

Spontaneous Programmed Cell Death of Peripheral Blood Mononuclear Cells from HIV-infected Persons is Decreased with Interleukin-15

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Abstract

Interleukin 15 (IL-15) is an important regulatory cytokine in cellular immunity. *In vitro* replacement of IL-15 has been shown to enhance immunity in Human immunodeficiency virus type 1 (HIV-1) infected lymphocytes. We evaluated the effect of IL-15 on the survival of peripheral blood mononuclear cells of HIV patients by examining *in vitro* lymphocyte apoptosis, and correlated the process with Bcl-2 and Fas gene regulation. Peripheral blood mononuclear cells (PBMC) from 21 HIV-infected adults and 24 HIV-seronegative healthy individuals were isolated and cultured to determine the effect of escalating doses of IL-15 (0, 1, 10, 100, 1000 ng/mL) on apoptosis. Lymphocyte proliferation assay with (^3H) TdR was measured and Bcl-2 and Fas gene regulation was observed. The results were as follows: 1) IL-15 reduced culture induced lymphocyte apoptosis in HIV patients in a dose dependent manner, and reached a plateau level at a concentration of 100 ng/ml; 2) IL-15 significantly reduced the level of apoptosis after 3 days (14%) and 5 days (15%) of culture in HIV patients, while no difference was observed in HIV (–) donors; 3) The percentage of viable cells among the total number of lymphocytes was significantly enhanced by 25% in HIV patients with IL-15; 4) Bcl-2 expression was decreased in HIV patients ($53.9 \pm 12.3\%$) compared to HIV (–) donors ($93.0 \pm 3.7\%$), and IL-15 increased Bcl-2 expression by $21.2 \pm 5.2\%$ in HIV patients; 5) Fas expression was increased in HIV patients ($70.2 \pm 4.6\%$) compared to HIV (–) donors ($32.4 \pm 4.3\%$), and IL-15 increased Fas expression by $8.4 \pm 1.2\%$ in HIV (–) donors. Our findings indicate that IL-15 may influence immunologic abnormalities in HIV infection, particularly its ability to prevent apoptosis of lymphocytes by suppressing the down-modulation of Bcl-2. This may provide an experimental basis for IL-15 immunotherapy.

Key Words: Interleukin 15, lymphocyte, apoptosis, Human Immunodeficiency Virus

INTRODUCTION

Infection by the human immunodeficiency virus (HIV) is characterized by an asymptomatic phase of variable length and a depletion in CD4+ T cells, eventually leading to the acquired immune deficiency syndrome (AIDS).¹ In asymptotically-infected patients, functional abnormalities of T cells are demonstrated followed by a decline in the number of cells, and a peculiar phenomenon is that with a comparatively smaller number of infected T cells in the beginning of the infection, a larger number of cells are involved in functional and quantitative deter-

oration.² This may indicate that an immune process may be involved in the pathogenesis of AIDS. Several possible immunologic abnormalities in HIV infection have been investigated, including specific loss of memory cells, induction of anergy by antigen-presenting cell dysfunction, dysregulation of Th1/Th2 cytokine balance and HIV-induced apoptosis.³ Many studies have been performed on HIV-induced apoptosis thereafter, and this may account for the CD4+ T cell depletion and loss of cellular immune function in HIV-infected individuals.⁴

Apoptosis is a physiologic cell death process that is genetically controlled. It is crucial for the proper development and homeostasis of many tissues, including the nervous and immune systems.^{5,6} Apoptosis was induced *in vitro* in both CD4 and CD8 T cells following a short-term culture of HIV-infected lymphocytes,^{7,8} and later it was also demonstrated *in vivo* in lymph nodes of HIV-infected persons.^{9,10} Another interesting discovery was that apoptosis occurred

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predominantly in the bystander cells rather than the infected CD4+ cells in HIV Infection.¹¹ Some proposed mechanisms of increased susceptibility to apoptosis of lymphocytes from an HIV infected person are direct virus infection of lymphocytes, in vivo priming of CD4+ T cells by gp 120,¹² defective antigen presenting cell function¹³ and abnormal patterns of cytokine expression.¹⁴ Some immunomodulating effects of IL-15 are promotion of survival and proliferation of T lymphocytes.¹⁵ By exogenously supplying IL-15 to the immunologic microenvironment of HIV infection, apoptosis may be regulated, thus affecting the survival of the lymphocytes. In the present study, we evaluated the effect of IL-15 on the survival of lymphocytes in HIV patients by examining in vitro lymphocyte apoptosis, and correlated the process with Bcl-2 and Fas gene regulation. By correlating Bcl-2 and Fas expression, as well as the apoptosis level with the application of IL-15 in HIV-infected lymphocytes, we attempted to observe if there were Bcl-2 and Fas gene regulations involved in the pathogenic process with the hope that there may be a way for therapeutic intervention by regulating apoptosis to preserve deteriorating immune function in HIV infection.

MATERIALS AND METHODS

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) from 24 HIV-infected patients (range of CD4 counts 5 to 704/ μ L.) and from HIV-seronegative healthy individuals were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech, Gaithersburg, MD, USA). Except for proliferation assay, cells (1×10^6 /mL) were cultured in round-bottom 12 \times 75 mm polystyrene tubes (Falcon 2054, Becton Dickinson Labware, Bedford, MA, USA) in the presence or absence of human rIL-15 (Boehringer Mannheim, Indianapolis, IN, USA). RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated FCS (Life Technologies), 2 mmol/L L-glutamate (Whittaker Bioproducts, Walkersville, MD, USA), 100 U/mL penicillin G, and 100 μ g/mL streptomycin was used for all cultures.

Lymphocyte proliferation assay and cell count analysis

To examine lymphocyte proliferation, 2×10^6 cells were seeded in 96-well, round-bottom plates (Becton Dickinson) with complete medium in the presence or absence of IL-15 (100 ng/mL) for 1, 3, and 5 days followed by a 16-hour pulse of [methyl-³H]TdR (Amersham life science, Buckinghamshire, England). Incorporation of [methyl-³H]TdR was assessed in a liquid scintillation counter (Pharmacia LKB, Gaithersburg, MD, USA). To assess the surviving cell numbers, 1×10^6 /mL of cells were resuspended in complete medium in the presence or absence of IL-15 (100 ng/mL) and were cultured for the indicated periods. Total cells and live cells were counted on a hemocytometer by trypan blue exclusion.

Lymphocyte Bcl-2 and Fas staining

FITC-conjugated anti-human Fas/Apo-1 clone DX2 (Dako, Carpinteria, CA, USA) was stained to 1×10^6 cells and put in the dark for 30 minutes and washed with 2% FCS (Life Technologies). Paraformaldehyde (0.25%) was added while vortexing. After fixing the cells with 70% methanol, the cells were washed with PBS and 2% FCS (Life Technologies) for flow cytometric analysis (Becton Dickinson, San Jose, CA, USA). FITC-conjugated anti-human bcl-2 clone 124 (Dako, Carpinteria, CA, USA) was stained by the following procedure. Paraformaldehyde (0.25%) were added to 1×10^6 cells while vortexing and put in the dark for 15 minutes. After washing with PBS, the cells were fixed with 70% methanol and washed with PBS. FITC-anti-human bcl-2 was stained to the cells and washed with PBS and 2% FCS (Life Technologies) for flow cytometric analysis (Becton Dickinson).

Measurement of apoptosis

Propidium iodide (PI) staining and Annexin V binding, were employed to measure the percentage of cells undergoing apoptosis.

PI staining was performed as follows: Cells were fixed in 70% ethanol for 1 hour at 4°C, washed, and resuspended in HBSS containing PI (50 μ g/mL, Molecular probes, Eugene, OR) and ribonuclease A (type I-A, Sigma, 250 μ g/mL). The PI fluorescence of

individual cells was measured at 72 hours poststaining by flow cytometry. This method was employed to measure total cellular apoptosis.

Annexin V binding detects phosphatidylserine, which is preferentially expressed on the outer surface of cells undergoing apoptosis, and was performed utilizing a commercially available kit (Annexin V-FITC, Trevigen, Gaithersburg, MD, USA).

Statistical analysis

Statistical significance was assessed by student's t test. Paired samples were also assessed for differences with the Wilcoxon Signed Rank test and considered significant at $p < 0.05$.

RESULTS

Response of IL-15 on lymphocyte apoptosis

We examined the effect of exogenous IL-15 on the degree of apoptosis by assessing the emergence of sub-diploid nuclei in PI-stained samples of 3-day cultured lymphocytes. As shown in Fig. 1, ex-

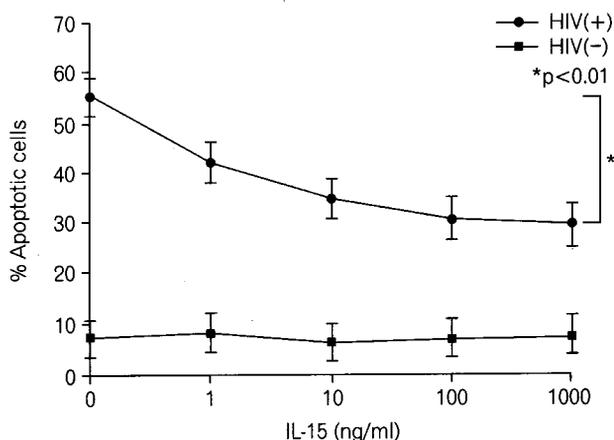


Fig. 1. Dose response of interleukin-15 on lymphocyte apoptosis. PBMC from HIV (-) donors (n=13) and HIV (+) patients (n=20) were cultured for 3 days in the absence or presence of various concentrations of IL-15. Cells were stained with PI and the percentage of sub-diploid nuclei (% apoptotic cells) were evaluated by flow cytometry. IL-15 blocked culture-induced lymphocyte apoptosis in a dose-dependent manner, demonstrated by the reduction of apoptotic cells. The level of apoptosis was significantly decreased in HIV patients at an IL-15 concentration of 100 ng/mL ($p < 0.01$). Values are expressed as mean \pm SD.

genously-provided IL-15 blocked culture-induced lymphocyte apoptosis in a dose-dependent manner, demonstrated by the reduction of apoptotic cells. The level of apoptosis was significantly decreased in HIV patients at an IL-15 concentration higher than 100 ng/mL ($p < 0.01$).

We examined the time course effect of IL-15 100 ng/mL on lymphocyte apoptosis for a 5-day culture period. We confirmed that a significant amount of spontaneous apoptosis was observed after 3 days in the lymphocytes of patients, and a significant decrease in the apoptosis level was seen on day 3 (14%) and day 5 (15%) by administering IL-15 in the patients' lymphocytes, while no difference was observed in HIV (-) donors (Fig. 2) ($p < 0.01$). Fig. 3 shows a flow cytometric analysis of a double-staining of Annexin V and PI of an HIV (-) donor and an HIV (+) patient with or without IL-15 administration. IL-15 administration in the HIV (+) patient increased the number of viable cells.

Effect of IL-15 on lymphocyte proliferation and lymphocyte counts

The effect of IL-15 on the number of live cells, the

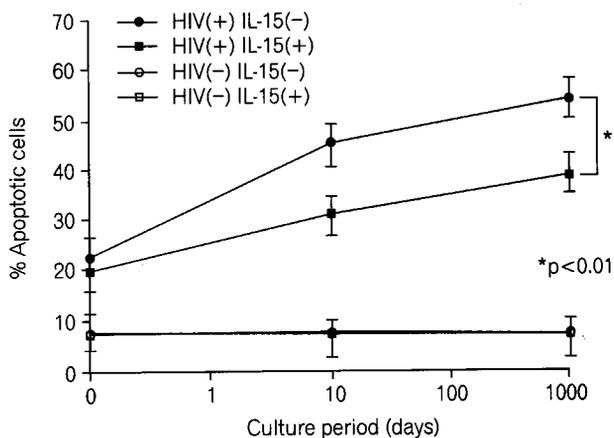


Fig. 2. Time course study of the effect of IL-2 (100 ng/mL) on lymphocyte apoptosis. PBMC from HIV (-) donors (n=13) and HIV (+) patients (n=20) were cultured for 5 days. Cells were harvested 1, 3, and 5 days after culture initiation and analyzed by flow cytometry to determine the percentage of apoptotic cells. A significant amount of spontaneous apoptosis was observed after 3 days in the lymphocytes of patients, and a significant decrease in the apoptosis level was seen on day 3 (14%) and day 5 (15%) by administering IL-15 in the patients' lymphocytes, while no difference was observed in HIV (-) donors ($p < 0.01$). Values are expressed as mean \pm SD.

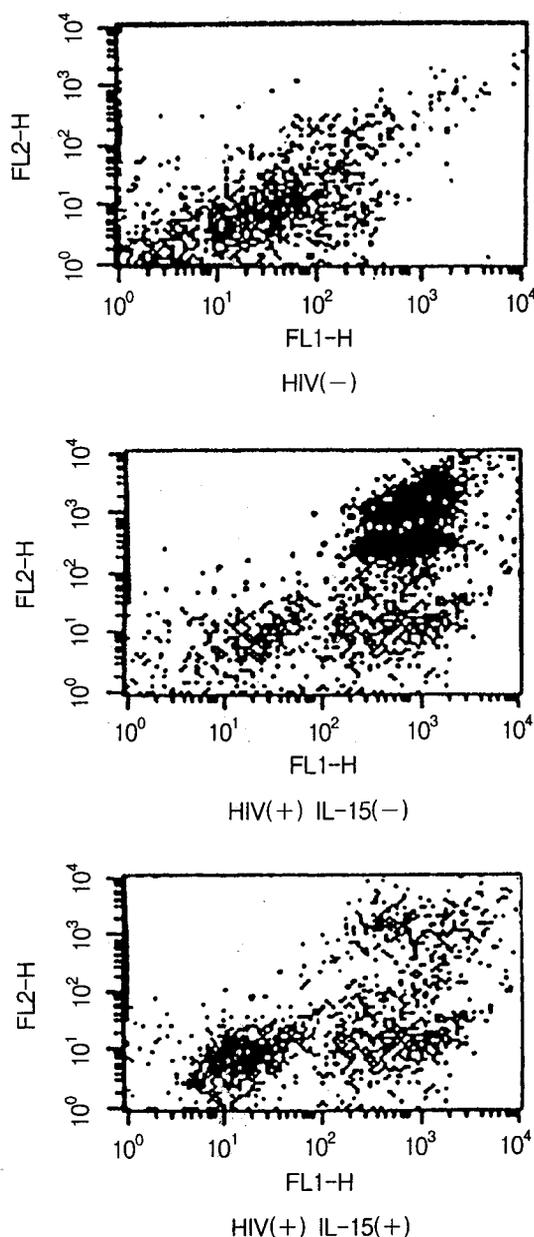


Fig. 3. Representative cytogram showing the effect of interleukin-15 on lymphocyte apoptosis shown by flow cytometry after Annexin V-FITC binding (x-axis) and PI staining (y-axis). PBMC from an HIV (-) donor and an HIV (+) patient were cultured for 5 days in the absence or presence of IL-15 (100 ng/mL). Cells positively stained with both Annexin V and PI increased in HIV (+) compared to HIV (-) and decreased after the addition of IL-15.

amount of apoptosis and lymphocyte proliferation in HIV patients and controls during the culture period was evaluated by conducting a simultaneous comparison of lymphocyte proliferative responses and cell

count analysis. As shown in Table 1, the addition of IL-15 did not result in a net increase of cell counts during the culture period up to 5 days either in cells from controls or patients. The total cell count declined progressively in HIV patients and on day 5 it was 49% of the initial cell count. This decline in cell number was significantly blocked by IL-15 in HIV patients resulting in a cell count of 73% of the initial cell count. Cellular viability was also significantly improved by 25% with the addition of IL-15 ($p < 0.05$). The addition of IL-15 led to significant [methyl- ^3H]TdR incorporation on days 3 and 5, both in control and patient cells. Therefore, in HIV patients, a smaller decrease of viable cell count after the addition of IL-15 was due to both the increase in cellular proliferation and the decrease in the percentage of apoptosis.

Correlation of Bcl-2 and Fas expression with lymphocyte apoptosis

To determine whether the ability of IL-15 to rescue cells from apoptosis is associated with its regulatory effects of bcl-2 or Fas expression, we compared bcl-2 and Fas expression in lymphocytes of controls and patients cultured with or without IL-15. As shown in Table 2, the addition of IL-15 resulted in a significant reduction of the down modulation of bcl-2 expression in the patients' lymphocytes by 21.2% ($p < 0.01$). Fas expression was increased significantly in control lymphocytes by 8.4% ($p < 0.05$) with IL-15 administration, but there was no significant correlation in the patient lymphocytes, of which Fas expression was higher compared to control lymphocytes.

DISCUSSION

The role of IL-15 on T cell apoptosis is controversial. Reports on the effect of IL-15 in the immune system describe that it either promotes or blocks apoptosis. In this study, we examined the effects of IL-15 on spontaneous lymphocyte apoptosis of HIV-infected patients. We demonstrated that in most patients IL-15 can significantly reduce the degree of apoptosis, thus leading to increased lymphocyte survival. This effect was attributable to the combined effect of its apoptosis-preventing ability and cellular proliferation during the culture period.

Table 1. Kinetics of Cell Number, Cell Viability, and [methyl-³H]TdR Uptake of Lymphocytes

	Total lymphocytes (viable cells) × 10 ⁴ /ml (mean ± SD) ^a		
	Day 1	Day 3	Day 5
HIV (-) IL-15 (-)	98 ± 4 (98 ± 2)	87 ± 4 (76 ± 5)	83 ± 6 (73 ± 8)
HIV (-) IL-15 (+)	99 ± 5 (90 ± 3)	95 ± 8 (84 ± 10)	94 ± 9 (82 ± 9)
HIV (+) IL-15 (-)	72 ± 11 (67 ± 12)	61 ± 10 (45 ± 11)	49 ± 12 (30 ± 8)
HIV (+) IL-15 (+)	85 ± 15 (75 ± 20)	75 ± 15 (60 ± 21)	73 ± 15 (55 ± 12)

	[methyl- ³ H]TdR incorporation (cpm) (mean ± SD) ^b		
	Day 1	Day 3	Day 5
HIV (-) IL-15 (-)	106 ± 13	289 ± 79	388 ± 61
HIV (-) IL-15 (+)	152 ± 20	2455 ± 249	4113 ± 491
HIV (+) IL-15 (-)	121 ± 35	166 ± 51	164 ± 69
HIV (+) IL-15 (+)	241 ± 71	909 ± 217	1710 ± 214

^a PBMC were obtained from HIV (-) donors (n=10) and HIV (+) patients (n=16). A total of 1 × 10⁶/mL cells were cultured with or without IL-15 (100 ng/mL) for indicated periods. Total cells (live and dead cells) and live cells (assessed by trypan blue exclusion) were determined.

^b PBMC were obtained from HIV (-) donors (n=10) and HIV (+) patients (n=16). Lymphoproliferative assays were performed by seeding 2 × 10⁶ cells in 96-well, round-bottom plates in the presence or absence of IL-15 (100 ng/mL) for 1, 3, and 5 days followed by a 16-hour pulse of [methyl-³H]TdR. Incorporation of [methyl-³H]TdR was assessed in a liquid scintillation counter. Viable cells decreased more in HIV (+) patients compared to HIV (-) donors, while IL-15 stimulation significantly improved viability in HIV (+) patients (* p < 0.05). [methyl-³H]TdR incorporation was increased in cells of both HIV (-) and (+) patients after IL-15 stimulation with no significant difference.

Table 2. Effect of IL-15 on Bcl-2 and Fas Expression in HIV (-) Donors and HIV (+) Patients

	Bcl-2 (%)	Fas (%)
HIV (-) IL-15 (-)	93.0 ± 3.7	32.4 ± 4.3
HIV (-) IL-15 (+)	91.3 ± 5.2	40.8 ± 5.4
HIV (+) IL-15 (-)	53.9 ± 12.3	70.2 ± 4.6
HIV (+) IL-15 (+)	75.1 ± 11.0	72.0 ± 4.1

PBMC from HIV (-) donors (n=10) and HIV (+) patients (n=16) were cultured for 3 days in the presence or absence of IL-15 (100 ng/mL). Fas and bcl-2 expression was determined by staining 1 × 10⁶ cells with FITC-conjugated anti-human Fas/Apo-1 clone DX2 and FITC-conjugated anti-human bcl-2 clone 124, respectively and then they were analyzed by flow cytometric analysis. IL-15 stimulation resulted in a significant reduction of the down-modulation of bcl-2 expression in the patients' lymphocytes by 21.2% (* p < 0.01), and Fas expression was increased significantly in control lymphocytes by 8.4% († p < 0.05). Values are expressed as mean ± SD.

Bcl-2 inhibits apoptosis by enhancing cell survival rather than by accelerating the rate of cellular proliferation,¹⁶ and its product plays a key role in the

control of cell death of T and B cell lineages during lymphoid development, ensuring their appropriate selection.¹⁷ The mechanism of how Bcl-2 prevents apoptosis is described by sequestering proforms of death-driving cysteine proteases called caspases or by prevention of the release of mitochondrial apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF) into the cytoplasm. After entering the cytoplasm, cytochrome c and AIF directly activate caspases that cleave a set of cellular proteins to cause apoptotic changes.¹⁸ Another gene product also involved in the apoptotic death program of lymphoid cells is Fas/Apo-1 (CD95) Ag, a transmembrane protein.¹⁹ Ligation of Fas by a specific antibody²⁰ or by Fas ligand²¹ has been shown to transduce a potent apoptotic signal in sensitive target cells, and it is thought that this Fas mediated cell death might play a significant role in the development and function of the immune system.²² By correlating Bcl-2 and Fas expression and the apoptosis level with the application of IL-15 in HIV-infected lymphocytes, we attempted to observe if there were any gene regulations involved in the pathogenic process with the hope that there

may be some way for therapeutic intervention by regulating apoptosis to preserve deteriorating immune function in HIV infection. There have been some reports on the protective effect of IL-15 in association with bcl-2 induction.²³ Our study revealed that the apoptosis-preventing effect of IL-15 was associated with reduced down-modulation of bcl-2 expression, and this suggested that IL-15 mediated its apoptosis-blocking effects by suppressing the down-modulation of bcl-2. Interleukin-15 shares many biological activities with IL-2 and signals through the IL-2 receptor beta and gamma chains. However, IL-15 and IL-2 differ in their controls of expression and secretion, their range of target cells and their functional activities. These dissimilarities may include differential effects on apoptosis. IL-2 has been previously shown to up-regulate bcl-2 expression in a variety of cell culture systems, including an IL-2 dependent CD8+ T-cell line,²⁴ an IL-2R β -chain-expressing hemopoietic cell line,²⁵ and PHA-stimulated T-cell blasts that had been maintained with IL-2.²⁶ The exact nature of culture-induced down-modulation of bcl-2 is not known, and IL-2 failed to up-regulate bcl-2 expression in normal lymphocytes. Thus, IL-2 appears to mediate its effect either by signals that counteract negative regulatory signals for bcl-2 expression or by providing signals that are required for bcl-2 expression.

The death-mediating activity of Fas and its apoptosis regulation in HIV infection is an interesting aspect. In HIV infection, Fas expression in lymphocytes increases with disease progression,²⁷ and patients' lymphocytes have been shown to be extremely sensitive to apoptosis in response to cross-linking an anti-Fas antibody.²⁸ However, there is a report that IL-15 stimulation has only minor effects on mRNA and protein level expression of Fas compared to bcl-2 or bax in culture conditions.²⁹ In our study, the up-regulation of Fas by IL-15 was only detected in control lymphocytes. Fas expression was higher in lymphocytes of patients compared to controls and was not further increased by IL-15. Based on our results, it appears unlikely that IL-15 affects apoptosis via regulation of the Fas system in HIV-infected patients, but further investigations by examining the effect of the antagonist of Fas and Fas Ligand may be necessary to provide more direct evidence of this apoptosis regulation. Moreover, the HIV itself might influence the Fas-mediated cell death pathway since

Westendorp et al.³⁰ recently reported that two HIV proteins, Tat and gp 120, accelerated Fas-mediated, activation-induced T-cell apoptosis.

An important still unresolved issue concerns the relevance of apoptosis for AIDS pathogenesis and whether cell death is the cause of AIDS or the consequence. Several mechanisms can be evoked to support apoptosis as a consequence of HIV infection. The chronic production of viral antigens would contribute to apoptosis either directly by inducing a cell death signal or indirectly by influencing the activation of the immune system. The suggested rapid turnover of CD4 T cells in HIV-infected persons due to an active lymphocyte regenerative process may contribute significantly to the rate of apoptosis in patients. A high expression of transglutaminase, a preapoptotic marker, in CD4 T cells but not CD8 T cells from a patient's lymph nodes is in agreement with such a mechanism.³¹ The impaired production of Th1 cytokines, such as IL-2 or IL-12 would contribute to the excess of cell death, since these cytokines have been shown to block apoptosis in patients' CD4 and CD8 lymphocytes.³² In other respects, apoptosis can also significantly contribute to AIDS. Since apoptosis was shown *in vivo* to involve activated CD4 and CD8 T cells^{9,10} and mostly noninfected lymphocytes,¹¹ it could be responsible for the clearance of activated but healthy T cells. Therefore, the uncontrolled and chronic immune activation occurring throughout HIV infection is probably the primary mechanism responsible for lymphocyte apoptosis, the deteriorating effects of which contribute to the collapse of the immune system.

In conclusion, our results show that the administration of IL-15 may have a positive influence on the immunological abnormalities in HIV infection, especially on the ability to prevent apoptosis. This may provide a basis for the clinical application of IL-15 administration to HIV patients in combination with antiretroviral drugs for correction of immunologic defects while suppressing virus replication.

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