yielded the following results. The mtDNA content in the activated $CD8^+$ T cell fraction (median, 1.60 $\log_{10}$ copies/cell [IQR, 1.53–1.78 $\log_{10}$ copies/cell]) was statistically significantly

lower than that in the nonactivated $CD8^+$ T cell fraction (median, 2.01 $\log_{10}$ copies/cell [IQR, 1.76–2.18 $\log_{10}$ copies/cell]) ($P = .043$) (figure 2). A similar trend, although not statistically significant ($P = .068$), could be seen for the comparison between activated and nonactivated $CD4^+$ T cells (figure 2).

DISCUSSION

HIV-1 infection is known to induce chronic immune activation, which is characterized by increased CD38 and HLA-DR antigen expression in T lymphocytes [15] and increased T lymphocyte turnover [15, 20]. Although the precise mechanism still needs to be clarified, chronic immune activation has been reported to accelerate HIV-1 disease progression [21].

Here, we investigated the effect of HIV-1 infection itself and of related immune activation on mtDNA content in PBMCs as well as in isolated $CD4^+$ and $CD8^+$ T cells. Our longitudinal assessment in documented, antiretroviral-naive seroconverters shows that mtDNA content decreased in the $CD8^+$ T cell compartment but not in the $CD4^+$ T cell compartment. Specifically, we observed mtDNA decline 5 years after seroconversion—that is, during chronic HIV-1 infection. No correlation was found between mtDNA content measured in the pool of $CD4^+$ and $CD8^+$ T cells and the proportion of $CD4^+$ and $CD8^+$ T cells expressing immune activation markers (CD38, HLA-DR, or both [i.e., $CD38^+HLA\text{-}DR^+$ cells]). Of note, however, is the fact that mtDNA content had not been measured separately in activated versus nonactivated $CD4^+$ and $CD8^+$ T cells.

As already mentioned, the $CD4^+$ and $CD8^+$ T cell pool has been reported to undergo considerable expansion and differentiation during chronic immune activation, and the T cell dynamics differ according to the activation state within these cell subsets [15, 22], leading to a changed metabolism [23]. That mitochondria play a key role in cellular metabolism led us to hypothesize that changes in the T cell activation state induced by HIV infection could affect mitochondria and possibly mtDNA content. We therefore proceeded to prospectively sample 5 additional HIV-1–infected patients to assess mtDNA content separately in activated and nonactivated subsets of $CD4^+$ and $CD8^+$ T cells.

A decrease in the mtDNA content in the activated ($CD38^+HLA\text{-}DR^+$) $CD8^+$ T cell fraction of these patients was found in comparison with the nonactivated ($CD38^-HLA\text{-}DR^-$) $CD8^+$ T cells. A similar trend could be seen in the $CD4^+$ T cell fraction, although it did not reach statistical significance ($P = .068$) (figure 2). This may be ascribed to the fact that, during chronic immune activation, the population of activated $CD4^+$ T cells expands to a lesser extent than the population of $CD8^+$ T cells [15]. Another factor that could be relevant was the somewhat smaller number of patients in whom we could assess activated $CD4^+$ T cells.

Several studies have reported a change in mtDNA content in PBMCs of HIV-infected antiretroviral-naive patients [11–13, 16, 24]. In the present study, in contrast to our previous longitudinal study, we did not observe a statistically significant decrease in mtDNA content in PBMCs. This may well have been caused by the lower number of samples analyzed in the present study, which may have resulted in low statistical power. Our present findings suggest that the decreases reported earlier in PBMCs may at least partly be driven by changes occurring in cell fractions that are affected most by immune activation. Hypothetically, the resulting elevated T cell activation and division could disrupt mtDNA replication as an indirect effect of mitochondrial hyperpolarization during chronic T cell activation [25]. Mitochondrial hyperpolarization is accompanied by increased reactive oxygen species production, which could lead to mtDNA depletion [26] and eventually result in mitochondrial dysfunction.

In conclusion, our results suggest that HIV-1–induced chronic immune activation may be responsible for a decrease in mtDNA content in $CD8^+$ T cells and, more specifically, in the activated ($CD38^+HLA\text{-}DR^+$) subfraction. Because blood is the most easily obtainable tissue, PBMCs have been widely assessed to study mitochondrial toxicity in the context of HIV-1 infection and antiretroviral therapy. Our study shows one of the limitations of such an approach and emphasizes the importance of taking into account not only differences between blood cell fractions but also their immune activation state.

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