Real-Time Nucleic Acid Sequence–Based Amplification Assay to Quantify Changes in Mitochondrial DNA Concentrations in Cell Cultures and Blood Cells from HIV-Infected Patients Receiving Antiviral Therapy

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Background: To study the clinical relevance of changes in mitochondrial DNA (mtDNA) in peripheral blood mononuclear cells (PBMCs) attributable to HIV infection and/or combination antiretroviral therapy (cART), a high-throughput molecular assay to quantify mtDNA is required.

Methods: We developed a quantitative real-time duplex nucleic acid sequence–based amplification assay in which both mtDNA and nuclear DNA are simultaneously amplified in 1 tube. The assay could accurately quantify mtDNA in a range of 15–1500 copies of mtDNA per 2 genomic copies with an intrarun variation of 11% and an interrun variation of 16%. We compared this real-time assay the lactate/pyruvate ratios in fibroblasts incubated with glucose and exposed to zalcitabine. Additionally, we studied the effects of platelet contamination and the in vivo effects of cART on mtDNA in PBMCs from a small group of patients.

Results: Decreases in mtDNA preceded the increase in lactate/pyruvate ratios and vice versa when zalcitabine was eliminated from the culture. Platelets affected the mtDNA in PBMCs if >5 platelets per PBMC were present. Within 12 weeks, mtDNA increased and remained increased in PBMCs from patients on continuous treatment with zidovudine/lamivudine/indinavir therapy (P = 0.03), but increased if patients were switched to stavudine/didanosine therapy (P = 0.008).

Conclusions: After drug exposure, the mtDNA assay can detect changes in mtDNA concentrations in cell lines and PBMCs, when properly controlled for platelet effects, earlier than traditional assays.

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Nucleoside reverse transcriptase inhibitors (NRTIs) inhibit, in addition to HIV-1 reverse transcriptase, the DNA polymerase-γ that is located in the mitochondria. These NRTIs form the backbone of most currently used combination antiretroviral therapies and have changed HIV infection to a chronic, manageable disease. However, long-term treatment with combination antiretroviral therapy often leads to adverse effects such as development of insulin resistance, lactic acidosis, and hyperlactatemia; cardiomyopathy; pancreatitis; peripheral neuropathy; increased lipids; and lipodystrophy (1). It has been hypothesized that inhibition of DNA polymerase-γ by NRTIs and the consequent depletion of mitochondrial DNA (mtDNA) are, at least in part, responsible for the onset of these clinical adverse events (2). To confirm this hypothesis, clinical studies should be performed, which require accurate assays that assess the mtDNA content in the cells of the body and can detect relatively small changes. To
facilitate these studies and the routine screening of the effects of antiviral drugs on cells of patients, the cells should preferentially be taken from easy accessible compartments of the body, for example, blood cells. Several clinical studies in this field have been conducted but without a consistent outcome. The effect of HIV infection itself on the mtDNA concentrations in various tissues from the body is not uniform: the results of both cross-sectional studies (3, 4) and a longitudinal study (5) have indicated a decrease in mtDNA in peripheral blood mononuclear cells (PBMCs) attributable solely to the HIV infection itself, whereas the authors of other studies have not been able to repeat these findings (6). Controversy between the correlation of mtDNA depletion and the development of clinically adverse events can also be found in the literature. Some studies have found relationships between mtDNA depletion and the development of clinically adverse events (3, 7–11), whereas other studies could not confirm these findings (6, 12, 13). To date, only 1 published study has investigated the effects of NRTIs on mtDNA in HIV-negative individuals (14). In that study, no depletion of mtDNA attributable to NRTIs was observed, which could be interpreted that HIV and NRTIs are both needed to cause mtDNA depletion in PBMCs (14). Further studies are needed to address the specific roles of HIV infection itself and the various NRTIs on the observed changes or lack of observed changes in the various studies.

To measure the mtDNA content of the cell, it is essential to determine the ratio of mtDNA over nuclear DNA (nDNA). We developed a real-time duplex nucleic acid sequence based amplification (NASBA) assay, in which both mtDNA and nDNA are simultaneously amplified in a 1-tube format instead of in separate reactions (3, 15), which increases reproducibility and productivity. We have chosen to express the number of mtDNA copies per 2 genomic molecules as the number of mtDNA copies per cell, recognizing that not all tissue compartments always contain 2 genomic copies. Because for this study we wanted to investigate the logic of evaluating easy-to-collect blood samples from patients vs complex and inconvenient biopsy samples, we selected PBMC samples, which contain 2 genomic copies per cell, which explains the reporting rationale. The dynamic range of the mtDNA quantification assay was optimized for PBMCs and fibroblasts. The assay was able to quantify changes in mtDNA content after in vitro treatment of fibroblasts and PBMC cultures with antiviral drugs. Because clinical samples often are contaminated with platelets, which contain mtDNA but no nDNA, it has been found that platelets influence the result of the mtDNA measurements (16). We have investigated the extent of contamination that is allowed before the platelets will have an effect on the mtDNA quantification. Additionally, to show the feasibility for use of the assay in a clinical setting, we evaluated the in vivo effects of initiating antiretroviral treatment with zidovudine (AZT) with lamivudine (3TC) and indinavir (IDV) and on the mtDNA content in PBMCs derived from patients over time after switching to stavudine (d4T) with didanosine (dDI) and IDV as well as in a study in which protease inhibitors were either continued or switched to non-NRTIs.

Materials and Methods

Patients

Cohort 1. PBMCs were collected from 14 patients who participated in AIDS Clinical Trial Group (ACTG) study 343 and who rolled over to ACTG study 5025 after a median of 89 weeks. The details of these studies and the demographics of the study populations have been published elsewhere (17, 18). Patients in ACTG 343 were antiretroviral naive or had minimal experience with AZT and received a combination of AZT, 3TC, and IDV. After maintaining an undetectable viral load, they were eligible to roll over to ACTG 5025 and were randomized to maintain the AZT + 3TC + IDV combination (n = 5) or switched to a combination of d4T + dDI + IDV with or without hydroxyurea (n = 9). PBMC specimens collected from the following time points and frozen until analysis were evaluated: week 0, week 12 of AZT + 3TC + IDV, baseline before the switch to d4T + dDI (median of 89 weeks after the initiation of antiretroviral therapy), and week 12 after the switch. The group of patients who continued on AZT + 3TC + IDV were evaluated at the same time points. This group allowed us to evaluate the mtDNA copy number per cell in HIV-infected individuals, the effects of initiating an antiretroviral regimen, and the subsequent substitution of the nucleoside analogs from AZT + 3TC to d4T + dDI while maintaining the protease inhibitor part of the combination.

Cohort 2. Samples were selected from 19 patients who were part of a clinical trial in which patients with undetectable viral load on a stable protease inhibitor regimen continued that regimen for 6 months or switched the protease inhibitor to nevirapine. Details of these studies have been published elsewhere (19). This group allowed us to evaluate the changes over time of mtDNA in a variety of antiretroviral regimens and the effects of substituting the protease inhibitor for nevirapine, maintaining the nucleoside analog part of the regimen.

Informed consent was given by all patients participating in the above-mentioned studies.

PBMC Preparations

PBMCs were isolated from whole-blood samples from the patients by centrifugation over a Ficoll-Paque (GE Healthcare) according to standardized protocols (20). After centrifugation over the Ficoll-Paque layer, the cells were 3 times washed with phosphate-buffered saline containing 20 mL/L fetal calf serum (FCS) before they were frozen viable and stored in liquid nitrogen. Before the mtDNA assay was performed on the cells, the number of platelets was determined and had to be ≤5 times the number of...
cells. The number of platelets vs cells was assessed by phase-contrast light microscopy after trypan blue staining of the samples (21). This method was compared with automated, quantitative hematologic analysis by a Cell-Dyn 4000 (Abbott Laboratories) as well as flow cytometry by FACScalibur (Becton Dickinson) with CD16 staining of the platelets. The results of counting platelets by light microscopy were similar to those obtained by both Cell-Dyn and flow cytometry (see Tables 1–3 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue6/). A clear difference could be observed between platelets and cells, even if the platelets adhered to the cells. PBMC preparations that contained an excess of 5-fold more platelets than PBMCs were washed 2 more times with a wash medium of phosphate-buffered saline containing 20 mM/L FCS and subsequently centrifuged for 15 min at 100 g [protocol adapted from Ref. (22)]. When used this protocol, all samples fulfilled the requirement of <5 times the number of platelets per cell.

**NASBA duplex reaction**

Nucleic acids from 50,000 or 500,000 cells were isolated by a silica-based method (23) in a final volume of 50 mL of HPLC-purified water (J.T. Baker). If nucleic acids from 500,000 cells were isolated, the eluates were diluted 10-fold in HPLC-purified water before addition of the reagents to the sample. The input range for number of cells without requiring dilution of the nucleic acids was large, between 10,000 and 100,000. If a low number of cells is used in the nucleic isolation, too little genomic DNA is present for accurate quantification, whereas if higher number of cells are used in the nucleic acid isolation, the amount of DNA is too high and the amplification reactions will proceed too rapidly. The NASBA reactions were performed in 200-L tubes (Greiner Bio-One) containing the following reagents in 10 mL: 40 mM Tris-HCl (pH 8.3); 12 mM MgCl$_2$; 90 mM KCl; 5 mM dithiothreitol; 1 mM DMSO; 2 U of MspI restriction enzyme (Roche Applied Science); 0.2 U of RNase H (0.08 U), T7 RNA polymerase (64 U), AMV-RT (9.6 U), and sorbitol (0.375 mol/L). The amplification was followed for 60 min at 41 °C in a fluorescence reader (Primagen RetinAlyzer) that measured the fluorescein (excitation at 485 nm, and emission at 518 nm) and rhodamine (excitation at 578 nm and emission at 604 nm) signals every 30 s. All samples were assayed in duplicate, and results were accepted only if the variation between the duplicates was <25% of the mean of the duplicates.

A calibration curve was constructed by use of plasmids that contained parts of the mtDNA and human nDNA (snRNP U1A; accession numbers HUMMTTCG and AC020945, respectively), the concentration of which was determined by the A$_{260}$/A$_{280}$ ratio. A series of calibrators containing 6 × 10$^0$ copies of nDNA and 9 × 10$^4$, 3 × 10$^5$, 9 × 10$^5$, and 3 × 10$^6$ mtDNA plasmid molecules (equivalent to 30, 100, 300, and 1000 mtDNA copies per 2 genomic copies, which equals the number of copies per cell) was included in each experiment, covering a linear range of at least 15 to 100 mtDNA copies per cell. The ratio of the slopes of the fluorescence curves of mtDNA and nDNA was calculated and after a logarithmic transformation was found to be linear with the logarithm of the known input ratio of mtDNA vs nDNA. This is a phe-

<table>
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<th>Name</th>
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<td>Primer P1</td>
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<td>U1A-beacon</td>
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<tr>
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<td>Primer P2</td>
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<tr>
<td>mtDNA-beacon</td>
<td>Probe-ROX</td>
<td>CGTACGCTGATATCAGCTCAACGTACAGCATCCTGCGATTCG</td>
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a The T7 RNA promoter sequence that is part of the P1 primer is shown in italics. The inserted transcription initiation site is shown underlined. The stem sequence of the molecular beacons is indicated in bold. The down arrow indicates the MspI restriction enzyme cleavage site.
b FAM, 6-carboxyfluorescein; ROX, 6-carboxy-X-rhodamine.
nomenon we have observed in multiplex quantitative systems in which the same reagents are used for amplification of 2 different targets. The number of mtDNA copies per cell in a sample was calculated from the calibration curve.

PRODUCTION OF LACTATE AND PYRUVATE FROM GLUCOSE IN CULTURED SKIN FIBROBLASTS AND THE LACTATE/PYRUVATE RATIO
To explore the relationship between a classic assay for mitochondrial function (lactate/pyruvate ratio) and the real-time duplex NASBA method, we cultured control human fibroblasts (purchased from the National Institute on Aging Cell Repository, Camden, NJ) in DMEM supplemented with 100 mL/L FCS and antibiotics in the presence of the antiretroviral drug zalcitabine (ddC; 30 μmol/L) for 4 weeks. After 4 weeks, the culture with ddC was split into a part in which ddC was maintained at 30 μmol/L NaCl; 5 mmol/L KCl; 1.2 mmol/L KH₂PO₄; 1.2 mmol/L MgSO₄; 25 mmol/L NaHCO₃; 20 mmol/L HEPES buffer, pH 7.4; 2.5 mmol/L CaCl₂) and into a part in which ddC was absent. During this culture period, we also measured the production of lactate and pyruvate from glucose by washing the cells once with 5 mL of Krebs–Henzelheit buffer (118 mmol/L lactate and pyruvate from glucose by washing the cells different conditions, we also measured the production of lactate and pyruvate from glucose by washing the cells once with 5 mL of Krebs–Henzelheit buffer (118 mmol/L NaCl; 5 mmol/L KCl; 1.2 mmol/L KH₂PO₄; 1.2 mmol/L MgSO₄; 25 mmol/L NaHCO₃; 20 mmol/L HEPES buffer, pH 7.4; 2.5 mmol/L CaCl₂) and subsequently resuspending them in 1 mL of Krebs–Henzelheit buffer supplemented with 10 mmol/L glucose. The cells were incubated for 2 h at 37 °C. At the end of the incubation period, lactate and pyruvate in the medium were measured by spectrophotometric (lactate) and fluorometric (pyruvate) assays (27). From these results the ratio of lactate to pyruvate was calculated. An increased lactate/pyruvate ratio is indicative of a dysfunctional mitochondrial respiratory chain (28).

CELL CULTURE OF PBMCs
PBMCs isolated from a healthy donor were stimulated with phytohemagglutinin in RPMI 1640 (Invitrogen) supplemented with 100 mL/L FCS and streptomycin sulfate (100 mg/L) and penicillin G (100 kilounits/L). After 3 days of stimulation with phytohemagglutinin (2 mg/L), the PBMCs were maintained in RPMI containing 100 mL/L FCS and 100 kilounits/L interleukin-2 in the presence of the NRTI ddC (5 μmol/L) dissolved in DMSO (1 mL/L) or with just the solvent DMSO (1 mL/L) as a control for 11 days. During this period of culture, every second day part of the cells was harvested and analyzed for the ratio of mtDNA to nDNA.

EFFECT OF PLATELETS ON THE mtDNA RESULT IN A PURIFIED PBMC ISOLATE
Platelets contain mtDNA but do not contain nDNA. Substantial platelet contamination of PBMCs from patients may lead to mtDNA copy number results higher than the real number (16). To investigate the effect of platelet contamination, we tested part of a highly purified PBMC preparation, which was concluded to have no platelet contamination based on light microscopy after trypan blue staining, for its mtDNA content. To this preparation without platelets, we added platelets at amounts 0.2, 1, 2, 5, 25, and 125 times the number of PBMCs. The platelets used were from a clinical preparation obtained from the Academic Medical Centre in Amsterdam.

STATISTICAL ANALYSIS
We evaluated the evolution over time of mtDNA. Comparisons within arms were done with one-way repeated-measure ANOVA, and if a significant effect of time was detected, we made multiple comparisons with the baseline copy number value, using the Bonferroni correction. Comparisons between the cohorts were performed with ANOVA. All statistical analyses were performed using SPSS, Ver. 11.5.

Results
CHARACTERISTICS OF THE REAL-TIME mtDNA/nDNA DUPLEX ASSAY
Because we used real-time detection with 2 target-specific molecular beacons, amplification curves in time could be plotted in which an increase of fluorescence was observed until most of the molecular beacon was hybridized and the fluorescence had reached a maximum (Fig. 1A). The ratio of the slopes between the 2 fluorescence curves reflected the ratio of mtDNA to nDNA in the initial sample and was linear with the input when both variables were plotted on logarithmic scales (Fig. 1B). In the standard format, the assay could accurately quantify mtDNA within a range of at least 15 to 1500 copies of mtDNA per 2 genomic copies, which is within the range of concentrations found in the cells in most tissues that have been used to date, including PBMCs. The assay could easily be adapted to a different calibration range by varying the primer concentrations to make the assay suitable for measuring mtDNA in various cell types with higher or lower amounts of mtDNA per cell (data not shown). In a collection of 452 samples that were assayed in duplicate, 419 samples had a CV <25% of the mean of the duplicates, with a median of 8.4% (data not shown). Repeat analysis of the 33 samples with a CV >25% yielded an additional 27 samples with a CV <25%. In an evaluation of the reproducibility of the assay, we assayed 1 sample 40 times in 2 runs; the mean (SD) was 159 (18) copies mtDNA/细胞, and the CV was 11%. The 95% confidence interval of the mean was 153–165 copies mtDNA/cell. We determined the interassay variation by assaying 12 PBMC samples in 7 runs, of which 5 samples were tested in all 7 runs, 6 samples in 6 runs, and 1 sample in 5 runs (see Table 4 in the online Data Supplement). The average interrun CV was 16%.
Fig. 1. Calibration results for the NASBA assay.

(A), typical calibration curves obtained with various plasmid mtDNA/nDNA mixtures. Shown is the relative fluorescence on the y axis for the 6-carboxyfluorescein (nDNA; gray line) and 6-carboxy-X-rhodamine (mtDNA; dashed black line) signals obtained with different ratios of mtDNA to nDNA (30, 100, 300, and 1000 copies per cell) as input. The x axis represents the time in increments of 30 s. (B), calibration curve calculated from the ratio of the slopes of the signal and the ratio of mtDNA to nDNA real-time amplification curves, showing the linear relationship between the logarithm of the ratio of the slopes of the signal and the logarithm of the ratio of mtDNA to nDNA.
RELATIONSHIP BETWEEN A CLASSIC ASSAY FOR MITOCHONDRIAL FUNCTION AND THE REAL-TIME DUPLEX NASBA ASSAY

To compare changes in mtDNA with changes in mitochondrial function, we have chosen to measure the production of lactate and pyruvate from glucose and calculate the corresponding lactate/pyruvate ratios as a general measure of function of the mitochondrial respiratory chain. A lactate/pyruvate ratio >50 indicates that the mitochondrial respiratory chain is malfunctioning (28). During the 4-week culturing of the fibroblasts in the presence of ddC, the lactate/pyruvate ratio increased from 10 to 120 (Fig. 2). In the cultures that were maintained in the presence of ddC, the lactate/pyruvate ratio remained >100, whereas in the cultures in which ddC was removed, the lactate/pyruvate ratio decreased to <50 at week 8. The decrease in mitochondrial respiratory chain function, as apparent from the increase in the lactate/pyruvate ratio, was preceded by a decrease in mtDNA content, which decreased from ~1300 copies/cell at the start of the culture to <15 copies/cell after 3 weeks of culture in the presence of ddC. After removal of ddC from the culture, mtDNA content returned to baseline values after 6 weeks of prolonged culture (10 weeks after the start of the experiment), whereas in the cultures with ddC, mtDNA concentrations remained <15 copies/cell, the lower limit of quantification for the mtDNA assay.

IN VITRO RESPONSE OF PBMCs TO ddC

PBMCs are cells of the blood compartment and are therefore relatively easy to obtain by venipuncture. We investigated whether PBMCs could be used in the real-time duplex NASBA and whether their mtDNA decreased as a result of exposure of the antiviral drug ddC. For each of the time points, the mtDNA concentration of the control culture without ddC was set at 100% (Fig. 3). In the cultures with ddC, the mtDNA content decreased and was calculated as percentage of the control culture. Thus, changes in the mtDNA copy numbers in these cultured PBMCs could be quantified by use of the duplex NASBA assay.

EFFECT OF PLATELET CONTAMINATION ON THE mtDNA COPY NUMBER RESULT

Platelets contain mtDNA but no nDNA. We determined the ratio of platelets to cells that was could be present before it started to influence the results. Platelets did not influence the results obtained for the mtDNA content of PBMCs if they were present in numbers <5 times the number of cells (Fig. 4). When platelet were present in concentrations between 5- and 25-fold higher than PBMC concentrations, the mtDNA copies present in platelets...
started to influence the results. From these addition experiments, we were able to calculate that a platelet contained 7 copies of mtDNA or less.

EFFECT OF ANTIVIRAL THERAPY ON THE mtDNA COPY NUMBER OF PATIENT PBMCs

We measured the number of mtDNA copies per PBMC in frozen samples that were obtained as part of 2 longitudinal studies of antiretroviral therapy. The samples were coded; therefore, the analytical laboratory had no patient or medication information.

After the initiation of antiretroviral therapy with AZT + 3TC + IDV, there was a significant increase in mtDNA per cell from a mean (SE) of 215 (22) copies/cell to 371 (27) copies/cell ($P = 0.003$) already within the first 12 weeks after initiation of therapy (Fig. 5). The concentrations plateaued and remained stable for a median of 89 weeks without significant changes while the patients remained on AZT + 3TC + IDV [309 (27) copies/cell at completion of the ACTG 343 study; $P = 0.03$ compared with baseline; not significant compared with week 24; Fig. 5].

After a median of 89 weeks, patients were randomized to continue AZT + 3TC + IDV or switched to d4T + ddI + IDV with or without hydroxyurea. The mtDNA copy number did not change in the patients who remained on AZT + 3TC + IDV; however, there was a significant decrease in the mtDNA copy number among the patients who switched to d4T + ddI + IDV with or without hydroxyurea, from 301 (36) copies/cell to 194 (13) copies/cell (week 12 after switch) and 227 (22) copies/cell (week 24 after switch; Wilks $\Lambda = 0.2; F = 13.4; P = 0.004$). The majority of the decrease in mtDNA copy number occurred within the first 12 weeks after switch. There was no difference between the patients who received and those who did not receive hydroxyurea.

The mean (SE) copy number of mtDNA per cell was significantly lower [215 (22) copies/cell] in HIV-infected naïve patients ($n = 14$; Fig. 5) compared with patients on stable protease inhibitor–based regimens [300 (18) copies/cell; $P = 0.01; n = 26$] or nevirapine-based regimens [351 (25) copies/cell; $P = 0.001; n = 16$]. These last 2 values were similar to the mtDNA copy number (321 copies/cell) observed in samples from a cohort of HIV-negative persons, as measured with the same assay (29). There was no significant difference in mtDNA copy number between protease inhibitor–based regimens vs nevirapine-based regimens over time (see Fig. 1 in the online Data Supplement).

Discussion

Mitochondrial dysfunction is the underlying cause of many diseases and can be caused by sequence changes in the mitochondrial genome [e.g., represented by diseases such as myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), and neurogenic weakness, ataxia, retinitis pigmentosa (NARP) (30)] as well as the nuclear genome. Mitochondrial dysfunction can also be caused by depletion of mtDNA. To quantify the mtDNA copy number in cells, we developed an assay that measured simultaneously the mtDNA and nDNA in a 1-tube format using a real-time duplex NASBA method. Because the amount of nDNA is directly proportional to the number of cells, the assay result could be expressed as mtDNA copy number per cell. The advantage of the 1-tube format is that variation in the assay attributable to the experimental procedure influences both mtDNA and nDNA amplification in the same manner, increasing reproducibility. In addition, the duplex format provides savings in materials and hands-on time, reducing the costs. The assay uses small quantities of valuable patient material: 50 000 cells as input in the isolation of nucleic acids and 5000 cell equivalents in the reaction.

We compared the assay with a classic assay for mitochondrial function, which involves incubation of cells in a glucose-containing medium followed by the measurement of the lactate and pyruvate produced from glucose. If mitochondria are dysfunctional as a consequence of an inherited defect in the respiratory chain, e.g., cytochrome c oxidase deficiency, the lactate/pyruvate ratio will be markedly increased. Incubation of human fibroblasts in the presence of the relatively toxic antiviral drug ddC showed a decrease in mtDNA copy numbers even before the lactate/pyruvate ratio increased. Although in the presence of ddC the mtDNA copy numbers decreased to <1% of the initial value, after removal of ddC, the mtDNA copy number rapidly increased to the pretreatment values. Again, recovery of mtDNA started earlier than the rebound of lactate/pyruvate ratios. An explanation for the differential response is that the mtDNA decrease is the first event induced by the presence of the antiviral drug ddC and that the functions mediated by mitochondrial proteins lags behind. The in vitro observa-

![Fig. 5. Effects of starting antiretroviral therapy on mtDNA (copy number per cell) in PBMCs.

After 89 weeks on AZT + 3TC + IDV ($n = 14$), patients were randomized to continue their antiretroviral regimen (■; $n = 5$) or switch to d4T + ddI + IDV with or without hydroxyurea (□; $n = 9$). Values are the mean (SE; error bars). After initiation of therapy, there was a significant increase in the amount of mtDNA, which decreased significantly after the switch of nucleosides. A 343 and A 5025 represent the ACTG study numbers.](image-url)
tions in fibroblasts indicated that changes in mtDNA content might be an early marker for the onset of mitochondrial dysfunction. Furthermore, if these in vitro data translate to HIV-positive individuals, the recovery of mtDNA copy numbers to pretreatment or preinfection values would be promising for the reversal of adverse effects.

Because fibroblasts are not conveniently obtained from patients, we investigated the use of easy accessible blood-derived cells: PBMCs. In vitro, we showed that PBMCs responded to ddC by a large decrease in mtDNA copy number. Similarly, PBMCs also respond in vivo to antiviral drugs and could serve as a monitoring tool reflecting the mtDNA content of tissues of interest. However, one concern in the use of PBMCs is the contamination with platelets, which contain mtDNA but not nDNA. Contamination with various amounts of platelets might distort the measured mtDNA content per cell. The addition experiment with different amounts of platelets added to PBMCs showed that the outcome of the assay will be substantially affected only if at least a 5-fold excess of platelets relative to cells is present. Routine microscopic examination of the PBMCs can confirm whether such an extent of contamination is present.

Changes in the mtDNA concentrations in PBMCs of patients can indeed be observed in response to antiviral therapy with nucleoside analogs, as was shown in the 2 series of samples of patients on various treatments and has also been reported in various other studies (3, 25, 31–34). The reason to develop an assay that can detect small changes in mtDNA copy numbers was to allow verification of studies that have not been able to find changes in mtDNA attributable to antiretroviral treatment (14). As has been reported previously (3–5), our studies confirm that antiretroviral-naive HIV-infected individuals have lower mitochondrial DNA per PBMC than do HIV-negative persons and that effective antiretroviral therapy may restore mtDNA concentrations at least to some extent. On the other hand, we observed that therapy that is effective in suppressing viral replication could lead to both a decrease in mtDNA copy number (ddT+ ddI+ IDV with or without hydroxyurea), but also in increases in mtDNA copy number (AZT+ 3TC+ IDV), which also has been observed previously (29, 34, 35).

In the cohorts on stable protease inhibitor or nevirapine therapy, the mtDNA copy number per cell remained stable without significant changes over time, suggesting that the changes observed in the cohort on NRTI-containing therapy were associated with changes in the nucleoside analog component of the antiretroviral regimen and not in cumulative toxicity of the antiretroviral treatment. Although correlation does not imply causation, there is growing evidence that mitochondrial toxicity may play a significant role in the metabolic complications associated with anti-HIV therapy, e.g., insulin resistance and lipodystrophy. This NASBA assay is useful for studying possible relationships between changes in mtDNA attributable to antiretroviral therapy and the development of adverse events. If it turns out that the concentrations of mitochondrial DNA correlate with the development or onset of adverse events, then it is possible to create individualized regimens that are less toxic to the mitochondria and potentially prevent the development of these complications.

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