

CASE REPORT

Mitochondrial disruption and apoptosis in lymphocytes of an HIV infected patient affected by lactic acidosis after treatment with highly active antiretroviral therapy

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Aims: Highly active antiretroviral therapy (HAART) can induce an increase in lactic acid concentrations that seems to be caused by mitochondrial dysfunction induced by the interaction of nucleoside reverse transcriptase inhibitors (NRTIs) with DNA polymerase γ in the mitochondria. Mitochondrial alterations have been described in liver and muscle cells of NRTI treated human immunodeficiency virus (HIV) infected patients. Because lymphocytes are the main target for HIV and because mitochondria are involved in apoptosis, we studied mitochondrial morphology and apoptosis in the lymphocytes of an HIV infected patient with severe lactic acidosis after treatment with stavudine, didanosine, and indinavir.

Methods: The patient was a 39 year old woman. After two years of treatment she developed rapid weight loss with severe fat wasting, peripheral neuropathy, and hyperlacticaemia, which persisted after treatment withdrawal. The numbers and the morphology of the mitochondria were evaluated by electronic microscopy; the percentage of apoptotic cells was calculated by flow cytometry after staining with annexin V and by fluorescent microscopy after staining with ethidium bromide and acridine orange.

Results: The numbers of mitochondria in the lymphocytes were greatly decreased when compared with the lymphocytes of healthy individuals. The most important mitochondrial morphological alterations were swelling and the disruption of cristae and internal mitochondrial structure. These alterations were more evident during the period in which lactic acid values were very high. Moreover, a high percentage of apoptotic lymphocytes was seen. Morphological examination conducted one week after the normalisation of lacticaemia showed a pronounced increase in the number of mitochondria. The morphological alterations were no longer evident, although the size of each mitochondrion was smaller than normal. Moreover, the percentage of apoptotic cells was lower than 5%.

Conclusions: This report describes important morphological alterations in lymphocyte mitochondria in an HIV infected patient during a severe phase of HAART induced hyperlacticaemia. These alterations persisted for several weeks after treatment withdrawal and were associated with an increase in lymphocyte apoptosis. Considering the important role of mitochondria in the apoptotic pathway, the increase in lymphocyte apoptosis may be a consequence of proapoptotic factors released from altered mitochondria.

Nucleoside reverse transcriptase inhibitors (NRTIs) are important drugs in the treatment of human immunodeficiency virus (HIV) infection. They disrupt the function of the viral reverse transcriptase enzyme, which results in the premature termination of viral DNA synthesis. NRTIs do not affect cellular DNA duplication because the enzymes implicated in this process have a proofreading mechanism. However, NRTIs can affect the mitochondrial genes.^{1,2} Mitochondria replicate in cells independently of cell proliferation, according to the energy needs of the cell. The high sensitivity of mitochondrial DNA to NRTIs depends on a particular DNA polymerase (DNA polymerase γ), which directs DNA mitochondrial duplication. DNA polymerase γ has no proofreading function, so NRTIs can inhibit this enzyme in a manner similar to their effect on viral reverse transcriptase. When about 70% of mitochondrial DNA is damaged, cells begin to suffer from energy deficiencies and increase their anaerobic processes (glycolysis), with the production of lactic acid.^{3,4} Lactic acidosis is a rare but serious side effect of HAART.⁵⁻⁹ The symptoms of NRTI induced lactic acidosis are nausea, abdominal pain, and fatigue.¹⁰ Together with the increase in lactic acid values, laboratory tests show a decrease in oxygen uptake and high concentrations of pancreatic, muscle, and liver enzymes as a consequence of tissue damage.^{11,12}

“Lactic acidosis is a rare but serious side effect of highly active antiretroviral therapy”

Here, we report a case of severe lactic acidosis in an HIV infected patient treated with a combination of stavudine, didanosine, and indinavir. We observed important mitochondrial morphological alterations in lymphocytes and an increase in lymphocyte apoptosis, which persisted for one month after treatment withdrawal.

METHODS

Cells

Peripheral blood was layered on to a Histopaque gradient (density, 1.077 g/ml), centrifuged at 400 \times g for 30 minutes, and the interface cells collected. The interface cells were washed twice in phosphate buffered saline (PBS).

Cell surface phenotypic characterisation

Flow cytometry was performed on peripheral whole blood samples after staining with each monoclonal antibody (monoclonal antibodies to CD3, CD4, and CD8; Becton

Abbreviations: Fas-L, Fas ligand; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; NRTI, nucleoside reverse transcriptase inhibitor; PBS, phosphate buffered saline

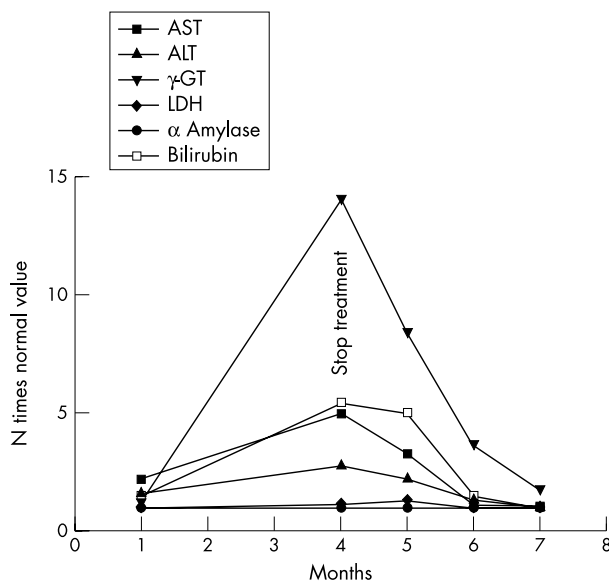


Figure 1 Blood chemical and enzyme values. ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ glutathione; LDH, lactate dehydrogenase.

Dickinson, San Jose, California, USA) using a Becton Dickinson FACScan flow cytometer. A two colour panel of monoclonal antibodies for HIV monitoring was used to measure each lymphocyte subset.

Viral load

Plasma HIV RNA values were measured using the Roche Amplicor RNA monitor assay (ultra sensitive test; Roche Diagnostic System, Branchburg, New Jersey, USA) with a lower limit of detection of 50 copies/ml.

Electronic microscopy

Thin sections (80 nm) were prepared from peripheral blood mononuclear cells, fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M Millonig's buffer, and embedded in Epon 812 resin. Polymerisation was accomplished at 60°C. Sections mounted on formavar pretreated nickel grids were counterstained with 2% uranyl acetate (10 minutes) and lead citrate (10 minutes), then analysed by electron microscopy (Jeol Jem 1220).

Apoptosis

Apoptosis was evaluated by electronic microscopy (see above), fluorescence microscopy after staining with acridine orange and ethidium bromide, and by flow cytometry after staining

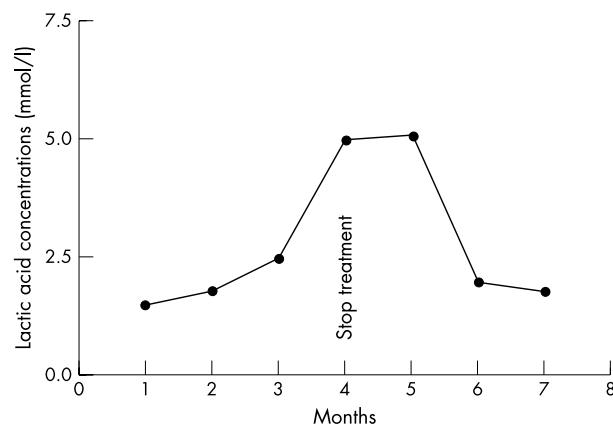


Figure 2 Trend in lactic acid concentrations.

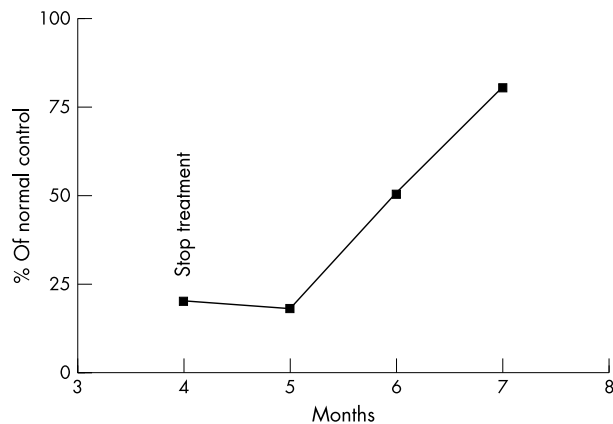


Figure 3 Quantitative mitochondrial alterations in lymphocytes. Mitochondria were counted in 100 lymphocytes and expressed as percentages of the number of mitochondria counted in lymphocytes of healthy donors.

with annexin V. Cells (2×10^5) were centrifuged ($300 \times g$) and the pellet was resuspended in 25 μ l of a dye mixture made up of acridine orange and ethidium bromide. The mixture (10 μ l) was examined under oil immersion with a $\times 100$ objective using a fluorescence microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and the exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labelling of the cells with ethidium bromide. For the annexin V test, the cells (1×10^6) were washed with PBS and centrifuged at $200 \times g$ for five minutes. The cell pellet was suspended in 100 μ l of staining solution containing annexin V-fluorescein labelling reagent (annexin V-fluos staining kit; Roche Molecular Biochemicals, Mannheim, Germany) and CD3 fluoresceinated monoclonal antibodies (Becton Dickinson) and incubated for 15 minutes at 20°C. Annexin V-CD3 positive cells were evaluated by flow cytometry.

RESULTS

Patient presentation

The patient is a 39 year old woman with known HIV seropositivity since 1994, hepatitis B surface antigen and hepatitis C virus antibody negative, treated with HAART (stavudine, didanosine, and indinavir) since 1998, with a viral load of < 50 copies/ml and a CD4 count of > 500 cells/ml. On follow up, after two years of HAART treatment, aspartate aminotransferase was higher than the baseline value (90 U/litre *v* 23 U/litre). The patient remained healthy until seven months later, when she complained of the onset of back pain, fever, weakness, headaches, and vomiting. The presence of kidney stones was demonstrated. Despite treatment for nephrolithiasis and the replacement of indinavir with nelfinavir, the clinical picture worsened. She developed rapid weight loss with severe fat wasting and neurological abnormalities related to peripheral neuropathy.

Blood chemical and enzyme values

Figure 1 shows the main laboratory test values. Alanine aminotransferase, aspartate aminotransferase, and conjugated bilirubin increased to values of approximately 5, 4.8, and 2.5 times higher than normal, respectively. At the time of treatment withdrawal, γ glutathione reached a value of 14 times higher than normal. No important alterations in α amylase and lactate dehydrogenase values were observed. Lactic acid concentrations remained high until one month after

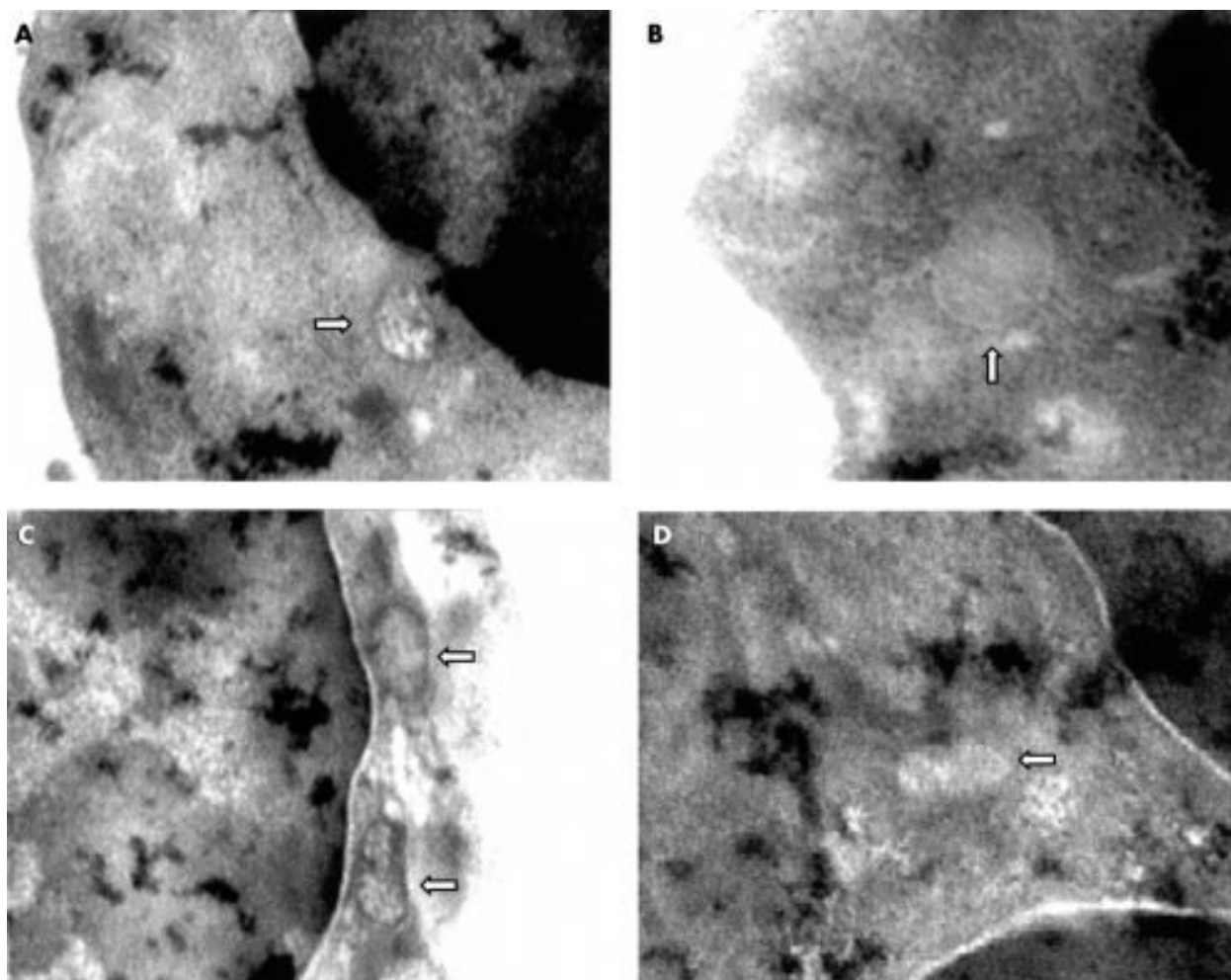


Figure 4 Morphology of mitochondria in lymphocytes one month after treatment withdrawal. Evaluation by electronic microscopy (original magnification, $\times 30\,000$).

HAART withdrawal and then decreased to reach normal values after two months (fig 2).

Mitochondria

The numbers of mitochondria were very much decreased in the patient's lymphocytes compared with the peripheral blood lymphocytes of healthy individuals (fig 3). The number of mitochondria increased one month after treatment withdrawal with a trend opposed to the lacticaemia trend (figs 2, 3). The most frequent mitochondrial morphological alterations were: swelling, disruption of cristae and internal mitochondrial structure, and a pronounced thinning of the mitochondrial membrane. In some mitochondria, these alterations were only partial; other mitochondria showed complete disruption of their internal structure.

Figure 4A and C shows two lymphocytes with partially disrupted cristae. In fig 4D cristae disruption is more pronounced and in fig 4B the mitochondria are swollen and the cristae have completely disappeared. Moreover, a pronounced thinning of the mitochondrial membrane was seen in several mitochondria (fig 4D). These alterations were more evident in the phases in which lacticaemia was very high. Morphological examination conducted one week after normalisation of lacticaemia showed a normalisation in the numbers of mitochondria (figs 3, 5A–D). However, in some cells there was a residue of disrupted mitochondria (fig 5C). Moreover, the size of each mitochondrion was smaller than normal.

Apoptosis

Figure 6 shows the percentage of apoptotic CD3+ lymphocytes over three months after treatment withdrawal. The apoptosis trend was similar to the lactaemia trend. The highest percentage of apoptosis was seen one month after treatment withdrawal. The percentage of CD3+ lymphocyte apoptosis decreased over the following two months, with the decrease of lacticaemia, and despite the increase in viral load (from < 50 to 21 000 copies/ml) caused by treatment withdrawal. No important alterations in CD4+ lymphocyte numbers were seen after treatment withdrawal.

DISCUSSION

Several studies have reported the effects of antiviral analogues on mitochondrial functions. Severe lactic acidosis and macrovesicular steatosis accompanied by hepatic failure have been described in patients treated with AZT, ddC, ddI, and d4T as a consequence of mitochondrial disruption.^{13–18} Lactic acidosis is an infrequent side effect in patients with HIV who are treated with HAART. A five year review of patients from the Johns Hopkin's HIV clinic recorded an incidence of 1.3/1000 patient years for lactic acidosis.¹⁹ A more recent French study found a frequency of 8.4/1000 patient years for symptomatic high lactic acid values (L Maulin, *et al.* Emerging complication of antiretroviral therapy: symptomatic hyperlactatemia. 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, 26–29 September 1999, abstract 1285). The frequency

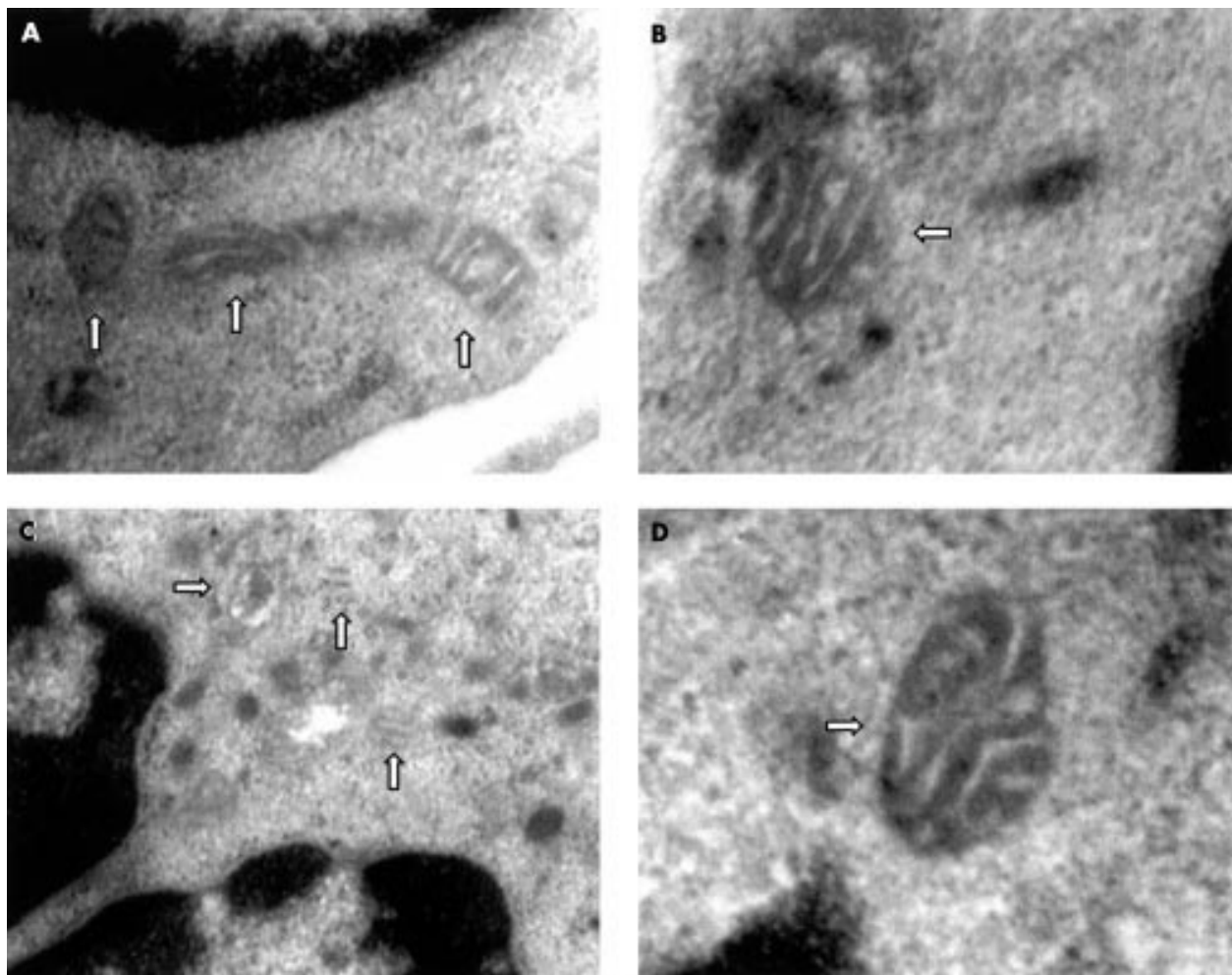


Figure 5 Morphology of mitochondria in lymphocytes three months after treatment withdrawal. Evaluation by electronic microscopy (original magnification, $\times 30\,000$).

of lactic acidosis in HIV infected patients seems, at present, to be increasing, probably because of the prolonged use of NRTIs, and the Food and Drug Administration continues to receive reports of deaths from lactic acidosis (DE Boxwell and BA Styr. Lactic acidosis (LA) in patients receiving nucleoside reverse transcriptase inhibitors (NRTIs). 39th Interscience Conference on Antimicrobial Agents and Chemotherapy. 26–29 September 1999, abstract 1284). Mitochondrial alterations have been described in the liver and muscle cells of NRTI

treated HIV infected patients with different morphological aspects.^{20–25} The examination of liver postmortem specimens from a patient treated with AZT showed only enlarged mitochondria, whereas the disruption of cristae was described in skeletal muscle mitochondria. More recently, Pan-Zhou *et al* observed disruption of mitochondrial cristae in a human hepatoma cell line treated in vitro with ddC and ddI. In contrast, AZT induced only a slight increase in mitochondrial size.²⁶

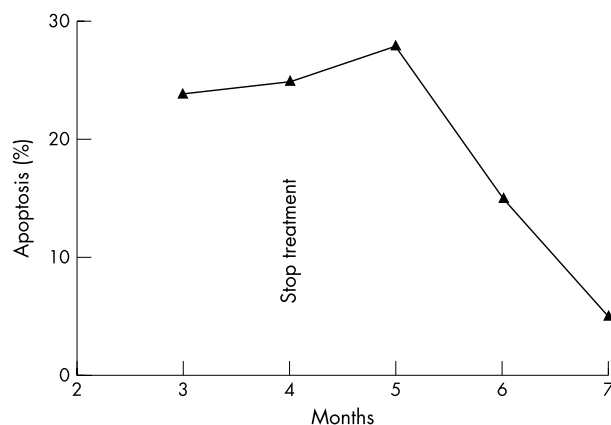


Figure 6 Percentage of apoptotic T cells before and after treatment withdrawal.

“Compared with other similar cases reported in the scientific literature, the mitochondrial alterations seen in our patient appeared to be more pronounced than those described for liver and muscle”

In our study, we have shown that important quantitative and qualitative alterations in mitochondria can also be present in the lymphocytes of a patient with severe lactic acidosis after treatment with NRTIs. Moreover, we found that these alterations can persist for several weeks after NRTI withdrawal. We think that this is important considering the fact that lymphocytes are the main targets of HIV. Compared with other similar cases reported in the scientific literature, the mitochondrial alterations seen in our patient appeared to be more pronounced than those described for liver and muscle. Other than swelling and cristae disruption, we observed in several lymphocytes the complete disappearance of internal mitochondrial structure, with a pronounced thinning of the

Take home messages

- We describe important morphological alterations in lymphocyte mitochondria in a human immunodeficiency virus infected patient during a severe phase of highly active antiretroviral therapy induced hyperlacticaemia
- These alterations persisted for several weeks after treatment withdrawal and were associated with an increase in lymphocyte apoptosis
- Because mitochondria play an important role in the apoptotic pathway, the increase in lymphocyte apoptosis may be a consequence of proapoptotic factors released from altered mitochondria

mitochondrial membrane, during which the identity of the outer and inner membranes was lost.

Another important event in our patient during the phase of hyperlacticaemia was the appearance of apoptosis in lymphocytes. The percentage of apoptotic cells decreased with the decrease in lactic acid concentrations. Although a decrease in CD4+ lymphocytes was seen before treatment withdrawal, no important alterations appeared in the next months, probably because of a balanced effect resulting from treatment withdrawal. It is noteworthy that apoptosis is a feature of HIV infection.²⁷ Increased lymphocyte apoptosis is involved in CD4 depletion, and the overexpression of Fas and the Fas ligand (Fas-L) in CD4+ cells seems to play a major role in this process.^{28,29} Several studies have shown an increase in the expression of membrane bound Fas-L and Fas in lymphocytes and monocytes and in amounts of the soluble forms of Fas-L and Fas in the plasma of HIV infected subjects.³⁰ In contrast, serum concentrations of Bcl-2 are often reduced.^{30,31} We do not know the causes of the increase in lymphocyte apoptosis, but considering the important involvement of mitochondria in the apoptotic pathway,³² it may be a consequence of proapoptotic factors released from disrupted mitochondria. In conclusion, we have documented important mitochondrial damage in lymphocytes from a patient treated with NRTIs; this damage is associated with an increase in apoptosis in lymphocytes and is not immediately reversible by treatment withdrawal.

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