

Circulating immune complexes from HIV-1⁺ patients induces apoptosis on normal lymphocytes

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SUMMARY

Isolated immune complexes from sera of 49 out of 67 human immunodeficiency virus-1-positive (HIV-1⁺) patients (CIC–HIV⁺), composed of anti-HIV–HIV-Ag, could induce apoptosis on normal phytohaemagglutinin (PHA)-activated lymphocytes. DNA degradation was detected by propidium iodide staining. This activity is directed against CD4⁺ lymphocytes as demonstrated by double binding of CIC–HIV⁺ and anti-CD4 on apoptotic cells. Expression of Fas antigen is prior to apoptotic phenomena. CIC–HIV⁺ apoptosis inducers belong mainly to asymptomatic HIV-infected patients, indicating that immune complexes from these patients can destroy CD4⁺ lymphocytes.

INTRODUCTION

Two distinct models of cell death, apoptosis and necrosis, have been described in eukaryotic cells. Apoptosis is involved in many normal biological processes such as embryonic and T-cell development and can be regarded as a mechanism of cell suicide, and is characterized biochemically by the fragmentation of DNA into oligonucleosomal fragments.¹ Apoptosis also occurs during pathological conditions such as human immunodeficiency virus (HIV) infection. Apoptosis has been well documented in peripheral blood mononuclear cell culture of HIV-infected individuals and has been proposed as a mechanism of T-cell dysfunction at the beginning of the disease, and depletion in the later stages.^{2–4} One of the mechanisms proposed for the cell apoptosis in HIV-1-infected patients is the cross-linking of bound glycoprotein 120 (gp120) on human CD4⁺ cells followed by signalling through the T-cell receptor.⁵ *In vivo*, the cross-linking of CD4 could be mediated by circulating gp120 bound to anti-gp120, which can be considered as circulating immune complexes.⁶ It is well known that patients infected with immunodeficiency virus present elevated levels of circulating immune complexes but their significance is not well understood; except for a few reported cases, those pathological features commonly associated with increased CIC levels are not found in HIV-infected patients. Euler *et al.* found a significant correlation between decreasing CD4 cells and the amount of CIC.⁷ The relationship among high levels of CIC and low CD4 cell count could be due to the down-regulation of surface CD4 expression by immune complexes.⁸ Naturally occurring circulating immune complexes in HIV infection have been well documented to

have anti-CD4 activity⁹ and to act as enhancers of the HIV-1 infection.¹⁰ In this paper we study the role of naturally occurring CIC from HIV⁺ patients in inducing the apoptosis on normal human CD4 lymphocytes and the expression and regulation of Fas (CD95) antigen in the induction of apoptosis.

MATERIALS AND METHODS

Patients

Sixty-seven patients (48 male and 19 female) positive for HIV-1 antibody were studied. The age range was from 21- to 40-years old. Clinically, 40 individuals were classified as group A (asymptomatic), seven as group B and 20 as group C following the classification for acquired immune deficiency syndrome (AIDS) of the Centre for Disease Control (CDC, Atlanta, GA).¹¹ Blood samples were collected at the time of diagnosis before previous anti-retroviral treatment.

Ten HIV seronegative patients with more than 200 µg/ml of CIC (with different pathologies such as chronic hepatitis B and C) were used as controls. The study was approved by the ethical committee of our hospital. Normal peripheral blood lymphocytes were drawn from normal volunteers.

Isolation of circulating immune complexes

CIC were isolated from a patient's sera by precipitation with 1.04 M ammonium sulphate as described previously.^{9,10} Anti-HIV antibody and p24 antigen in isolated CIC were performed by enzyme-linked immunosorbent assay (ELISA; Abbott, Germany).

Induction of apoptosis

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by means of Lymphoprep (Nyegaard, Norway) at 400 g for 20 min. PBMC were collected, washed

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and adjusted to 1×10^6 cells/ml in RPMI-1640 medium (Whitaker, Belgium) supplemented with 10% fetal calf serum (FCS; Whitaker), penicillin G (200 U/ml; Whitaker), streptomycin (200 U/ml; Whitaker) and L-glutamine (0.3 mg/ml; Whitaker). 1×10^6 cells were stimulated with 20 μ l of phytohaemagglutinin (PHA; 1 mg/ml; Gibco, Paisley, UK) in 24-well plates (Costar, Cambridge, MA) and incubated with 200 μ g/ml of isolated CIC from HIV-infected patients or controls for 72 hr at 37° in 5% CO₂ atmosphere. Cells were recovered for measuring apoptosis at times, 24, 48 and 72 hr from commencement of the experiment.

Detection of apoptosis

Cell apoptosis was measured by flow cytometry using an intercalating DNA dye, propidium iodide (PI; Sigma, St Louis, MO). Cells were treated with ribonuclease A (10 μ g/ml; Sigma) in order to remove RNA, and DNA was stained with propidium iodide (100 μ g/ml) in Nonidet P-40 (0.05%; Sigma). The stained specimen was kept in the dark at 4° before flow cytometry. Analysis was performed in an EPICS-XL flow cytometer (Coulter, Hialeah, FL). Analysis of apoptotic cell cycle was carried out using the Mcycle program. This is described as the easiest and most rapid method for measuring apoptosis.¹²⁻¹⁴ the apoptotic population showed a low stainability resulting in a quantifiable region below the G0/G1 peak. Also, apoptotic cells give lower forward scatter and higher side scatter values than viable cells, reflecting their smaller size and different consistency.

Expression of Fas (CD95) antigen

A total of 5×10^5 of cultured lymphocytes were incubated with 5 μ l of anti-CD95 fluorescein isothiocyanate (FITC)-monoclonal antibody (UB-2, non-inducer of apoptosis) (Kamiya, CA), for 30 min. Cells were washed twice with phosphate-buffered saline-bovine serum albumin (PBS-BSA) 0.1% and fluorescence was measured in an Epics-XL flow cytometer.

Binding of CIC to normal CD4+ lymphocytes

A total of 200 μ g of isolated CIC from HIV+ patients were incubated with 1×10^6 normal PHA-activated peripheral blood lymphocytes. After 72 hr, cells were collected and incubated with FITC-labelled F(ab)₂ goat-anti-human immunoglobulin G (IgG; Caltag, San Francisco, CA) and, simultaneously, with phycoerythrin (PE)-labelled anti-CD4 or anti-CD8 monoclonal antibody (Dakopatts, Glostrup, Denmark), for 30 min. After washing, fluorescence was measured in an Epics-XL flow cytometer.

Statistical analysis

Fisher's exact test and Mann-Whitney U-test were used for statistical purposes.

RESULTS

Induction of apoptosis by isolated CIC from HIV-1+ patients

Forty-nine out of 67 HIV-1+ patients (73%), possess circulating immune complexes able to induce apoptosis on normal PHA-activated lymphocytes after culturing for 72 hr. The percentage of apoptotic population induced by these CIC is $41.8 \pm 17\%$; however, CIC isolated from the others 18 patients gave very low levels of apoptosis, $2.3 \pm 2.8\%$. Levels of

apoptosis of lymphocytes incubated with PHA alone or with CIC non-related with HIV infection, were almost negative ($0.7 \pm 2.1\%$ and $2.2 \pm 2.9\%$). Two replicates from each patient were performed with the same results. When CIC-HIV+ were incubated with normal lymphocytes without PHA activation, apoptosis did not occur. All these results are depicted in Table 1. 64% of these 49 immune complexes belong to asymptomatic patients, whereas immune complexes that were not able to induce apoptosis belonged to AIDS patients (63%) ($P=0.0002$). There was no difference either in CD4+ counts in both groups of patients: patients in whom the CIC are apoptosis inducers, $CD4^+ = 290 \pm 205$ cells/ μ l versus patients in whom the CIC are non-apoptosis inducers, $CD4^+ = 263 \pm 219$ cells/ μ l ($P=0.6$), or in CIC levels (CIC apoptosis inducers, 614 ± 240 μ g/ml versus CIC non-apoptosis inducers, 616 ± 172 μ g/ml $P=0.9$). Regarding the composition of CIC (anti-HIV and HIV-Ag), all isolated CIC were anti-HIV+ and, probably, all CIC are HIV-Ag+, although we only detected p24 antigen in 33% of all the isolated CIC.

Expression of Fas antigen (CD95)

Expression of the Fas antigen was measured in lymphocytes from five normal donors activated with PHA. At 24 hr of culture, the expression is low ($2.8 \pm 0.5\%$ positive cells) (average \pm SD) and reaches the maximum at 48 hr of culture ($22.4 \pm 2.4\%$) with no variation at 72 hr ($23 \pm 6.1\%$). When normal lymphocytes were incubated with an apoptosis inducer CIC-HIV+ in the presence of PHA, the expression of Fas antigen, measured in whole population, increased at 24 hr above PHA-induced levels ($9.3 \pm 2.8\%$), with a high expression at 48 hr of culture ($21.5 \pm 9.5\%$) and a decrease at 72 hr below PHA-induced levels ($4.9 \pm 2.4\%$). Results are depicted in Table 2. At 24 hr of culture there was no apoptosis present but it became evident after 48 hr of incubation reaching the maximum at 72 hr. These results could indicate that Fas expression is prior to the apoptotic process and could be one of the events in the induction of apoptosis by these CIC-HIV+, but the down-regulation of Fas expression at the end of the culture could be due to the loss of CD95+ cells.

Table 1. Levels of apoptotic population on normal PHA-activated lymphocytes induced by isolated immune-complexes from HIV+ patients

CIC-HIV App inducers	CIC-HIV non-App inducers	CIC non-HIV related	Control PHA
$41.8 \pm 17.05\%$	$2.3 \pm 2.8\%$	$2.2 \pm 2.9\%$	$0.7 \pm 2.1\%$

Table 2. Levels of Fas (CD95) expression at different times of cell culture of PHA-activated lymphocytes with an apoptosis-inducer CIC-HIV

	24 hr	48 hr	72 hr
CIC-HIV	9.3 ± 2.8	21.5 ± 9.5	4.9 ± 2.4
Control PHA	2.8 ± 0.5	22.4 ± 2	23.6 ± 6.1
P	0.002	0.9	0.002

CIC-HIV⁺ induce apoptosis on CD4⁺ lymphocytes

In order to assess that CIC-HIV⁺ induced apoptosis on CD4⁺ T lymphocytes, cells were recovered after culture and labelled simultaneously with a PE-anti-CD4 monoclonal antibody and FITC-anti-human IgG. When viable cells were gated, only 6% of these cells bound to anti-human IgG; 1.2% were CD4⁺ and 4.5% were CD4⁻ (probably these cells could be B cells that express immunoglobulins on their surface). But when apoptotic cells were gated, 64% of the cells bound to anti-human IgG, 60% were CD4⁺, and 4% were CD4⁻ (Fig. 1). We can consider that non-CD4⁺ cells are mainly CD8⁺ cells. When apoptotic cells were gated, non-CD4⁺ cells (most of them CD8⁺) did not bind to anti-human IgG. In fact, when cells were labelled simultaneously with FITC-anti-human IgG and PE-anti-CD4, neither the viable nor the apoptotic population were positive for both markers. The apoptosis of CD8⁺ cells may follow a different mechanism. These results suggest that CIC-HIV⁺ bind preferentially to CD4⁺ lymphocytes and induce apoptosis on them.

DISCUSSION

Naturally occurring immune complexes isolated from HIV-1⁺ patients can activate cell death in normal activated peripheral blood CD4⁺ lymphocytes. These CIC are mainly composed of anti-HIV antibody and HIV antigen (CIC-HIV⁺), although we could not detect the presence of HIV proteins in all of CIC-HIV⁺ tested. The anti-HIV antibody present in CIC-HIV⁺ is anti-gp120 as we demonstrated by Western Blot in a previous paper.⁹

The induction of apoptosis is directed against normal activated CD4⁺ lymphocytes, as demonstrated by the double binding of apoptotic lymphocytes with isolated CIC-HIV⁺ and antibody anti-CD4⁺. When lymphocytes were not PHA activated, incubation with CIC-HIV⁺ did not induce apoptosis, this result indicates that two signals are necessary for the induction of apoptosis. One signal is ligation of the CD4 molecule that could result from interaction of the anti-CD4 activity present in these CIC-HIV⁺, as previously described,⁹ and a second signal is PHA activation.

These results partially agree with those from Oyaizu *et al.* in which cross-linking of the CD4 molecule, if performed in unfractionated peripheral blood mononuclear cells instead of on purified CD4⁺,⁵ induced apoptosis without secondary T-cell receptor (TCR) activation. In our case, we needed two stimuli to induce apoptosis, PHA and CIC-HIV⁺, probably binding CIC-HIV⁺ to the CD4 molecule is more physiological

than the binding of monoclonal anti-CD4 to the CD4 molecule. Also, these authors found evidence of apoptosis in normal PBMC stimulated with PHA for 3 days, we also observed these results in a few cases using normal PBMC from different individuals and discarded these samples, under the presumption that they had been previously activated. The apoptotic activity of these CIC-HIV⁺ against CD4⁺ lymphocytes could be due the interaction of gp120/anti-gp120 with the CD4 molecule; Siliciano *et al.* also reported that the cross-linking of CD4 with gp120 and anti-gp120 followed by cell activation, induced apoptosis *in vitro*.¹⁵ Our results could answer the question formulated in the paper of Siliciano *et al.*¹⁵ and from Banda *et al.*:⁶ does the *in vitro* priming for apoptosis by gp120-anti-gp120 have any *in vivo* relevance? The answer could be that naturally occurring circulating immune complexes in HIV⁺ patients could induce the apoptosis of CD4⁺ lymphocytes. The role of Fas antigen in the induction of apoptosis in HIV infection has been well documented by many authors; in the paper from Katsikis *et al.*¹⁶ CD4⁺ and CD8⁺ lymphocytes from HIV⁺ patients are primed to undergo apoptosis in response to Fas stimulation, and in Wang *et al.*¹⁷ the engagement of the CD4 molecule induced Fas antigen-dependent apoptosis *in vivo*. In the present report the expression of Fas antigen precedes the induction of apoptosis by CIC-HIV⁺ but, the down-regulation of Fas expression after 72 hr of culture could be due to the loss of these cells. The CIC-HIV⁺ able to induce apoptosis are mainly present in asymptomatic individuals and not in AIDS patients, as could be expected; however, in a recent paper, Groux *et al.* described activation-induced apoptosis of CD4⁺ cells from HIV-infected asymptomatic individuals.¹⁸ One could speculate that the induction of apoptosis in asymptomatic HIV-1⁺ individuals could be a mechanism for destroying infected cells and that this ability is lost in AIDS patients. However, several recent reports have provided compelling evidence that uninfected cells are the target for spontaneous apoptosis, rather than infected cells.^{19,20} We could therefore consider that spontaneous apoptosis in HIV⁺ patients, and induced apoptosis by CIC-HIV⁺, are different processes of destroying CD4⁺ cells. There are few reports on the therapeutic inhibition of apoptosis; Sekigawa *et al.*²¹ describe how the treatment of gp120-primed cells with the immunosuppressant (FK 506) before TCR signalling inhibited apoptotic cell death and suggests that FK 506 is a potentially useful drug in HIV infection. However, according to our results on the biological role of immune complexes in this infection, from a therapeutic point of view the clearance of these immune complexes could result in a benefit for patients.

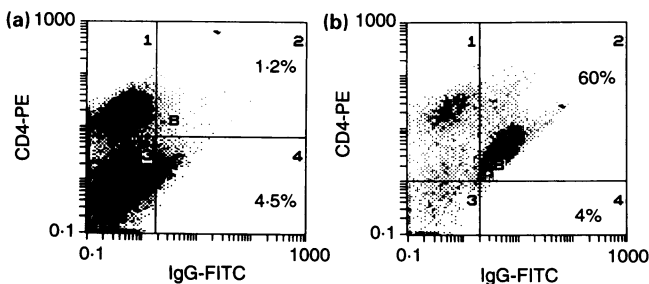


Figure 1. Apoptotic cells bind CIC-HIV⁺ and monoclonal anti-CD4. (a) Viable cells. (b) Apoptotic cells.

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