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$CD4^+$ lymphocytes from HIV-infected patients display impaired CD45-associated tyrosine phosphatase activity which is enhanced by anti-oxidants

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SUMMARY

It has been proposed that signal transduction defects may, at least partially, account for the functional impairment of $CD4^+$ lymphocytes during HIV-1 infection. Recently, we have demonstrated that unresponsive $CD4^+$ lymphocytes from these patients had reduced protein tyrosine phosphorylation after CD3 engagement, and that this defect was associated with constitutively altered levels of $p56^{lck}$ and $p59^{fyn}$ kinases. Since CD45 is essential for T cell receptor (TCR) and CD2-mediated activation of protein tyrosine kinases, we study here CD45-associated tyrosine phosphatase activity in resting and activated CD4 T cells from HIV-infected patients. We found a significant decrease in the basal and post-activation phosphatase activity of CD45 which correlated well with impairment of proliferative responses. In addition, decreased levels of cellular thiols observed in resting $CD4^+$ lymphocytes from these patients suggested a disturbed redox status. Although expression levels of CD45 were decreased in most patients, a significant recovery of phosphatase activity and proliferative responses was observed in most patients by preincubating cells with *N*-acetyl-L-cysteine and β_2-mercaptoethanol. In some patients, anti-oxidant treatment failed to significantly enhance phosphatase activity and proliferative responses. The low responses of purified $CD4^+$ lymphocytes from these patients were associated with a high ratio of apoptotic cell death which did not appear to be influenced by anti-oxidant treatment.

Keywords HIV $CD4^+$ lymphocytes CD45 tyrosine phophatase oxidative stress

INTRODUCTION

Progressive depletion of $CD4^+$ T cells is the hallmark of HIV infection [1]. However, in a number of asymptomatic HIV-infected individuals, despite adequate $CD4^+$ T cell counts, early impairment of immune responses may also occur [2–5]. These functional defects observed in both $CD8^+$ and $CD4^+$ T cells exhibit properties reminiscent of an anergic state.

It has been proposed that a signal transduction defect might at least partially account for lymphocyte dysfunction in HIV-infected patients [6]. This assumption is supported by reported alterations in Ca^{2+} mobilization, inositol polyphosphate generation and phospholipase C activation observed in T cells from infected patients and in T cells exposed to viral proteins [7–10]. In a recent work, we found that unresponsiveness of $CD4^+$ T cells from HIV-infected individuals after engagement of the CD3 complex was associated with reduced tyrosine phosphorylation and altered levels of $p56^{lck}$ and $p59^{fyn}$ tyrosine kinases [11]. CD45 tyrosine phosphatase, in concert with the $p50^{csk}$ tyrosine kinase [12–14], is essential for normal tyrosine phosphorylation response following T cell receptor (TCR) engagement by modulating the activity of src-like tyrosine kinases. The enzymatic activity of CD45 depends on the presence of adequate amounts of sulfhydryl compounds [15] and has important effects in levels of constitutive NFkB activity and thereby in HIV-1 replication [16]. In the present work, we have tried to assess the putative role of CD45-associated tyrosine phosphatase activity on functional impairment of $CD4^+$ T cells from asymptomatic HIV-infected individuals. Our results showed a significant decrease in the basal and post-activation tyrosine phosphatase activity of CD45 after CD3 engagement, which correlated well with impairment in proliferative responses. Although expression levels of CD45 were decreased in most patients, *in vitro* anti-oxidant pretreatment resulted in a significant improvement in proliferative responses and tyrosine phosphatase activity from most patients. Nevertheless, in the experimental conditions used in this work, the beneficial effects of anti-oxidant treatment were limited to anergic lymphocytes, and failed to rescue $CD4^+$ lymphocytes engaged in ongoing programmed cell death.

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SUBJECTS AND METHODS

Subjects

Peripheral blood was obtained from 23 asymptomatic HIV-1$^+$ homosexual men with CD4 counts $> 300/mm^3$ followed up at the Hospital of the Pasteur Institut. Seropositive status was defined by the presence of serum antibodies to HIV-1 using an ELISA (Elavia I; Diagnostics Pasteur, Marnes La Coquette, France) and was confirmed by Western blot analysis (Lav-blot; Diagnostic Pasteur). Among these 23 patients only five were under treatment. HIV 11, 13, 17, 18 patients were receiving at the moment of the study a combination of AZT 500 mg/day and 2·25 mg/day of DDC, whereas HIV 14 patient was receiving AZT 500 mg/day and DDI 400 mg/day. Controls ($n = 20$) were HIV$^-$ healthy volunteers.

Antibodies and reagents

Anti-CD16 (3G8, IgG1) and anti-CD56 (T199, IgG1) MoAbs used for panning and a rabbit polyclonal anti-mouse IgG (H + L) antibody used for immunoprecipitation of CD45 were purchased from Jackson Immunoresearch (Marseille, France). Murine anti-CD19 (HD37, IgG1), anti-CD8 (DK25, IgG1) and anti-CD14 (TUK4, IgG2a) MoAbs also used for panning, and anti-CD3 MoAb (UCHT1, IgG1) used for lymphocyte stimulation were purchased from Dako (Dakoppats AS, Glostrup, Denmark). Fluorescent MoAbs to CD45 and CD45RO were from Dako and anti-CD45RA from Coulter (Hialeah, FL). Sodium orthovanadate, Tris (hydroxymethyl) aminomethane (Tris buffer), Nonidet P-40, *N*-acetylcysteine (NAC), β_2-mercaptoethanol (β_2-ME) and dithiothreitol (DTT) were from Sigma (St Louis, MO).

Isolation of CD4 T lymphocytes

Purification of CD4 T lymphocytes was carried out as previously reported [11]. Briefly, peripheral blood lymphocytes (PBL), isolated in a Ficol gradient, were incubated in the presence of anti-CD8, CD19, CD16, CD56 and CD14 antibodies followed by incubation in the presence of sheep anti-mouse-coated immunomagnetic beads (Dynabeads; Dynal, Oslo, Norway), so as to achieve a 5:1 bead-to-cell ratio. After incubation for 60 min at 4°C, cell fractions were separated by application of a magnet to the outside of the tube for 3 min; unbound cells, enriched for CD4 T cells, were carefully aspirated and a second round of immunomagnetic separation was performed for an additional period of 60 min at 4°C with a bead-to-cell ratio of 10:1. Purity of subsets was evaluated at the end of the enrichment procedures. In all experiments, purity of cell preparations was $> 90\%$ for CD3$^+$ CD4$^+$ cells, as determined by immunofluorescence analysis using an EPICS 752 flow cytometer (Coulter).

Membrane preparation

Cells were washed twice in serum-free RPMI 1640 and resuspended at 3×10^6/ml in hypotonic lysis buffer (HLB; Tris–HCl 25 mM pH 7·5, sucrose 25 mM, EDTA 0·1 mM, $MgCl_2$ 5 mM, DTT 5 mM, PMFS 1 mM, leupeptin 10 μg/ml, aprotinin 10 μg/ml). Individual samples were made in duplicate in the presence and absence of 1 mM Na_3VO_4. Cells were sonicated and nuclei were removed by low-speed centrifugation. The membranes were sedimented at 100 000 ***g*** for 60 min at 4°C. The resulting pellet was resuspended in 200 μl of the hypotonic lysis buffer by sonication. Protein concentration was determined using the BioRad assay system.

In vitro *phosphatase assay*

Either 20 μg of membrane protein or CD45 immunocomplexes from 5×10^6 cells were incubated in a reaction mixture of 5 mM of *p*-nitrophenyl phosphate, 80 mM 2-(N-morpholino)ethane sulphonic acid pH 5·5, 10 mM EDTA and 10 mM DTT at 37°C. After 20 min incubation, reactions was stopped by addition of 1 ml of 0·2 N NaOH. The tyrosine phosphatase activity was expressed as absorbance units at 410 nm after removal of duplicate sample values in the presence of vanadate at 1 mM.

Immunoprecipitation

CD4 T cells (5×10^6), were washed twice in serum-free RPMI 1640 and cellular pellets were solubilized in 0·5 ml Nonidet lysis buffer (25 mM Tris–HCl pH 7·4, 1% Nonidet P-40, 150 mM NaCl, 5 mM DTT, 1 mM PMFS, leupeptin 10 μg/ml, aprotinin 10 μg/m) for 30 min at 4°C. Nuclei were then removed by low-speed centrifugation; 10 μg of anti-CD45 MoAb were added and the mixture was incubated for 2 h at 4°C. Supernatants were mixed with 10 mg of Protein A-coupled Sepharose beads pre-coated with 20 μg of rabbit anti-mouse IgG serum and further incubated for 60 min at 4°C. After three washes in Nonidet lysis buffer and three washes in HLB buffer the beads were resuspended in 200 μl of a reaction mixture of 5 mM of *p*-nitrophenyl phosphate, 80 mM 2-(N-morpholino)ethane sulphonic acid pH 5·5, 10 mM EDTA, and 10 mM DTT. After incubation for 30 min at 37°C, absorbance at 410 nm was recorded.

Anti-oxidant preincubation of CD4$^+$ lymphocytes

Addition of NAC (Sigma) was made from freshly prepared stock solutions in RPMI 1640 titrated to pH 7·2 with NaOH. For anti-oxidant treatment, cells were resuspended at 5×10^6/ml in serum-free RPMI 1640 and incubated with NAC (20 mM) and β_2-ME (50 μm) for 2 h at 37°C. Cells were then washed twice to remove excess of NAC and β_2-ME and finally resuspended in 10% fetal calf serum (FCS) RPMI 1640.

Cell proliferation assays

For ^{3}H-thymidine uptake, cells were cultivated in 96-well culture plates at 5×10^4 cells/well in a total volume of 200 μl of supplemented RPMI 1640 culture medium (10% human pooled AB serum (Jacques Boy, Paris, France), glutamine 4 mM, HEPES 20 mM, sodium pyruvate 10 mM and penicillin/streptomycin). Stimulation was carried out by the addition of immunomagnetic beads pre-coated with saturating doses of mouse anti-human CD3 IgG MoAb at a bead-to-cell ratio of 5:1. After 3 days, cultures were pulsed with 1 μCi ^{3}H-thymidine (CEA, Paris, France) during the last 18 h of culture and then harvested with a cell harvester system (Tomtec Inc., Orange, CT) and radioactivity was measured in a micro beta plate scintillation counter (LKB). All cultures were performed in quadruplicate, and for each experiment a control using only culture medium was performed.

Flow cytometry analysis

Flow cytometry analysis was performed on an EPICS 752 fluorescent activated cell sorter (Coulter) as previously reported. In order to standardize the assays and to correct for day-to-day variations in instrument performance, the mean fluorescence of calibration beads (Standard-Brite; Coulter) was used to adjust reference fluorescence. A total of at least 10 000 cells were analysed for each sample, and data were stored in a histogram mode.

Staining for intracellular low molecular weight thiols

Low molecular weight thiol (LMT) levels were estimated by flow cytometry using the 5-chloromethyl fluorescein diacetate (CMF) probe [17]. This non-fluorescent probe has been demonstrated to form fluorescent adducts with intracellular low molecular thiols, of which GSH is by far the most abundant. Intracellular thiol levels were measured by the method previously described by Poot *et al*. [18], with minor modifications. Briefly, a stock solution of 5-chloromethyl fluorescein diacetate was made up in DMSO at 5 μM and stored in the dark at -20°C. Cells were resuspended at 10^6/ml in serum-free medium and equilibrated at 37°C. CMF was added to obtain a final concentration of 50 nM and incubated for 10 min in the dark at 37°C . The cells were immediately analysed in the EPICS 752 flow cytometer.

Flow cytometric analysis of apoptotic cells

The fluorescent dye merocyanine 540 (MC 540; Fluka Chemical Corp., Ronkonkona, NY), is a lipid packing probe that binds avidly to the symmetric membranes with exposed phosphatidyl-serine, and has been successfully used as a fluorescent probe in cytometric analysis of apoptotic cells, characterized by high merocyanin expression [19,20]. For staining, a stock solution of merocyanine was prepared in 50% ethanol at 1 mg/ml and stored in the dark at -20°C. A working solution was freshly prepared by diluting merocyanin at 1: 500 (final concentration 2 μg/ml) in a HEPES-buffered salt solution with 0·1% bovine serum albumin (BSA; Sigma). Then, 10^6 cells were resuspended in 100 μl of working solution and incubated for 5 min in the dark at room temperature. Cells were then washed twice with HEPES-buffered salt solution without BSA and immediately analysed on the Epics 752 flow cytometer.

RESULTS

Decreased membrane-associated tyrosine phosphatase activity in CD4$^+$ T cells from HIV-infected individuals

The vanadate-sensitive membrane-associated tyrosine phosphatase activity was measured in resting CD4$^+$ T cells from 12 asymptomatic HIV-infected patients, and was compared with that of healthy volunteers. As depicted in Fig. 1a, a significant decrease in phosphatase activity was observed ($P < 0{\cdot}05$) in cells from HIV-infected individuals. Relative mean values of optical density for controls were 245 and ranged from 181 to 334. In the case of HIV-infected patients, mean values were 114 and ranged from 35 to 202. The time course of membrane-associated tyrosine phosphatase activity upon immobilized anti-CD3-mediated activation was examined in order to exclude a post-activation normalization of this activity. As depicted in Fig. 1b, phosphatase activity from normal lymphocytes upon CD3-mediated stimulation showed a typical but transient increase, reaching maximal values at 5 min, followed by a progressive decrease up to 60 min. Although a similar kinetic pattern was observed in HIV-infected individuals, absolute values of phosphatase activity remained significantly decreased and maximal values were achieved precociously (at 1 min post-activation). As reported, the proliferative responses of CD4 T cells from HIV-infected individuals were defective and ranged from about 10% to 90% of those observed in normal CD4 T cells and correlated well with impairment in membrane-associated tyrosine phosphatase activity (Fig. 1c).

When flow cytometric analysis of CD45 was performed, CD4 T cells from most HIV-infected patients displayed decreased expression of CD45 (Fig. 2). In patients with decreased CD45 expression, flow cytometry analysis using anti-CD45RA and anti-CD45RO fluorescent antibodies revealed a similar decrease in expression levels of both CD45RA^{high} and CD45RO^{high} isoforms (data not shown). No clear correlation with impairment in membrane-associated tyrosine phosphatase activity was observed, suggesting that decreased levels of CD45 can only partially explain decreased activity.

Enhancement of CD45 tyrosine phosphatase activity and proliferative responses of CD4 T cells following pre-incubation with NAC and β_2-ME

Recent reports have shown that the enzymatic activity of CD45 depends on the presence of sulfhydryl compounds for activity, and that alkylating agents irreversibly inactivate the enzyme [15,21]. Since recent work has indicated that oxidative stress may be important in the immunopathogenesis of HIV infection [22,23], the intracellular thiol levels of purified CD4 T cells from 11 additional HIV-infected patients were monitored by FACS using the chloromethyl-fluorescein probe and compared with levels of

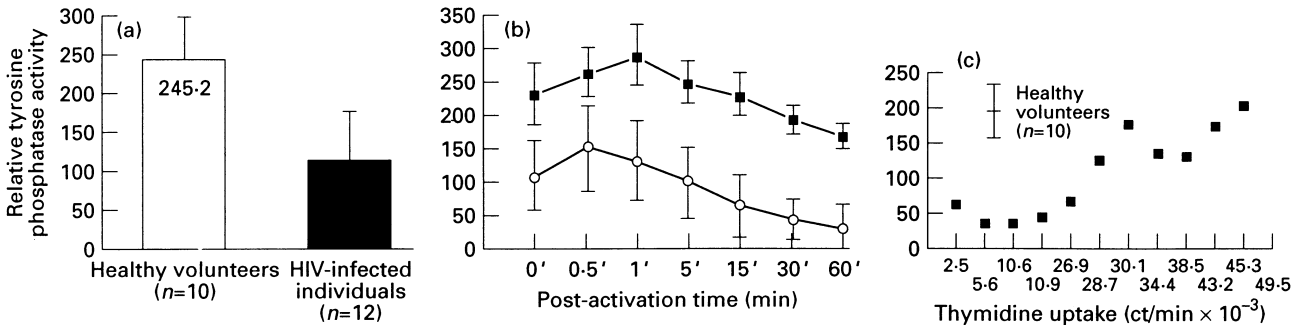

Fig. 1. Membrane-associated tyrosine phosphatase activity of purified CD4$^+$ lymphocytes from healthy volunteers and HIV-infected patients. Individual values of tyrosine phosphatase activity are expressed as absorbance units at 410 nm after removal of duplicate sample values in the presence of vanadate. (a) Membrane-associated tyrosine phosphatase activity in resting non-activated CD4 lymphocytes. Bars represent mean values from 12 HIV-infected patients (○) and 10 healthy volunteers (■). (b) Time course of membrane-associated tyrosine phosphatase activity of CD4$^+$ T cells after immobilized anti-CD3 MoAb-mediated activation. Individual values represent the mean and s.d. from 12 HIV-infected patients and 10 healthy volunteers. (c) Correlation between basal membrane-associated tyrosine phosphatase activity *versus* thymidine uptake after 72 h of culture. Thymidine uptake values are expressed in ct/min $\times 10^{-3}$. ■, HIV-infected individuals.

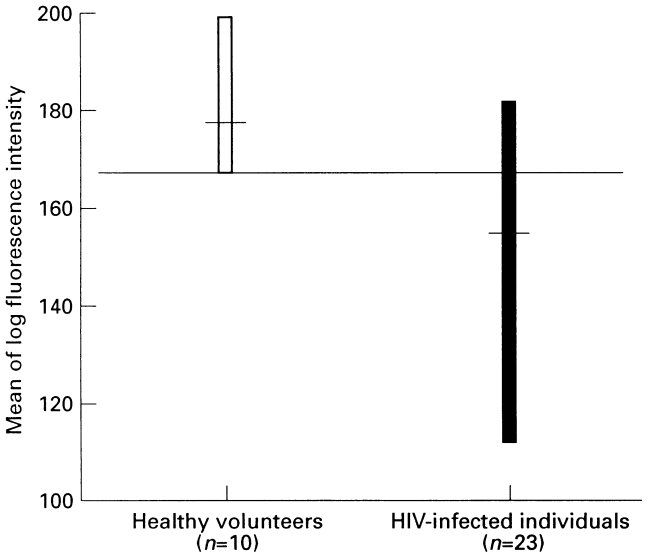

Fig. 2. Flow cytometric analysis of CD45 expression levels in $CD4^+$ T cells from HIV-infected patients and healthy volunteers. Schematic representation of mean and range of CD45 intensity values from 23 HIV-infected patients and 10 healthy volunteers. In all HIV-infected patients tested, the percentage of $CD4^+$ $CD45^+$ cells was identical to normals (> 96%), as assessed by flow cytometry.

their normal counterpart. Resting CD4 T cells from HIV-infected individuals showed a decrease of about 15% in mean values of CMF fluorescence, indicating a disturbed redox status (Table 1).

To evaluate the role of changes in intracellular thiol levels on proliferative responses and CD45 phosphatase activity, purified normal CD4 T cells from these 11 patients were either untreated or pre-incubated with NAC (20 mM) plus β_2-ME (50 μM) as anti-oxidant treatment or H_2O_2 (50 μM) as pro-oxidant treatment. Phosphatase activity from CD45 immunoprecipitates was measured after 2 h of culture at 37°C; and proliferative responses to immobilized anti-CD3 were assessed at day 3. As shown in Table 1 and Fig. 3, preincubation with NAC and β_2-ME significantly enhanced the proliferative responses and basal phosphatase activity of CD45 immunocomplexes of normal CD4 T cells compared with untreated cells. In contrast, pre-incubation with hydrogen peroxide at 50 μM almost completely inhibited thymidine uptake and basal CD45-phosphatase activity (data not shown).

Next, purified $CD4^+$ T cells from the same HIV-infected patients were assessed for proliferative responses and CD45 phosphatase activity before and after anti-oxidant pre-incubation. According to the degree of proliferative response recovery following anti-oxidant treatment (Table 1), HIV^+ patients were divided into two groups: low anti-oxidant responders (LAR; three out of 11) and high anti-oxidant responders (HAR; eight out of 11). In this latter group, proliferative responses and CD45 tyrosine phosphatase activity were restored to values close to those achieved with normal CD4 T cells after similar experimental conditions (Fig. 3). On the other hand, in patients with low responses to anti-oxidant treatment, defective proliferative responses and CD45 phosphatase activity were only slightly augmented.

Anti-oxidant treatment restored CD45-associated phosphatase activity and proliferative responses of unresponsive $CD4^+$ T cells but failed to affect cells undergoing programmed cell death

Programmed cell death (PCD) has been proposed to play an important role in CD4 depletion during HIV infection [24–26] and there is growing evidence indicating that oxidative stress may be involved in this process [27]. Thus, in order to elucidate whether the weak responses to anti-oxidant treatment were related to ongoing apoptotic cell death, we measured the ratio of apoptotic

Table 1. Values of ^{3}H-thymidine uptake and low molecular weight thiols of purified $CD4^+$ lymphocytes from HIV-infected individuals and healthy volunteers*

		Culture media		Anti-oxidant treatment		Basal thiols levels† (LTM)
		Controls	CD3 MoAb	Controls	CD3 MoAb	
HIV-infected patients (HAR)	HIV-01	0·14	21·1	0·22	76·7	158·2
	HIV-02	0·12	24·2	0·26	69·6	157·8
	HIV-03	0·13	24·2	0·21	60·3	143·9
	HIV-04	0·11	16·7	0·15	73·9	153·7
	HIV-05	0·17	34·4	0·16	71·6	154·5
	HIV-06	0·15	34·2	0·18	81·6	147·9
	HIV-07	0·16	15·9	0·19	69·8	153·1
	HIV-08	0·19	27·4	0·18	65·3	145·8
			24·7 ± 7·0		**69·6 ± 5·4**	**151·8 ± 5·4**
HIV-infected patients (LAR)	HIV-09	0·12	4·1	0·17	7·6	154·8
	HIV-10	0·12	6·2	0·21	12·5	150·2
	HIV-11	0·13	8·3	0·17	19·4	133·5
			6·2 ± 2·9		**13·5 ± 8·3**	**146·2 ± 11·2**
Healthy volunteers ($n = 6$), mean ± s.d.		0·12 ± 0·05	**50·4 ± 5·7**	0·22 ± 0·04	**73·8 ± 7·4**	**180·5 ± 9·8**

* Values of thymidine uptake are depicted as ct/min $\times 10^{-3}$ and were performed after 72 h of cultures.
† Thiol levels were estimated by flow cytometry and are expressed as the mean log of relative fluorescence intensity.
HAR, High anti-oxidant responders; LAR, low anti-oxidant responders.

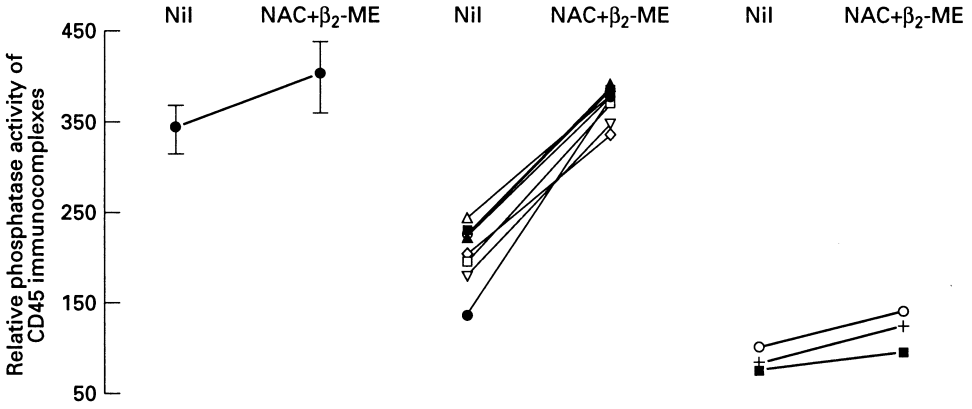

Fig. 3. Immunocomplex assay of immunoprecipitated CD45 in resting CD4 T cells before and after anti-oxidant treatment. Values of tyrosine phosphatase activity are expressed as absorbance units at 410 nm after removal of duplicated sample values in the presence of vanadate (1 mM). NAC, *N*-acetyl-L-cysteine; β_2-ME, β_2-mercaptoethanol. ○, HIV 13; □, HIV 15; △, HIV 17; ▽, HIV 23; ◇, HIV 14; ●, HIV 16; ■, HIV 19; ▲, HIV 25; ▼, HIV 18; ◆, HIV 20; +, HIV 24.

cells by flow cytometry [19,20]. Results depicted in Fig. 4 show that in the low anti-oxidant responder group, a substantial fraction of the cells ($>$ 40%) underwent PCD (merocyanin high) after 24 h culture and that anti-oxidant treatment failed to influence this process. These results suggest that anti-oxidant treatment was useful in restoring proliferative responses and CD45-mediated signalling in a high proportion of asymptomatic HIV-infected individuals by acting essentially on anergic non-apoptotic lymphocytes.

DISCUSSION

In this work, we show that purified $CD4^+$ T cells from asymptomatic HIV-1-infected individuals have decreased basal and post-activation membrane tyrosine phosphatase activity, which was associated with impairment of proliferative responses upon CD3-mediated activation. Interestingly, *in vitro* anti-oxidant treatment was able to significantly improve both defective tyrosine phosphatase activity and proliferative responses in most patients.

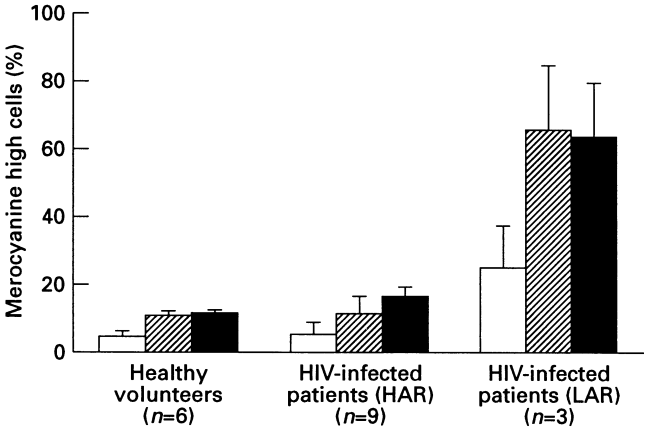

Fig. 4. Flow cytometric analysis of apoptotic cells. The ratio of apoptotic cells was estimated using the fluorescent dye merocyanin 540. Resting CD4 T cells from representative HIV-infected individuals and healthy volunteers were assessed for the percentage of merocyanin high cells (basal). Next, cells were preincubated either in culture media alone or in the presence of *N*-acetyl-L-cysteine (NAC; 20 mM) + β_2-mercaptoethanol (β_2-ME; 50 μM) for 2 h at 37°C. At the end of the pre-incubation time cells were washed and further cultivated overnight in culture media alone. HAR, High anti-oxidant responders; LAR, low anti-oxidant responders. Abscissa represents merocyanin intensity, whereas ordinate represents cell numbers. Cells undergoing programmed cell death are characterized by high fluorescence intensity expression. □, Basal; ▨, cultured overnight (without treatment); ■, cultured overnight (pretreated with NAC + β_2-ME).

Defects in signal transduction pathways have been postulated to play an important role in the impaired functional responses of T cells during HIV infection [6]. Recently, we have demonstrated that early tyrosine phosphorylation was defective in purified $CD4^+$ T cells from HIV-infected individuals, and was frequently associated with altered levels of two scr-family tyrosine-kinases, $p59^{fyn}$ and $p56^{lck}$ [11]. The phosphoinositide pathway, Ca^{2+} mobilization and phospholipase C activation have also been reported to be affected in T cells from HIV-infected individuals, as well as in T cells exposed to viral proteins [8,9,28–32]. The critical role of CD45 in regulating early signalling events after TCR engagement depends on its ability to regulate tyrosine kinase activity and phospholipase C activation, thereby affecting Ca^{2+} mobilization [21,33–35]. Indeed, $CD45^-$ cell lines show defective tyrosine phosphorylation and IL-2 secretion as well as impaired proliferative responses [12,36,37]. Thus, affected CD45 activity could at least partially explain the reported signalling defects in lymphocytes from HIV-infected individuals.

Although, as previously reported for HIV-infected T cell lines [16], a moderate reduction in cell surface expression of CD45 was observed in $CD4^+$ T cells from HIV-infected patients, there was no clear correlation between CD45 expression and impairment of phosphatase activity, suggesting that reduction in CD45 expression might only partially explain decreased phosphatase activity. Recent work [38] has reported that binding of surface CD45 by specific MoAbs can be regulated by modification of the accessibility of plasma membrane-associated CD45. Further studies are needed to answer these questions.

As concerns CD45 involvement in HIV infection, $CD45^-$ T cell lines have been shown to direct higher expression levels of HIV-1 long terminal repeat (LTR) due to the presence of constitutively active NFkB in the nucleus [16]. On the other hand, antibodies against CD45, which probably induce an increase in tyrosine phosphatase activity, were demonstrated to decrease post-activation levels of viral expression. These reports suggest that CD45

tyrosine phosphatase is able to regulate NFkB activation, and thereby levels of lymphocyte responsiveness and virus expression. According to these data, defective CD45 tyrosine phosphatase activity in CD4 T cells during HIV infection might induce a state of lymphocyte unresponsiveness, with concomitant enhanced virus replication.

The mechanisms regulating the enzymatic activity of CD45 *in vivo* are not yet completely known. It has recently been demonstrated, however, that this activity can be modulated either by tyrosine phosphorylation or by the redox status of the cell. Since transient phosphorylation of tyrosine residues of CD45 upon phytohaemagglutinin (PHA) or CD3 stimulation results in enhanced activity [39,40], the possibility exists that defective tyrosine phosphorylation previously reported in CD4 T cells from HIV-infected individuals [11] could explain the impaired enzymatic activity of CD45. However, this appears unlikely, since basal activity which was found defective in this work is not regulated by this mechanism. On the other hand, the optimal activity of CD45 is dependent on the presence of adequate levels of sulfhydryl compounds. In this respect, oxidants or SH-reactive agents are able to oxidize essential cysteinyl residues inhibiting enzymatic activity, which in turn is only partially recovered by adding back anti-oxidants [15].

Recent reports suggested a role for oxidative stress and GSH depletion in HIV-induced immunopathogenesis [22,23,41]. We examined the influence of oxidative stress in impairing CD45 tyrosine phosphatase activity and proliferative responses of isolated CD4 cells from these patients. Our results showing the presence of decreased low molecular thiols in $CD4^+$ T cells from most asymptomatic HIV-infected individuals suggest the existence of oxidative stress *in vivo*. However, the reduced thiol levels described here were not clearly correlated with severity of impairment in the proliferative responses and phosphatase activity. Since glutathione levels are submitted to tight regulation, this apparent discrepancy may be accounted for by assuming that low molecular weight thiol levels are not an ideal marker to identify cells which have undergone a redox imbalance *in vivo*, and that the actual values may reflect spontaneous partial restoration of low molecular weight levels prior to the study.

To better assess the role of oxidative stress, we studied the effect of 2 h pre-incubation of $CD4^+$ T cells in the presence or the absence of NAC plus β_2-ME. NAC is known to regenerate normal levels of GSH by providing newly converted cysteine, the limiting precursor of GSH, and through its sulphur, can act directly as a free radical scavenger. An important recovery of proliferative activity and of CD45-associated tyrosine phosphatase activity was observed for most patients. Only three out of the 11 patients failed to increase significantly their proliferative and phosphatase activity following NAC + β_2-ME treatment. The poor recovery of CD45 phosphatase activity in the LAR group is in agreement with previous reports indicating that only partial recovery of this activity could be achieved following stronger oxidant stress [15].

These results suggest that an imbalance in redox equilibrium may at least partially explain impaired tyrosine phosphatase activity and proliferative responses in CD4 cells from HIV-infected patients. Intracellular LMT, of which glutathione (GSH) is by far the most abundant, play a key role in proliferative responses, DNA and protein synthesis as well as in protection of T cells from oxidative damage. NAC is also a potent inhibitor of HIV-1 replication by blocking activation of NFkB transcription factor, has beneficial effects on CD4 lymphocyte survival when administered to HIV-1-infected individuals [42] and inhibits tumour necrosis factor-alpha (TNF-α) and phorbol myristate acetate (PMA)-induced cell death in infected U937 cell lines [43].

Our results show that restoration of defective proliferative responses by anti-oxidant treatment is mainly exerted on unresponsive cells. However, we failed to demonstrate this effect on cells engaged in ongoing PCD, which suggests that the anti-oxidant-mediated effect is limited to non-apoptotic unresponsive cells, though it remains unclear whether this effect could be exerted at very initial stages of this process and whether the NAC schedule used in this work could not be adequate in inhibiting cell death. An alternative hypothesis is that *in vitro* cell death in these patients is the consequence of an irreversible process triggered *in vivo*, which is not modified by anti-oxidant treatment. Studies on the redox status of T lymphocytes have suggested that the final outcome of T cell activation (proliferation, TCR/CD3 unresponsiveness or cell death) will be dictated by the ability of a cell to maintain an appropriate oxidant–anti-oxidant balance [27]. Thus, the severity of the oxidant insult will determine the cellular response (anergy *versus* cell death).

In conclusion, our results demonstrate that *in vitro* anti-oxidant treatment of CD4 cells from HIV-1 individuals is able to recover, at least in some cases, the impaired CD45-associated phosphatase activity and the defective proliferative activity of these cells.

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