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Phenotypic characteristics and tendency to apoptosis of peripheral blood mononuclear cells from HIV+ long term non progressors

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Abstract

The aim of this study was to analyze (i) phenotype, (ii) *in vitro* spontaneous and induced apoptosis, (iii) glutathione (GSH) intracellular content and (iv) inhibitors of apoptosis of potential therapeutical use in peripheral blood mononuclear cells (PBMC) from HIV+ long term non progressors (LTNP), in comparison with progressors (HIV+P) and seronegative controls (HIV−). Three groups of subjects were studied: 15 HIV+P (patients losing >150 CD4+/year), 9 LTNP (subjects infected by HIV for at least 7 years without clinical and immunological signs of progression, with a mean of 898 CD4+/μL) and 18 HIV−. All subjects were living in a large community for former drug addicts, and were matched for age and sex.

We used flow cytometry for analyzing PBMC phenotype and apoptosis; high performance liquid chromatography for measuring intracellular GSH content. PBMC phenotype of LTNP shared characteristics with those of both HIV− and HIV+P. Indeed, LTNP showed a normal number CD4+ cells (an inclusion criteria), but significantly increased numbers of CD8+ lymphocytes, activated T cells, CD19+, CD5+ B lymphocytes and CD57+ cells, as well as a decrease in CD19+, CD5− B lymphocytes and CD16+ cells. In LTNP, spontaneous apoptosis was similar to that of HIV− and significantly lower than that of HIV+P. Adding interleukin-2 (IL-2) or nicotinamide (NAM) significantly decreased spontaneous apoptosis in LTNP and HIV+P. Pokeweed mitogen-induced apoptosis was also similar in LTNP and HIV−, but significantly lower than that of HIV+P. In HIV+P, but also in LTNP, spontaneous apoptosis was inversely correlated to the absolute number and percentage of CD4+ cells and directly correlated to the number and percentage of activated T cells present in peripheral blood. GSH intracellular content was greatly decreased in PBMC from HIV+P and slightly, but significantly, reduced in LTNP. Adding 2-deoxy-D-ribose, an agent provoking apoptosis through GSH depletion, to quiescent PBMC resulted in similar levels of massive cell death in the three groups. This phenomenon was equally prevented in the three groups by N-acetyl-cysteine but not by IL-2.

A complex immunological situation seems to occur in LTNP. Indeed, PBMC from LTNP are characterized by a normal *in vitro* tendency to undergo apoptosis despite the presence of a strong activation of their immune system, unexpectedly similar to that of HIV+P. Our data suggest that NAM and IL-2 are possible candidates for reducing spontaneous apoptosis in HIV infection.

Keywords: HIV; AIDS; long term non progressors; glutathione; apoptosis

Abbreviations: LTNP, long term non progressors; HIV+P, seropositive progressor; PBMC, peripheral blood mononuclear cells; HIV, human immunodeficiency virus-1; D-RIB, 2-deoxy-D-ribose; GSH, glutathione; IL-2, interleukin-2; NAM, nicotinamide

Introduction

The gradual depletion of peripheral blood T lymphocytes, either CD4+ or CD8+, during the progression of human immunodeficiency virus (HIV) infection, may be due, at least in part, to apoptosis (Ameisen and Capron, 1991; Laurent-Crawford *et al*, 1991; Terai *et al*, 1991). The molecular and cellular mechanisms of this phenomenon are still poorly understood, even if several data suggest that apoptosis is linked to several events typical of HIV infection, including cell activation, cytopathic effect of HIV, syncytia formation and can be triggered – directly or indirectly (production of cytokines) – by HIV gene products such as gp120 and gp41, *tat* protein, or by antigenic stimulations (Groux *et al*, 1992; Finkel and Banda, 1994; Ameisen *et al*, 1995; Li *et al*, 1995; Pantaleo and Fauci, 1995a; Pantaleo and Fauci, 1995b). In particular, it has been reported that, when cultured *in vitro*, peripheral lymphocytes from HIV+ subjects in different stages of the

infection undergo apoptosis either spontaneously or following stimulation with polyclonal mitogens or superantigens. Such a phenomenon, which is present at a very low level also in cells from normal subjects, is dramatic during acute HIV infection (Cossarizza *et al*, 1997), and increases in peripheral blood mononuclear cells (PBMC) from HIV-infected patients with progression of the disease (Gougeon, 1995; Gougeon *et al*, 1996). However, the relationship(s) between *in vitro* tendency to undergo apoptosis and the phenomena responsible *in vivo* for the progressive deterioration of the immune system are far from being clear (Baltimore, 1995a; Pantaleo and Fauci, 1995a). In order to gain further insight on this important topic, we thought worthwhile to study a group of *'long term non progessors'* (LTNP), i.e. HIV+ patients whose main feature is the lack of clinical signs of disease progression and of the characteristic decrease of peripheral CD4+, despite being infected by HIV since several years (Levy, 1993; Sheppard *et al*, 1993). Recent data on LTNP indicate that they have low levels of HIV-1 in the presence of strong virus-specific immune responses, combined with some degree of viral attenuation (Cao *et al*, 1995). Similar observations concerning low plasma levels of HIV-1 RNA and viral burden in PBMC from LTNP have been reported by Pantaleo *et al*, who also showed that lymph-node architecture and immune function appear to remain intact in these subjects (Pantaleo *et al*, 1995).

Accordingly, the main aims of this study were the following: (i) to ascertain whether PBMC from LTNP have a different tendency to undergo apoptosis either spontaneously or after treatment with a mitogen (pokeweed mitogen, PWM), or with anti-CD95 monoclonal antibodies, in comparison with cells from subjects who show signs of progression of the infection (HIV+P) and from seronegative controls; (ii) to identify the phenotype of apoptotic cells, in order to see whether in LTNP different lymphocyte subsets have a different propensity to cell death; (iii) to ascertain whether spontaneous or induced apoptosis can be modulated by agents such as growth factors (interleukin-2, IL-2), molecules able to inhibit poly(ADP-ribosyl)polymerase (nicotinamide, NAM; 3-aminobenzamide, 3-ABA), or to increase intracellular reduced glutathione (GSH) content (N-acetyl-L-cysteine, NAC) or energy charge (L-acetyl-carnitine, LAC).

Moreover, we addressed another problem regarding the intracellular glutathione content in PBMC from LTNP. The critical role of intracellular thiols, and in particular of glutathione, for several immune phenomena (reviewed by Dröge *et al*, 1994) and for the survival of HIV-infected cells (Eck *et al*, 1991, 1992; Staal *et al*, 1992a) as well as for the survival of HIV+ patients (Herzenberg *et al*, 1997) has been reported. Thus, we thought it worthwhile to measure this thiol, and to study the sensitivity of PBMC from LTNP, as well as HIV− and HIV+P, to 2-deoxy-D-ribose (D-RIB), a simple sugar which induces apoptosis and a marked decrease in GSH intracellular content in human PBMC, whose action can be completely inhibited by NAC (Barbieri *et al*, 1994).

Results

Table 1 shows the main phenotypic characteristics of lymphocytes from the patients and controls we have studied. LTNP present a significant increase in CD8+ cells, which are likely to be mainly responsible for the altered CD4/CD8 ratio. Moreover, most CD8+ T cells, either from LTNP or HIV+P, also expressed CD57 and CD3 molecule (not shown), indicating that these cells were cytotoxic T lymphocytes (Lanier and Loken, 1984). A relevant percentage of T cells from LTNP, similar to that of HIV+P, expressed markers of activation such as HLA-DR molecules, and the main activated T cells were CD8+ (not shown). The total number of B cells was similar in the three groups, even if we observed an increase in the subset of CD5+ B cells either in LTNP or in HIV+P, and a decrease in CD5− B cells. Cells with markers related to natural killer (NK) activity had a different behaviour. Indeed, CD16+ and CD56+ cells, i.e. those with higher cytotoxic activity, were overall decreased in LTNP as well as in HIV+P, while CD57+ cells (among which there was a consistent amount of CD8+, CD16− cells) were significantly increased in all HIV infected subjects.

Table 1 Absolute number and percentages of peripheral blood leukocytes, monocytes, lymphocytes and lymphocyte subpopulations in control seronegative subjects (HIV−), seropositive progressors (HIV+P) and long term non progressors (LTNP)

	HIV−	**HIV+P**	**LTNP**
Subjects (N.)	18	15	9
Age (years)	29.9±1.4	28.1±1.1	32.0±1.7
Leukocytes/μl	7,304±316	5,671±391**	6,422±401
Monocytes %	6.7±0.5	6.6±0.6	6.0±0.7
monocytes/μl	495±44	376±45	398±61
Lymphocytes %	35.8±1.7	36.9±2.6	44.2±2.8*
lymphocytes/μL	2,584±116	2,089±237	2,785±122°
CD3 %	75.2±1.5	78.7±2.6	80.3±3.0*
CD3/μL	1,937±95	1,675±225	2,285±153
CD4 %	40.2±1.2	18.7±2.5**	32.6±2.6**°°
CD4/μL	1.030±50	404±76**	898±67°°
CD8 %	33.6±1.6	59.1±3.2**	50.1±3.5**
CD8/μL	870±67	1,248±177*	1,401±130**
CD4/CD8 *ratio*	1.27±0.10	0.37±0.05**	0.69±0.09**°°
Act. T cells %	9.2±0.9	37.1±3.5**	30.4±4.8**
act. T cells/μL	241±28	814±178**	863±150**
Total CD19+ %	13.7±1.3	13.1±1.5	13.7±2.6
total CD19+/μL	359±39	250±27*	376±64°
CD19+, CD5− %	10.4±1.0	6.8±0.7*	6.8±1.3*
CD19+, CD5−/μL	265±24	139±21**	190±47
CD19+, CD5+ %	3.4±0.7	6.3±1.2*	6.9±1.4*
CD19, CD5+/μL	90±19	111±16	186±37*
Total CD16+ %	7.6±1.2	3.3±0.7**	3.8±0.9*
total CD16+/μL	207±35	65±12**	110±36*
Total CD57+ %	14.3±1.5	24.5±2.8**	22.0±3.0*
total CD57+/μL	388±50	482±62	601±92*
CD16+, CD57− %	3.6±0.4	2.1±0.4*	2.0±0.5*
CD16+, CD57−/μL	96±12	42±8**	54±14*
CD16+, CD57+ %	4.0±0.9	1.3±0.3*	1.8±0.7
CD16, CD57+/μL	110±24	27±5**	51±21
CD16-CD57+ %	10.3±1.1	23.1±2.7**	20.5±0.5**
CD16-CD57+/μL	264±36	457±60**	556±96**
Total CD56+ %	14.3±1.8	8.1±1.1**	9.9±2.1
total CD56+/μL	380±59	156±20**	279±63°
CD56+, CD3− %	9.3±1.4	4.9±0.8*	6.8±1.7
CD56+, CD3−/μL	246±42	95±12**	196±46°

Data indicate mean±SEM; act. T cells=T activated lymphocytes, i.e. CD3+, HLADR+cells. *=P<0.05 and **=P<0.01 *vs.* HIV−; °=P<0.05 and °°=P<0.01 *vs.* HIV+P.

Table 2 Propensity to apoptosis of peripheral blood mononuclear cells from control seronegative subjects (HIV−), seropositive progressors (HIV+P) and long term non progressors (LTNP)

Apoptosis	HIV−	HIV+P	LTNP
Spontaneous	6.3±0.8	15.8±2.3**	8.9±1.3°
Spont.+IL-2	3.8±0.5§	7.4±1.2**§§	3.8±0.5°§§
Spont.+NAM	5.3±0.6	9.9±1.7*§§	5.8±1.2§
Spont.+NAC	8.7±0.8	15.5±2.0**	10.3±1.2
Spont.+LAC	7.6±1.2	21.3±3.0**	8.9±1.2°°
Spont.+3-ABA	7.7±1.1	21.5±3.6**	10.3±1.1°
PWM-induced	14.2±1.0§§	23.5±2.2**§§	15.1±1.5°§§
PWM-ind.+NAC	12.4±1.2§§	23.8±2.8**§§	15.0±2.0°§
D-RIB-induced	53.7±3.2§§	64.1±3.3*§§	52.4±3.2°§§
D-RIB-ind.+NAC	9.4±1.4#	15.6±1.4**#	10.6±0.8°#
D-RIB-ind.+IL-2	48.4±2.8§§	65.0±2.7**§§	51.9±3.0°°§§
Anti-CD95-induced	9.4±1.0	20.4±3.0*§	12.0±1.4

Data indicate mean±SEM, and are referred to the percentage of apoptotic cells after 72 h of culture. Subjects are those as in Table 1, except for the study of anti-CD95-induced apoptosis, where we could analyze only four donors per group. *=P<0.05 and **=P<0.01 *vs.* HIV−; °=P<0.05 and °°=P<0.01 *vs.* HIV+P; §=P<0.05 and §§P<0.01 *vs.* spontaneous apoptosis; #P<0.01 *vs.* D-RIB-induced apoptosis.

Table 2 reports data concerning PBMC tendency to undergo apoptosis, and the effects of various agents on this phenomenon. To investigate different possible pathways, we used a variety of apoptotic stimuli or molecules capable of inhibiting such a process. At the beginning of the culture, no differences in cell viability were observed among the subjects of the three groups. Moreover, no apoptotic cells nor cells with mitochondrial alterations were present in HIV+ donors (data not shown). After 72 h of culture, spontaneous apoptosis was much higher in HIV+P than in controls, as expected and already reported by other authors (Gougeon *et al*, 1993, 1996). Cells from LTNP showed a tendency to undergo apoptosis not significantly different from that of HIV− donors. In contrast with what was observed during primary, acute HIV syndrome (Cossarizza *et al*, 1997), adding NAC at the beginning of the culture did not modify this parameter. The presence of IL-2 provoked a marked and statistically significant ($P<0.01$) reduction of apoptosis in all groups. NAM caused a moderate protective effect on spontaneous apoptosis, while LAC or 3-ABA were ineffective. Due to lack of cells, anti-CD95-induced apoptosis was studied in four donors per group. No main differences were found between LTNP and HIV− donors, while in HIV+P apoptosis was significantly higher than in the other two groups.

Adding 10 mM D-RIB, an agent capable of provoking a dramatic decrease of intracellular GSH (D. Monti *et al*, submitted), caused a relevant increase of apoptosis in all the groups studied (P always <0.001). Interestingly, such increase was of the same magnitude in all groups (about 40–42%). In D-RIB-treated cells, adding IL-2 had no effect, while NAC consistently decreased the percentage of apoptotic cells to an extent similar to that of basal values. If cells were treated with lower doses of D-RIB (1 and 5 mM), the percentages of apoptotic cells increased over spontaneous apoptosis in a similar manner (about 8–15%) in all the groups and, again, NAC decreased such value to those of control, untreated cultures (not shown). PWM was able to significantly increase apoptosis in all groups ($P<0.01$ *vs* untreated cultures), and cells from HIV+P showed the highest sensitivity to the mitogen.

The analysis of spontaneous apoptosis after 24, 48 and 72 h of culture showed that a similar kinetics was present among the three groups, even if at different absolute values of apoptosis (not shown).

Figure 1 shows that, considering all HIV+ subjects, i.e. both HIV+P and LTNP, a significant correlation was observed when the percentage of CD4+ T (negative correlation) or the percentage of T cells expressing HLA-DR molecules (positive correlation) were plotted against the percentage of cells undergoing spontaneous apoptosis, measured with the PI method. The same significant correlations were observed when these two groups were analyzed separately, or if the absolute number of circulating CD4+ or CD8+ T cells were plotted (not shown).

We were interested in evaluating the modifications of lymphocyte subpopulations during the apoptotic process. For this reason, by using a cytofluorimetric technique based upon the combined evaluation of physical parameters and CD45 expression, we analyzed separately viable (Figure 2) and apoptotic (Figure 3) cells in the same samples. Figure 2 shows a representative experiment out of four where the

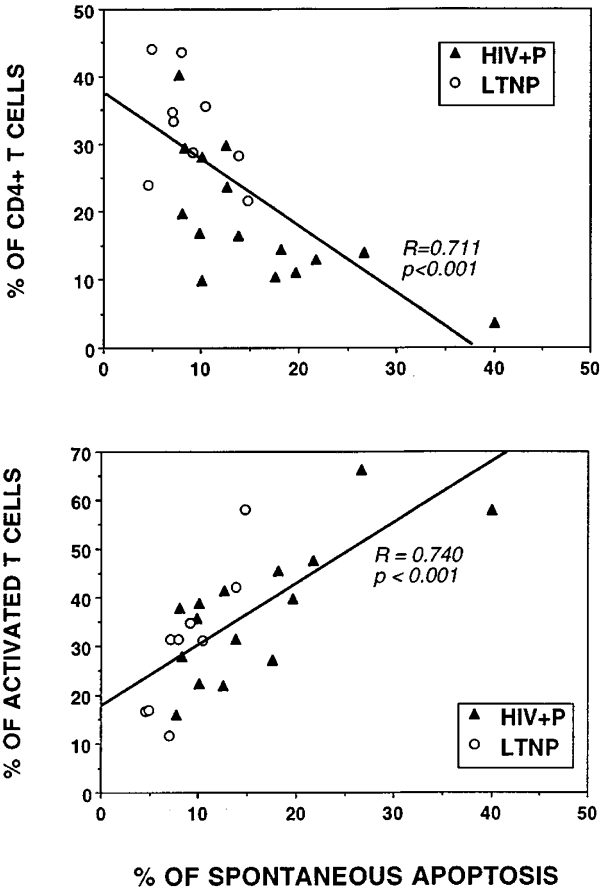

Figure 1 Inverse correlation between the percentage of CD4+ cells and spontaneous apoptosis (measured in total PBMC by the PI method), and direct correlation between the percentage of activated T cells (i.e. those expressing HLA-DR antigens) and spontaneous apoptosis in LTNP and HIV+P

phenotypes of lymphocytes still viable at different time points were analyzed. As in the other experiments, cells were from one HIV−, one LTNP and one HIV+P. In the region of viable lymphocytes (i.e. where, in parallel samples, cells did not incorporate PI, and maintained normal physical parameters and CD45 expression), we noted that the percentage of CD3+, CD4+ and CD8+ lymphocytes did not vary over time, while cells with NK markers (CD16, CD57) and B lymphocytes tended to increase and decrease, respectively. The pattern of these changes was similar in all the subjects of the three groups. The analysis of the phenotype of apoptotic cells, performed after 24 h of culture, confirmed the trend indicated by the data obtained analyzing viable cells. Figure 3 shows that, in that particular experiment, the phenotype of apoptotic cells was similar in the three donors studied, i.e. one LTNP, one HIV+P and one HIV− control. Most apoptotic cells were CD3+ or CD4+, but also CD16+ or CD19+. Similar results were obtained in the other three experiments. For technical reasons, i.e. because of the relevant autofluorescence of apoptotic cells, it was not possible to perform such analyses at 48 or 72 h.

Table 3 shows the intracellular GSH content in PBMC from HIV−, HIV+P and LTNP. LTNP had an intracellular GSH content which was significantly lower than that of HIV− and significantly higher than that of HIV+P. Linear regression analysis in all HIV+ donors, including LTNP, showed that spontaneous apoptosis was inversely correlated with intracellular GSH content (Figure 4). We could measure intracellular GSH and spontaneous apoptosis in

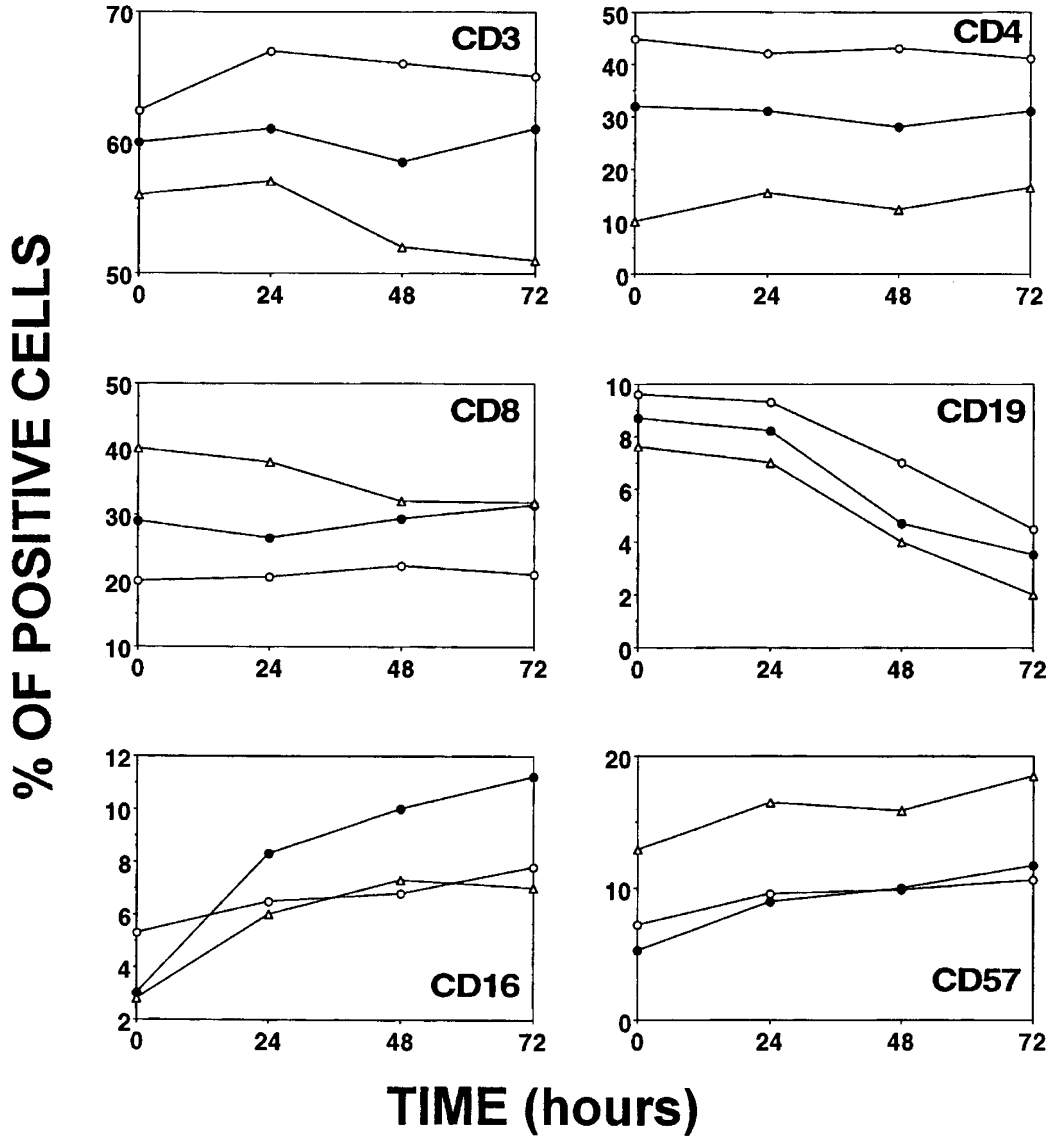

Figure 2 The phenotype of viable lymphocytes at different time points is similar in cells from one HIV− donor (open circles), one HIV+P (open triangles) and one LTNP (closed circles). Data refer to one representative experiment out of four performed on cells from one subject of each group. The phenotype of viable cells was performed by flow cytometry, using the PI exclusion method and anti-CD45 mAb. See text for details

PBMC collected from three HIV+P donors and 4 HIV− controls (not included in this paper) once a week for three times. In these samples, we could find only negligible variations of the parameters under investigations (data not shown). This indicates that the measures reported in this study are reliable and representative of each single donor.

Discussion

The scientific community is paying a growing attention to those subjects who have been infected by HIV since several years (at least seven) but in whom the infection seems unable to progress, i.e. the so-called LTNP (Levy, 1993; Baltimore, 1995a,b; Klein and Miedema, 1995). They represent a natural model for understanding the mechanisms which are able to contrast the progression of infection. Indeed, apart from the lack of clinical signs, the main inclusion criteria for being considered a LTNP is a high, or at least stable level of CD4+T cells. The available data indicate that LTNP have strong virus-inhibitory CD8+ lymphocyte and neutralizing-antibodies responses, and low levels of virion replication (Cao *et al*, 1995; Pantaleo *et al*, 1995). We found that a low propensity to undergo apoptosis in PBMC can be added to the characteristics of LTNP. However, they have a complex immunological derangement, regarding in particular their phenotype and their PBMC intracellular glutathione content.

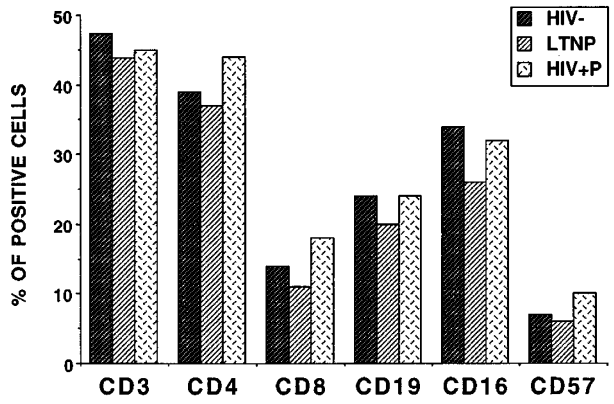

Figure 3 The phenotype of apoptotic lymphocytes after 24 h of culture is similar in one HIV− donor, one HIV+P and one LTNP. Data refer to one representative experiment out of four, performed on cells from one subject of each group. The phenotype of apoptotic cells was studied by flow cytometry, analyzing changes in FSC, SSC and in CD45 fluorescence. See text for details

Table 3 GSH content in peripheral blood mononuclear cells from control seronegative subjects (HIV−), seropositive progressors (HIV+P) and long term non progressors (LTNP)

	HIV−	HIV+P	LTNP
N. of subjects	11	10	7
GSH (nmol/10^6 PBMC)	4.65 ± 0.37	1.70 ± 0.20	3.03 ± 0.63

Data are expressed as mean ± SEM; statistical analysis by 2-tail Student's *t*-test: HIV− *vs.* HIV+: t=6.813, P<0.001; HIV− *vs.* LTNP: t=2.379, P=0.030; LTNP *vs.* HIV+: t=2.322, P=0.035.

In LTNP, spontaneous, Fas-induced and mitogen-induced apoptosis of PBMC, was of the same order of that found in HIV−, and significantly lower than that of HIV+P. This phenomenon is in apparent contrast with a variety of immunological alterations regarding PBMC phenotype. Indeed, apart from the inclusion criteria, in LTNP most parameters were strikingly similar to those found in HIV+P. The main alterations were a significant expansion in CD8+ T cells, a high number and percentage of T cells expressing HLA-DR molecules (classical markers of activation), a decrease in cells with markers characteristic of NK subset with high cytotoxic activity (CD16+, CD57−), and an increase in CD5+ B lymphocytes, i.e. B cells that are likely involved in autoimmune phenomena (Chen and Kearney, 1996).

An apparent discrepancy exists between the low level of apoptosis and the high level of activation of LTNP's immune system. One possible explanation is that the expression of HLA-DR is not a fully reliable marker of T cell activation if compared, for example, to CD38, whose expression increases in parallel with the progression of the infection and has a good prognostic value (Levacher *et al*, 1992; Giorgi *et al*, 1993). Another possibility is that, as MHC class II molecules are mainly expressed by CD8+ T lymphocytes, these cells are less prone to apoptosis in LTNP than in HIV+P. However, the fact that we found no main differences in the phenotypes of cells undergoing apoptosis among LTNP, HIV+P and HIV− does not support this explanation. In any case, further studies are needed to better identify the phenotype and clarify the role and destiny of activated T cells in LTNP.

A dynamic perspective can also be considered. According to the recent hypothesis of *'blind T cell homeostasis'* (Adleman and Wolsfy, 1993; Margolick *et al*, 1993, 1995; Roederer, 1995), the continuous erosion of CD4 compartment by apoptosis and/or other mechanisms would accel-

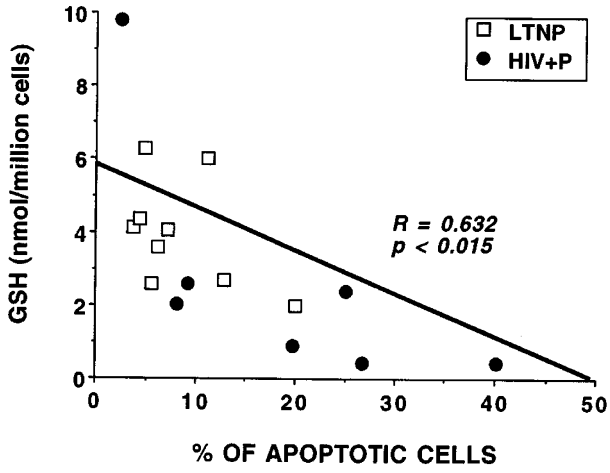

Figure 4 In LTNP and HIV+P, an inverse correlation was found between spontaneous apoptosis, measured in total PBMC after 72 h of culture in the absence of any stimulus by the cytofluorimetric method of the hypodiploic peak revealed by PI, and intracellular glutathione content

erate the normal homeostatic mechanism devoted to the maintenance of a constant level of circulating T cells, without regard to the phenotype (CD4+ or CD8+) of the T cell. One of the main consequences of this hypothesis is that it is possible to explain the concomitant decrease in CD4+ and increase in CD8+ lymphocytes along the years from seroconversion, or present in the acute primary infection (Cossarizza *et al*, 1995a,b). Assuming that what happens *in vitro* is a reliable mirror of the situation existing *in vivo*, the scenario likely present in LTNP could be the following: a low level of apoptosis, and the consequent reduced rate of CD4+ loss, likely accompanied by a low viral burden or by the presence of a defective virus (Cao *et al*, 1995; Kirchhoff *et al*, 1995), would trigger a renewal of CD4+ and CD8+ T cells lower than that present in HIV+P. However, owing to the chronicity of the process and to the length of the period in which this phenomenon occurs, an activation of the T cell compartment, and particularly an expansion of CD8+ lymphocytes, could be expected also in LTNP. In other words, the immunological consequences of HIV infection could be qualitatively similar in HIV+P and LTNP, but different from a quantitative point of view (rate of cell loss/rate of cell renewal). Accordingly, in LTNP the eventual failure of the T cell homeostasis would be significantly delayed, or even absent (Margolick *et al*, 1995). Independent support for this hypothesis is given by the correlations between spontaneous apoptosis and phenotype. An inverse correlation between apoptosis and the number of CD4+ cells/μL of blood or the percentage of CD4+ cells present at the beginning of the culture, and a direct correlation between apoptosis and the percentage of HLA-DR+ T cells, were found in both HIV+P and LTNP.

HIV-infected patients are in oxidative imbalance, which starts early in the course of the disease and they display low levels of serum and tissue antioxidants, as well as elevated concentrations of peroxidation products and clastogenic factors (Dröge *et al*, 1988; Eck *et al*, 1989; Roederer *et al*, 1991; Staal *et al*, 1992a,b; Fuchs *et al*, 1995). Several studies suggest that in HIV patients a significant decrease in intracellular GSH content occurs (Eck *et al*, 1989; Buhl *et al*, 1990; de Quay *et al*, 1992), and that this is a good prognostic marker of the infection (Herzenberg *et al*, 1997). It has also been demonstrated that cells with high GSH content, either CD4+ or CD8+, are selectively lost during HIV infection, even in the symptom free stages of the disease (Roederer *et al*, 1991; Staal *et al*, 1992a). Thus, also the immune system of LTNP could be under an oxidative stress, and it is reasonable to hypothesize that in LTNP the same selective loss of high-GSH cells occurs. In any case, the decrease in GSH content in PBMC from LTNP likely does not reach the threshold necessary to significantly increase spontaneous cell death. The lack of an effect of NAC on spontaneous apoptosis supports this interpretation.

PBMC from LTNP were not more sensitive to D-RIB, a potent inducer of apoptosis in human lymphocytes (Barbieri *et al*, 1994), whose action is mediated by an early and marked decrease in intracellular GSH content (unpublished observations). NAC was able to fully prevent D-RIB-induced apoptosis, but not spontaneous apoptosis, in all groups, suggesting that cells from HIV$-$, HIV+P and LTNP are equally sensitive to a strong GSH depletion, notwithstanding their different basal intracellular GSH content. Thus, the effect of NAC on T cell functions cannot be simply related to the changes in GSH levels, as also observed by other authors (Kinscherf *et al*, 1994).

Our data offers further information on apoptosis in LTNP. First, it is interesting to note that PWM-induced apoptosis was increased in HIV+P, as expected, but not in LTNP. This would suggest that, even if it has been shown that activation-induced apoptosis equally regards CD4+ and CD8+ T cells (Katsikis *et al*, 1996), the T cell activation in LTNP is likely to be different from that of HIV+P.

Second, the results concerning Fas-induced apoptosis are consistent with the observations that: (i) in peripheral lymphocytes from HIV+ patients the expression of CD95 increases with the progression of the disease, and is correlated with the percentage of CD4+ T lymphocytes (Gehri *et al*, 1996). CD95 was found expressed in CD4+ or CD8+ T cells; (ii) activated CD8+ T cells present lower levels of the anti-apoptotic protein bcl-2 and, subsequently, higher levels of CD95 (Boudet *et al*, 1996). Low bcl-2 expressing cells were negatively correlated with the percentage of CD4+ T cells, and this is clearly consistent with the well known evidence that T cell activation increases with the progression of the disease; and (iii) Fas-induced apoptosis is modulated by intracellular glutathione content (Chiba *et al*, 1996). Indeed, in comparison with HIV+P, PBMC from LTNP had less activation, more intracellular GSH, and were less sensitive to Fas-induced apoptosis. However, it has been shown that CD4+ cells from HIV+ subjects are more sensitive to Fas-induced apoptosis than CD8+ (Katsikis *et al*, 1996), and further studies are in progress to ascertain whether such phenomenon is present also in cells from LTNP. Moreover, studies on CD95L will be crucial as well to clarify the role of this pathway in LTNP.

Third, our data show that, as for subjects with primary, acute HIV infection (Cossarizza *et al*, 1997), several agents can be used to modulate apoptosis, even if their effects and mechanisms of action is likely quite different. In all groups, IL-2 was able to decrease spontaneous apoptosis of about 50%. This confirms, and extends to LTNP, previous observation on the role of CD4+ and IL-2 production in apoptosis of HIV patients (Pandolfi *et al*, 1995) and gives a possible explanation for the recent report on the capability of IL-2 to increase *in vivo* CD4+ cells (Kovacs *et al*, 1995).

A similar, even if less marked effect on spontaneous apoptosis was observed with NAM, a molecule that is able to increase intracellular levels of NAD and ATP (Monti *et al*, 1992). NAM is able to exert its action through the inhibition of poly(ADP-ribose)polymerase (PARP), and has been shown to protect cells from death induced by oxygen free radicals, cytotoxic effector cells and tumour necrosis factor (TNF)-α (Agarwal *et al*, 1988; Marini *et al*, 1990; Monti *et al*, 1994). However, the fact that 3-ABA, a classical inhibitor of PARP, was not capable of reducing spontaneous apoptosis could indicate that the effect of NAM was likely to be PARP-independent and mediated by other structures, such as cytoskeleton (Malorni *et al*, 1994).

In conclusion, a normal *in vitro* propensity to undergo spontaneous and induced apoptosis appears to be a major feature of PBMC from LTNP. However, many other immune parameters, such as the increase in CD8+ and activated T cells, the expansion of CD5+ B cells, the decrease in 'true' NK cells, as well as some signs of oxidative stress are similar in LTNP and HIV+P, suggesting that the immune system of LTNP is quite activated, and can be modulated by several agents such as NAM and IL-2, among others. The data here reported may contribute to understanding the fact that such subjects remain asymptomatic for many years. Thus, apoptosis can be considered an important host factor which, together with other relevant virologic and imunologic characteristics (low levels of HIV-1, a combination of strong virus-specific immune responses with some degree of attenuation of the virus) (Cao *et al*, 1995; Pantaleo *et al*, 1995), can play a role in the balance between virologic factors and immune responses peculiar of LTNP. A fundamental question remains open, concerning the possible occurrence of low tendency to apoptosis in lymph-nodes or in other immune organs of LTNP.

Materials and Methods

Subjects

This study was performed with the informed consent of the donors. Peripheral blood samples were obtained from 42 subjects (19 females and 23 males) living in San Patrignano Community (Rimini, Italy) – the biggest Italian community for former drug addicts. They have been selected among more than 500 HIV+ subjects or 1500 seronegative persons who are living in optimal and controlled environmental condition (adequate nutrition, lack of drug assumption or alcohol, etc.), and are continuously followed and monitored from both a clinical and laboratory point of view. They represent a unique homogeneous consistent population of HIV+ people, which we are studying for many years.

Donors were subdivided in three groups with approximately the same number of males and females. The first group was formed by 15 HIV+P, defined as those patients losing more than 150 CD4+cells/μL/year in the last 5 years; the second by 9 LTNP, i.e. subjects infected by HIV for at least 7 years, but who do not present clinical or immunological signs of progression of the infection, and with a stable number of CD4+ cells in the last years, as described elsewhere (Klein and Miedema, 1995; Pantaleo *et al*, 1995). All LTNP had >640 CD4+ cells/μL (range: 640 – 1209). In these subjects, in the last year the modification of circulating CD4+ lymphocytes was $+50.2 \pm 34.6$ cells/μL (range: -92, +153). The third group was formed by 18 seronegative controls (HIV$-$) with a history of drug abuse over the past years similar to that of HIV+ subjects. Blood samples were taken between 7:00 and 9.00 a.m., coded, immediately brought to Modena and processed in a single-blind manner, so that the operators did not know if a given sample was from a HIV$-$, a HIV+P or a LTNP. A minimum of three blood samples (one HIV$-$, one HIV+ and one LTNP) were studied in each experiment.

All donors were tested for several bacterial and viral pathogens, and serological evaluation for hepatitis B virus, Epstein-Barr virus, cytomegalovirus, Chlamidia, Mycoplasma and Toxoplasma infection were all negative. Syphilis serologic findings were non-reactive. No subject had herpesvirus infections.

Chemicals and monoclonal antibodies

NAC (final concentration: 10 mM), D-RIB (1 – 10 mM), NAM (5 mM), 3-ABA (5 mM), PWM (2.5 μg/mL) and propidium iodide (PI) were purchased from Sigma Chem. (St. Louis, MO, USA); human recombinant IL-2 (20 U/mL) from Boehringer (Mannheim, Germany). LAC (5 mM) was kindly provided by Sigma Tau SpA, Pomezia, Roma, Italy.

We used the following commercially available monoclonal antibodies (mAb), purchased from Becton Dickinson (San José, CA, USA), or Sigma Chem., directly conjugated with fluorescein, phycoerythryn or quantum red: anti-CD3, anti-CD4, anti-CD5, anti-CD8, anti-CD16, anti-CD19, anti-CD45, anti-CD56, anti-CD57, anti-HLA-DR. Anti-CD95 (clone APO1-3) was from Bender (Vienna, Austria), and goat-anti-mouse antibodies from Dako (Glostrup, Denmark).

Blood collections and PBMC cultures

PBMC were separated by discontinuous density gradient centrifugation, following standard methods. In all cases, the percentage of granulocytes after the separation was negligible ($<1\%$). Cultures were then prepared by placing 1×10^6 cells/mL in 24 well plates (Costar, Cambridge, MA, USA), in complete culture medium, i,e., RPMI-1640 medium (Flow Laboratories, Rockville, MD, USA) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, USA), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. In each well, we also added one or a combination of the above mentioned chemicals, reaching a total volume of 2 mL. PBMC were then incubated for different times (up to 72 h) in humidified 5% CO_2 and 95% air at 37°C. At the end of incubation periods, cells were collected, washed twice with cold phosphate buffered saline (PBS), and prepared for flow cytometric analysis. For the analysis of Fas-induced apoptosis, PBMC were incubated for 1 h at 4°C with 0.1 μg/mL anti-CD95 mAb, washed, incubated for 30 min at 4°C with the secondary mAb, washed and cultured for 24 h.

Detection of apoptosis

Apoptosis was detected in individual cells by reduced fluorescence of the PI – a DNA binding dye – in the apoptotic nuclei, according to Nicoletti *et al*. (Nicoletti *et al*, 1991). Briefly, cells were collected and washed, and the 200 g centrifuged cell pellet was gently resuspended in 1 mL hypotonic fluorochrome solution (PI 50 μg/mL in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma). Cells were analyzed after a miminum of 30 min of incubation in the dark at room temperature in this solution.

Cell viability

Cell viability was cytofluorimetrically analyzed by the method of PI exclusion (Cook and Mitchel, 1989). Samples were incubated with 10 μg/mL PI for 5 min at room temperature in the dark and immediately analyzed.

Phenotype analysis and immunofluorescence staining

Phenotypic analyses of lymphocyte subpopulations were performed on the same blood samples collected for the study of PMBC tendency to undergo apoptosis. PBMC were obtained by density gradient centrifugation following standard methods, and cells were then used

immediately for dual and triple colour cytofluorimetric analysis, performed on a FACScan (Becton Dickinson), as described previously (Cossarizza *et al*, 1991). Viable lymphocytes, i.e. those capable of excluding PI in parallel samples, were electronically gated on the basis of their physical parameters (FSC and SSC), and a minimum of 10 000 cells per sample was acquired in list mode and analyzed by Lysys II software program.

As far as the study of the modifications of lymphocyte phenotype during spontaneous apoptosis are concerned, we used the following approach. Cells were cultured, collected at different time points, and stained with different mAbs. Then, first, viable lymphocytes were electronically gated taking into account their physical parameters. The analysis of parallel samples revealed that these cells had a normal CD45 membrane expression and were capable to exclude PI. A minimum of 10 000 cells were acquired and analyzed. Second, an electronic gate was set in the physical region of apoptotic cells, i.e. those with lower forward and higher side angle scatter, and with reduced CD45 membrane expression, as previously reported (Carbonari *et al*, 1994). In this case, a minimum of 5000 cells per sample were acquired in list mode and analyzed as described above. Due to shortage of cells, we could investigate four subjects in each group. Four experiments were thus performed, in which cells from one donor per group were studied.

Intracellular reduced glutathione content

The measure of PBMC intracellular GSH content was performed according to the method proposed by Reed *et al*, (1980). Due to shortage of cells, we could analyze 7 LTNP, 10 HIV+P and 11 controls. Briefly, 10^6 PBMC were resuspended in a 0.9 mL of 10% trichloroacetic acid water solution to remove protein and centrifuged at 5000 rpm for 5 min. The supernatant was treated immediately with 50 μl of a fresh iodoacetic acid aqueous solution (4 μM), and then the obtained mixture was neutralised with an excess of $NaHCO_3$ dry powder to form S-carboxymethyl derivatives. After 60 min in the dark at room temperature, 0.1 mL of analcoholic solution of 1-fluoro 2,4-dinitrobenzene was added. The provoked reaction was allowed to proceed for 24 h in the dark, and this led to the formation of N-DNP derivatives that were resolved by high performance liquid chromatography (HPLC) analysis. The samples were stored at -20°C until analysis by HPLC. A Beckman Gold HPLC equipped with a Waters Bondapak amine column was used to resolve DNP derivatives. Solvent A was 4:1 methanol:water (v/v) and solvent B was prepared as follows: 272 mL sodium acetate trihydrate, 122 mL water and 378 mL glacial acetic acid were mixed and 200 mL of the resulting solution was added to 800 mL of solvent A. An isocratic period of 10 min at 25% of B was followed by a programmed linear gradient from 25–95% solvent B over a 20 min period. An aliquot of 40 μL of the sample was usually injected in the column using an autosampler Beckman mod. 507, and GSH was determined at 365 nm.

Statistical analysis

Statistical analysis was performed by two tails paired or unpaired Student's *t*-test and by linear regression analysis, using SPSS® for Windows software. A *P* value lower than 0.05 was considered significant.

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