Human immunodeficiency virus-1 (HIV-1) infection leads to CD4⁺-T-cell depletion associated with high viral turnover and precede by a progressive loss of T-cell-mediated immunity. We have proposed that T-cell defects in HIV-1-infected persons may be related to the inappropriate induction of T-cell death by apoptosis through HIV-mediated interference with cellular signaling. This model has been supported by several findings showing that abnormal levels of apoptosis occur both in vitro and in vivo in uninfected CD4⁺ and CD8⁺ T cells from HIV-1-infected persons. Studies performed in pathogenic and nonpathogenic primate models of HIV or Simian immunodeficiency virus (SIV) infection have further suggested a correlation between the induction of enhanced in vitro T-cell apoptosis and the in vivo pathogenic nature of the retroviral infection. Candidate mechanisms for apoptosis induction in HIV-infected persons include HIV proteins such as the gp120 envelope and the Tat regulatory gene product that induce or enhance in vitro apoptosis of T cells from controls through mechanisms that may at least partly involve an enhanced expression of the Fas or Fas ligand molecules, respectively. In vitro, T cells from HIV-infected persons undergo apoptosis in response to either T-cell receptor (TCR) stimulation or antibody-mediated Fas ligation. Our recent finding that the interleukin-12 (IL-12) cytokine could prevent the abnormal levels of apoptosis of CD4⁺ T cells from HIV-infected persons induced either by TCR stimulation or by antibody-mediated Fas ligation led us to investigate the hypotheses (1) that TCR-induced and Fas-mediated stimulation of T cells from HIV-infected persons share common downstream death effector pathways; (2) that cytokines play a general role in the modulation of Fas-mediated death signaling in T cells from HIV-infected persons; and (3) that TCR-induced apoptosis of T cells from HIV-infected persons occurs through a Fas-mediated death process.

**MATERIALS AND METHODS**

**Reagents, antibodies, and cytokines.** Unlabeled murine anti-human monoclonal antibodies (MoAbs) used were CD4 (Leu 3a); CD8 (Leu 2a; Becton Dickinson & Co, Mountain View, CA); CD14 (IOM2); CD19 (I0B4); CD56 (IOT56); CD3 (IOT3b); the agonistic CD95 IgM MoAb (CH11) and control IgM MoAb (GC3c); the antagonistic CD95 IgG1 MoAb (ZB4) and control IgG1 MoAb (679.1MC7; Immunotech, Marseille, France); and the antagonistic CD95 MoAb (Becton Dickinson); fluorescein isothiocyanate (FITC)-labeled CD95 MoAb (I0B4); phycoerythrin (PE)-labeled CD45 RA MoAb or CD45 R0 MoAb (Becton Dickinson); and unconjugated CD4 MoAb (Leu 2a) or CD8 MoAb (Leu 2a; Becton Dickinson); and phosphothiorate oligonucleotide (5'-GGT GTG CGT CAA GCT-3' and phosphothiorate antisense oligonucleotide (5'-GGT GTG CGT CAA GCT-3').

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**RAPID COMMUNICATION**

**Fas-Mediated Apoptosis of CD4⁺ and CD8⁺ T Cells From Human Immunodeficiency Virus-Infected Persons: Differential In Vitro Preventive Effect of Cytokines and Protease Antagonists**

By Jérôme Estaquier, Masato Tanaka, Takashi Suda, Shigekazu Nagata, Pierre Golstein, and Jean Claude Ameisen

Human immunodeficiency syndrome (HIV) infection leads to a progressive loss of T-cell-mediated immunity associated with T-cell apoptosis. We report here that CD4⁺ and CD8⁺ T cells from HIV-1-infected persons are sensitive to Fas- and Fas ligand-mediated death either by an agonistic anti-Fas antibody or by the physiologic soluble Fas ligand, although showing no sensitivity to tumor necrosis factor α-induced death. CD4⁺ and CD8⁺ T-cell apoptosis induced by Fas ligation was enhanced by inhibitors of protein synthesis and was prevented either by a soluble Fas receptor decoy or an antagonistic anti-Fas antibody. Fas-mediated apoptosis could also be prevented in a CD4⁺ or CD8⁺ T-cell-type manner by several protease antagonists, suggesting the involvement of the interleukin-18 (IL-18)-converting enzyme (ICE)-related cysteine protease in CD4⁺ T-cell death of both a CPP32-related cysteine protease and a calpain protease in CD8⁺ T-cell death; and (2) by three cytokines, IL-2, IL-12, and IL-10, that exerted their effects through a mechanism that required de novo protein synthesis. Finally, T-cell receptor (TCR)-induced apoptosis of CD4⁺ T cells from HIV-infected persons involved a Fas-mediated death process, whereas TCR stimulation of CD8⁺ T cells led to a different Fas-independent death process. These findings suggest that Fas-mediated T-cell death is involved in acquired immunodeficiency syndrome (AIDS) pathogenesis and that modulation of Fas-mediated signaling may represent a target for new therapeutic strategies aimed at the prevention of CD4⁺ T-cell death in AIDS.

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inhibitor Acetyl-YVAD-aldehyde (Ac-YVAD-CHO), and the reversible CPP32 inhibitor Acetyl-DEVAD-aldehyde (Ac-DEVAD-CHO; Bâle Biochimie, Voisins-le-Bretonneaux, France). Additional protease inhibitors that do not inhibit ICE or CPP32 activity23-25 included acetyl n-tosyl-lys-chloromethylketone (Ac-Tos-CMK), leupeptin (Ac-LLR-CHO), and E64 (Sigma, La Verpillière, France). Cytokines included recombinant human (rh) IL-2 (provided by Roussel-Uclaf, Paris, France), rhIL-4, rhIL-7, rhIL-10, rhIL-12 (R & D Systems, Abingdon, UK), and rh tumor necrosis factor α (rhTNFα; Genzyme, Cambridge, MA). Anti-TNFα antibodies were rabbit polyclonal anti-human TNFα neutralizing antibodies (Genzyme). Other reagents were cycloheximide (CHX; Sigma), acridine orange dye (Immuno-tech), YO-PRO-1 dye (Molecular Probes, Inc, Eugene, OR), Hoechst 33342 (Bisbenzimide; Sigma), and Calcein AM (Molecular Probes).

Cells and culture conditions. Heparinized venous peripheral blood was obtained from 45 asymptomatic HIV-1-seropositive adults (Service des Maladies Infectieuses, Hôpital Drou, Tourcoing, France) with CD4+ T-cell counts higher than 400/μL and from 15 HIV-seronegative healthy controls. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation and cultured in RPMI 1640 (GIBCO, Courbevoie, France) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim, Meylan, France), 2 mM/L L-glutamine, 1 mM/L sodium pyruvate (GIBCO), and gentamicin (Gentalline; 8 μg/mL; Schering-Plough, Levallois-Perret, France). Three-color flow cytometer analysis (FACScan; Becton Dickinson) of freshly isolated PBMC was performed by co-staining cells using MoAbs (including isotype controls) directly labeled with FITC, RA-PE, and PercP. Lymphocytes were gated under forward and size scatter light parameters. Purified CD4+ or CD8+ T cells, as assessed by flow cytometry, were directly labeled with FITC, RA-PE, and PercP. Lymphocytes were had lost trypan blue exclusion capacity, as previously described.23,24 Condensation and fragmentation as well as already dead cells that apoptotic included cells with characteristic nuclear chromatin dye YO-PRO-1, as described,27 or by UV fluorescent microscopy (FACSscan; Becton Dickinson) at 2.5 × 105/mL. Percentages of apoptotic cells were measured in duplicate under the light microscope. Cells counted as apoptotic included cells with characteristic nuclear chromatin condensation and fragmentation as well as already dead cells that had lost trypan blue exclusion capacity, as previously described.22,23 Percentages of apoptotic cells counted under the light microscope were confirmed by flow cytometry analysis after incubation with the nuclear dye acridine orange, as described,18 with the nuclear dye YO-PRO-1, as described,24 or by UV fluorescent microscopy (3CCD; Leica, Rueil Malmasion, France) analysis of cells co-stained with Calcein AM24 and with Hoechst 33342.21

Percentages of apoptosis prevention expressed in Figs 1C and 2A, B, D, and F were calculated as follows: ([Fas-Mediated Apoptosis] – (Fas-Mediated Apoptosis After Pretreatment With x)]/[Fas-Mediated Apoptosis] – (Spontaneous Apoptosis in Medium Alone)] × 100.

Percentages of apoptosis prevention expressed in Fig 3B were calculated as follows: ([TCR-Induced Apoptosis] – (TCR-Induced Apoptosis After Pretreatment With x)]/[TCR-Induced Apoptosis] – (Spontaneous Apoptosis in Medium Alone)] × 100.

Fold increases in apoptosis in Fig 2C were calculated as follows: ([Fas-Mediated Apoptosis After CHX Treatment] – (Fas-Mediated Apoptosis)]/[Fas-Mediated Apoptosis] – (Spontaneous Apoptosis in Medium Alone)] × 100.

Statistical significance. P was assessed using the Student’s t-test (Fig 1A) or the paired Student’s t-test in the other figures (NS, no significant statistical differences).

RESULTS AND DISCUSSION

In HIV-infected persons, the percentage of peripheral blood T cells expressing the Fas receptor is higher than in uninfected controls.21,22 We observed a significant increase in Fas-positive T cells in both the memory CD4+ T cells (that normally express Fas)26 and in the CD45 RA naive CD4+ and CD8+ T cells (that normally include few Fas-positive T cells; Fig 1A), suggesting that HIV infection induces a global immune activation process that also involves the naive or virgin T-cell subpopulation. In contrast to T cells from controls, freshly isolated CD4+ and CD8+ T cells from HIV-infected persons were sensitive to apoptosis induction through Fas receptor engagement (Fig 1B), either by an agonistic anti-Fas antibody, as previously reported,21,22 or by a soluble form the physiologic ligand of the Fas receptor, a recombinant human Fas ligand. The percentages of apoptosis induced by the agonistic anti-Fas antibody in CD4+ and CD8+ T cells (mean, 13% and 14.1%, respectively; with maximal values of 29.5% and 37.6%) were higher than that induced by the soluble recombinant Fas ligand, suggesting that anti-Fas antibody, as the cell surface-expressed Fas ligand, may be more effective in achieving Fas receptor aggregation than the secreted form of the Fas ligand. The selective involvement of Fas-mediated signaling in T-cell apoptosis was indicated (1) by the prevention of soluble Fas ligand-mediated apoptosis by the antagonistic anti-Fas antibody ZB4 and by a soluble Fas receptor decoy (human Fas Ig fusion protein) that both block Fas-Fas ligand interactions,21,22 (Fig 1C); and (2) by the absence of T-cell apoptosis induction in response to TNFα (Fig 1D), another member of the Fas ligand family whose p55 receptor has a Fas-related death domain,25 even in the presence of the protein synthesis inhibitor CHX (data not shown) that had an enhancing effect (from 58% to 95%) on TNFα-mediated death of the TNFα-sensitive cell line L929.

In freshly isolated T cells from healthy individuals, antibody-mediated Fas ligation does not induce apoptosis9,26 (Fig 1B) and even provides in some cases costimulatory signals to concomitant TCR stimulation, leading to an enhancement of TCR-induced T-cell proliferation8 (Fig 1E). However, in T cells from HIV-infected persons, antibody-mediated Fas ligation led not only to a significant induction of T-cell apoptosis (Fig 1B) but also to a significant reduction in the TCR-induced T-cell proliferation (Fig 1E) that was already severely impaired when compared with that of controls. Thus, CD4+ and CD8+ T cells from HIV-infected persons behave as potential targets for a Fas-mediated bystander death process that could be triggered by the Fas ligand expressed or secreted by activated neighboring immune cells. In this respect, T cells from HIV-infected persons resemble preactivated T-cell lines,27 suggesting that HIV infection induces a general state of immune activation that primes large numbers of T cells for death by apoptosis in response to further interaction with the Fas ligand.

Fas antibody-mediated and soluble Fas ligand-mediated apoptosis of CD4+ and CD8+ T cells from HIV-infected
persons could be prevented by pretreatment with the AC-YVAD-CMK peptide, an irreversible competitive inhibitor of the ICE cysteine protease family (Fig 2A and B), whereas the control Ac-TOS-CMK protease inhibitor peptide had no effect (Fig 2A), suggesting that Fas-mediated death signaling in T cells from HIV-infected persons shared the ICE-dependent downstream effector pathway recently described in T-cell lines. The family of ICE-related cysteine proteases, involved to various degrees in the effector pathway of apoptosis, has now been shown to include at least 6 members, ICE, CPP32/YAMA, ICH-1/NEDD-2, ICH-2/TX, MCH-2, and ICE rel III. The chloromethylketone (CMK) YVAD peptide is an irreversible competitive inhibitor of ICE protease activity and may therefore be less selective than the reversible aldehyde (CHO) peptide antagonists. Experiments using reversible aldehyde competitive inhibitors of cysteine protease activity that have been reported to be specific for either ICE (Ac-YVAD-CHO) or for CPP32 (Ac-DEVD-CHO) suggested that Fas-mediated death signaling in T cells from HIV-infected persons may involve the preferential activation of ICE in the CD4+ T cells and of CPP32 in the CD8+ T cells (Fig 2A). The leupeptin (Ac-LLR-CHO) protease antagonist that does not inhibit ICE or CPP32 had no inhibitory effect on Fas-mediated apoptosis of CD4+ and CD8+ T cells (Fig 2A). As also shown in Fig 2A, E64, an inhibitor of the calcium-dependent protease calpain that does not inhibit ICE or CPP32, was as effective as Ac-DEVD-CHO in inhibiting Fas-mediated CD8+ T-cell apoptosis, although having no effect on Fas-mediated CD4+ T-cell death. Together, these findings suggest that the Fas-induced apoptosis involves an ICE-related cysteine protease in CD4+ T cells; and in CD8+ T cells, both a CPP32-related cystein protease and a calpain-related protease that are either functioning downstream from one another or are each required for the activation of downstream effectors of apoptosis. Although further work will be needed
to confirm the precise nature of the proteases involved, our findings strongly suggest, at this stage, that the Fas-mediated death pathway requires the activation of different proteases in CD4+ and CD8+ T cells from HIV-infected persons.

The magnitude of Fas-mediated T-cell apoptosis (the net difference between the percentages of Fas-induced apoptosis and of spontaneous apoptosis) showed individual variations (Fig 1B), but the levels of the preventive effect achieved by protease antagonists did not depend on the magnitude of Fas-mediated apoptosis. For example, in the experiments shown in the Fig 2A, the mean preventive effect of Ac-YVAD-CMK on Fas-induced apoptosis of CD4+ T cells from 14 HIV-infected persons was of 58%. In the 2 of 14 cases in which Fas ligation induced the highest percentages of CD4+ T-cell apoptosis (29.5% and 16.7%), the preventive effects of Ac-YVAD-CMK were 58% and 67%, respectively. Also, T-cell treatment with the protein synthesis inhibitor greatly enhanced the magnitude of Fas-mediated apoptosis (Fig 2C). In such conditions, Ac-YVAD-CMK conserved its preventive effect. For example, in the experiments shown in Fig 2F, in the absence of CHX, the mean percentage of Fas-induced apoptosis in CD4+ T cells from 4 HIV-infected persons was 14% and the mean preventive effect of Ac-YVAD-CMK was 43%; in the presence of CHX, the mean percentage of Fas-induced apoptosis increased to 25%, and the mean preventive effect of Ac-YVAD-CMK remained at 35%.

The finding that Fas-mediated death of CD4+ and CD8+ T cells was enhanced by the protein synthesis inhibitor CHX (Fig 2C) suggested that Fas-induced death signaling in T cells from HIV-infected persons was partly under an active repression by newly synthesized proteins of short half life. Cytokines are a family of soluble mediators of cell signaling that are potent regulators of T-cell activation and differentiation and exert most of their effects on target cells through

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de novo protein synthesis as a consequence of cytokine receptor-mediated nuclear translocation of the signal transducers and activators of transcription (Stat) proteins. We have previously shown that IL-12, a cytokine that upregulates CD4+ Th1-cell functions, has a preventive effect on Fas-mediated apoptosis of CD4+ T cells from HIV-infected persons. As shown in Fig 2D, preincubation of T cells from HIV-infected persons for 2 hours with three different cytokines (IL-2, IL-10, or IL-12) had a highly significant preventive effect on death of CD4+ and/or CD8+ T cells. IL-2, a cytokine that is produced by activated CD4+ T cells and is involved in T-cell-mediated immunity, had a preventive effect on Fas-mediated death of both CD4+ and CD8+ T cells. Two other cytokines secreted by immune accessory cells (monocytes/macrophages and/or B cells), IL-12, and IL-10, acted in a cell-type specific way. IL-12 selectively prevented Fas-mediated CD4+ T-cell death, although having no preventive effect on CD8+ T-cell death; IL-10 prevented Fas-mediated CD8+ T-cell death, although having no preventive effect on CD4+ T-cell death (Fig 2D). Like that of protease antagonists, the preventive effect of cytokines did not depend on the magnitude of Fas-mediated T-cell death. As shown in Fig 2E, IL-10 was able to almost completely prevent apoptosis in a case in which the percentage of Fas-induced apoptosis in CD8+ T cells (36 hours after incubation with the anti-Fas antibody) reached 41%. Although these preventive effects of IL-10 (Fig 2E), IL-2, and of IL-12 lasted for more than 36 hours, IL-10 during this period also showed a delayed enhancing effect on Fas-mediated apoptosis of CD4+ T cells (data not shown). Flow cytometry analysis of purified CD4+ and CD8+ T cells from HIV-infected persons using double-color analysis with FITC-labeled anti-Fas MoAb and PE-labeled anti-CD4 or CD8 MoAb indicated that none of these cytokines induced any significant modification in the percentage of CD4+ and CD8+ T cells expressing the Fas molecule or in the densities of Fas molecules expressed on the CD4+ and CD8+ T-cell surface. For example, the mean fluorescence intensity of Fas labeling on CD4+ T cells was 8.29 ± 6.26 and 8.68 ± 6.93 after preincubation with medium alone or IL-12, respectively, and the mean fluorescence intensity of Fas labeling on CD8+ T cells was 8.43 ± 5.13 or 8.08 ± 5.09 after preincubation with medium alone or IL-10, respectively. These data indicated that cytokines exerted their effect through modulation of Fas-mediated signal transduction. The IL-2, IL-4, and IL-7 cytokines bind to the same type 1 cytokine receptor family that share one common receptor subunit, the IL-2Rγc, as well as the IL-13 receptor a1 and 3 signal transduction pathways, but do not activate the same Stat proteins. Although IL-7 showed a small preventive effect on Fas-mediated death of CD4+ T cells from HIV-infected persons (Fig 2D), the absence of preventive effect of IL-7 on Fas-mediated death of CD8+ T cells as well as the absence of preventive effect of IL-4 on Fas-mediated death of CD4+ and CD8+ T cells (Fig 2D) underlines the selectivity of the effect of cytokines on Fas-mediated death signaling. In contrast to the ICE family protease antagonist that retained its preventive effect on Fas-mediated T-cell apoptosis in the presence of the protein synthesis inhibitor CHX (Fig 2F), the preventive effect of cytokines on T-cell sensitivity to Fas ligation was completely abrogated by CHX treatment (Fig 2F). These data suggest that cytokines do not exert a direct inhibitory effect on ICE family protease activity, but rather act by activating selective transcription factors inducing de novo synthesis of proteins that interfere with Fas-mediated death signaling.

CD4+ and CD8+ T cells from HIV-infected persons undergoing apoptosis not only in response to direct Fas ligation, but also to TCR stimulation (2,9,12) (Fig 3A). As shown in Fig 3B, TCR-induced apoptosis of CD4+ T cells was prevented by either a Fas ligand antisense phosphothiorate oligonucleo-
In contrast, treatment with the Fas ligand antisense oligonucleotide with the antagonistic Fas antibody 4B4 or with Ac-YVAD-CMK had no significant preventive effect on TCR-induced apoptosis of CD8+ T cells (Fig 3B). Ac-YVAD-CHO and CPP32 Ac-DEVD-CHO were also devoid of preventive effect on TCR-induced CD8+ T-cell death (data not shown). These findings are consistent with our observation that IL-10 that prevents CD8+ T-cell apoptosis induced by Fas ligation (Fig 2D) is devoid of any preventive effect on CD8+ T-cell apoptosis induced by TCR stimulation (data not shown).

It has been recently reported that TCR-induced death of preactivated mature murine CD4+ T cells occurs through a Fas-mediated mechanism, whereas TCR-induced death of preactivated CD8+ T cells occurs through a p75 TNF receptor-mediated mechanism. However, the addition of TNFα did not induce apoptosis in CD4+ or CD8+ T cells from HIV-infected persons (Fig 1D), and the addition of anti-TNF antibodies showed no preventive effects on TCR-induced apoptosis of either CD4+ or CD8+ T cells from HIV-infected persons (data not shown). Therefore, our findings suggest that TCR-induced CD8+ T-cell apoptosis in HIV infection occurs through a Fas-independent, TNF-independent, CD8+ T-cell killing process whose mechanism remains to be assessed.

Together, our findings imply (1) that Fas-mediated death signaling could play an important role in a bystander death process of both CD4+ and CD8+ T cells from HIV-infected persons through contacts with the Fas ligand that may be expressed or secreted by activated T cells or NK cells, a situation that could occur in vivo in lymphoid organs and tissues that contain high densities of activated immune cells; (2) that several protease antagonists and cytokines have the capacity to prevent T-cell sensitivity to Fas-mediated death signals. Finally, beyond HIV infection, it will be important to explore whether the recently described protective effect on CD8+ T-cell apoptosis induced by Fas ligation (Fig 2D) is devoid of any preventive effect on CD8+ T-cell apoptosis induced by TCR stimulation after encounter with specific antigens.

Our findings suggest that CD4+ T cells from HIV-infected persons are in an activation and/or a differentiation stage very similar to that of preactivated murine CD4+ T-cell lines and therefore imply a role for immune activation in the pathogenesis of acquired immunodeficiency syndrome (AIDS). They also suggest several potential strategies for the control of Fas-mediated death of CD4+ T cells in HIV infection, including the use of soluble Fas receptor decoys, of inhibitors of the ICE protease family (that have been recently shown to prevent apoptosis during development in vivo), and of the IL-2 or IL-12 cytokines. In this respect, it will be important to explore whether the recently described beneficial in vivo effect of IL-2 immunotherapy on CD4+ T-cell counts in HIV-infected persons may at least partly involve the prevention of CD4+ T-cell sensitivity to Fas-mediated death signals. Finally, beyond HIV infection, a general implication of our findings is that the general and potent regulatory role of cytokines on the activation and differentiation of defined T-cell populations may operate through the regulation of T-cell sensitivity to Fas-mediated death signaling.
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Fas-mediated apoptosis of CD4+ and CD8+ T cells from human immunodeficiency virus-infected persons: differential in vitro preventive effect of cytokines and protease antagonists

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