Activation-induced Death by Apoptosis in CD4$^+$ T Cells from Human Immunodeficiency Virus–infected Asymptomatic Individuals

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Summary

In immature thymocytes, T cell receptor for antigen (TCR) mobilization leads to an active T cell suicide process, apoptosis, which is involved in the selection of the T cell repertoire. We have proposed that inappropriate induction of such a cell death program in the mature CD4$^+$ T cell population could account for both early qualitative and late quantitative CD4$^+$ T lymphocyte defects of human immunodeficiency virus (HIV)-infected individuals (Ameisen, J. C., and A. Capron. 1991. *Immunol. Today.* 4:102). Here, we report that the selective failure of CD4$^+$ T cells from 59 clinically asymptomatic HIV-infected individuals to proliferate in vitro to TCR mobilization by major histocompatibility complex class II–dependent superantigens and to pokeweed mitogen (PWM) is due to an active CD4$^+$ T cell death process, with the biochemical and ultrastructural features of apoptosis. Activation-induced cell death occurred only in the CD4$^+$ T cell population from HIV-infected asymptomatic individuals and was not observed in T cells from any of 58 HIV-seronegative controls, including nine patients with other acute or chronic infectious diseases. Activation-induced CD4$^+$ T cell death was prevented by cycloheximide, cyclosporin A, and a CD28 monoclonal antibody (mAb). The CD28 mAb not only prevented apoptosis but also restored T cell proliferation to stimuli, including PWM, superantigens, and the tetanus and influenza recall antigens. These findings may have implications for the understanding of the pathogenesis of acquired immune deficiency syndrome and for the design of specific therapeutic strategies.

HIV-infected individuals present early CD4$^+$ T cell functional defects (1–7) that precede the quantitative reduction in this cell population which leads to acquired immune deficiency syndrome (AIDS). These functional defects are detected while less than 1/1,000 helper T cells are infected (8–10), and are characterized by a selective loss of ability to proliferate in vitro to self-MHC class II–restricted recall antigens and to PWM (1–7), and the capacity to proliferate to the PHA mitogen is maintained. CD4$^+$ T cell dysfunction and depletion have been attributed to a wide range of distinct mechanisms. In particular, the early qualitative defects have been related to T cell suppression (7, 11), clonal anergia (7), autoimmune responses (12), inhibitory effects of HIV proteins (13, 14), or selective HIV-mediated destruction of memory T cells, leading to the presence of only naive CD4$^+$ T cells (15, 16).

Ameisen and Capron (17) have proposed the hypothesis that a single mechanism, the inappropriate reemergence of a T cell death program in response to activation, could account for both early qualitative and late quantitative CD4$^+$ T cell defects from HIV-infected individuals. Programmed cell death, activation-induced cell death, or apoptosis, is an active cell suicide mechanism of widespread biological importance (18) that constitutes the physiological response of normal immature thymocytes to activation (18–23). This process is involved in the negative selection of the T cell repertoire, which leads to the clonal deletion of autoreactive T cells, and to the establishment of self-tolerance (24). This cell suicide mechanism occurs in the absence of bystander cell destruction, requires cell activation, initiation of protein synthesis, and involves the activation of an endogenous endonuclease that results in a characteristic regular fragmentation of the entire cellular DNA into multiples of an oligonucleosome unit of 200 bp (18–28). In immature thymocytes, apoptosis is not an obligatory response to TCR stimulation, but is the consequence of incomplete signal transduction related to the nature of the antigen-presenting cell and to the ab-

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sence of some cosignals (22, 23, 29, 30). A major question in T cell biology has been whether TCR mobilization may also lead in some circumstances to the reemergence of a functional cell death program in mature T cells. Recent studies involving mature murine T cells indicate that this is indeed the case (31, 32).

We have investigated whether in vitro activation of T cells from clinically asymptomatic HIV-1-infected individuals, including individuals with normal CD4+ T cell counts, and from controls with polyclonal activators and self-MHC class II-dependent recall antigens may lead to T cell death. Since memory T cells specific for a given recall antigen are rare, and might be depleted in HIV-infected individuals, we also investigated the T cell response to the self-MHC class II-dependent staphylococcal enterotoxin B (SEB)[1] superantigens (33), which had not been previously explored in HIV-infected individuals. These superantigens bind to MHC class II molecules and interact with defined Vβ TCR molecules expressed by up to 30% of human T cells, inducing proliferation in normal mature CD4+ T cells (33) and apoptosis in immature thymocytes (21).

Materials and Methods

Study Subjects. Peripheral blood was obtained from 109 HIV-infected asymptomatic individuals in the Service des Maladies Infectieuses, Centre Hospitalier de Tourcoing, France. Of 75 men and 34 women, all clinically asymptomatic (stage II of the Center for Disease Control [CDC], Atlanta, classification), 79 were CDC stage IIA (no biological abnormalities, CD4 >500/mm^3, mean 884) and 30 were CDC stage IIB (biological abnormalities, CD4 <500/mm^3, mean 342). HIV infection was related to homosexuality (n = 49), heterosexual contact (n = 30), intravenous drug use (n = 28), or blood transfusion (n = 2). Controls (n = 58) were 49 HIV-seronegative healthy donors and nine HIV-seronegative patients with infectious or autoimmune diseases. These included, respectively: symptomatic infectious mononucleosis, genital herpes, *Candida albicans* spondylitis, cutaneous staphylococcal infection with staphylococcal septic arthritis in a diabetic patient, evolutive *Loa loa* filariasis, evolutive onchocercosis with eosinophilia, falciparum malaria, Gram-negative pneumopathy, and periarthritis nodosa.

Cell Preparations. PMBC were obtained on Ficoll-Hypaque, and cells were cultured as previously described (34).

In some experiments, CD4+ or CD8+ T cells were purified by negative selection with magnetic beads coated by anti-mouse IgG (Dynal, Biosys, Compiègne, France). Cells (50 × 10^6) were plated to plastic petri dishes to harvest adherent cells by scraping. Nonadherent cells were incubated with 5 μg/ml of CD20, CD56, MHC class II, CD4, or CD8 mAbs in a volume of 5 ml in RPMI for 30 mn. Subsequently, excess antibody was removed by washing twice in RPMI. The cells were then resuspended in 5 ml RPMI with magnetic beads, according to the manufacturer's instructions. This mixture was rotated in the cold for 30 min and the cells were passed through a magnetic field twice to remove the cells that had bound to the magnetic beads. Cells were 98% pure as assessed by cytofluorometry.

[1] *Abbreviations used in this paper:* CsA, cyclosporin A; SEB, staphylococcal enterotoxin B; TT, tetanus toxoid.

In some experiments, PBMC were depleted either in CD4+ or CD8+ T cells by using the same general method, cytofluorometry analysis revealing less than 2% contaminating cells.

Cell Proliferation Assays. Cell proliferation assays were performed in 96-well culture plates (Nunc, Roskilde, Denmark) in a final volume of 200 μl, as previously described (34). PBMC (2.5 × 10^5/ml) were cultured in RPMI/10% FCS. Mitogens (purchased from Sigma, La Verpillière, France) were used, respectively, at the following final concentrations: PHA, 10 μg/ml; Con A, 10 μg/ml; PWM, 10 μg/ml; staphylococcal enterotoxin B (SEB) superantigens, 1 μg/ml; the CD3 mAb was used at 1 μg/ml. Tetanus toxoid (TT) recall antigen (Biomerieux, Lyon, France) was used at 10 μg/ml; and influenza A hemagglutinin (Infl) recall antigen (Eurobio, Paris, France) at 10 μg/ml. After 3 d for mitogens or 6 d for antigens, cultures were pulsed with 1 μCi of [^{3}H]thymidine (1 or 25 Ci/mmol, depending on the experiments; Amersham, les Ulis, France) during the final 15 h of incubation, and harvested.

Evaluation of Cell Death by Trypan Blue Exclusion. Cells were incubated in 96-well plates with various stimuli in the same conditions as for proliferation assays. They were harvested by pipetting and diluted 1:2 with 0.1% trypan blue in PBS. The live and dead cells were counted in a hemocytometer.

DNA Fragmentation Assays. DNA fragmentation was determined according to the methods of Wyllie and Morris (28), and Newell et al. (31), with slight modifications. In brief, 10^7 cells were collected by centrifugation at 200 g for 10 min, and lysed in 1 ml hypotonic lysing buffer (5 mM Tris, pH 7.4, 5 mM EDTA, 0.5% Triton X-100). The lysates were centrifuged at 13,000 g for 15 min. Supernatants were deproteinized by extraction once in phenol/chloroform and twice in chloroform/isoamyl alcohol (24:1) and precipitated at −20°C in 50% isopropanol, 130 mM NaCl. After electrophoresis on 2% agarose slab gels, DNA was stained by ethidium bromide.

Electron Microscopy. Cells were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h at +4°C. Pellets were postfixed in 1% aqueous osmium tetroxide for 1 h, en bloc stained in 1% aqueous uranyl acetate for 6 h, and embedded in araldite. Sections were stained with uranyl acetate and lead citrate before examination with a Philips (Mahwah, NJ) EM 420 electron microscope.

Monoclonal Antibodies and Chemicals. The mAbs used in this study were CD3 (X35-7, IgG2a), generous gift from Dr. Bourel, Centre Régional de Transfusion Sanguine, Rennes, France; CD28 (9.3, IgG2a), generous gift from Oncogen Corp., Seattle, WA; CD28 (CLB 28/1, IgG1), purchased from Janssen, Beerse, Belgium; CD20 (IOB20, IgM), CD56 (IOT56, IgG1), HLA-DR (IOT2a, IgG2b), CD4 (IOT4, IgG2a), CD8 (IOT8, IgG2a), were all purchased from Immunotech, Marseille, France; CD5 (A50, IgG1), CD44 (P245, IgG2a), generous gifts from Dr. A. Bernard, Centre Hospitalier Régional, Nice, France. CsA was purchased from Sandoz, Rueil-Malmaison, France; cycloheximide from Sigma, and azidothymidine (AZT) from Burroughs Wellcome, Paris, France.

Statistical Analysis. Statistical significance was assessed by using Student's t test.

Results

Proliferative Response of T Cells from HIV-infected Asymptomatic Individuals and from Controls. We investigated the in vitro proliferation of T cells from 109 HIV-infected asymptomatic individuals and 59 controls in response to the polyclonal activators PHA, Con A, PWM, CD3 mAb, to the

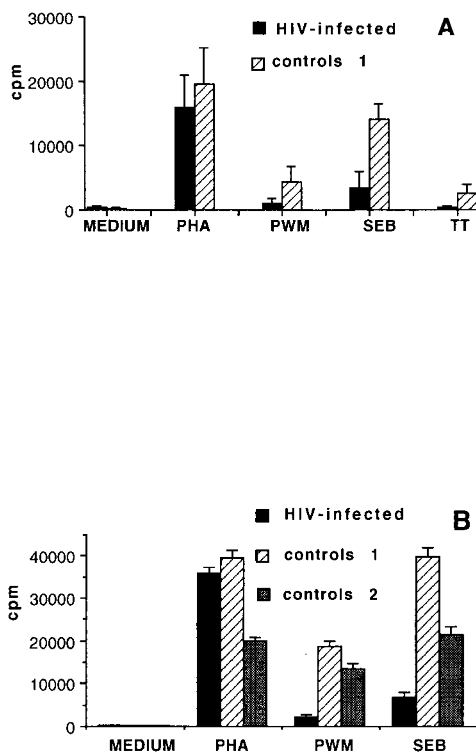

Figure 1. Proliferative response of T cells from HIV-infected asymptomatic individuals and from controls. (*A*) Histograms represent the mean ± SD of measurement of [^{3}H]thymidine incorporation of T cells from 100 HIV-infected asymptomatic individuals (HIV-infected) and from 40 HIV-seronegative healthy controls (controls *1*) incubated with medium alone, PHA (10 μg/ml), PWM (10 μg/ml), superantigens (SEB) (1 μg/ml), and the tetanus toxoid (TT) recall antigen (10 μg/ml). The specific activity of the [^{3}H]thymidine used was 1 Ci/mmol. (*B*) Histograms represent the mean ± SD of measurement of [^{3}H]thymidine incorporation of T cells from nine HIV-infected asymptomatic individuals (HIV-infected), from nine HIV-seronegative healthy controls (controls 1), and from nine HIV-seronegative patients suffering from acute or chronic infectious diseases (controls *2*) incubated with medium alone, PHA, PWM, and SEB as described in *A*. The specific activity of the [^{3}H]thymidine used was 25 Ci/mmol.

tetanus recall antigen, and to the SEB superantigens. T cells from all healthy controls proliferated to all stimuli, and T cells from HIV-infected asymptomatic individuals showed a selective defect in their response to SEB, to recall antigen, and to PWM (Fig. 1 *A*).

Proliferation of T cells from HIV-infected individuals to Con A and to CD3 mAb was only slightly reduced (not shown), as was proliferation to PHA (Fig. 1 *A*). This was consistent with the fact that all HIV-infected individuals studied were clinically asymptomatic, with few or no biological abnormalities, since defective proliferations to CD3 mAb and to PHA have been reported to be predictive markers of evolution towards AIDS in long-term infected individuals or patients (35).

As shown in Fig. 1 *B*, T cells from nine HIV-seronegative controls with acute or chronic infectious diseases proliferated less well to PHA than T cells from HIV-infected individuals, but better to PWM and to SEB.

Cell Death in PBMC and Purified T Cells from HIV-infected Asymptomatic Individuals and from Controls. Cell viability was investigated in 59 of the 109 HIV-infected asymptomatic individuals explored in the study. Addition of PWM or SEB to PBMC from the 59 HIV-infected asymptomatic individuals was followed by cell death after 48 h of about 40% and 20%, respectively, of PBMC, whereas no cell death was observed at 48 h in the unstimulated PBMC from HIV-infected asymptomatic individuals. No cell death was detected in the unstimulated and stimulated PBMC from the 49 HIV-seronegative healthy controls (Fig. 2 *A*) and from the nine HIV-seronegative controls with acute or chronic infectious diseases (Fig. 2 *B*).

To identify the cell population undergoing cell death after activation, PBMC from 12 HIV-infected asymptomatic indi-

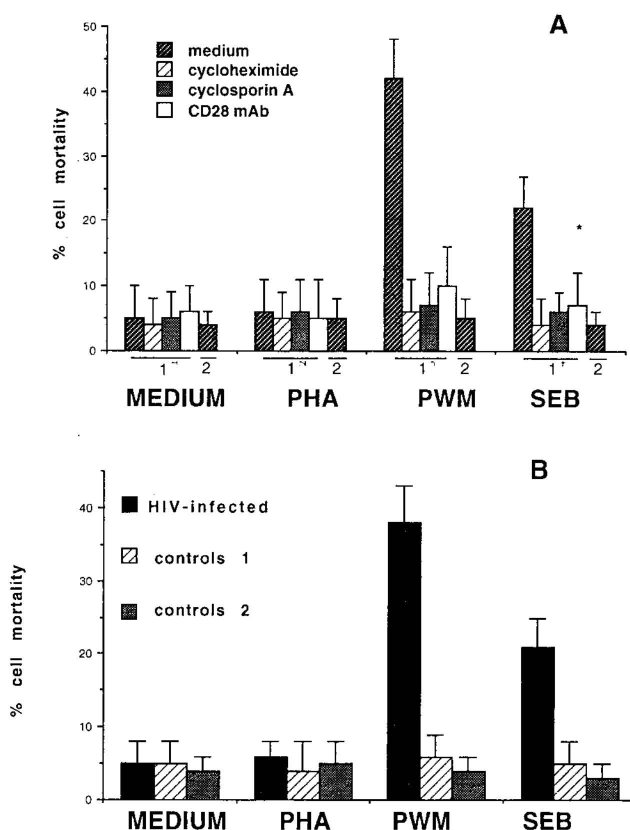

Figure 2. Cell death in PBMC from HIV-infected asymptomatic individuals and from controls after in vitro culture with PWM and SEB. (*A*) Histograms represent the mean ± SD of triplicate measurements of the percentage of cell death in PBMC from 50 different HIV-infected asymptomatic individuals (*1*) and 40 controls (*2*), 48 h after incubation with medium alone, PHA (10 μg/ml), PWM (10 μg/ml), or SEB (1 μg/ml), in the absence or in the presence of cycloheximide (50 μg/ml), CsA (200 ng/ml), or CD28 mAb (10 μg/ml). Cell death was assessed by trypan blue permeability. (*B*) Histograms represent the mean ± SD of triplicate measurements of the percentage of cell mortality in PBMC from nine different HIV-infected asymptomatic individuals (HIV-infected), nine HIV-seronegative controls (controls *1*), and nine HIV-seronegative patients suffering from acute or chronic infectious diseases (controls *2*), 48 h after incubation with medium alone, PHA, PWM, or SEB as described in *A*.

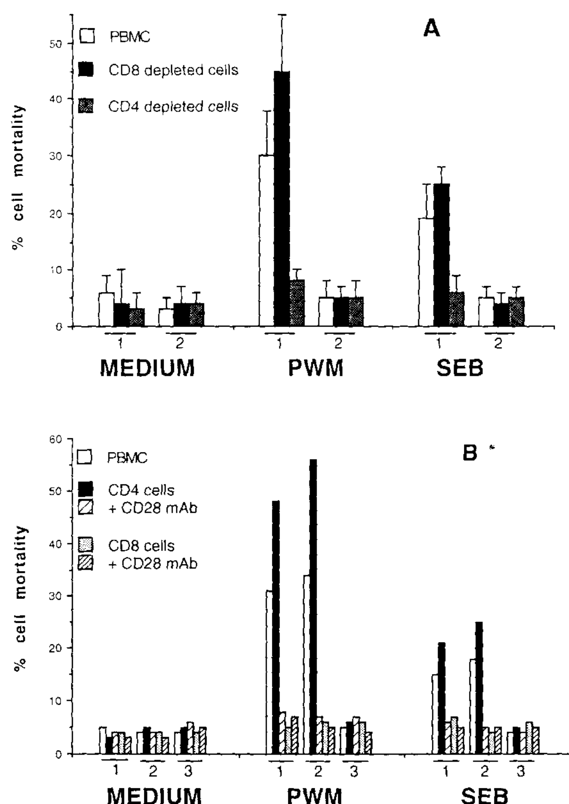

Figure 3. Cell death in purified T cell populations from HIV-infected asymptomatic individuals and from controls after in vitro culture with PWM and SEB. (*A*) Histograms represent the mean ± SD of triplicate measurement of cell mortality in $CD8^+$ T cell- or $CD4^+$ T cell-depleted PMBC from 12 HIV-infected asymptomatic individuals (*1*) and 4 controls (*2*). T cell proliferation was also measured by [^{3}H]thymidine (25 Ci/mmol) incorporation after 3 d; the $CD8^+$ T cell-depleted PBMC from controls proliferated to PWM (16,828 ± 4,247 cpm) and to SEB (31,327 ± 8,624 cpm), whereas neither the $CD4^+$ T cell-depleted PBMC from the control, nor the $CD8^+$ or $CD4^+$ T cell-depleted PBMC from the HIV-infected asymptomatic individuals proliferated to either stimuli. (*B*) Histograms represent the mean ± SD of triplicate measurement of cell mortality in purified (98% pure) $CD4^+$ and $CD8^+$ T cells from two HIV-infected asymptomatic individuals (*1* and *2*) and a control (*3*). Percentage of $CD4^+$ T cells in unpurified PBMC was 50% in individual *1*, and 52% in individual *2*. T cell proliferation was also measured by [^{3}H]thymidine (1 Ci/mmol) incorporation after 3 d; purified $CD4^+$ and $CD8^+$ T cells from HIV-infected individuals and from the control proliferated normally to PHA. $CD4^+$ T cells from control proliferated in response to PWM (4,817 ± 351 cpm) and to SEB (21,892 ± 1,628 cpm), whereas $CD8^+$ T cells from the control and $CD4^+$ and $CD8^+$ T cells from the HIV-infected individuals did not proliferate to either stimuli.

viduals and four healthy controls were depleted of either $CD4^+$ T cells or of $CD8^+$ T cells. After addition of PWM or SEB, cell death was observed only in the $CD8^+$ T cell–depleted PBMC population (containing the $CD4^+$ T cell population) from HIV-infected asymptomatic individuals (Fig. 3 *A*), suggesting that neither $CD8^+$ T cells, B cells, natural killer cells, nor monocytes underwent activation-induced cell death in the absence of $CD4^+$ T cells. 98% pure $CD4^+$ T cells and $CD8^+$ T cells were also prepared from PBMC from two HIV-infected asymptomatic individuals and a control. As shown in Fig. 3 *B*, activation-induced cell death in response to PWM or SEB was only observed in the $CD4^+$ T lymphocyte population from the two HIV-infected asymptomatic individuals. The percentages of cell death induced by PWM stimulation (% PWM-induced death – % background death in medium) were 45% and 52%, respectively, in the purified $CD4^+$ T cell populations of the two HIV-

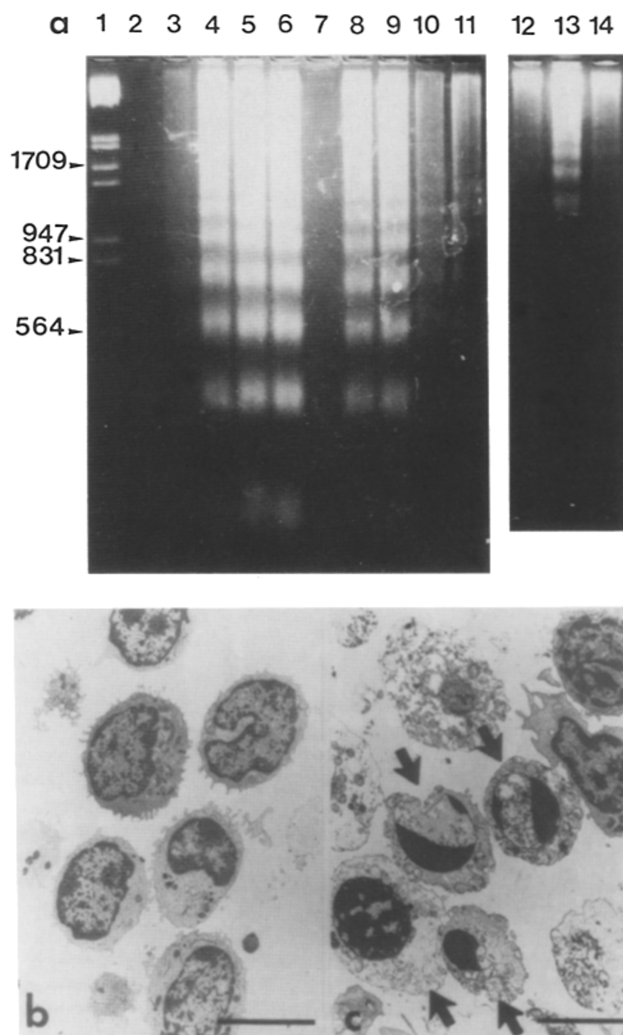

Figure 4. Apoptosis of T cells from HIV-infected asymptomatic individuals in response to PWM or SEB. (*a*) DNA fragmentation in PBMC from HIV-infected asymptomatic individuals and controls after overnight incubation with medium alone, PWM (5 μg/ml), or SEB (1 μg/ml) in the absence or presence of CsA (200 ng/ml) or CD28 mAb. DNA weight markers (lane *1*); control PBMC with PWM (lane *2*); PBMC from eight HIV-infected asymptomatic individuals with: medium (lanes *3* and *12*), PWM (lanes *4–9* and *13*), or SEB (lanes *10* and *11*). Lanes *3* and *4* and *12–14* show cells from the same individuals. Lanes *4–6*, *8–11*, and *13* show a clear ladder of degraded DNA bands which are multiple of 200 bp, characteristic for apoptosis (*10–13* and *17–19*). DNA fragmentation was suppressed when CsA (lane 7, cells from same individual as lane *6*) or CD28 mAb (lane *14*, cells from same individual as lane *13*) was added to PWM. (*b* and *c*) Electron micrographs of PBMC from a HIV-infected asymptomatic individual after 24 h of incubation with (*b*) medium or (*c*) PWM (5 μg/ml). Cells showing various stages of chromatin condensation, characteristic for apoptosis (10, 17, 19, 20), can be seen in (*c*) (*arrows*). Bars = 5 μm.

Table 1. In vitro *Effect of AZT on T Cell Proliferation and Death of T Cells from HIV-infected Asymptomatic Individuals and Controls*

	Proliferation			Mortality		
	Medium	PWM	PHA	Medium	PWM	PHA
		cpm			%	
Patient A	180	3,604	44,567	7	34	7
+ AZT	152	2,846	46,567	10	32	6
Patient B	395	2,138	51,299	6	28	5
+ AZT	432	3,027	47,321	7	31	6
Patient C	204	1,873	17,556	6	23	5
+AZT	396	1,846	19,122	5	29	7
Patient D	818	2,491	25,284	4	38	5
+ AZT	396	3,936	33,336	5	36	4
Control A	321	13,227	17,754	6	5	6
+ AZT	244	12,521	14,081	4	6	5
Control B	467	18,532	37,317	5	4	7
+ AZT	383	21,123	34,821	6	5	7

Results represent mean ± SD of triplicate measurements of [^{3}H]thymidine incorporation of PBMC and the percentage of cell mortality at 48 h from four HIV-infected asymptomatic individuals and from two controls in response to medium alone, PHA, and PWM in the presence or absence of 10^{-5} M of AZT. At 10^{-5} M, AZT inhibited syncitia formation in the MT2 CD4$^+$ T cell line infected with 100 × TCID 50 of a purified preparation of the HTLV-IIIB strain of HIV-1.

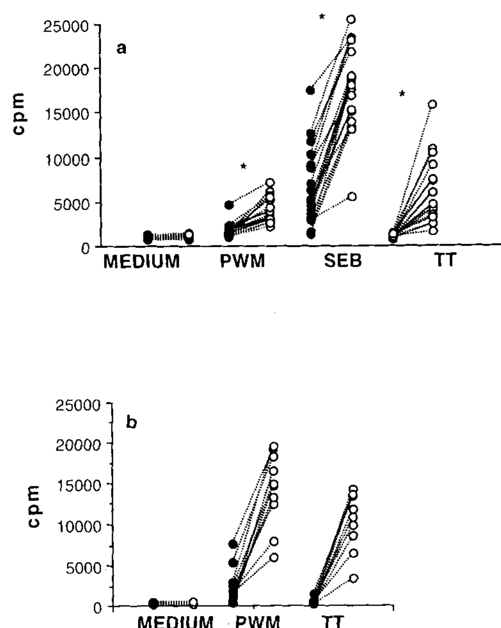

Figure 5. Effect of CD28 mAb on the proliferative response of T cells from HIV-infected asymptomatic individuals. Circles represent the mean ± SD of triplicate measurement of [^{3}H]thymidine incorporation of T cells from (*a*) each of 19 HIV-infected asymptomatic individuals incubated with PWM, SEB, TT, or medium alone, in the absence (●) or presence (○) of CD28 mAb (specific activity of [^{3}H]thymidine used, 1 Ci/mmol), and from (*b*) each of 15 HIV-infected asymptomatic individuals incubated with PWM, SEB, TT, or medium alone, in the absence (●) or presence (○) of CD28 mAb (specific activity of [^{3}H]thymidine used, 25 Ci/mmol). Certain circles representing similar values are superimposed. $^*p < 0.00004$.

infected individuals. Since activation-induced cell death was only observed in the CD4$^+$ T cell population, and since the percentages of CD4$^+$ T cells present in unpurified PBMC were, respectively, 50 and 52%, one would expect around 22 and 27% of cell death in PWM-stimulated unpurified PBMC. These percentages are close to the 27 and 30% actually observed. In superantigen-stimulated unpurified PBMC, expected percentages of cell death were 9 and 10%, and observed percentages were 10 and 14%. Thus, slightly more cells died in the stimulated unpurified PBMC than in the purified stimulated CD4$^+$ T cell populations. This suggests either that stimulation of the whole PBMC population leads to more CD4$^+$ T cell death, or alternatively that, when CD4$^+$ T cells are present, a fraction of cells other than CD4$^+$ T lymphocytes also undergo activation-induced death.

Mechanism and Prevention of T Cell Death. Death of T cells from HIV-infected asymptomatic individuals involved two features characteristic of apoptosis (18–21, 25–28, 36). First, gel electrophoresis of the DNA of PBMC from HIV-infected asymptomatic individuals, performed 18 h after addition of PWM or SEB, showed a DNA fragmentation pattern in multiples of a 200-bp oligonucleosome length unit (Fig. 4 *a*). Second, electron microscopy of PBMC from HIV-infected asymptomatic individuals 18 h after the addition of PWM revealed nuclear chromatin condensation (Fig. 4 *c*).

Activation-induced T cell death appeared unrelated to HIV-mediated cytopathogenic effects. Indeed, first, electron microscopy of PWM- or SEB-activated PBMC did not reveal the presence of HIV viral particles. Second, as shown in Table 1, activation-induced cell death in PBMC from HIV-infected asymptomatic individuals was not modified in the presence of the reverse transcriptase inhibitor azidothymidine (AZT), a concentration that prevented syncitia formation in the MT2 CD4$^+$ T cell line when added together with 100 × TCID 50 of the HTLV-IIIB stain of HIV-1 (Table 1).

An essential characteristic of apoptosis in various cell populations is its dependence on cell activation, gene transcription, and protein synthesis in the dying cell (25–27; reviewed in reference 18). Addition of the protein synthesis inhibitor cycloheximide, or of cyclosporin A (CsA), both of which prevent activation-induced apoptosis in immature thymocytes (20), prevented activation-induced death by apoptosis of T cells from HIV-infected asymptomatic individuals in response to PWM and SEB (Fig. 2 *A*, and Fig. 4 *a*, lane 7).

Restoration of the Capacity of CD4$^+$ T Cells To Proliferate to Stimuli. Protein synthesis inhibitors and CsA, which prevented apoptosis, also prevented T cell proliferation in response to stimuli. It has been shown in immature thymocytes that the addition of certain cosignals such as IL-1, IL-2, and phorbol esters not only prevent apoptosis, but also allow a proliferative response to stimuli (22, 24, 29, 30). Addition

to T cells from HIV-infected asymptomatic individuals of IL-1, IL-2, or phorbol esters neither prevented apoptosis nor allowed proliferation in response to PWM or SEB (not shown). The CD28 mAb cosignal, that enhances the stability and transcription of several lymphokine mRNAs (37, 38) in normal mature activated $CD4^+$ T cells, has been reported to allow proliferation of normal immature thymocytes to stimuli (39), and to enhance in T cells from HIV-infected individuals the proliferative response to the CD3 antibody (15). As shown in Figs. 2 *A*, 3 *B*, and 4*a* (lane *14*), addition of CD28 mAb to PBMC or purified $CD4^+$ T cells from HIV-infected asymptomatic individuals prevented PWM- and SEB-induced death by apoptosis and, as shown in Fig. 5, also restored T cell proliferation in response to PWM and SEB. CD28 mAb alone did not induce proliferation of T cells from HIV-infected asymptomatic individuals (Fig. 5), and CD28 mAb did not enhance control T cell proliferation to PWM, SEB, or TT. CD28 mAb restored proliferation to PWM and SEB of purified $CD4^+$ T cells, but not of purified $CD8^+$ T cells from an HIV-infected asymptomatic individual (not shown). Two different CD28 mAb (CLB28/1, IgG1, and 9.3, IgG2a isotype) were used and had the same effect, while control mAbs of the same isotype (CD5, IgG1, and CD44, IgG2a) had no effect.

As shown in Fig. 5, CD28 mAb not only restored proliferation of T cells from HIV-infected individuals to PWM and SEB, but also to the tetanus recall antigen, indicating that tetanus-specific memory T cells were present in the HIV-infected individuals, and suggesting that in vitro antigen-mediated induction of apoptosis might account for their in vitro proliferative defect to the recall antigen.

Antigen-mediated Functional Deletion of Antigen-specific T Cells. Whether antigen may induce selective in vitro deletion of specific memory T cells was further investigated. PBMC from four HIV-infected asymptomatic individuals and from two controls were first incubated for 10 d with the tetanus antigen in the absence of CD28 mAb. At day 10, cell death was 12–15% in PBMC from HIV-infected asymptomatic individuals and from controls. As shown in Table 2, cells were then layered on Ficoll/Hypaque, washed twice, and incubated for 3 d with PHA or for 6 d with the tetanus or the influenza recall antigens, in the absence or presence of the CD28 mAb. Cells from HIV-infected asymptomatic individuals that had been treated first with tetanus recall antigen retained their capacity to proliferate to the influenza recall antigen in the presence of CD28 mAb, but selectively lost their subsequent capacity to proliferate to tetanus in the presence of CD28 mAb, suggesting that tetanus-specific memory T cells had been selectivity deleted during the first incubation with the tetanus antigen. As also shown in Table 2, preincubation of PBMC from controls with the tetanus antigen did not lead to any subsequent functional impairment.

Table 2. *Antigen-mediated Functional Deletion of Antigen-specific Memory T Cells from HIV-infected Asymptomatic Individuals*

		Before stimulation with:						
	Preincubation	Medium	CD28	TT	TT+CD28	Infl.	Infl. + CD28	PHA
					cpm			
HIV^+1	No preincubation	121 ± 52	314 ± 87	236 ± 78	2,617 ± 321	341 ± 58	3,217 ± 206	18,621 ± 817
	with TT	614 ± 87	258 ± 94	318 ± 97	415 ± 87	721 ± 75	6,731 ± 345	17,182 ± 751
HIV^+2	No preincubation	447 ± 34	313 ± 57	317 ± 27	5,736 ± 187	151 ± 57	3,842 ± 137	17,980 ± 243
	with TT	221 ± 67	318 ± 61	176 ± 50	118 ± 21	345 ± 46	5,351 ± 142	16,441 ± 419
HIV^+3	No preincubation	324 ± 38	249 ± 64	437 ± 31	3,781 ± 108	438 ± 51	6,242 ± 107	28,143 ± 243
	with TT	479 ± 61	408 ± 37	459 ± 61	438 ± 71	386 ± 48	2,674 ± 157	24,597 ± 267
HIV^+4	No preincubation	350 ± 217	405 ± 104	157 ± 117	1,549 ± 817	108 ± 64	1,560 ± 258	28,232 ± 6,712
	with TT	103 ± 37	207 ± 83	483 ± 121	342 ± 106	435 ± 265	2,170 ± 598	23,315 ± 4,612
Control 1	No preincubation	680 ± 217	354 ± 70	3,930 ± 881	3,290 ± 721	2,314 ± 567	3,254 ± 432	31,217 ± 2,642
	with TT	569 ± 258	208 ± 62	7,091 ± 932	4,382 ± 792	2,090 ± 589	1,850 ± 678	27,218 ± 4,812
Control 2	No preincubation	477 ± 81	387 ± 73	4,830 ± 523	4,087 ± 238	5,721 ± 267	4,239 ± 540	20,587 ± 267
	with TT	297 ± 54	579 ± 69	5,761 ± 287	5,983 ± 345	3,549 ± 138	4,820 ± 534	31,589 ± 354

Results represent mean ± SD of triplicate measurements of [^{3}H]thymidine incorporation of PBMC from four HIV-infected asymptomatic individuals and from two controls. Results showing functional deletion are underlined. Preincubation with TT: PBMC (2.5 × 10^6/ml were cultured for 10 d with the tetanus recall antigen (TT) (10 μg/ml). Cells were then incubated with different stimuli, and proliferation measured by [^{3}H]thymidine incorporation after 3 d for PHA or PWM, and 6 d for TT or influenza A (Infl.) recall antigens (10 μg/ml), in the presence or absence of CD28 mAb (10 μg/ml).

Discussion

Our results support the hypothesis (17) that the selective in vitro proliferative defect of CD4$^+$ T cells from HIV-infected asymptomatic individuals to TCR mobilization by self-MHC class II–dependent superantigens, and to PWM, is related to the induction by these stimuli of an active CD4$^+$ T cell death process by apoptosis. Cell death was not observed in the absence of stimuli nor in response to stimuli that induced proliferation (PHA and the CD3 mAb). Cell death did not occur in mononuclear cells depleted in CD4$^+$ T lymphocytes, and was observed in purified CD4$^+$ T cell populations, suggesting that the presence of CD4$^+$ T cells was both necessary and sufficient for the induction of this cell death process. No cell death was detected in stimulated or unstimulated T cells from 49 healthy HIV-seronegative controls, nor from nine HIV-seronegative patients with various acute or chronic infectious diseases. Although CD4$^+$ T cells from HIV-infected individuals failed to proliferate to TCR mobilization by the self-MHC class II–dependent tetanus and influenza recall antigens, activation-induced CD4$^+$ T cell death in response to these antigens could not be detected. This is consistent with the fact that memory T cells specific for a given recall antigen are rare and that activation-induced cell death spares bystander cells (18), but could also be related, as previously suggested, to an early in vivo depletion of antigen-specific memory T cells (15, 16). We observed however that addition of a CD28 mAb cosignal that prevented apoptosis and restored T cell proliferation in response to PWM and to superantigens, also restored T cell proliferation to the tetanus and influenza recall antigens, indicating thus that the specific memory T cells were present in HIV-infected asymptomatic individuals. Preincubation of T cells with the tetanus antigen in the absence of CD28 mAb led to a subsequent selective loss of their capacity to proliferate to this antigen in the presence of the antibody, while the T cell proliferative response to the influenza antigen in the presence of CD28 was not impaired. This suggested that antigen-specific activation-induced CD4$^+$ T cell death was the mechanism most likely to account for the failure of the memory T cells to proliferate to these recall antigens.

After our work was completed and submitted for publication, two independent reports (40, 41) also suggested the existence of a relationship between HIV-1 infection and apoptosis. One study (40) showed that in vitro activation of PBMC from HIV-infected individuals with calcium ionophore leads in 24 h to death by apoptosis of a significant proportion of cells that were not characterized. That study also reported spontaneous death by apoptosis of PBMC from HIV-infected individuals after a 3-d incubation in the absence of any stimuli. Our assays, which were all performed after a 2-d incubation, showed the absence of spontaneous cell death in unstimulated PBMC from all 59 HIV-infected asymptomatic individuals tested. Additional vital dye exclusion assays that we performed in PBMC from 18 HIV-infected asymptomatic individuals did not reveal cell death after 3 d of incubation in the absence of stimuli. We do not know whether this discrepancy may be related to differences in cell culture conditions, or to the biological or clinical status of HIV-infected individuals tested.

The other report, which does not concern T cells from HIV-infected individuals, shows that the in vitro cytopathogenic effect of some HIV-1 strains, which follows in vitro HIV-1 infection of T cell lines or mononuclear cells, is related to the induction of apoptosis (41). In HIV-infected asymptomatic individuals, less than 0.1% of peripheral blood CD4$^+$ T cells are infected (8–10). Therefore, since we observed that in vitro activation with PWM resulted in the deaths of ~40% of the CD4$^+$ T cells, the possibility that apoptosis occurred only in HIV-infected CD4$^+$ T cells appeared most unlikely. This was confirmed by electron microscopy studies revealing the absence of viral particle production, and by in vitro experiments performed in the presence of the reverse transcriptase inhibitor AZT, showing that AZT did not affect activation-induced T cell death.

The recent observations of apoptosis in mature murine CD4$^+$ T cells (31, 32) suggest at least two indirect mechanisms that may account for the reemergence in the mature CD4$^+$ T cell population of an activation-induced death program. First, CD4$^+$ T cells from HIV-infected asymptomatic individuals may be primed in vivo for apoptosis upon further activation. Pretreatment of mature murine CD4$^+$ T cells with CD4 antibody has been shown to prime them for apoptosis upon selective mobilization of their TCR-α/β, but not of their CD3 complex (31), a response resembling that of CD4$^+$ T cells from HIV-infected individuals. Obvious candidates for such an in vivo priming include the binding to CD4 of secreted HIV-gp120 envelope protein released in serum or lymph (42), gp120–anti-gp120 antibody immune complexes, or anti-CD4 autoantibodies. However, preliminary results obtained in our laboratory suggest that the sole preincubation of normal mature human CD4$^+$ T cells with CD4 antibody or gp120, whether crosslinked or not, does not lead to apoptosis upon further stimulation.

A second possibility is that CD4$^+$ T cells from HIV-infected asymptomatic individuals have no intrinsic abnormalities, but that defective antigen-presenting cell function accounts for induction of T cell apoptosis. Whether antigen-presenting cells from HIV-infected individuals are unable to provide relevant cosignals required for a proliferative response to PWM, or to MHC class II-dependent TCR mobilization, is currently under investigation. It has been shown that restimulation of a mature murine CD4$^+$ T cell clone by crosslinked CD3 antibodies in the absence of antigen-presenting cells results in an active cell death process that involves INF-γ, and is prevented by anti-INF-γ antibody (32). Our preliminary data indicate that anti-INF-γ antibody does not prevent activation-induced death of CD4$^+$ T cells from HIV-infected individuals.

A third possible interpretation of our findings, which cannot be completely excluded, is that apoptosis is the consequence of a CD4$^+$ T cell/CD4$^+$ T cell–killing process. Induction of apoptosis in their target cells is one of the means by which CTL kill their targets (18, 43). Although cytotoxic proper-

ties have been mainly ascribed to a subpopulation of CD8$^+$ T cells, CD4$^+$ CTL clones have been described (43). Since CsA, which does not prevent CD8$^+$ CTL- or CD4$^+$ CTL-mediated apoptosis of target cells (43), prevented apoptosis of CD4$^+$ T cells from HIV-infected asymptomatic individuals, we think that an activation-induced CD4$^+$ T cell suicide process, in the absence of any participation of CD4$^+$ killer T cells, represents the simplest explanation for our observations at this stage.

Our findings suggest the possibility that activation-induced T cell death might also occur in vivo and account for the progressive depletion of CD4$^+$ T cells that leads to AIDS (17). This implies that the rate of in vivo CD4$^+$ T cell depletion might directly depend on the percentage of activated T cells and not on the percentage of HIV-infected T cells. In fact, since CD4$^+$ T cell activation is required for HIV-provirus integration (44), and since activation will result in a rapid cell deletion process by apoptosis, apoptosis could also account for the very low percentage of HIV-infected T cells in HIV-infected individuals.

We have observed that the CD28 mAb prevents apoptosis and allows in vitro proliferation of memory T cells to recall antigens. Therefore, in vitro proliferation assays of CD4$^+$ T cells in response to various recall antigens in the presence of CD28 mAb should allow to assess the extent of the memory CD4$^+$ T cell repertoire that is remaining in vivo at any given time. This should allow to test whether CD4$^+$ T cells specific for pathogens continuously present in HIV-infected individuals, such as HIV itself, herpes virus, or cytomegalovirus, disappear earlier in vivo than CD4$^+$ memory T cells specific for pathogens that have been rarely (influenza) or never (tetanus) encountered subsequent to HIV infection. However, additional mechanisms may lead to a progressive enhancement of antigen-mediated T cell loss in vivo. First, once T cell clones specific for a given pathogen are depleted, the persistence of the pathogen may progressively lead to the recruitment and deletion of low-affinity crossreactive T cell clones, that are specific for other antigens. Second, superinfection by pathogens that express superantigens, such as staphylococcus, streptococcus, or mycoplasma (45), could induce the deletion of a large number of CD4$^+$ T cells expressing the matching Vβ molecules, independent of their antigen specificity. Since murine retroviruses have been recently shown to encode superantigens (46), one cannot exclude the possibility that HIV itself might lead to the expression of molecules with similar properties during the course of the disease.

Our observation that CsA and the CD28 mAb prevent activation-induced death of CD4$^+$ T cells from HIV-infected asymptomatic individuals, and that CD28 mAb also restores their proliferative response to stimuli, might have implications for the design of specific therapeutic strategies aimed at the prevention of AIDS. Animal models of AIDS-related diseases should allow testing the potential beneficial effect of early in vivo correction of CD4$^+$ T cell apoptosis on the further evolution of CD4$^+$ T cell counts, and on the course of the disease.

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