Clin Exp Immunol 2000; **122**:364–373

Apoptosis and apoptosis-associated perturbations of peripheral blood lymphocytes during HIV infection: comparison between AIDS patients and asymptomatic long-term non-progressors

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(Accepted for publication 25 July 2000)

SUMMARY

This study was designed to compare the degree of lymphocyte apoptosis and Fas–Fas ligand (FasL) expression in AIDS patients and long-term non-progressors (LTNPs) and correlate these parameters with apoptosis-associated perturbations in lymphocyte function. LTNPs had a lower frequency of apoptotic $CD4^+$ and $CD8^+$ T cells compared with subjects with AIDS. This correlated with a lower frequency of cells expressing Fas and FasL. The frequency of selected lymphocyte populations exhibiting a disrupted mitochondrial transmembrane potential ($\Delta\Psi_m$) and increased superoxide generation was lower in LTNPs than in patients with AIDS; these abnormalities were associated with lower levels of caspase-1 activation in LTNPs. The results indicate a significantly reduced level of apoptosis and apoptosis-associated parameters in LTNPs than in patients developing AIDS. Based on these findings, a crucial role for mitochondria can be predicted in the process of lymphocyte apoptosis during the evolution of AIDS.

Keywords long-term non-progressors lymphocyte apoptosis mitochondria

INTRODUCTION

An enhanced apoptotic turnover contributes to the progressive decline in $CD4^+$ T lymphocyte numbers characteristic of AIDS [1–3]. A small subset of untreated HIV-infected subjects, who are referred to as long-term non-progressors (LTNPs), show little or no net T cell loss and remain asymptomatic for periods longer than at least 7 years after seroconversion with a relatively low viral burden in the peripheral blood and lymphoid organs [4,5]. The reasons for such an extraordinarily benign disease course of an otherwise rapidly evolving infection are not completely understood, but there is some evidence suggesting that peripheral lymphocytes from LNTPs have a relatively low level of apoptosis compared with lymphocytes from subjects progressing to AIDS [6–8].

Several different factors account for the increased lymphocyte apoptosis seen in HIV-infected progressors. These factors include increased transduction of the apoptotic signal of the Fas–Fas ligand (FasL) pathway [9–12] through the endogenous mediators ceramide [13–15] and IL-1β-converting enzyme (ICE, caspase-1) [16–18], as well as the generation of oxidant stress [19–21].

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Since the mechanisms responsible for the slow rate of lymphocyte apoptosis in LTNPs are largely unknown, this study was designed to compare the degree of lymphocyte apoptosis and Fas–FasL expression and correlate them with apoptosis-associated perturbations in lymphocyte function in AIDS patients and in a group of LTNPs who remained clinically asymptomatic with stable $CD4^+$ T counts for at least 7 years after seroconversion. Our results demonstrate that LTNPs have lower frequencies, compared with AIDS patients, of (i) apoptotic $CD4^+$ and $CD8^+$ T cells; (ii) lymphocytes expressing Fas and FasL; and (iii) selected lymphocyte populations exhibiting a disrupted mitochondrial transmembrane potential ($\Delta\Psi_{m)}$ and increased superoxide generation.

MATERIALS AND METHODS

Patients and lymphocyte isolation

Peripheral blood mononuclear cells (PBMC) were obtained from 12 male HIV^+ LTNPs and from 12 consecutively recruited male patients with clinical or laboratory parameters of AIDS (Centers for Disease Control and Prevention 1993, C3) living in the Community of San Patrignano (Rimini, Italy). All patients with AIDS were receiving zidovudine (600 mg daily) and trimethoprim-sulfamethoxazole (oral 15 mg/kg daily of the trimethoprim component) for prophylaxis of *Pneumocystis carinii* pneumonia.

None of the patients had an opportunistic infection within 1 month of the tests being done. The demographic and clinical characteristics of LTNPs and AIDS patients who entered this study are summarized in Table 1.

PBMC were separated from heparinized peripheral blood by Lymphoprep gradient centrifugation (Nycomed, Oslo, Norway), washed twice with PBS and resuspended in RPMI 1640 (Life Technologies, Inc., Paisley, UK) medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies), 10 U/ml penicillin/streptomycin (Life Technologies), 10 mM HEPES (Sigma Chemical Co., St Louis, MO), and 1 mM L-glutamine (Life Technologies) (complete medium). In the apoptosis assay, PBMC (5×10^5/ml) were cultured in complete medium for 12 h at 37°C in a 5% CO_2-humidified atmosphere. In addition, for the analysis of mitochondrial functions, aliquots of cells were isolated and maintained in complete culture medium at 4°C until labelling.

Expression of surface and intracellular antigens

The absolute counts of cells bearing either the CD4, CD8 or the Fas/FasL phenotype were determined by flow cytometry. PBMC were stained with the following antibodies: PE-labelled anti-hCD4 or anti-hCD8 (Becton Dickinson Immunocytometry Systems, San Josè, CA), anti-hCD95/Fas/APO1 (Upstate Biotechnology Inc., New York, NY) and FITC-labelled anti-mouse IgM (Sigma), anti-hCD95L/FasL (PharMingen, San Diego, CA) and anti-mouse IgG FITC conjugate (Sigma). For staining of surface antigens 5×10^5 PBMC were washed in PBS containing 1% bovine serum albumin (BSA; Sigma) and 0·1% sodium azide (PBS–BSA–NaN_3) followed by incubation for 20 min at 4°C with the MoAbs previously described. For determination of background staining, cells were incubated with 20 μl each of mouse IgG1 FITC and mouse IgG1 PE (Becton Dickinson Immunocytometry Systems). Then, after washing twice with PBS–BSA–NaN_3 containing 2% FCS, the labelled cells were analysed by flow cytometry using a FACScan flow cytometer (Becton Dickinson). For each sample 10 000 viable lymphocytes were gated, according to size (forward scatter, FSC) and granularity (side scatter, SSC) parameters.

Assessment of cells undergoing apoptosis

Staining of apoptotic nuclei with propidium iodide. Lymphocyte apoptosis was quantified as the percentage of cells with hypodiploid DNA using the technique of Nicoletti *et al.* [22–24]. Briefly, following a short-term culture, cell suspensions were centrifuged at 200 ***g*** for 10 min. For staining of surface antigens, aliquots of 1×10^6 cells were incubated with FITC-conjugated MoAbs as previously described and, after washing, the pellet was gently resuspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide (PI) in 0·1% sodium citrate plus 0·1% Triton X-100, 0·05 mg/ml RNase A; Sigma). Cells were kept overnight at 4°C, then analysed in their staining solution using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with a 15-mW air-cooled 488-nm argon–ion laser. Apoptotic nuclei appeared as a broad hypodiploid DNA peak which was easily distinguished from the narrow peak of nuclei with normal (diploid) DNA content in the red fluorescence channel. Orange PI fluorescence was collected after a 585/42 nm band pass (BP) filter and was displayed on a four-decade log scale. Acquisition on the flow cytometer was done in the low sample flow rate setting (12 μl/min) to improve the coefficient of variation on the DNA histograms. Lymphocytes, including live, early apoptotic and late apoptotic cells, were gated on the basis of their FSC and SSC parameters, and fluorescence data were gated on FSC *versus* PI fluorescence dual-parameter contour plots for exclusion of monocytes, debris and clumps. This method of gating allowed ready discrimination of debris (very low FSC and decreased PI fluorescence) from dead cells (low FSC and

Table 1. Demographic and clinical characteristics of long-term non-progressors (LTNPs) compared with AIDS patients (CDC stage 3)

Characteristic	LTNPs	AIDS	*P*
No	12	12	NS
M/F sex ratio	9/3	10/2	NS
Age, years			
Median ± s.d.	36 ± 5	33 ± 4	NS
Range	30–44	24–42	
Years since seroconversion			
Median ± s.d.	10 ± 2	3 ± 1	< 0·000 01
Range	8–12	1–5	
Viraemia, particles/μl			
Median ± s.d.	2100 + 1451	53 800 + 27 432	0·0004
Range	900–8300	3200–104 500	
$CD4^+$ T cells, no./μl			
Median ± s.d.	804 ± 83	35 ± 16	< 0·0001
Range	680–911	21–67	
$CD8^+$ T cells, no./μl			
Median ± s.d.	1351 ± 142	280 ± 82	< 0·000 01
Range	1287–1789	173–460	
Total lymphocytes, no./μl			
Median ± s.d.	2630 ± 172	411 ± 114	< 0·000 01
Range	2383–2885	269–633	

NS, Not significant.

high PI fluorescence). A minimum of 10 000 events was collected on each sample.

Phenotypic analysis of apoptotic T cells. Quantification and phenotypic analysis of apoptotic cells from the short-term cultured lymphocytes were performed by staining apoptotic cells with 7-amino-actinomycin D (7-AAD; Sigma) as reported by Schmid *et al*. [25]. This method was shown to discriminate between early and late apoptotic cells due to the increased membrane permeability of the latter group. Cultured lymphocytes were first incubated with FITC-conjugated MoAbs against surface antigens as described above, and washed cells were then incubated with 20 μg/ml of 7-AAD for 20 min at 4°C protected from light. Stained cells were further fixed with 1% paraformaldehyde in PBS in the presence of 20 μg/ml of non-fluorescent actinomycin D (Sigma) to block 7-AAD staining within apoptotic cells and avoid non-specific labelling of living cells. Finally, the double-stained cells were incubated overnight at 4°C in the dark and were then analysed in their staining solution by a FACScan flow cytometer (Becton Dickinson). The green fluorescence was collected after a 530/30 BP nm filter, the red fluorescence from 7-AAD was filtered through a 650 long pass filter. Scattergrams were generated by combining FSC with 7-AAD fluorescence, and regions were drawn around clear-cut populations having either negative (live cells), dim (early apoptotic cells), or bright fluorescence (late apoptotic cells). A minimum of 10 000 events was collected on each sample.

Analysis of mitochondrial functions

For the simultaneous determination of surface markers and $\Delta\Psi_m$, cells were first stained with PE-labelled anti-hCD4 or anti-hCD8 (Becton Dickinson) and anti-Fas (PharMingen) antibodies (30 min on ice). Cells were washed (5 min; 600 ***g***; 4°C) in ice-cold staining buffer (PBS, pH 7·2, supplemented with 2% BSA) (Sigma) followed by exposure for 15 min at 37°C to 40 nmol/*l* 3,3′-dihexyloxacarbo-cyanine iodide (3) ($DiOC_6$; Molecular Probes, Eugene, OR) [26]. For the simultaneous assessment of surface markers and mitochondrial reactive oxygen species (ROS) generation, such as superoxide and hydroxyperoxide, cells were first stained with PE-labelled anti-hCD4 or anti-hCD8 antibodies and then exposed for 15 min at 37°C to 2 mmol/*l* hydroethidine (HE; Molecular Probes) [27] or for 1 h at 37°C to 5 mM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) [27,28], respectively. In control experiments, cells were labelled after preincubation with the uncoupling agent carbonyl cyanide m-chlorophenyl-hydrazone (mClCCP; 50 mmol/*l*, 37°C, 30 min; Sigma), or the ROS-generating agent menadione (1 mmol/*l*, 37°C, 1 h; Sigma). For DCFH-DA, a positive control (cells kept for 2 min in 15 mM H_2O_2 and washed three times) was inserted. Analysis were performed using a FACScan cytofluorometer (Becton Dickinson). FSC and SSC parameters were gated on the major population of normal-sized lymphoid cells. After suitable compensation, fluorescence was recorded at different wavelengths: FITC, $DiOC_6$(3) and DCFH-DA at 525 nm (FL-1), PE at 575 nm (FL-2) and HE at 600 nm (FL-3).

Statistical analysis

The primary purpose of this study was to compare several parameters, both actual values and percentages, between the patients with AIDS and LTNPs. Individual *P* values for each separate parameter comparison were obtained using the Wilcoxon's rank sum test, and the unadjusted *P* values are reported. Multiple *P* values were adjusted following the method developed by Hochberg [29], and a non-parametric procedure for simultaneously evaluating multiple endpoints was used [30]. All *P* values are two-tailed.

RESULTS

Fas–FasL expression and lymphocyte apoptosis

Patients with AIDS had lower $CD4^+$ and $CD8^+$ T counts in comparison with LTNPs ($P < 0{\cdot}0001$ for CD4 and $< 0{\cdot}000\ 01$ for CD8) (Table 1). Furthermore, expression of both Fas and FasL was found to be significantly increased among $CD4^+$ and $CD8^+$ T cell subsets from the AIDS subjects with respect to LTNPs ($P = 0{\cdot}0002$ and $< 0{\cdot}000\ 01$ for Fas-positive $CD4^+$ and Fas-positive $CD8^+$ T cells, respectively; $P < 0{\cdot}000\ 01$ and $= 0{\cdot}002$ for FasL-positive $CD4^+$ and FasL-positive $CD8^+$ T cells, respectively) (Table 2).

These findings were associated with an increased susceptibility of lymphocytes to apoptosis. In fact, an increased number of lymphocytes were undergoing apoptosis in AIDS patients compared with LTNPs. This was established by staining apoptotic nuclei with PI [22], which detects late events of apoptosis such as chromatin condensation and DNA fragmentation [24]. Following 12 h of incubation in complete medium, the percentage of spontaneous apoptosis was significantly increased in $CD4^+$ and $CD8^+$ T lymphocytes from patients at the AIDS stage compared

Table 2. Apoptotic $CD4^+$ and $CD8^+$ T lymphocytes and Fas–FasL expression (see Materials and Methods for details)

Parameter	LTNPs	AIDS	*P*
Apoptotic CD4 (%) (stained with PI)			
Median ± s.d.	3·4 ± 0·7	31·8 ± 5	< 0·000 01
Range	2·3–4·4	21·4–36·5	
Apoptotic CD8 (%) (stained with PI)			
Median ± s.d.	5·5 ± 1·1	27·7 ± 3	< 0·000 01
Range	3·5–7·6	20·5–31·2	
Apoptotic CD4 (%) (stained with 7-AAD)			
Median ± s.d.	3·3 ± 0·8	31·7 ± 5	< 0·000 01
Range	2·1–4·5	20·7–38·9	
Apoptotic CD8 (%) (stained with 7-AAD)			
Median ± s.d.	5·4 ± 1·1	26·9 ± 2·4	< 0·000 01
Range	3·7–7·8	22·6–30·2	
Fas CD4 (%)			
Median ± s.d.	38 ± 8·2	71 ± 18	0·0002
Range	16–51	31–85	
Fas CD8 (%)			
Median ± s.d.	26·7 ± 6	52·7 ± 12	< 0·000 01
Range	16–35	22–67	
FasL CD4 (%)			
Median ± s.d.	29·3 ± 11	70 ± 17	< 0·000 01
Range	1–46	33–98	
FasL CD8			
Median ± s.d.	39·7 ± 11	71 ± 2	0·002
Range	13–50	10–88	

PI, Propidium iodide; 7-AAD, 7-amino-actinomycin D; LTNP, long-term non-progressor.

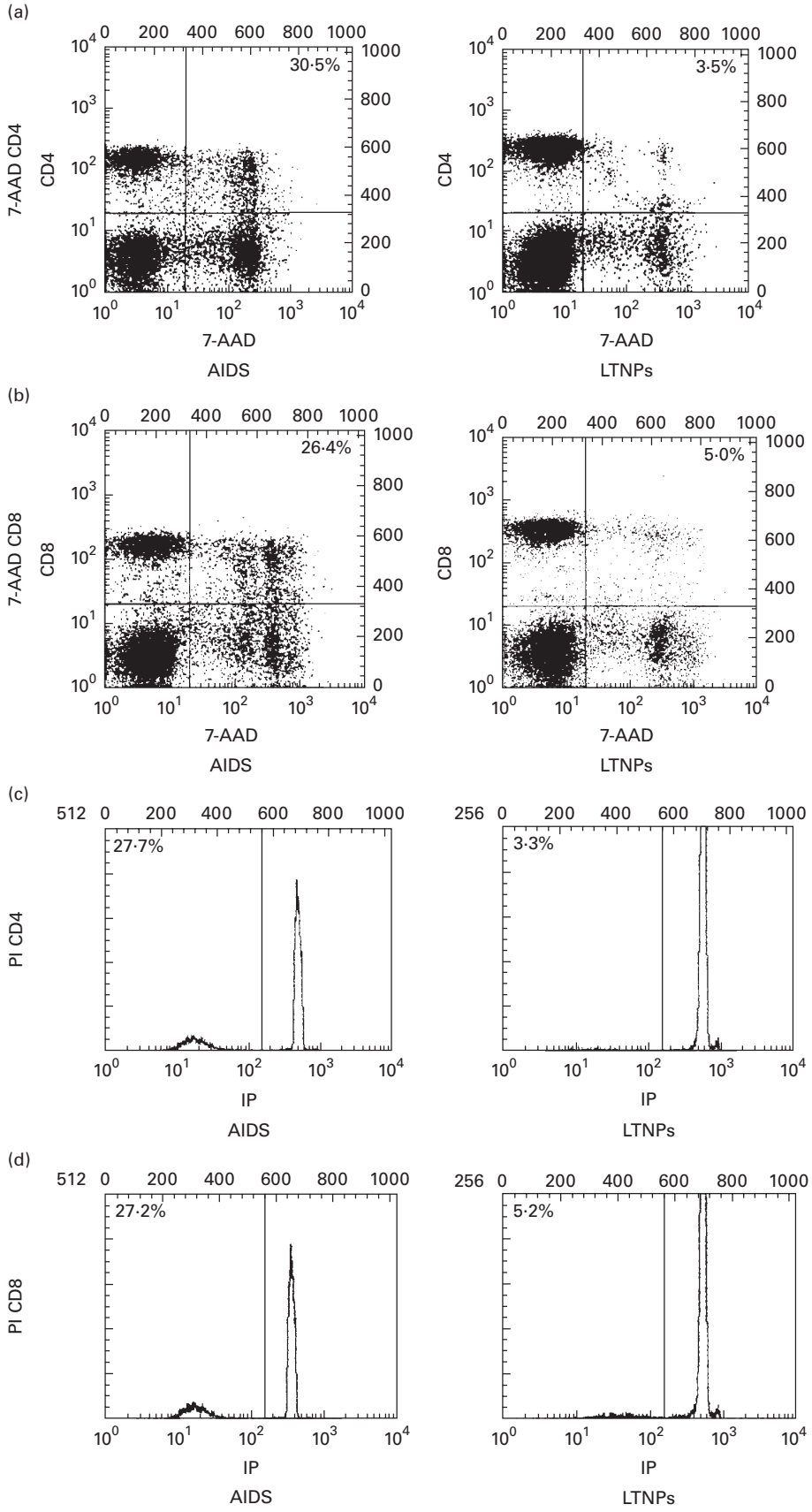

Fig. 1. (See next page for caption)

with the rate in LTNPs (31·8 ± 5·3 and 27·7 ± 2·4 in AIDS patients, 3·4 ± 0·7 and 5·5 ± 1·1 in LTNPs, respectively, for $CD4^+$ and $CD8^+$ T cells; $P < 0{\cdot}000\ 01$ for both parameters), whereas the level of apoptosis was only 1% in lymphocytes from control donors (Table 2). We confirmed these results by measuring apoptosis also with 7-AAD, a fluorescent DNA-intercalating agent which only penetrates the membrane of cells undergoing apoptosis and thus exhibiting a shrunken phenotype (reduced FSC) ($P < 0{\cdot}000\ 01$ for both parameters) (Table 2). Furthermore, this staining discriminates between early and late apoptotic cells [25], and Fig. 1 shows that both methods produced comparable results when measuring the extent of lymphocyte apoptosis in lymphocytes from AIDS patients and LTNP individuals. The FSC and SSC characteristics of cells stained with PI and 7-AAD confirmed that cells were indeed undergoing apoptosis (data not shown).

Generation of ROS

As shown in Table 3, circulating lymphocytes from HIV^+ donors contained a fraction of cells which are able to oxidize the non-fluorescent lipophilic (i.e. membrane-permeable) dye HE into the hydrophilic fluorescent product Eth. Since HE is particularly sensitive to the superoxide anion, this change is thought to reflect the generation of this anion [27]. Moreover, lymphocytes were labelled using DCFH-DA, a fluorochrome that detects hydroperoxide generation [27,28]. We found that the percentage of cells bearing an Eth^{high} and DCFH-DA^+ phenotype was elevated in AIDS patients compared with LTNPs. Statistical analysis revealed a highly significant difference between the two groups with respect to $CD4^+$ and $CD8^+$ T cells stained with DCFH-DA ($P < 0{\cdot}000\ 01$ for both parameters) (Table 3). A significant increase in the Eth^{high} $CD4^+$ T cell subset was also found in the AIDS group compared with LTNPs ($P < 0{\cdot}000\ 01$) (Table 3). There was a similar trend for the Eth^{high} $CD8^+$ T cell subset, although the difference was only marginally significant ($P = 0{\cdot}03$) (Table 3).

Typical cytofluorographic plots using staining with Eth and DCFH-DA are shown in Figs 2 and 3.

Mitochondrial activity

The low incorporation of $DiOC_6(3)$, a cationic lipophilic fluorochrome that allows for the assessment of mitochondrial $\Delta\Psi_m$, is thought to reflect the dissipation of mitochondrial $\Delta\Psi_m$ [26]. This change constitutes an early and irreversible step in the effector phase of apoptosis [31–34]. A relatively high percentage of peripheral blood T lymphocytes from HIV^+ donors incorporated low levels of $DiOC_6(3)$. However, there was no correlation between the clinical status of HIV carriers and the level of $DiOC_6(3)$ incorporation when we investigated the $CD4^+$ and $CD8^+$ T populations. Indeed, the differences observed between AIDS subjects and LTNPs were significant with respect to the $DiOC_6(3)^{low}$ $CD4^+$ T cells ($P = 0{\cdot}008$) but not to $CD8^+$ T cells ($P = 0{\cdot}24$) (Table 4). By contrast, we observed that patients with AIDS had a higher percentage of $DiOC_6(3)^{low}$ lymphocytes expressing the Fas antigen compared with such lymphocytes from LTNPs ($P = 0{\cdot}002$) (Table 4).

Typical cytofluorographic plots using staining with $DiOC_6(3)$ are shown in Fig. 4.

DISCUSSION

We report here that LTNPs had a lower degree of lymphocyte apoptosis, with regard to either $CD4^+$ or $CD8^+$ T cells, compared with subjects progressing to AIDS. Previous studies have suggested that apoptosis is a critical contributory component to HIV disease progression, and data provided so far are consistent with the view that the persistent viraemia or the chronic state of immune activation that characterize HIV infection might be the primary mechanism responsible for the accelerated rate of apoptotic lymphocyte death in AIDS [1–3]. A strong correlation appears indeed to link the intensity of lymphocyte apoptosis and the degree of cell activation [6,35].

The reduced rate of apoptosis that we measured in lymphocytes from LTNPs in comparison with AIDS patients was associated with lower expression of Fas, which is both an activation marker and a death factor, and FasL. These results correlate well with previous evidence of significantly lower levels of lymphocyte-associated ceramide, an endogenous mediator of apoptosis which has been shown to play a crucial role in Fas-induced apoptotic signalling [13,14], in LTNPs in comparison with AIDS patients [7,36]. Other studies have recently suggested an involvement of the Fas–FasL system in the pathophysiology of the HIV-related $CD4^+$ T lymphocyte decline, and up-regulation of Fas expression on both $CD4^+$ and $CD8^+$ T cells, as well as increased sensitivity of lymphocytes to Fas-induced apoptosis [9–12], have been reported in patients with HIV infection.

The increased level of lymphocyte apoptosis that we found in subjects with AIDS was associated with altered mitochondrial function. We measured a higher proportion of cells incorporating low levels of the $DiOC_6(3)$ fluorochrome among peripheral blood lymphocytes from AIDS patients than from LTNPs; in particular, $DiOC_6(3)^{low}$ cells with decreased $\Delta\Psi_m$ were significantly more frequent among the Fas^+ subset irrespective of a $CD4^+$ or $CD8^+$ phenotype. In our opinion, these data further highlight, although indirectly, the relationship linking the expression of Fas with the increased rate of lymphocyte apoptosis in HIV progressors.

It is known that lymphocytes undergoing apoptosis suffer a sequential dysregulation of mitochondrial function early during the apoptotic process, and this implicates some functional influence of mitochondria on the regulation of apoptotic cell death [31–34]. In all experimental models of apoptosis, including those associated with Fas–FasL activation and those induced by oxidant stress, the alterations in nuclear morphology and degradation of chromosomal DNA, the hallmarks of the apoptotic process are invariably preceded by a step-wise dysregulation of mitochondrial function [31–34]. Lymphocytes exhibit first a reduction in mitochondrial $\Delta\Psi_m$, as quantifiable by means of suitable fluorochromes such as $DiOC_6(3)$, and then an additional decrease in $\Delta\Psi_m$ as evidenced by increased superoxide anion-mediated oxidation of

Fig. 1. (See previous page) Assessment of apoptotic peripheral blood T lymphocytes from AIDS patients and long-term non-progressors (LTNPs). Two-colour staining was performed for the simultaneous determination of apoptosis and CD4/CD8 surface phenotype. Quantification of apoptosis was performed by staining either apoptotic cells with 7-amino-actinomycin D (7-AAD) (a,b) or apoptotic nuclei with propidium iodide (PI) (c,d), as described in Materials and Methods. Numbers refer to the percentage of apoptotic cells among the $CD4^+$ (a,c) and $CD8^+$ (b,d) T lymphocytes. Results are representative of two independent experiments each performed on two different subjects.

Table 3. Generation of reactive oxygen species by $CD4^+$ and $CD8^+$ T lymphocytes as assessed with staining with the fluorochromes hydroethidine (HE) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (see Materials and Methods for details)

Parameter	LTNPs	AIDS	*P*
HE CD4 (%)			
Median ± s.d.	13·1 ± 3	22·1 ± 2·6	< 0·000 01
Range	9–18·8	18–25	
HE CD8 (%)			
Median ± s.d.	14·7 ± 2·3	17 ± 6·2	0·03
Range	12–19	13–34	
DCFH-DA CD4 (%)			
Median ± s.d.	26·3 ± 6·5	41·4 ± 4·2	< 0·000 01
Range	10–30	31–46	
DCFH-DA CD8 (%)			
Median ± s.d.	13·4 ± 3·6	31·1 ± 4·2	< 0·000 01
Range	4–21	29–40	

LTNPs, Long-term non-progressors.

HE into the fluorescent product Eth [31–34]. The collapse in $\Delta\Psi_m$ constitutes an early and irreversible step in the apoptotic effector phase and allows us to identify an additional pool of lymphocytes that are irreversibly committed to undergo apoptosis despite still lacking the characteristic morphological nuclear changes and the degradation of internucleosomal DNA associated with apoptosis [31–34].

The altered mitochondrial function, as shown by the disruption of $\Delta\Psi_m$ that we determined in patients with AIDS rather than in LTNPs, appeared to be associated with oxidant stress and the increased generation of ROS. In fact, we found a greater frequency of $(HE\rightarrow Eth)^{high}$ or $DCFH\text{-}DA^{high}$ cells among $CD4^+$ and $CD8^+$ T lymphocytes from patients with AIDS compared with LTNPs. Thus, based on our findings, the pool of lymphocytes with abnormal mitochondrial function and enhanced generation of ROS appears to be significantly greater in subjects progressing to AIDS than in LTNPs. Taking into account all our findings, a crucial role for mitochondria in the process of lymphocyte apoptosis during the evolution of the disease towards AIDS can be predicted.

Different mechanisms may contribute to the mitochondrial alterations associated with HIV infection. HIV-specific gene

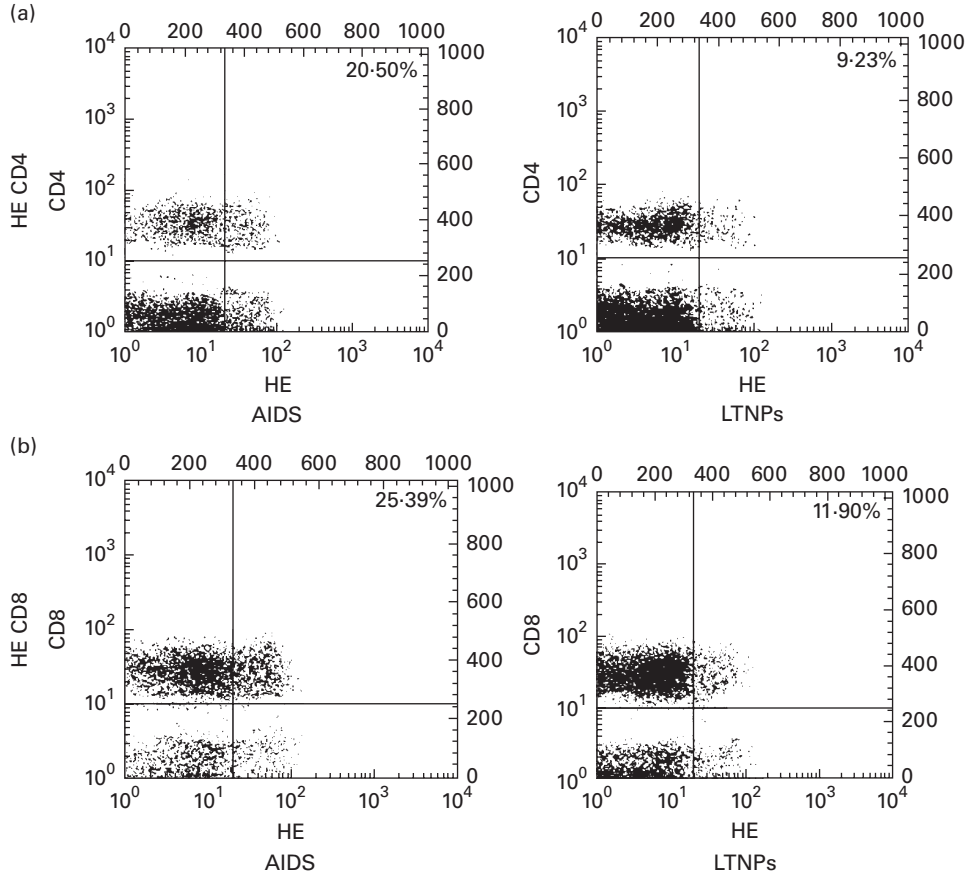

Fig. 2. Assessment of superoxide anion generation in peripheral blood T lymphocytes from AIDS patients and long-term non-progressors (LTNPs). Two-colour staining was performed for the simultaneous determination of CD4/CD8 surface phenotype and superoxide anion generation. Cells from representative AIDS patients and LTNPs were labelled, as described in Materials and Methods, using markers for superoxide anion generation (hydroxyethidine (HE)) and MoAbs to CD4/CD8 antigens (a/b, respectively). Numbers refer to the percentage of cells bearing an $HE\rightarrow Eth^{high}$ phenotype among the $CD4^+$ and $CD8^+$ T lymphocytes. In the FACScan profiles, apoptotic cells were identified either by forward light scatter (FSC)/side scatter (SSC) criteria or by positive staining with 7-amino-actinomycin D (7-AAD), as described in Materials and Methods. The cursors were set according to the background staining defined with mouse IgG isotypic controls. Results are representative of two independent experiments each performed on two different subjects.

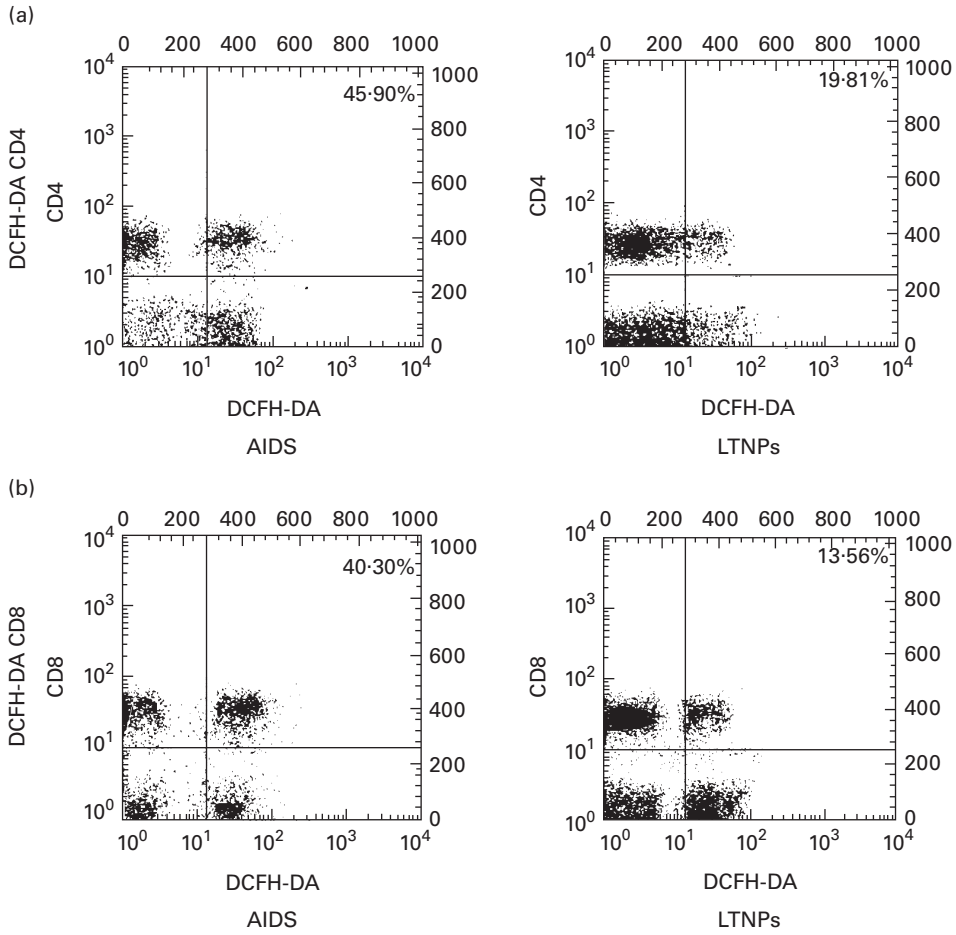

Fig. 3. Assessment of hydroxyperoxide generation in peripheral blood T lymphocytes from AIDS patients and long-term non-progressors (LTNPs). Two-colour staining was performed for the simultaneous determination of CD4/CD8 surface phenotype and hydroxyperoxide generation. Cells from representative AIDS patients and LTNPs were labelled, as described in Materials and Methods, using markers for hydroxyperoxide generation (2′,7′-dichlorofluorescein diacetate (DCFH-DA)) and MoAbs to CD4/CD8 antigens (a/b, respectively). Numbers refer to the percentage of DCFH-DA-positive cells among the $CD4^+$ and $CD8^+$ T lymphocytes. Results are representative of two independent experiments each performed on two different subjects.

Table 4. $CD4^+$ and $CD8^+$ T lymphocytes and Fas-positive lymphocytes with disrupted mitochondrial transmembrane potential as shown by the incorporation of the (3,3′-dihexyloxacarbo-cyanine iodide (3) (DiOC6(3)) fluorochrome (see Materials and Methods for details)

Parameter	LTNPs	AIDS	*P*
$DiOC6(3)^{low}$ CD4 (%)			
Median ± s.d.	27·6 ± 8	28·3 ± 2·5	0·008
Range	23–47	34–42	
$DiOC6(3)^{low}$ CD8 (%)			
Median ± s.d.	33·5 ± 10·3	41·5 ± 5	0·24
Range	24–62	28–45	
DiOC6(3) Fas (%)			
Median ± s.d.	27·8 ± 13·2	44·8 ± 5·9	0·002
Range	5–57	36–59	

LTNPs, Long-term non-progressors.

products could directly affect mitochondrial function [19–21, 37–40]; however, it is more likely that mitochondrial function is predominantly altered indirectly by HIV infection. The relatively small proportion of lymphocytes actually infected with HIV *in vivo* and the observation that apoptosis occurs in both $CD4^+$ and $CD8^+$ T lymphocytes support this view. An additional mechanism of mitochondrial damage could be the oxidation of mitochondrial DNA caused by the use of anti-retroviral drugs, in particular zidovudine [41].

One point to be noted about this study is that the AIDS patients we investigated were fast progressors with a median time from seroconversion to the development of AIDS significantly shorter than that usually seen in developed countries [42]. We do not know whether this discrepancy has influenced the differences in the degree of apoptosis between the LTNPs and AIDS patients determined in this study. On the basis of our findings, the only conclusion we can draw is that once HIV infection has progressed to AIDS patients have a greater degree of lymphocyte apoptosis compared with asymptomatic LTNPs. Whether this is also influenced by the rate of disease progression remains to be established. Our work was not designed to test this hypothesis, and longitudinal investigations will be helpful to clarify this problem.

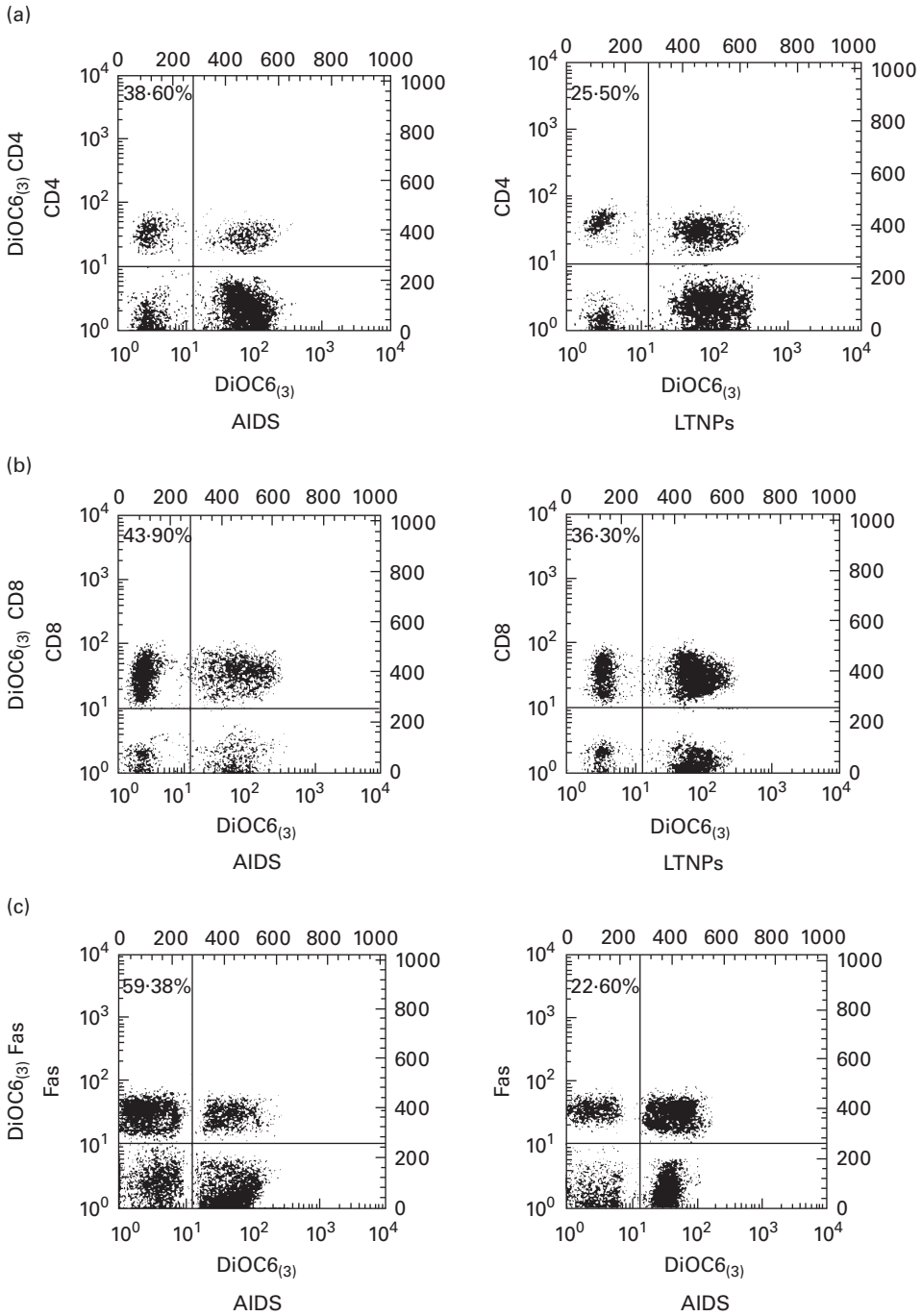

Fig. 4. Assessment of mitochondrial transmembrane potential ($\Delta\Psi_m$) in peripheral blood T lymphocytes from AIDS patients and long-term non-progressors (LTNPs). Two-colour staining for the simultaneous determination of surface phenotype and $\Delta\Psi_m$. Cells from representative AIDS patients and LTNPs were labelled, as described in Materials and Methods, using markers for $\Delta\Psi_m$ (3,3′-dihexyloxacarbo-cyanine iodide (3) ($DiOC_6(3)$)) and for CD4, CD8 and Fas antigens (a,b,c, respectively). Numbers refer to the percentage of lymphocytes with disrupted mitochondrial transmembrane potential ($DiOC_6(3)^{low}$) among the CD4, CD8 and Fas-positive cells. Results are representative of three independent experiments each performed on two different subjects.

An additional point is that all the patients with AIDS in this study were on zidovudine therapy at the time of cell sampling for apoptosis analysis. The relative contribution of zidovudine to the increased lymphocyte apoptosis is unclear. Zidovudine and other nucleoside analogues have been shown to induce apoptosis in several *in vitro* systems [43–46]. In clinical studies of anti-retroviral therapy however, the suppression of viral replication is not clearly associated with a decline in lymphocyte apoptosis [47–49]. Rather, the marginal improvement in indices of apoptosis afforded by anti-retroviral therapy appears to be in part independent of the suppression of viral replication. Even though these data are somewhat conflicting, in our opinion the direct

pro-apoptotic action of nucleoside analogues is probably marginal *in vivo* compared with the pro-apoptotic potential either directly or indirectly exploited by the virus. It is also likely that even low levels of HIV replication are sufficient to induce immune activation and lymphocyte apoptosis.

In conclusion, our data indicate a significantly reduced level of apoptosis and apoptosis-associated parameters in LTNPs compared with patients developing AIDS. Even though a number of questions remain, it appears plausible that a lower degree of Fas/FasL expression and/or enhanced apoptosis resistance associated with a lower degree of mitochondrial superoxide anion generation may contribute to delayed AIDS progression and improving the long-term outcome of HIV infection.

ACKNOWLEDGMENTS

Informed consent was obtained from all patients, and human experimentation guidelines of the Ethical Committee of the Community of San Patrignano were followed in the conduct of clinical research.

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