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Analysis of lymphocyte cell death and apoptosis in HIV-2-infected patients

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SUMMARY

Recent evidence suggests that T cell apoptosis could be involved in the pathogenesis of HIV-1 infection. As the progression of HIV-2 associated disease appears to be slower than that of HIV-1, we investigated whether there were differences in the degree of T cell death and apoptosis in peripheral blood mononuclear cell (PBMC) cultures from patients with HIV-1 or HIV-2 infection. PBMC from healthy controls ($n = 28$) and patients infected with HIV-1 ($n = 26$: asymptomatic (ASY)/persistent generalized lymphadenopathy (PGL), $n = 16$; and AIDS-related complex (ARC)/AIDS $n = 10$) or HIV-2 ($n = 30$: ASY/PGL, $n = 16$; ARC/AIDS, $n = 14$) were cultured in the absence or presence of mitogens (PHA, PWM) or superantigen (SEB). After 48 h, cell death (CD) was assessed by trypan blue exclusion and in some patients programmed cell death (PCD) was quantified in flow cytometry by measuring the percentage of hypodiploid nuclei corresponding to fragmented DNA, after treating the cells with a propidium iodide hypotonic solution. HIV-1 and HIV-2 ARC/AIDS patients and ASY/PGL HIV-1^+ patients had significant increases in cell death percentages compared with controls, both in unstimulated and stimulated lymphocyte cultures. However, HIV-2^+ ASY/PGL patients did not exhibit significant increases of cell death in unstimulated cultures. In addition, the comparison between HIV-1 and HIV-2 infected subjects in similar stages of disease, showed no significant differences in CD in the ARC/AIDS patients, although ASY/PGL HIV-2-infected subjects had lower levels of CD than the HIV-1^+ ASY/PGL (3·4% ± 0·6 s.e.m. *versus* 6·8% ± 1·1 s.e.m., $P < 0.01$). PCD was significantly increased both in ASY/PGL (14·3% ± 2·2 s.e.m., $n = 8$, $P < 0.005$) and in ARC/AIDS (25·3% ± 4·5 s.e.m., $n = 9$, $P < 0.001$) HIV-1^+ patients compared with healthy controls (5·8% ± 1·7 s.e.m., $n = 11$). This contrasts with HIV-2 infected subjects where the ASY/PGL patients (10·0% ± 2·8 s.e.m., $n = 6$) did not differ significantly from healthy controls, although ARC/AIDS patients (27·2% ± 4·2 s.e.m., $n = 9$, $P < 0.001$) had significantly increased levels of PCD. In conclusion, this is the first report describing the occurrence of spontaneous and activation-induced lymphocyte death by apoptosis in HIV-2 infected subjects. The lower levels of PCD in ASY/PGL HIV-2 infected patients compared with HIV-1^+ patients at a similar stage justify further investigation to define whether these differences have any role in the putative slower progression of HIV-2 disease.

Keywords HIV-1 infection HIV-2 infection apoptosis cell death flow cytometry AIDS programmed cell death HIV-2 natural history

INTRODUCTION

Infection with the immunodeficiency virus type 1 (HIV-1) affects T helper lymphocytes both quantitatively with a progressive depletion of this subset and qualitatively with a variety of functional defects such as reduced lymphocyte responses to antigens, alloantigens and mitogens [1]. Several mechanisms have been proposed to account for these alterations, namely single-cell killing, formation of syncytia [2], autoimmune mechanisms [3,4], anergy [5] and superantigen-mediated perturbation of T cell subsets [6]. Recently, a single mechanism, apoptosis, was proposed to account for both functional and numerical abnormalities of T cells in HIV-1-infected patients [7].

Programmed cell death (PCD) or apoptosis is a cell death mechanism morphologically and biochemically distinct from necrosis, characterized by chromatin condensation, membrane blebbing and fragmentation of DNA into oligonucleosome fragments following the activation of a calcium-dependent endogenous endonuclease [8,9]. This phenomenon constitutes a physiological mechanism involved in the negative intrathymic

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selection and in the establishment of self-tolerance, although it is not observed in normal mature T lymphoctes [10]. However, some authors have documented the occurrence of T cell apoptosis in HIV-1 infection, and proposed that the abnormal induction of this mechanism is relevant to the pathogenesis of AIDS and could play a role in the evolution of the disease [11–13].

HIV-2 is a retrovirus with a high prevalence in Western African countries [14] and extremely rare in the USA and Europe, with the exception of Portugal, where it is responsible for 10% of the reported AIDS cases [15]. Although HIV-2 is capable of producing a spectrum of diseases similar to HIV-1 infection, some evidence suggests that the period between primary infection and development of AIDS is longer in HIV-2 infection [16,17]. Nevertheless, there are very few studies investigating possible immunological explanations for this putative lower pathogenicity of HIV-2.

In view of the possible role of T cell apoptosis in the pathogenesis of HIV-1 infection, we investigated in this study whether patients with HIV-2 infection had different levels of T cell apoptosis compared with HIV-1-infected patients.

MATERIALS AND METHODS

Subjects

Twenty-six patients seropositive for HIV-1 infection with a mean age of 36 years (range 22–53) and 30 patients seropositive for HIV-2 infection with a mean age of 42 years (range 20–61) were studied. Seropositivity was determined by ELISA and confirmed by Western blot analysis. HIV^+ patients were divided into two subgroups based on clinical data and on the Center for Disease Control (CDC) classification criteria. There were 16 asymptomatic (ASY)/persistent generalized lymphadenopathy (PGL) (ASY = 12, PGL = 4), and 10 AIDS-related complex (ARC)/AIDS (ARC = 2, AIDS = 8), $HIV\text{-}1^+$ patients and 16 ASY/PGL (ASY = 14, PGL = 2) and 14 ARC/AIDS (ARC = 2, AIDS = 12) $HIV\text{-}2^+$ patients. At the time of the study, 75% of the ARC/AIDS patients and 25% of the ASY/PGL patients were receiving zidovudine. Twenty-eight seronegative healthy subjects with a mean age of 34 years (range 20–65) were also studied as a control group.

Isolation of mononuclear cells

Heparinized venous blood was collected from patients and controls. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll–Hypaque (Gibco, Grand Island, NY) density gradient. Cells were washed three times in PBS (Biomérieux, Marcy L'Etoile, France) and resuspended in RPMI 1640 (Gibco) supplemented with 10% inactivated fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Flow, Mclean, VA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco).

Cell viability

PBMC (1×10^5 cells/well) were cultured in triplicate in round-bottomed 96-well microtitre plates (Costar, Cambridge, MA) in the absence or presence of pockweed mitogen (PWM, 5 μg/ml), phytohaemagglutinin (PHA, 30 μg/ml) or staphylococcal enterotoxin B (SEB, 2 μg/ml). Cultures were incubated for 48 h at 37°C in a 5% CO_2 humidified atmosphere. Cell viability was then assessed by trypan blue exclusion and a minimum of 100 cells was counted in a haemocytometer.

Quantification of apoptosis

Aliquots of 1 ml PBMC (1×10^6 cells/test tube) were incubated for 48 h at 37°C in 5% CO_2 humidified atmosphere either in the absence or presence of PHA (30 μg/ml). The percentage of intact and fragmented DNA in the samples was then determined by flow cytometry according to the method described by Nicoletti *et al.* [18]. Briefly, the cells were centrifuged at 200 *g* for 10 min, and for the fluorochrome staining the cell pellet was resuspended in 1 ml propidium iodide (PI) hypotonic solution (PI 50 μg/ml in 0·1% sodium citrate plus 0·1% Triton X-100 plus 0·05% RNAse, Sigma). The cell suspensions were incubated overnight at 4°C in the dark and flow cytometric analysis was then performed in an EPICS Profile flow cytometer (Coulter, Hialeah, FL).

Lymphocyte subset analysis

T cell subsets in PBMC were determined by flow cytometry using directly labelled anti-CD4 and anti-CD8 (Dako, Dakopatts, Denmark) monoclonal antibodies as previously described [19].

Statistical analysis

Statistical comparisons were made by Student's *t*-test for the analysis of both cell death and apoptosis. Correlations were calculated by linear regression analysis. A P value less than 0·05 was considered significant.

RESULTS

Cell death in PBMC from HIV-infected subjects and healthy controls

Table 1 shows the percentages (mean ± s.e.m.) of cell deaths in lymphocyte stimulated and unstimulated cultures of HIV-1- and HIV-2-infected patients as well as of healthy controls. ASY/PGL HIV-1 patients had significant increases ($P < 0·001$) in the percentage of cell death, both in unstimulated and in stimulated lymphocyte cultures, compared with healthy controls. In contrast, no significant increases were found in the percentage of cell death in unstimulated cultures of ASY/PGL HIV-2 infected subjects. In addition, the comparison of HIV-1 and HIV-2 ASY/PGL patients (unstimulated lymphocytes) showed that there were statistically significant differences between the two populations. Both HIV-1 and HIV-2 ARC/AIDS patients had significant increases in the percentages of cell death ($P < 0·01$) and these levels are higher than the ones observed in ASY/PGL subjects (Table 1). No differences between the two infections were found in ARC/AIDS patients. Finally, the percentages of cell death were significantly higher in stimulated cultures than in the unstimulated ones in all groups of patients studied (Table 1). Comparison between the degree of $CD4^+$ T cell depletion in ASY/PGL HIV-1 *versus* ASY/PGL HIV-2 patients and in ARC/AIDS HIV-1 *versus* ARC/AIDS HIV-2 patients revealed that the CD4 levels were similar (ASY/PGL HIV-1: 33·2% ± 9·5 s.d., ASY/PGL HIV-2: 33·1% ± 9·6 s.d. and ARC/AIDS HIV-1: 13·9% ± 10·5 s.d., ARC/AIDS HIV-2: 14·8% ± 12·5 s.d.).

Table 1. Cell death percentage (± s.e.m.) in HIV-1 and HIV-2 infection

		(0 μg/ml)	PWM (5 μg/ml)	PHA (30 μg/ml)	SEB (2 μg/ml)
Healthy controls ($n = 28$)		2·4 ± 0·5	3·7 ± 0·3	6·7 ± 0·6	4·4 ± 0·5
ASY/PGL ($n = 32$)	HIV-1 ($n = 16$)	6·8 ± 1·1*	11·3 ± 1·6*	17·9 ± 1·5*	11·5 ± 1·8*
		$P < 0·01$	NS	NS	NS
	HIV-2 ($n = 16$)	3·4 ± 0·6	8·2 ± 1·1*	15·7 ± 2·2*	9·0 ± 1·0*
ARC/AIDS ($n = 24$)	HIV-1 ($n = 10$)	7·4 ± 1·1*	12·0 ± 1·5*	16·6 ± 1·7*	14·1 ± 2·2*
		NS	NS	NS	NS
	HIV-2 ($n = 14$)	6·6 ± 0·9*	10·2 ± 1·6*	15·1 ± 2·2*	13·1 ± 1·7*

* Cases where there is a statistically significant difference between HIV patients and controls.
Statistical analysis performed by Student's *t*-test.
NS, Not significant.

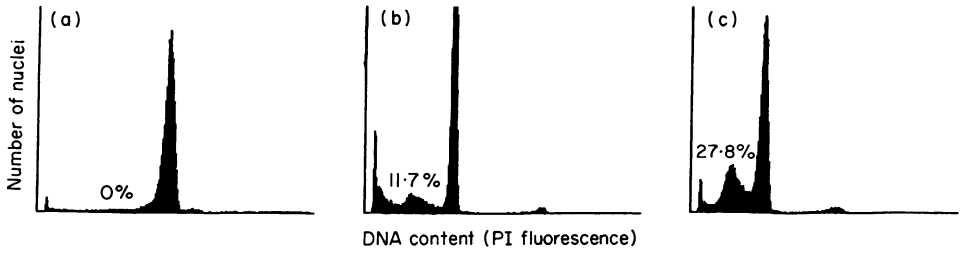

Fig. 1. Quantification of apoptosis by flow cytometric analysis of DNA fragmentation which appears as a hypodiploid peak. Three representative experiments performed in the absence of stimuli are shown. (a) Healthy subject. (b) HIV-2 asymptomatic patient. (c) HIV-1 asymptomatic patient.

Mechanism of in vitro *cell death: evidence for apoptosis*

Flow cytometric analysis of propidium iodide-stained cells allows their characterization in terms of DNA content, namely the quantification of apoptotic cells [18]. Thus, the internucleosomal DNA cleavage that seems to be the major event in apoptosis is detectable in flow cytometric analysis as a broad hypodiploid peak (Fig. 1). DNA fragmentation is expressed in Table 2 as a percentage (mean ± s.e.m.) of apoptotic cells in unstimulated and PHA-stimulated lymphocyte cultures of healthy individuals, HIV-1- and HIV-2-infected subjects. Apoptosis was significantly increased in unstimulated cultures of HIV-1 ASY/PGL patients, whereas in HIV-2 ASY/PGL patients no significant increases were found either in unstimulated or in stimulated lymphocyte cultures in comparison with healthy controls. However, the differences between HIV-1 and HIV-2 patients in the same clinical stage did not reach statistical significance (Table 2). The increases in the levels of apoptosis were more pronounced in ARC/AIDS patients than in ASY/PGL patients both in HIV-1 (unstimulated, $P < 0·05$; stimulated, $P < 0·005$) and in HIV-2 (unstimulated, $P < 0·005$; stimulated, $P < 0·01$) infection. Finally, the increases in the percentages of apoptosis are more pronounced in PHA-stimulated cultures than in unstimulated ones, but the differences do not reach statistical significance (Table 2).

Table 2. Apoptosis in HIV-1 and HIV-2 infected patients

		Percentage of apoptotic cells (mean ± s.e.m.)	
		PHA (0 μg/ml)	PHA (30 μg/ml)
Healthy controls ($n = 11$)		5·8 ± 1·7	9·5 ± 3·2
ASY/PGL ($n = 14$)	HIV-1 ($n = 8$)	14·3 ± 2·2*	16·2 ± 2·8
		NS	NS
	HIV-2 ($n = 6$)	10·0 ± 2·8	14·5 ± 5·1
ARC/AIDS ($n = 18$)	HIV-1 ($n = 9$)	25·3 ± 4·5*	30·8 ± 2·9*
		NS	NS
	HIV-2 ($n = 9$)	27·2 ± 4·2*	34·7 ± 4·3*

Details as Table 1.

Correlation between apoptosis and T cell subsets

In order to characterize the T cell composition of PBMC from HIV-infected patients we determined the $CD4^+$ and $CD8^+$ T cell subsets in all patients studied. As expected, patients with ARC/AIDS had a marked depletion of CD4 T lymphocytes (13·6% ± 11·5 s.d.) as well as increases in $CD8^+$ cells (29·3% ± 13·4 s.d.) while ASY/PGL subjects showed a less

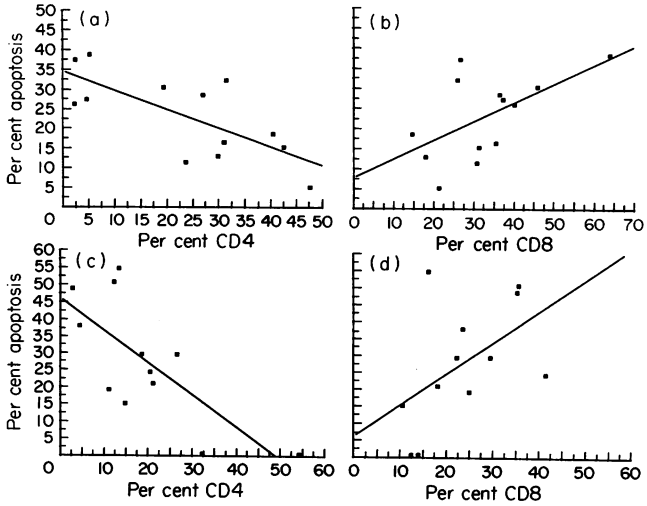

Fig. 2. Correlation between the levels of apoptosis and the percentages of $CD4^+$ and $CD8^+$ T cells in HIV-1 (a and b) and HIV-2 (c and d) infection. (a) Correlation coefficient (r) = −0·72, $P < 0·01$; (b) $r = 0·57$, $P < 0·05$; (c) $r = -0·73$, $P < 0·01$; (d) $r = 0·52$, NS.

pronounced alteration of these T cell subsets (CD4, 31·7% ± 11·1 s.d., CD8, 24·0% ± 8·6 s.d.).

As shown in Fig. 2(a and b), the analysis of correlation between the levels of apoptosis in PHA-stimulated cultures and the percentage of $CD4^+$ or $CD8^+$ T cell subsets in HIV-1 infection revealed a negative correlation between the percentage of apoptosis and the percentage of $CD4^+$ lymphocytes ($r = -0·72$, $P < 0·01$) and a positive correlation between the percentage of apoptosis and the percentage of $CD8^+$ lymphocytes ($r = 0·57$, $P < 0·05$). In HIV-2 infection (Fig. 2c and d), there was also a negative correlation between the levels of apoptosis and the percentage of $CD4^+$ T cells ($r = -0·73$, $P < 0·01$) and a trend for a positive correlation between the percentage of apoptosis and the percentage of $CD8^+$ T cells ($r = 0·52$) which did not reach statistical significance. Analysis of the correlations between apoptosis in unstimulated lymphocyte cultures and the percentages of $CD4^+$ or $CD8^+$ lymphocytes revealed similar trends, although statistical significance was not reached (data not shown).

DISCUSSION

The main objective of our study was to determine whether T cell apoptosis was present in PBMC cultures from HIV-2-infected subjects as has been described in HIV-1 infection [11–13]. Our results demonstrated that PBMC from HIV-2-infected patients display a loss of viability, as tested by simple dye exclusion assays, when cultured either in the absence or presence of stimuli. Furthermore, we investigated whether cell death occurred via apoptosis by flow cytometric analysis of isolated nuclei stained with propidium iodide according to a recently described method [18]. The rationale for this approach is based on the evidence that fragmented DNA in apoptotic nuclei, a characteristic feature of apoptosis, results in a quantifiable hypodiploid DNA peak which correlates well with other methods for measuring apoptosis [18]. Increased levels of apoptosis were found in unstimulated and stimulated lymphocyte cultures from HIV-2-infected subjects. However, HIV-2 ASY/PGL patients did not display significantly increased levels of apoptosis and cell death in unstimulated cultures, in contrast with HIV-1 patients who had significant increases in all stages of disease both in unstimulated and stimulated PBMC cultures. Our observations in HIV-1 infection are in agreement with previous studies from other authors. Thus, both Gougeon *et al.* [11] and Meyaard *et al.* [13] reported the occurrence of spontaneous and activation-induced death by apoptosis in $CD4^+$ and $CD8^+$ T cells from asymptomatic HIV-1-infected individuals, although in another study, apoptosis of $CD4^+$ T cells from asymptomatic HIV-1 patients could only be observed after stimulation [12].

The comparison between HIV-1 and HIV-2 ASY/PGL patients revealed a trend for higher levels of apoptosis and cell death in HIV-1 infection. This finding can be analysed having in consideration the two main mechanisms which were recently proposed for the induction of apoptosis in T lymphocytes of HIV-1-infected subjects. Thus, it has been suggested that the cross-linking of CD4 molecules by HIV-1 gp120 or gp120/anti-gp120 immune complexes primes T cells for PCD in response to subsequent T cell receptor (TCR) stimulation [7,12,20]. It is conceivable that differences in the sequence of the envelope proteins between HIV-2 and HIV-1 could determine distinct abilities of these proteins to induce an apoptotic mechanism. On the other hand, it was recently shown by Simon *et al.* [21] that HIV-2 infection appears to be associated with lower levels of plasma viral load compared with HIV-1 and, thus, it is possible that such differences could contribute to explain distinct levels of PCD. Another recently proposed mechanism for controlling apoptosis involves antigen-presenting cells (APC); inappropriate co-signal delivery or absence of signals provided by APC would be responsible for the abnormal induction of this cell death mechanism in the context of HIV-1 infection. Thus, HIV-1 appears to interfere in the interaction between APC and T cells either by infecting monocytes and dendritic cells or by subverting the expression of accessory molecules on these cells [7,22,23]. Investigation of antigen presentation via either dentritic cells or monocytes in HIV-2 infection will therefore be relevant to further characterize the putative APC-dependent apoptotic mechanism in HIV-2-infected subjects.

Although the lower degrees of cell death and apoptosis in HIV-2-infected individuals in the asymptomatic stages could have a role in the reported slower progression of HIV-2-associated disease, sequential prospective studies on HIV-2 infection will be required to investigate the relationship between the levels of apoptosis and subsequent disease progression. It is worth noting that the differences in the levels of cell death and apoptosis between HIV-1- and HIV-2-infected subjects are observed only in ASY/PGL patients, whereas in advanced stages of disease no differences are found between the two infections. It is possible to speculate on several explanations for this apparent discrepancy. Firstly, we may hypothesize that low levels of apoptosis could influence disease progression during the asymptomatic period, whereas in patients reaching an advanced stage, where a severe $CD4^+$ cell depletion and an overall dysfunction of the immune system are observed, no differences in the rate of progression would occur. Alternatively, the lower levels of apoptosis in HIV-2 infection could be the result of subgroups of patients with low

apoptosis, who would be under-represented in the advanced stages as consequence of a lower progression rate. In view of the importance of T cell activation in the pathogenesis of HIV infection, it will also be important to investigate whether the degree of T cell apoptosis in HIV-2 infection correlates with the magnitude of T cell activation disturbances.

Most of the previous studies on T cell apoptosis in HIV-1 infection investigated only asymptomatic patients [12,13]. Therefore, it is important to stress our results showing that apoptosis is clearly more pronounced in advanced stages of disease both in HIV-1 and HIV-2 infection. Furthermore, our observation of a negative correlation between the percentage of $CD4^+$ cells and apoptosis and a positive correlation between the percentage of $CD8^+$ cells and apoptosis in both HIV-1 and HIV-2 infection, is consistent with the hypothesis of an association between the occurrence of this phenomenon and the progression of HIV-associated disease. In this respect, it is interesting that Gougeon *et al.* [24] suggested a correlation between PCD and AIDS pathogenesis based on the comparison of the lymphocytes from lentivirus-infected primates, namely SIV-infected macaques that are susceptible to AIDS, and HIV-infected chimpanzees that are resistant.

In conclusion, we have shown that lymphocyte apoptosis, recently implicated in the pathogenesis of HIV-1 disease, also occurs in HIV-2 infection, although asymptomatic HIV-1 patients appear to have higher levels of apoptosis than asymptomatic HIV-2 patients. Further investigation of apoptosis in HIV-2 infection, including prospective studies and correlations with the rate of CD4 decline, is justified to assess whether these differences could explain the slower progression of HIV-2 disease.

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