All-Trans Retinoic Acid Inhibition of Anti-CD3–Induced T Cell Apoptosis in Human Immunodeficiency Virus Infection Mostly Concerns CD4 T Lymphocytes and Is Mediated via Regulation of CD95 Ligand Expression

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This study analyzes the influence of all-trans retinoid acid (tRA) on apoptosis of peripheral lymphocytes from human immunodeficiency virus (HIV)-positive patients. tRA inhibits the ex vivo apoptosis in T cells; a more potent effect was observed on activation-induced apoptosis. Phenotypic characterization of T cell subsets prevented from anti-CD3–induced apoptosis by tRA revealed a more potent effect on CD4 T cells. A central regulatory system for apoptosis is the CD95 system, and inappropriate induction of this pathway is thought to contribute to AIDS pathogenesis. In investigation of CD95-based apoptosis, tRA had no effect on activation-dependent induction of CD95 on T lymphocytes, but it inhibited the induction of CD95 ligand expression on anti-CD3–activated T cells. The previously reported in vivo effect of tRA inhibiting HIV-associated apoptosis and the present observations suggest that tRA could be considered to down-regulate apoptosis associated with AIDS pathogenesis.

Physiologic cell death is primarily mediated through apoptosis. One of the central regulatory systems for apoptosis is the CD95 receptor/ligand system [1, 2]. The important role played by CD95-mediated death in T cell homeostasis was supported by the finding that CD95 and the CD95 ligand (CD95L) are mutated in mouse strains with severe autoimmune diseases and lymphoproliferation [3, 4]. Thus, mutations of the CD95 molecule in lpr mice and mutations of the CD95L in gld mice constitute the first genetically defined syndromes of defective apoptosis. Recently, human counterparts of the lpr mutation in mice have been identified [5–7].

The CD95 molecule is a cell surface receptor, of the tumor necrosis factor receptor superfamily, that is readily expressed in peripheral T cells after activation. To induce apoptosis, CD95 receptors on the cell surface have to oligomerize [8, 9]. CD95-mediated death depends on an apoptosis-sensitive phenotype that is acquired during prolonged activation of T cells. Sensitivity or resistance toward CD95-induced apoptosis may depend on the ability to transmit the death signal or may be modulated by differential expression of anti-apoptotic proteins of the Bcl-2 family [10, 11]. The CD95L is a type II transmembrane protein expressed by activated T cells, and it is also produced in soluble form by proteolytic cleavage. T cell receptor (TCR) triggering in activated peripheral T cells may induce apoptosis that involves autocrine suicide or paracrine death mediated via CD95 receptor/ligand interaction [12–14]. Just as a defect of the CD95 system is intimately linked to an impaired removal of autoreactive lymphocytes, inappropriate induction of apoptosis may lead to various pathologic conditions [15]. This is exemplified in chronic virologic diseases such as AIDS [16, 17].

Indeed, T cells from human immunodeficiency virus (HIV)-infected persons are highly prone to in vitro spontaneous and activation-induced apoptosis [18–22]. Although apoptosis is not detected in freshly isolated peripheral blood lymphocytes, a high level of apoptosis may be induced in both CD4 and CD8 T cells from patients after overnight culture in the presence of mitogens or superantigens or after triggering of the TCR [18, 19, 21]. Recent characterization of apoptotic cells (from patients) indicated that the majority were in an activated state [22, 23], and the intensity of activation-induced apoptosis in T cells correlated with the degree of immune activation in the patients [22, 23] and with disease progression [23].

The in vivo involvement of the CD95 pathway in T cell apoptosis during HIV infection is supported by reports showing an increased expression of the CD95 receptor [24, 25] and cell surface CD95L [26] on CD4 and CD8 T lymphocytes of HIV-infected patients. This is associated with an increased susceptibility of patients’ T cells to apoptosis following ligation of CD95 by agonist antibodies or soluble human CD95L and the susceptibility of activation-induced apoptosis to inhibition by
antagonist anti-CD95 antibodies [25–29]. Moreover, the proportion of compliant CD95-expressing cells dramatically increases with disease progression [23, 25, 29]. Of interest, exacerbated CD95 expression and CD95 sensitivity seem to be confined to primed/memory (CD45R0) T cells, supporting the idea that virus-driven T cell activation is responsible for the increased apoptosis and contributes to the loss of memory cells throughout HIV infection [30].

Some have speculated that inappropriate activation-induced apoptosis of T cells from HIV-infected patients contributes to the decline of CD4 T cell number in HIV infection [16, 17]. The finding that a massive cell death is detected in situ in lymph nodes from patients, affecting all lymphocyte subsets [22], including CD4 T cells and dendritic cells [31], and concerning mostly noninfected cells [32], argues for this hypothesis. The mechanisms contributing to the priming for apoptosis of noninfected patients’ lymphocytes are actually misunderstood, but they might involve some viral gene products, such as gp120 and Tat, which accelerate activation-induced apoptosis in normal T cells via triggering of the CD95 pathway [33].

A recent study evaluating the influence of 6 HIV-infected persons of all-trans retinoic acid (tRA) on ex vivo T cell apoptosis showed that peripheral blood mononuclear cells (PBMC) from these patients exhibited a reduced spontaneous and anti-CD3–induced apoptosis [34]. This finding was completed by in vitro studies demonstrating that tRA prevented activation-induced apoptosis of patients’ PBMC, whereas no effect was observed on lymphocytes from control donors [34]. These observations prompted us to determine whether the inhibitory effect of tRA on apoptosis was similar on CD4 and CD8 T cells from HIV-infected persons at different stages of the disease and to characterize the molecular mechanism involved in this inhibition.

Materials and Methods

Blood samples. Peripheral blood was obtained from HIV-infected persons at various stages of the disease in the Service for Infectious Diseases (R. Roué), Bégin Military Hospital, Saint Mandé, France. Patients were classified according to their ex vivo CD4 cell percentage (>29%, 14%–29%, <14%), following the revised classification of HIV infection (CDC, Atlanta). Control blood samples were drawn from HIV-seronegative healthy donors (Centre National de Transfusion Sanguine, Paris).

Monoclonal antibodies (MAbs). The mouse MAbs specific for human surface antigens used in this study included anti-CD4 (IgG1k, clone SK3) and anti-CD8 (IgG1k, clone SK1) conjugated with fluorescein isothiocyanate (FITC; Becton Dickinson, San Jose, CA). Anti-CD95 (IgG1k, clone UB2) MAb conjugated with FITC was purchased from Immunotech (Marseille, France). Two anti-CD95L MAbs were used, clone 4A5 (Immunotech) to block activation-induced apoptosis and clone 33 (IgG1k; Transduction Laboratories, Pantin, France), in Western blot analysis. Control antibodies for cell surface labeling included mouse FITC or phycoerythrin-conjugated IgG1k (Becton Dickinson). Lymphocyte isolation and stimulation. Human PBMC were isolated from heparinized blood by centrifugation on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient. Cells were washed and resuspended in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Institut Jacques Boy, Reims, France), 10 IU/mL penicillin, 10 mg/mL streptomycin, 20 mM HEPE, and 2 mM L-glutamine (complete medium).

In the apoptosis assay, PBMC (5 × 10⁶/mL) were cultured for 24 h in complete medium alone or in the presence of an optimal concentration of soluble (0.1 μg/mL) or coated (10 μg/mL) 1-h 30-min incubation in complete medium at 4°C anti-CD3 MAb (IoT-3; Immunotech) as previously described [23]. tRA (Sigma, St. Louis) diluted in dimethyl sulfoxide (DMSO) was added in some cultures at 1 μL of tRA/mL of culture, and control cultures received 1 μL of DMSO. In some experiments, anti-CD95L MAbs (clone 4A5) were added at the initiation of the culture at 1 μg/mL. After 24 h, cells were harvested and studied for apoptosis quantification.

Phenotypic analysis of apoptotic cells. In recent comparative analyses of flow cytometric methods for apoptosis quantification [35], we found that the most reliable way to phenotype lymphoid apoptotic cells was a combination of cell surface staining and 7-amino actinomycin D (7-AAD) incorporation. 7-AAD discriminates between early and late apoptotic cells and indicates the increase of fluorescence of apoptotic cells due to alteration in their membrane permeability [35].

Phenotypic analysis of apoptotic cells was performed as follows: 5 × 10⁶ cultured lymphocytes were washed in PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBS-BSA-Na₃) and incubated 20 min at 4°C with MAbs conjugated to FITC specific for surface antigens CD4 or CD8. After being washed, lymphocytes were incubated with 20 μg/mL 7-AAD (Sigma) in PBS for 20 min at 4°C in the dark, as described [35]. Stained cells were further fixed with 1% paraformaldehyde in PBS in the presence of 20 μg/mL nonfluorescent actinomycin D (Sigma) to block 7-AAD staining within apoptotic cells and to avoid nonspecific labeling of living cells [35]. Finally, the double-stained lymphocytes were incubated overnight at 4°C in the dark.

For each sample, 20,000 lymphocytes were acquired for flow cytometry (FACScan; Becton Dickinson). The spectral properties of 7-AAD allow staining of apoptotic cells by fluorescence emission in the red channel, FL-3 (wavelength, 650–850 nm), and the easy and simultaneous labeling of cell surface antigens (FL-1 and FL-2). In some experiments done to test the influence of tRA on apoptosis, data are given as the percentage of inhibition of apoptosis calculated as follows: % inhibition of spontaneous apoptosis = [(% apoptosis in medium – (% apoptosis + tRA)) / % apoptosis in medium) × 100; for anti-CD3–induced apoptosis, [% apoptosis with anti-CD3 – (% apoptosis with anti-CD3 + tRA)] / [% apoptosis with anti-CD3 – % apoptosis in medium] × 100.

CD95 detection on the cell surface of CD4 and CD8 T lymphocytes. Lymphocytes stimulated for 24 h by soluble anti-CD3 MAbs (1 μg/mL) in the presence or absence of tRA (10 μM) were washed twice and resuspended in ice-cold PBS-BSA-Na₃, and stained with phycoerythrin-conjugated anti-CD8, phycoerythrin-conjugated anti-CD4, and FITC-labeled anti-CD95, or control MAbs. The cells were incubated 30 min at 4°C, further fixed with 1% paraformaldehyde, and acquired for flow cytometry (FAC-
CD95L bioassay. CD95L on activated lymphocytes was detected using, as a target, radiolabeled U937 cells (incubated for 12 h with 5 mCi/mL [3H]thymidine; Amersham, Amersham, UK), which had been preincubated for 12 h with IFN-γ (200 U/mL; gift of Ara Hovanessian, Institut Pasteur, Paris). Interferon-γ (IFN-γ) treatment of U937 cells induced up-regulation of the CD95 molecule and rendered the cells sensitive to CD95L-induced apoptosis [36]. IFN-γ–treated radiolabeled U937 cells were then washed and divided into 2 groups: The first was incubated at 38.1°C for 1 h with neutralizing anti-CD95 MAb (ZB4; 70 ng/mL) to cover the CD95 receptor; the other group was kept at 38.1°C for 1 h and used as a control. The human lymphocyte population (PBMC from control or HIV-positive donors) to be tested for CD95L expression was preactivated for 3 h with anti-CD3 MAb (1 μg/mL), in the presence or absence of tRA (10 μM), and then incubated for 15 h with either anti-CD95–treated or control U937 cells in the presence of 1 μM cold thymidine. Apoptosis of U937 cells was measured following cell lysis, and the released radioactivity associated with fragmented DNA and that of the intact chromatin were measured in counts per minute (cpm) as previously described [36]. The percentage of fragmented DNA was expressed as (cpm released/total cpm) × 100. Data are expressed as the percentage of fragmented DNA released above the background level detected in cocultures of U937 cells with nonstimulated PBMC.

CD95L detection by Western blot analysis. PBMC were stimulated for 24–48 h with coated anti-CD3 MAbs in the presence of the metalloprotease inhibitor, BB3103 (1 μg/mL; gift of Alan H. Drummond, British Biotech Pharmaceuticals, Oxford, UK). Cell lysates (100 μg of total proteins/sample) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane, which was then blocked with blocking buffer (5% nonfat milk in 10 mM Tris [pH 7.5], 100 mM NaCl, and 0.1% Tween 20) for 1 night at 4°C with agitation. The membrane was then incubated with primary anti-CD95L MAbs (Transduction Laboratories, Pantin, France; 1/1000 in blocking buffer) for 60 min at room temperature. Six washes were performed in washing buffer (10 mM Tris [pH 7.5], 100 mM NaCl, and 0.1% Tween 20), and protein was detected using the ECL protein detection system (Amersham, Courtaboeuf, France). Briefly, peroxidase-conjugated secondary anti–mouse IgG antibodies were added and incubated for 30 min at room temperature. Again six washes were performed; substrate was added and left for 1 min. The chemiluminescent blots were exposed to Kodak XAR-5 film for recording; exposure times ranged from 5 to 15 min. Positive control for CD95L expression consisted of lysates from PBMC cultured at 37°C for 3 days with 5 μg/mL concanavalin A and 10 IU/mL interleukin (IL)-2 in the presence of BB-3103, then stimulated with 10 ng/mL PMA and 500 ng/mL ionomycin for 1 day, according to the report of Tanaka et al. [37].

Results

tRA inhibits both the spontaneous and activation-induced apoptosis of PBMC of HIV-infected persons. We and others previously reported that a short-term incubation in medium alone of PBMC from HIV-infected persons induced a rapid spontaneous apoptosis that could be detected by several features, including morphologic criteria, alteration in membrane permeability, condensed chromatin, or the presence of fragmented DNA on agarose gels [18–21]. Cytocfluorometric analysis permitted the quantification and characterization of apoptotic cells [23], and, as is shown in figure 1A, the level of spontaneous apoptosis in lymphocytes from HIV-infected persons, detected with 7-AAD staining, was significantly higher than that of lymphocytes from HIV-seronegative persons. A significant increase of apoptosis could be induced in lymphocytes from HIV-infected persons after 24 h of stimulation with anti-CD3 MAbs

Figure 1. Effect of tRA on ex vivo spontaneous and anti-CD3–induced apoptosis of peripheral blood mononuclear cells (PBMC) from healthy controls or HIV-infected donors. PBMC (5 × 10^6/mL) from controls (n = 7) or HIV-infected persons at different stages of disease (CD4 cells >29%, n = 7; 14%–29%, n = 7; <14%, n = 7) were cultured overnight in medium or activated by 0.1 μg/mL anti-CD3 monoclonal antibodies, in presence or absence of various concentrations of tRA (0.01–10 μM). Quantification of apoptosis and calculations to obtain % of inhibition of apoptosis were performed as described in Materials and Methods. Means of % of apoptosis or of % of inhibition of apoptosis in total lymphocytes are shown; bars represent SE.
Influence of tRA on anti-CD3-induced apoptosis of CD4 and CD8 T cells from HIV-infected donor. Peripheral blood mononuclear cells (5 x 10^6/mL) from HIV-infected donor were stimulated overnight with 0.1 μg/mL anti-CD3 monoclonal antibodies (MAbs), in presence or absence of 10 μM tRA, as indicated. Quantification of apoptotic cells in each subset was performed following dual staining with anti-CD4 or anti-CD8 MAbs and with 7-amino actinomycin D (7AAD) for detection of apoptotic lymphocytes. Percentage of apoptotic lymphocytes among each subset is indicated on dot blots. FSC, forward scatter.

Figure 2. Influence of tRA on anti-CD3-induced apoptosis of CD4 and CD8 T cells from HIV-infected donor. Peripheral blood mononuclear cells (5 x 10^6/mL) from HIV-infected donor were stimulated overnight with 0.1 μg/mL anti-CD3 monoclonal antibodies (MAbs), in presence or absence of 10 μM tRA, as indicated. Quantification of apoptotic cells in each subset was performed following dual staining with anti-CD4 or anti-CD8 MAbs and with 7-amino actinomycin D (7AAD) for detection of apoptotic lymphocytes. Percentage of apoptotic lymphocytes among each subset is indicated on dot blots. FSC, forward scatter.
Figure 3. Preferential inhibitory effect of tRA on activation-induced apoptosis of CD4 T lymphocytes from HIV-infected persons. A, Peripheral blood mononuclear cells (5 × 10^6/mL) from control donors (n = 7) or HIV-infected persons at different stages of disease (CD4 cells >29%, n = 7; 14%-29%, n = 7; <14%, n = 7) were cultured overnight in medium or activated by 0.1 μg/mL of anti-CD3 monoclonal antibodies. Apoptosis was quantified in CD4 and CD8 T cell subsets. Data are mean ± SE of % of apoptosis in each subset. B, Cultures were performed as indicated above, in presence of 10 μM tRA. Percentage of inhibition of apoptosis in each subset was calculated as described in Materials and Methods. Data are mean ± SE of % of inhibition of apoptosis in each subset.

Patients with more advanced disease (CD4 cells <14%), the effect of tRA on spontaneous apoptosis was selectively observed on the CD4 T cell subset (figure 3B). When anti-CD3-induced apoptosis was studied, a strong inhibitory effect of tRA on apoptosis of CD4 T cells was also observed, even in the early stages of HIV-infection (CD4 cells >29%) (figure 3B, 3C). Strikingly, tRA had no effect on anti-CD3-induced apoptosis of CD8 T cells in most of the patients, whatever the stage of the disease (figure 3B, 3C). Moreover, it must be noticed that not only CD4 and CD8 T cells are involved in the process of apoptosis in HIV-infected persons, but also B and NK cells [23]. Of interest, we observed that tRA also inhibited the spontaneous apoptosis of B cells (data not shown). Together, these data reveal that tRA has a selective inhibitory effect on apoptosis of CD4 T lymphocytes from HIV-infected persons, and this inhibition is particularly strong on activation-induced apoptosis.

TtRA does not inhibit activation-dependent CD95 expression on CD4 and CD8 T cells. The CD95 receptor has been reported to be a potent inducer of apoptosis [1, 2], to be mainly present
Figure 3. Continued. C, Influence of 10 μM tRA on spontaneous and anti-CD3–induced apoptosis of CD4 and CD8 T cells is shown for individual patients, separated according to their ex vivo % of CD4 T cells.

on activated human T cells [38], and to be highly expressed on peripheral T cells from HIV-infected children [24]. We and others recently reported that CD95 expression was increased in T cells from adult HIV-infected persons, and the cells were highly sensitive to apoptosis triggered through ligation of CD95 [25–30]. One of the possible mechanisms of tRA-dependent inhibition of activation-induced apoptosis is interference in the induction of CD95 expression on T cells following anti-CD3 stimulation. Therefore, CD95 expression was analyzed on CD4 and CD8 T cells from HIV-infected persons and controls after 24 h of activation with anti-CD3 MAbs in the presence or absence of tRA (10 μM). Anti-CD3 activation of T cells from patients induced an increase in the mean fluorescence intensity of CD95, which was unchanged when tRA was added to the stimulated culture (figure 4B). In addition, comparison of the percentages of CD4+CD95+ or CD8+CD95+ cells among patients’ or controls’ total CD4 or CD8 T cells in unstimulated or stimulated cultures indicated that anti-CD3 activation induced an increase in the percentage of CD95+ cells that was not affected by tRA (figure 4A). The absence of effect of tRA on anti-CD3–induced CD95 expression was observed on T cells from control donors and from HIV-infected persons, whatever the stage of the disease (figure 4A). To address whether tRA affected CD95 signaling, PBMC from patients were incubated in the presence of the agonistic anti-CD95 MAbs (CH-11, 1 μg/mL), and tRA (10 μM) was added simultaneously or 6 h later. The rate of apoptosis was similar in the presence or absence of tRA, suggesting that tRA had no influence on the CD95 signaling pathway (data not shown).

tRA prevents anti-CD3–induced apoptosis in patients’ T lymphocytes through inhibition of CD95L expression. Since tRA did not affect CD95 expression, we tested whether its inhibitory effect on anti-CD3–induced apoptosis of patients’ T cells was related to interference of CD95L expression. As reported by
Figure 4. Absence of effect of tRA on activation-induced CD95 expression on CD4 and CD8 T lymphocytes from HIV-infected donors. A. Peripheral blood mononuclear cells (5 × 10^6/mL) from control donors (n = 4) or HIV-infected persons at different stages of disease (CD4 cells >29%, n = 9; 14%-29%, n = 9; <14%, n = 9) were cultured overnight in medium or activated by 0.1 μg/mL anti-CD3 monoclonal antibodies in presence or absence of 10 μM tRA. Percentage of CD95 expressing cells among CD4 and CD8 T lymphocytes was determined by cytofluorometry at end of culture. Data represent mean ± SE of % of CD95<sup>+</sup> cells in each subset. B. Comparative flow cytometry profiles of CD95 expression on CD4 T cells from representative HIV-infected donor, following overnight stimulation under conditions described above.

Tanaka et al. [37], sequential activation with concanavalin A plus IL-2 and subsequently with PMA plus ionomycin induces CD95L expression in PBMC from healthy donors (figure 5A). Similarly, CD95L can be detected in PBMC from HIV-infected persons that are activated under the same conditions (figure 5A). With this protocol of activation, CD95L induction was observed for all patients tested. Anti-CD3 activation could also induce CD95L expression on PBMC from healthy and infected donors, but a significant increase in CD95L in stimulated cultures compared with nonstimulated ones was optimally detected after 48 h of stimulation (figure 5B, lanes 4 and 5). This specific inhibitory effect of tRA was confirmed in a functional assay. PBMC from control donors or HIV-infected persons were stimulated for 3 h with anti-CD3 MAb to induce CD95L expression and were cocultured for 15 h with radio-labeled U937 cells, which had been pretreated with IFN-γ (200 U/mL) to induce CD95 expression. This coculture induced cell death by apoptosis of CD95<sup>+</sup> U937 cells through a CD95-based mechanism, provided that CD95L was expressed by anti-CD3-activated lymphocytes.
Taken together, our data show that tRA inhibited the activation-dependent induction of CD95L expression on T cells and support the hypothesis that the prevention of anti-CD3-induced apoptosis of patients’ T cells under the influence of tRA is the consequence of decreased CD95–CD95L interaction.

Discussion

In the present study, we investigated the influence of tRA on ex vivo spontaneous and activation-induced apoptosis of CD4 and CD8 T lymphocytes from HIV-infected persons at different stages of the disease. Retinoids are a group of natural and synthetic vitamin A derivatives that modulate the growth and differentiation of many different cells in vivo and in vitro. tRA is the high-affinity ligand for retinoic acid receptors, and it was found to prevent specifically TCR-induced apoptosis of murine T cell hybridomas and thymocytes, while not affecting TCR-mediated cellular activation itself [39]. tRA was also found recently to inhibit ex vivo activation-induced apoptosis of PBMC from HIV-infected persons [34]. However, the cell subsets involved were not studied, and the mechanism of inhibition not addressed.

In the present report, we confirm the ability of tRA to prevent ex vivo activation-induced apoptosis of PBMC from HIV-infected persons, and we show that the inhibitory effect of tRA concerns essentially the CD4 T cell subset. Moreover, the prevention of apoptosis in anti-CD3–stimulated T cells from patients was found to be associated with the inhibition of CD95L induction following TCR triggering. Therefore tRA prevents ex vivo activation-induced apoptosis in patients’ PBMC by inhibiting the CD95 pathway.

The reason why not only T cells but also B cells, NK cells [22, 23], and accessory cells [31] from HIV-infected patients undergo apoptosis is not clear, but several mechanisms have been proposed. The in vivo priming of CD4 T cells by gp120 and subsequent deletion following T cell triggering [33, 40] would represent a CD4-specific mechanism. The possible defective antigen-presenting cell function [41] or alteration in the pattern of cytokines produced [42, 43] in patients might contribute to apoptosis of both CD4 and CD8 T cells. However, recent studies, including analyses of patients’ lymph-nodes, suggested that apoptosis detected in B and T cell subsets was related and caused by the continuous stimulation of the immune system observed throughout HIV infection [22, 23, 31]. This hyperactivation causes the dysregulation of the expression of survival (Bcl-2) and death (CD95, CD95L) molecules, inducing a preapoptotic state in patients’ circulating lymphocytes [22–31].

We recently reported that tissue transglutaminase (tTG) is highly expressed in lymph nodes of HIV-positive patients, on lymphocytes as well as dendritic cells, while undetectable in lymph nodes of control donors; it is also selectively expressed in blood CD4 T cells from patients, thus defining a preapoptotic state.

Quantification of apoptosis in U937 cells was performed by calculating the percentage of fragmented DNA released, as described in Materials and Methods, and the results were expressed as the increase in fragmented DNA released above the background level of apoptosis (10%–15%) in cocultures of U937 cells and unstimulated PBMC. Cocultures of IFN-γ–pretreated U937 cells with anti-CD3–stimulated PBMC of control donors were associated with a slight increase of apoptosis in the U937 cells (figure 6). This was more pronounced when anti-CD3–stimulated lymphocytes came from HIV-infected patients (figure 6). This induction of apoptosis in U937 cells was CD95-mediated, since it was almost completely prevented by preincubation of IFN-γ–pretreated U937 cells with anti-CD95 neutralizing antibodies (figure 6). Patients’ PBMC that were stimulated with anti-CD3 in the presence of tRA became unable to induce apoptosis in IFN-γ–pretreated U937 cells, in a manner similar to that occurring when U937 were protected by anti-Fas neutralizing antibodies (figure 6). For a control, we checked that neither tRA nor anti-CD95 neutralizing antibodies had effect when added to cocultures of IFN-γ–pretreated U937 cells and unstimulated PBMC (figure 6). We also observed that tRA had no blocking effect on CD95-induced death of U937 cells, whether tRA was added at the same time as agonistic anti-CD95 MAbs (CH-11, 1 μg/mL) or 6 h later (anti-CD95 MAbs induced 24.5% ± 2.2% of U937 apoptosis vs. 26.5% ± 1.8% and 24.2% ± 3.1% for anti-CD95 plus 10 μM tRA added at time 0 or 6 h later, respectively). These data suggest that tRA does not influence CD95 signaling.

Figure 5. Inhibition by tRA of CD95L expression by anti-CD3–stimulated lymphocytes. A, Peripheral blood mononuclear cells (PBMC) from control donor (lane C) and 3 HIV-infected patients (P1–P3) were sequentially stimulated with concanavalin A plus interleukin-2 and PMA plus ionomycin to induce CD95L expression [47]. CD95L was detected by Western blot analysis. B, PBMC from HIV-infected patient were cultured in medium (lanes 1 and 4), stimulated with coated anti-CD3 monoclonal antibodies in absence (lanes 2 and 5) or presence (lanes 1 and 6) of 10 μM tRA. Western blot analysis of CD95L was performed after 24 h (lanes 1–3) or 48 h (lanes 4–6) of stimulation. Representative experiment is shown.
Figure 6. Inhibition by tRA of CD95L activity of anti-CD3–stimulated lymphocytes from control donors or HIV-infected patients. Radiolabeled interferon (IFN)-γ–pretreated U937 cells were cocultured with peripheral blood mononuclear cells (PBMC) from control donors (n = 3) or HIV-infected patients (n = 6), previously activated for 3 h by 0.1 μg/mL anti-CD3 monoclonal antibodies (MAbs), in presence or absence of 10 μM tRA. In some experiments, IFN-γ–pretreated U937 cells were preincubated with neutralizing anti-Fas MAb (70 ng/mL), as indicated. Data are mean ± SE % of fragmented DNA released above background level, as indicated in Materials and Methods.

The present report suggests that tRA prevents anti-CD3–induced apoptosis of patients’ lymphocytes by interfering in the CD95 pathway. CD95 is highly expressed on PBMC from HIV-infected persons, and at the AIDS stage, around 80%–100% of these cells are CD95-positive [24–30]. Increased expression of CD95 is detected on both CD4 and CD8 T cells from patients, and those cells are susceptible to apoptosis induced by CD95 triggering [25, 28–30]. CD95L expression can be induced following T cell activation [14], and we show here that the increased apoptosis induced by anti-CD3 stimulation in patients’ PBMC is associated with an induction of CD95L expression. Moreover anti-CD95L MAbs could inhibit anti-CD3–induced apoptosis (data not shown), in agreement with previous observations of Bäumler et al. [45], who reported similar effects of anti-CD95 MAbs. Therefore, activation-induced apoptosis in patients’ PBMC is probably mediated via the engagement of the CD95 receptor by activation of up-regulated CD95L.

The CD95 pathway might also be involved in spontaneous apoptosis. Thus, neutralizing anti-CD95 MAbs inhibited partially spontaneous apoptosis of T cells from HIV-positive patients in the late stage of disease, whereas no effect was observed on patients with earlier disease stages (data not shown). This would suggest that, in AIDS, spontaneous apoptosis is partially mediated by CD95L, which is released by the patient’s hyper-
activated lymphocytes and promotes apoptosis on CD95-positive susceptible lymphocytes in this patient.

tRA inhibited activation-induced apoptosis by blocking the activation-dependent induction of CD95L expression on T cells. These findings are consistent with several recent reports demonstrating that tRA inhibits TCR-mediated apoptosis in T cell hybridomas by blocking the expression of CD95L [46–48]. The selective effect of tRA on the CD4 T cell subset from patients is intriguing. It would suggest that apoptosis of patients’ CD4 T cells preferentially involves the CD95 death pathway. This is indeed compatible with observations of Katsikis et al. [25] and our own, showing that, following ligation of the CD95 receptors with agonistic antibodies, patients’ CD4 T lymphocytes are more susceptible than CD8 T lymphocytes to CD95-based apoptosis. This is also in agreement with a recent study of the normal immune system, which showed that the CD95 pathway mediates TCR-induced apoptosis of most CD4 T cells, while tumor necrosis factor mediates the death of CD8 T cells [49].

tRA is currently used to treat acute promyelocytic leukemia and has recently been investigated as a possible treatment for AIDS-related Kaposi’s sarcoma [50]. Yang et al. [34] recently administered tRA orally to 6 HIV-infected patients and observed a reduction in ex vivo activation-induced apoptosis of PBMC from treated patients compared with nontreated ones. No overall elevation of circulating CD4 T cells was noted in patients undergoing tRA therapy [34] but, as discussed by the authors, such an effect would not be expected from this small and time-limited study.

The in vitro influence of tRA on activation-induced CD95L expression makes it attractive as a means of affecting pathologic apoptosis. The in vivo persistence of HIV associated with the unceasing expression of retroviral antigens is probably the primary mechanism for the chronic stimulation of the immune system. This mechanism is responsible for the dysregulation in the expression of molecules involved in the balance between cell survival and cell death, such as Bel-2 and CD95, respectively down-regulated and up-regulated in patients’ lymphocytes [23, 24–30]. It also triggers the expression of CD95L [26, 45], thus creating appropriate conditions for excessive CD95-based peripheral T cell deletion throughout HIV infection.

Recently, combination antiretroviral therapies, including anti-HIV proteases, have been shown to be quite efficient in reducing the plasmatic virus load in HIV-infected persons [51, 52] and to limit the development of AIDS. A decrease in lymphocyte activation and in the ex vivo expression of CD95L [53] was reported in T cells of patients under combination antiretroviral therapy, and we found that the rate of spontaneous and activation-induced apoptosis was significantly reduced in treated patients, concomitant with decreased CD95 expression (Gougeon ML, et al., unpublished data). However, these combined therapies are not always successful from an immunologic point of view, and integrated treatments that will help interrupt the viral triggering of apoptotic cell death should be considered. The coadministration of tRA, involved in the regulation of T cell apoptosis [44], with antiretroviral therapy might represent a strategy to sustain T cell numbers and recover immune functions in AIDS.

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