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Immunology; or Cell Biology; or Medical Sciences

**Activation-induced death by apoptosis in CD4 T cells
from HIV-infected asymptomatic individuals**

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Abbreviations: PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor; PHA, phytohemagglutinin A; PWM, pokeweed mitogen; SEB, staphylococcal enterotoxin superantigens; TT, tetanus toxoid recall antigen; Infl, Influenza A hemagglutinin recall antigen; IL, interleukin; mAb, monoclonal antibody.

ABSTRACT In immature thymocytes, T-cell receptor (TCR) mobilization leads to an active T-cell suicide process, apoptosis, 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. Here, we report that the selective failure of CD4⁺ T cells from 38 clinically asymptomatic HIV-infected individuals to proliferate *in vitro* to major histocompatibility complex class II (MHC-II)-dependent TCR mobilization and to pokeweed mitogen is due to an active CD4⁺ T-cell death process, with biochemical and ultrastructural features of apoptosis, including DNA fragmentation in multiples of 200 base pairs, and chromatin condensation. Activation-induced cell death was not detected in T cells from any of 20 controls, and occurred in purified CD4⁺ T cells from HIV-infected asymptomatic individuals. Activation-induced CD4⁺ T-cell death was prevented by cycloheximide, cyclosporin A, and a CD28 monoclonal antibody. CD28 monoclonal antibody not only prevented apoptosis but also restored T-cell proliferation to stimuli, including pokeweed mitogen, superantigens, and the tetanus and influenza recall antigens. These findings provide new insights into the pathogenesis of AIDS and may represent a basis 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 that leads to AIDS. These functional defects are detected while less than 1/1,000 TH cells are infected (8-10), and are characterized by a selective loss of ability to proliferate *in vitro* to self MHC-II-restricted recall antigens and to pokeweed mitogen (PWM) (1-7). 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). We have proposed the hypothesis (17) that a single mechanism, the inappropriate re-emergence 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, or 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, the deletion of autoreactive T-cell clones, and 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 base pairs (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 absence of certain co-signals (22,23,29,30). A major question in T-cell biology is thus whether TCR mobilization may also lead in certain circumstances to the re-emergence of a functional cell death program in mature T cells.

We have investigated whether *in vitro* activation of T cells from clinically asymptomatic HIV1-infected individuals - including individuals with normal CD4⁺ T-cell counts - and from controls with polyclonal activators and self-MHC-II-dependent recall antigens may lead to T-cell death. Since memory T cells specific for a given recall antigens are rare, and might be depleted in HIV-infected individuals, we also investigated the response to the self-MHC-II-dependent staphylococcal enterotoxin B superantigens (SEB) (31). These superantigens bind to MHC-II molecules and interact with defined V β TCR molecules expressed by up to 30% of normal human T cells, inducing proliferation in normal mature CD4⁺ T cells (31) and apoptosis in immature thymocytes (21).

MATERIALS AND METHODS

Study subjects. Peripheral blood was obtained from 38 HIV-infected individuals in the Service des Maladies Infectieuses, Centre Hospitalier de Tourcoing, France. They were 26 males and 12 females, all clinically asymptomatic (stage II of the Center for Disease Control (CDC) classification); 25 were CDC stage IIA (no biological abnormalities, $CD4 > 500/mm^3$, mean 856); 13 were CDC stage IIB (biological abnormalities, $CD4 < 500/mm^3$, mean 345). HIV infection was related to homosexuality (n=15), heterosexual contact (n=14), intravenous drug use (n=7), or blood transfusion (n=2). Controls were 20 HIV-seronegative donors from the medical staff.

Cell preparations. Peripheral blood mononuclear cells (PBMC) were obtained on Ficoll-Hypaque, and cells were cultured as previously described (32).

In some experiments, $CD4^+$ or $CD8^+$ T cells were purified by negative selection with magnetic beads coated by anti mouse IgG (Dynal, Biosys, France). Cells ($50 \cdot 10^6$) were plated to plastic Petri dishes in order to harvest adherent cells by scraping. Non adherent cells were incubated with $5\mu g/ml$ of CD20, CD56, MHC-II, CD4 or CD8 monoclonal antibodies (mAb) in a volume of 5ml in RPMI for 30mn. Subsequently, excess antibody was removed by washing twice in RPMI. The cells were then resuspended in 5ml RPMI with magnetic beads (according to the manufacturer's instructions).

This mixture was rotated in the cold for 30mn 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.

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) in a final volume of 200 μ l as previously described (33). PBMC (2.5×10^5 /ml) were cultured in RPMI/10% fetal calf serum. Mitogens (purchased from Sigma, France) were used respectively at the following final concentrations: phytohemagglutinin (PHA), 10 μ g/ml; concanavalin A (ConA), 10 μ g/ml; pokeweed mitogen (PWM), 10 μ g/ml; staphylococcal enterotoxin B superantigens (SEB), 1 μ g/ml; the CD3 mAb was used at 1 μ g/ml. Tetanus toxoid (TT) recall antigen (Biomerieux, France) was used at 10 μ g/ml; and Influenza A hemagglutinin (Infl) recall antigen (Eurobio, France) at 10 μ g/ml. After 3 days for mitogens or 6 days for antigens, cultures were pulsed with 1 μ Ci of ³[H]-thymidine (5 Ci/mmol, Amersham, France) during the final 15h 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. (33) with slight modifications. In brief, 10^7 cells were collected by centrifugation at 200g for 10mn, and lysed in 1ml hypotonic lysing buffer (5mM Tris Ph 7.4, 5 mM EDTA, 0.5% Triton X 100). The lysates were centrifuged at 13,000g for 15mn. 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.

Electronmicroscopy. Cells were fixed in 1% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4 for 2h at $+4^{\circ}\text{C}$. Pellets were post fixed in 1% aqueous osmium tetroxide for 1h, en-block stained in 1% aqueous uranyl acetate for 6h and embedded in araldite. Sections were stained with uranyl acetate and lead citrate before examination with a Philips EM 420 electron microscope.

Monoclonal antibodies and chemicals. The monoclonal antibodies (mAb) used in this study were CD3 (X35-7, IgG2a), generous gift from Dr. Bourel (France); CD28 (9.3, IgG2a), generous gift from Oncogen corp.; CD28 (CLB 28/1, IgG1), purchased from Jansen; CD20 (IOB20, IgM), CD56 (IOT56, IgG1), HLA-DR (IOT2a, IgG2b), CD4 (IOT4, IgG2a), CD8 (IOT8, IgG2a), were all purchased from Immunotech, France; CD5 (A50, IgG1), CD44 (P245, IgG2a), generous gifts from Dr. A. Bernard, France.

Cyclosporin A was purchased from Sandoz, France; and Cycloheximide from Sigma.

Statistical analysis. Statistical significance was assessed by using a Student 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 38 HIV-infected asymptomatic individuals and 20 healthy controls in response to the polyclonal activators PHA, ConA, PWM, CD3 mAb, to the tetanus recall antigen, and to the SEB superantigens. T cells from all controls proliferated to all stimuli, while T cells from HIV-infected asymptomatic individuals showed a selective defect in their response to PWM, to SEB and to recall antigen (Fig. 1). Proliferation of T cells from HIV-infected individuals to ConA and to CD3 mAb was only slightly reduced (not shown), as was proliferation to PHA (Fig. 1). This was consistent with the fact that all HIV-infected individuals studied were clinically asymptomatic, with few or no biological abnormalities, since defective proliferation to CD3 mAb and to PHA have been reported to be predictive markers of evolution towards AIDS in long term infected individuals or patients (34,35).

Cell death in PBMC and purified T cells from HIV-infected

asymptomatic individuals and from controls. Addition of PWM or SEB to PBMC from 20 different HIV-infected asymptomatic individuals was followed by cell death after 48h of about 40% and 20% respectively, of PBMC, whereas no cell death was observed at 48h in the unstimulated PBMC from HIV-infected asymptomatic individuals, nor in the unstimulated and stimulated PBMC from 20 different controls (95% viability) (Fig. 2).

In order to identify the cell population undergoing cell death after activation, PBMC from a HIV-infected individual and a control were depleted either of CD4⁺ T cells or of CD8⁺ T cells. After addition of PWM or SEB, cell death was only observed in the CD8⁺ T-cell depleted PBMC population from HIV-infected asymptomatic individuals (Fig. 3a), suggesting that neither CD8⁺ T cells, B cells, natural killer cells nor monocytes underwent activation-induced cell death. Purified CD4⁺ T cells and CD8⁺ T cells were also prepared from PBMC from a HIV-infected asymptomatic individual and a control. Activation-induced cell death was only observed in response to PWM or SEB in the CD4⁺ T lymphocyte population from HIV-infected asymptomatic individuals (Fig. 3b).

Mechanism and prevention of T-cell death. Death of T cells from HIV-infected asymptomatic individuals involved 2 features characteristic of apoptosis (18-21,25-28,36). First, gel electrophoresis of the DNA of PBMC from HIV-infected asymptomatic individuals, performed 18h after addition of PWM or SEB, showed a DNA fragmentation pattern in multiples of a 200 base pair oligonucleosome length unit (Fig. 4a). Second, electron

microscopy of PBMC from HIV-infected asymptomatic individuals 18h after the addition of PWM revealed nuclear chromatin condensation (Fig. 4c).

An essential characteristic of programmed cell death in various cell populations is its dependence on cell activation, gene transcription, and protein synthesis in the dying cell (25-27; reviewed in 18). Addition of the protein synthesis inhibitor cycloheximide, or of Cyclosporin A, 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, 4a).

Restoration of the capacity of CD4⁺ T cells to proliferate to stimuli. Protein synthesis inhibitors and Cyclosporin A, 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 Interleukin (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 in normal mature activated CD4⁺ T cells the stability and transcription of several lymphokines mRNA (37,38), 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 Fig. 5, addition of CD28 mAb to PBMC or purified CD4⁺ T cells from HIV-infected asymptomatic individuals prevented PWM- and SEB-induced death by apoptosis (Fig. 2, 3b, and 4a) and restored T-cell proliferation in response to PWM and SEB (Fig. 5). 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). 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. 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, IgG2) had no effect. CD28 mAb not only restored proliferation of T cells from HIV-infected individuals to PWM and SEB, but also to the tetanus recall antigen (Fig. 5), indicating that tetanus-specific memory T cells were present in the HIV-infected individuals, and suggesting that induction of apoptosis might account for their *in vitro* unresponsiveness 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 4 HIV-infected asymptomatic individuals and from 2 controls were first incubated for 10 days with the tetanus antigen in the absence of CD28 mAb. At day 10, cell mortality was 12 to 15% in PBMC from HIV-infected individuals and from

controls. Cells were then layered on Ficoll Hypaque, washed twice and incubated for 3 days with PHA or for 6 days with the tetanus or the influenza recall antigens, in the absence or in the presence of the CD28 mAb. Cells from HIV-infected asymptomatic individuals that had been first treated 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 selectively deleted during the first incubation with the tetanus antigen (Table 1). As also shown in Table 1, preincubation of PBMC from controls with the tetanus antigen did not lead to any subsequent functional impairment.

DISCUSSION

Our results show that the selective *in vitro* proliferative defect of CD4⁺ T cells from HIV-infected individuals to PWM and to self-MHC-II-dependent TCR mobilization by superantigens is related to the induction by these stimuli of an active CD4⁺ T-cell death process by apoptosis. Activation-induced cell death was not observed in mononuclear cells depleted in CD4⁺ T lymphocytes, and occurred 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. Although CD4⁺ T cells failed to proliferate to TCR mobilization by the self-MHC-II-dependent tetanus and influenza recall antigens, activation-induced CD4⁺ T-cell death in response to these antigens could not be detected. This may be related to the fact that memory T cells specific for a given recall antigen are rare and that activation-induced cell death spares bystander cells (18), or alternately, as previously suggested, that the antigen-specific memory T cells have been already depleted *in vivo* (15,16). We observed that addition of a CD28 mAb co-signal 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.

In HIV-infected asymptomatic individuals, less than 0.1% of peripheral blood CD4⁺ T cells are infected (8-10). Since *in vitro* activation with PWM resulted in death of around 40% of the CD4⁺ T cells, the possibility that apoptosis occurred only in HIV-infected CD4⁺ T cells could be excluded. Recent observations of apoptosis in mature murine CD4⁺ T cells (33,40) suggest at least two indirect mechanisms that may account for the re-emergence in the CD4⁺ T-cells 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 $\alpha\beta$ TCR but not of their CD3 complex (33), 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 (41), gp120-anti-gp120 antibody immune complexes, or anti-CD4 autoantibodies. However, preliminary results obtained in our laboratory suggest that preincubation of normal mature human CD4⁺ T cells

with CD4 antibody or gp120, whether cross-linked or not, does not lead to apoptosis upon further simulation.

A second possibility is that CD4⁺ T cells from HIV-infected individuals have no intrinsic abnormalities, but that defective antigen presenting cell function accounts for induction of T-cell apoptosis. Whether antigenpresenting cell from HIV-infected individuals are unable to provide relevant co-signals required for a proliferative response to PWM, or to MHC-II-dependent TCR mobilization, is currently under investigation. It has been shown that restimulation of a mature murine CD4⁺ T-cell clone by cross-linked CD3 antibodies in the absence of antigen presenting cell results in an active cell death process that involves interferon γ , and is prevented by anti-interferon γ antibody (40). Our preliminary data, however, indicate that anti-interferon γ antibody does not prevent activation-induced death of CD4⁺ T cells from HIV-infected individuals.

A third possible interpretation of our findings, that 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 cytotoxic T lymphocytes (CTL) kill their targets (18,42). Although cytotoxic properties have been mainly ascribed to a sub-population of CD8⁺ T cells, CD4⁺ CTL clones have been described (42). Since cyclosporin A, which does not prevent CD8⁺ CTL- or CD4⁺ CTL-mediated apoptosis of target cells (42), prevented apoptosis of CD4⁺ T cells from HIV-infected individuals, we think that an activation-induced CD4⁺ T-cell

suicide process, in the absence of any participation of CD4⁺ killer T cells, represents at this stage the simplest explanation for our observations. Our findings suggest that activation-induced T-cell death may occur *in vivo* and account for the progressive depletion of CD4⁺ T cells that leads to AIDS. *In vitro* proliferation assays of CD4⁺ T cells in response to various recall antigens in the presence of CD28 mAb, should allow one to assess at any given time the extent of the memory CD4⁺ T-cell repertoire that is remaining *in vivo*. In particular, it will be possible to test whether CD4⁺ T cells specific for pathogens continuously present in HIV-infected individuals, such as HIV itself, herpes virus, or cytomegalovirus, are deleted earlier *in vivo* than CD4⁺ memory T cells specific for pathogens that have been rarely (influenza) or never (tetanus) encountered subsequent to HIV infection. Over the years, CD4⁺ T-cell depletion may extend beyond the T-cell repertoire specific for antigens present in the patient. Infection by pathogens that produce superantigens, such as staphylococci or streptococci (31), could induce the deletion of a large number of CD4⁺ T cells expressing the matching V β molecules, independently of their antigen-specificity. Whatever the stage of the disease, the *in vivo* rate of CD4⁺ T-cell depletion should 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 (43), and since activation will result in a rapid cell deletion through apoptosis, this T-cell suicide process could provide an

explanation for the very low percentage of HIV-infected T cells in HIV-infected individuals (8-10).

Finally, our observation that cyclosporin A 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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LEGENDS

FIG. 1. Proliferative response of T cells from HIV-infected asymptomatic individuals and from controls.

Histograms represent the mean \pm S.D. of triplicate measurement of $^3\text{[H]}$ -thymidine incorporation of T cells from 38 HIV-infected asymptomatic individuals () and from 20 HIV-seronegative controls () incubated with medium alone, phytohemagglutinin (PHA) ($10\mu\text{g/ml}$), pokeweed mitogen (PWM) ($10\mu\text{g/ml}$), superantigens (SEB) ($1\mu\text{g/ml}$), and the tetanus toxoid (TT) recall antigen ($10\mu\text{g/ml}$). * = $p < 0.00004$.

FIG. 2. Cell death in peripheral blood mononuclear cells from HIV-infected asymptomatic individuals and from controls after *in vitro* culture with PWM and SEB.

Histograms represent the mean \pm S.D. of triplicate measurements of the percentage of cell mortality in PBMC from 20 different HIV-infected asymptomatic individuals (1) and 20 controls (2), 48hr after incubation with medium alone, PWM (10 μ g/ml), or SEB (1 μ g/ml), in the absence or in the presence of cycloheximide (50 μ g/ml), cyclosporin A (Cyclosporin A) (200ng/ml), or CD28 mAb (10 μ g/ml). Cell death was assessed by trypan blue permeability. * = $p < 0.001$

FIG. 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 \pm S.D. of triplicate measurement of cell mortality in CD8⁺ T cell- or CD4⁺ T cell-depleted PBMC from a HIV-infected asymptomatic individual (1) and a control (2). T-cell proliferation was also measured by ³[H]-thymidine incorporation after 3 days; the CD8⁺ T cell-depleted PBMC from a control proliferated to PWM (5,250 \pm 651cpm) and to SEB (21,642 \pm 2,317cpm), 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 individual proliferated to either stimuli.

b) Histograms represent the mean \pm S.D. of triplicate measurement of cell mortality in purified CD4⁺ and CD8⁺ T cells from a HIV-infected asymptomatic individuals (1) and a control (2). T-cell proliferation was also measured by ³[H]-thymidine incorporation after 3 days; purified CD4⁺ and CD8⁺ T cells from the HIV-infected individual and from the control proliferated normally to PHA. CD4⁺ T cells from the control proliferated in response to PWM (4,817 \pm 351 cpm) and to SEB (21,892 \pm 1,628 cpm), whereas CD8⁺ T cells from the control and CD4⁺ and CD8⁺ T cells from the HIV-infected individual did not proliferate to either stimuli.

FIG. 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 μ g/ml) or SEB (1 μ g/ml) in the absence or presence of Cyclosporin A (200ng/ml) or CD28 mAb. DNA weight markers (lane 1); control PBMC with PWM (lane 2); PBMC from 8 HIV-patients with: medium (lanes 3, 12), PWM (lanes 4-9, 13) or SEB (lanes 10,11). Lanes 3-4 and lanes 12-14: cells from the same patients. Lanes 4-6 and 8-11 and 13 show a clear ladder of degraded DNA bands which are multiple of 200 base pairs, characteristic for apoptosis (10-13, 17-19). DNA fragmentation was suppressed when Cyclosporin A (lane 7, cells from same patient as lane 6) or CD28 mAb (lane 14, cells from same patient as lane 13) were added to PWM.

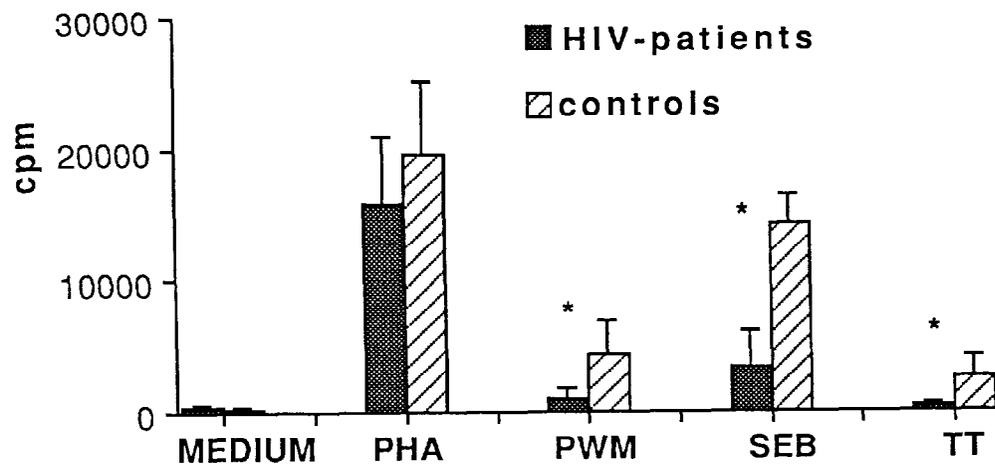
b and c) Electron micrographs of PBMC from a HIV-patient after 24h incubation with **b**: medium or **c**: PWM (5 μ g/ml). Cells showing various stages of chromatin condensation, characteristic for apoptosis (10, 17, 19, 20), can be seen in **c** (arrows). Bars= 5 μ m.

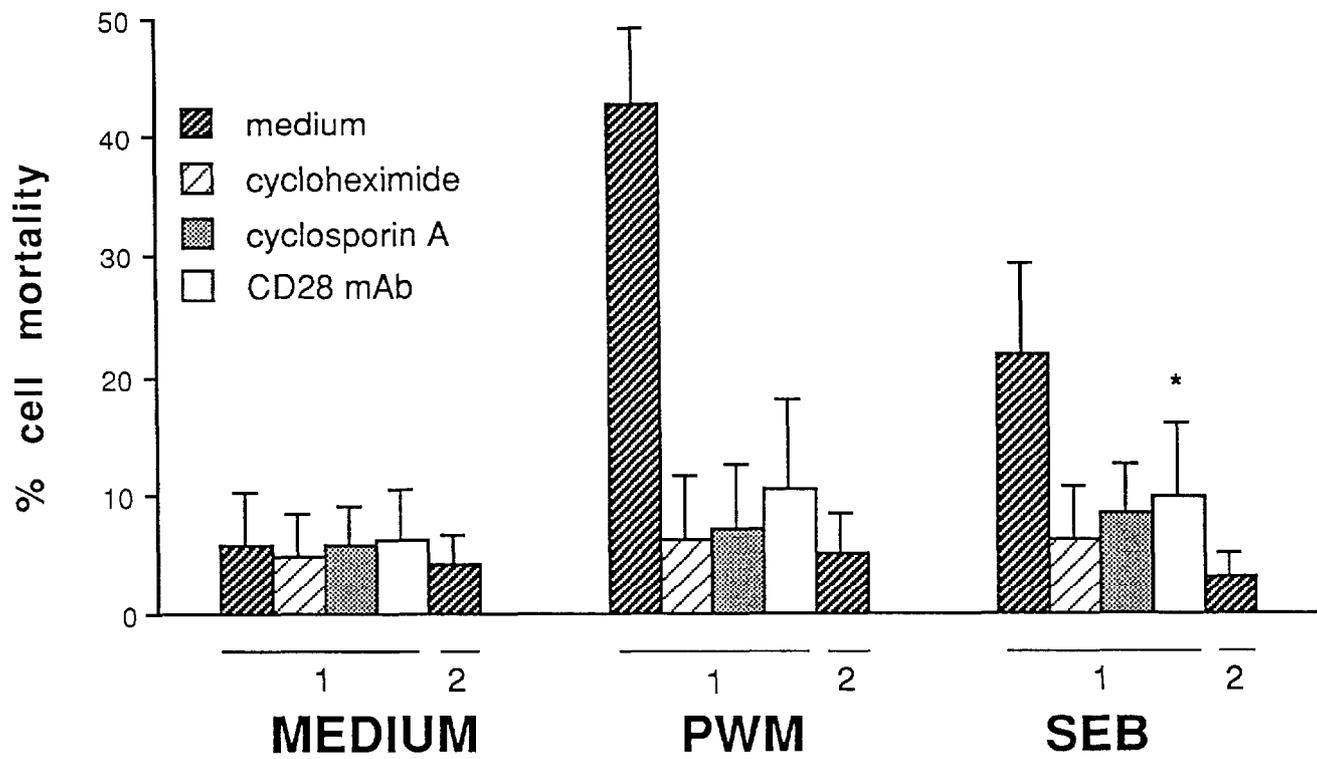
FIG. 5. Effect of CD28 mAb on the proliferative response of T cells from HIV-infected asymptomatic individuals.

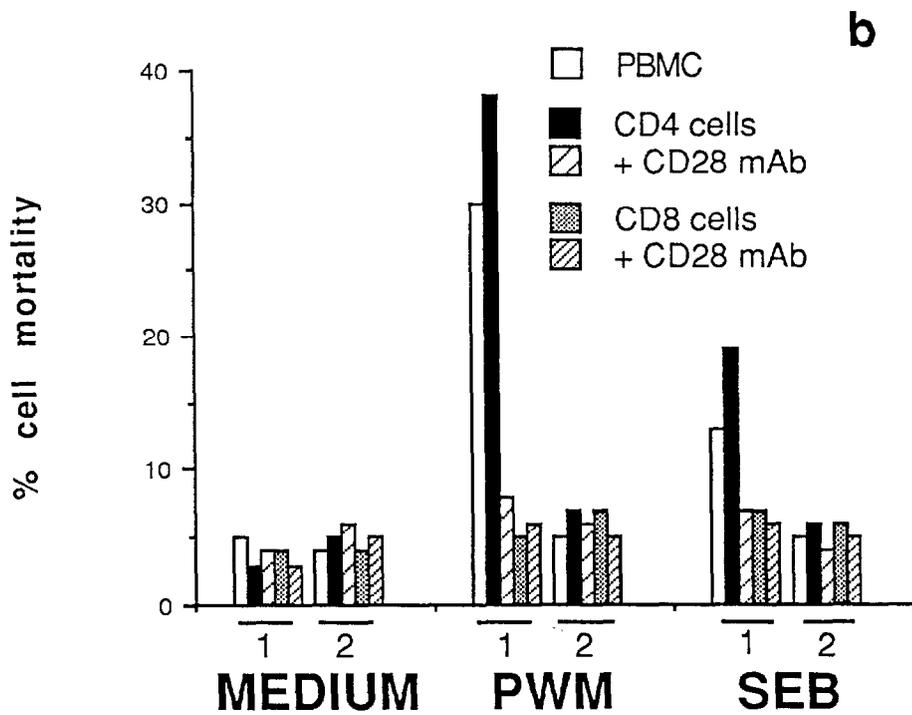
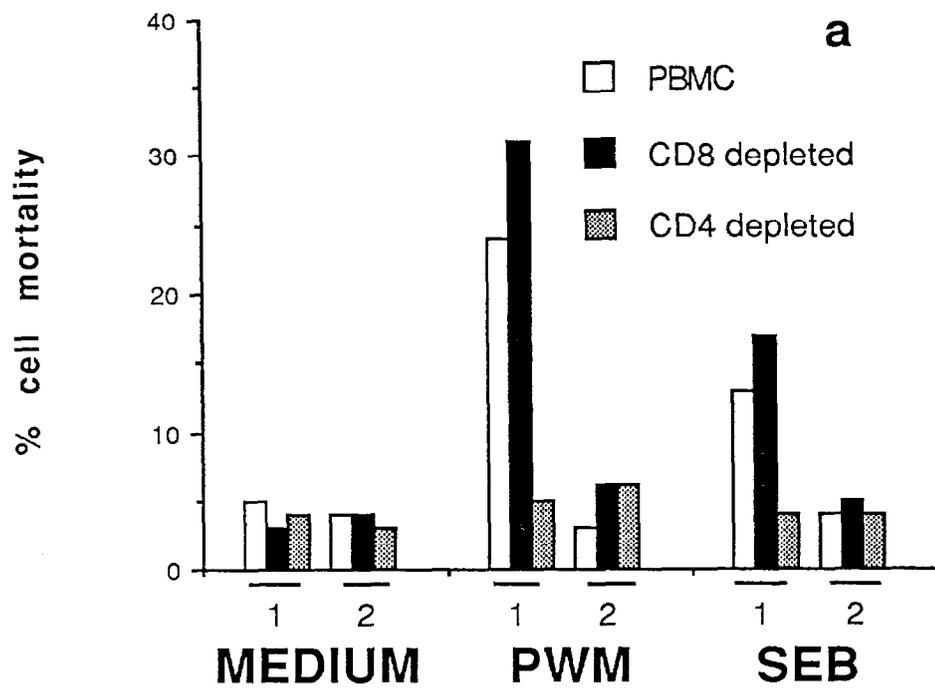
Circles represent the mean \pm S.D. of triplicate measurement of ^3H -thymidine incorporation of T cells from each of 19 HIV-infected asymptomatic individuals incubated with PWM, SEB, TT, or medium alone, in the absence () or presence () of CD28 mAb. (Certain circles representing similar values are superposed). * = $p < 0.00004$

TABLE 1. Antigen-mediated functional deletion of antigen-specific memory T cells from HIV-infected asymptomatic individuals.

Results represent mean \pm SD of triplicate measurements of $^3\text{[H]}$ -thymidine incorporation of PBMC from 4 HIV-infected asymptomatic individuals and from 2 controls. Results showing functional deletion are underlined. Preincubation with TT: PBMC ($2.5 \times 10^6/\text{ml}$) were cultured for 10 days with the tetanus recall antigen (TT) ($10\mu\text{g}/\text{ml}$). Cells were then incubated with different stimuli, and proliferation measured by $^3\text{[H]}$ -thymidine incorporation after 3 days for PHA or PWM and 6 days for TT or Influenza A recall antigens (Infl) ($10\mu\text{g}/\text{ml}$), in the presence or absence of CD28 mAb ($10\mu\text{g}/\text{ml}$).







Preincubation		Thymidine incorporation (cpm)						
		followed by stimulation with						
		medium	CD28	TT	TT+CD28	Infl	Infl+CD28	PHA
HIV+ 1	No preincubation with TT	121±52	314±87	236±78	2617±321	341±58	3217±206	18621±817
		614±87	258±94	318±97	<u>415±87</u>	721±75	6731±345	17182±751
HIV+ 2	No preincubation with TT	447±34	313±57	317±27	5736±187	151±57	3842±137	17980±243
		221±67	318±61	176±50	<u>118±21</u>	345±46	5351±142	16441±419
HIV+ 3	No preincubation with TT	324±38	249±64	437±31	3781±108	438±51	6242±107	28143±243
		479±61	408±37	459±61	<u>438±71</u>	386±48	2674±157	24597±267
HIV+ 4	No preincubation with TT	350±217	405±104	157±117	1549±817	108±64	1560±258	28232±6712
		103±37	207±83	483±121	<u>342±106</u>	435±265	2170±598	23315±4612
Control 1	No preincubation with TT	680±217	354±70	3930±881	3290±721	2314±567	3254±432	31217±2642
		569±258	208±62	7091±932	4382±792	2090±589	1850±678	27218±4812
Control 2	No preincubation with TT	477±81	387±73	4830±523	4087±238	5721±267	4239±540	20587±267
		297±54	579±69	5761±287	5983±345	3549±138	4820±534	31589±354

TABLE 1