Apoptosis induced by HIV-gp120 in a Th1 clone involves the generation of reactive oxygen intermediates downstream CD95 triggering

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Abstract HIV-gp120 sensitizes Th1 clones from seronegative donors to apoptosis, which occurs through two distinct events: expression of CD95L followed by its interaction with CD95 to trigger cell death. gp120-apoptosis of the Th1 clone 103 was inhibited by Cyclosporin A, the PTK inhibitors Genistein and PNU152518, as well as the anti-oxidants Ascorbic Acid and Glutathione. Cyclosporin A interfered with CD95L expression, Ascorbic Acid and Glutathione inhibited cell death triggered by CD95/CD95L interaction; Genistein and PNU152518 acted on both steps. The occurrence of oxidative stress during CD95-dependent apoptosis was supported by the direct evidence of ROI production.

Key words: HIV; gp120; Apoptosis; ROI; PTK; $CD4^+$ Th1 clone

1. Introduction

We have previously reported [1] that, following HIV-gp120/CD4 cross-linking and CD3/TCR activation, a human Th1 clone (clone 103) from an HIV-seronegative donor underwent apoptosis (thereafter indicated as gp120-apoptosis). In this system, either gp120/CD4 cross-linking or CD3/TCR stimulation alone did not induce relevant cell death. These observations were consistent with results obtained by others using polyclonal $CD4^+$ populations and suggested that apoptosis of uninfected $CD4^+$ cells could contribute to $CD4^+$ lymphocyte depletion in AIDS patients [2]. The CD95 (Fas/APO-1)/CD95L (Fas-L) cell death pathway was related to AIDS pathogenesis as suggested by the increased CD95 expression and increased susceptibility to CD95-mediated apoptosis observed on T lymphocytes from HIV-infected individuals [3–5].

More recently [6], we obtained evidence for a differential susceptibility of Th1 and Th2 clones to gp120-apoptosis depending on different expression of CD95L, which is upregulated in Th1 but not Th2 clones. A two-step model for gp120-apoptosis in Th1 clones could be formulated: treatment with gp120/anti-gp120/anti-TCR induces significant expression of membrane-anchored and soluble functional CD95L (step 1); the subsequent interaction (in autocrine or paracrine fashion) of CD95L with its receptor, CD95, triggers cell death (step 2).

The aim of the present work was to identify agents able to rescue Th1 clones from gp120-apoptosis, interfering with either step 1 or step 2. Several different potential inhibitors, chosen because effective in other models of apoptosis [7], were tested.

Cyclosporin A is an immunosuppressant affecting the calmodulin/calcineurin signal transduction pathway and already known to block activation-induced cell death by interfering with CD95L induction [8,9]; thus, it was expected to inhibit gp120-apoptosis, which represents a form of activation-induced cell death [6].

Genistein is a natural, broad spectrum inhibitor of PTKs [10], whereas PNU152518, previously named TMO [11] or FCE26806 [1,12], is a PTK inhibitor with a more restricted activity, known to block Met [11] and Abl [12]. The involvement of PTKs in TCR-dependent signal transduction and in apoptosis has been shown in a variety of systems [7], but there is not general consensus on whether they are required for CD95-mediated apoptosis [13–19].

The involvement of oxidative injure in apoptosis has been reported in various models [20] and the water-soluble anti-oxidants Ascorbic acid and Glutathione are known to block some forms of apoptotic cell death [7]. Nonetheless, the role of oxidative stress in CD95-mediated cell death is still debated [15,21–27].

After identification of agents blocking gp120-apoptosis, we determined which of them act by inhibiting induction of functional CD95L and which act downstream the triggering of CD95.

Inhibition of CD95-mediated death in our Th1 clone by anti-oxidants indicated a role for oxidative damage downstream CD95 triggering. To confirm this hypothesis, the generation of ROI following CD95 crosslinking was directly indagated.

Our data could contribute to the comprehension of cellular mechanisms involved in gp120-apoptosis, as well as suggest the use of anti-oxidants as adjuvants in therapeutic strategies aimed at counteracting the depletion of uninfected $CD4^+$ lymphocytes during AIDS progression.

2. Materials and methods

2.1. Cells

Clone 103, a human $CD3^+$ $CD4^+$ Th1 clone, has been previously described [1]. It was cultured at 37°C in a 5% $C0_2$ atmosphere, in complete medium composed of RPMI 1640 (MA Bioproducts, Walkersville, MD) supplemented with 2 mM glutamine (MA Bioprod-

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Abbreviations: TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; DHR, dihydrorhodamine-123; MFI, mean fluorescence intensity; PTK, protein tyrosine kinase; ROI, reactive oxygen intermediates

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ucts), 20 mM HEPES buffer (MA Bioproducts), 100 U/ml penicillin and 100 μg/ml streptomycin (Pharmacia, Milan, Italy) plus 10% pooled human heat-inactivated serum. IL-2 (Proleukin, Eurocetus, Amsterdam, The Netherlands) was added at a final concentration of 180 IU/ml. Clone 103 cells (2×10^5/well) were cultured in 24 well plates (Costar, Cambridge, MA) and weekly restimulated with irradiated allogeneic PBL (4×10^5 cells/well) and 1 μg/ml PHA (Murex, Temple Hile Dartford, UK).

Six days after the last restimulation, cells were washed twice with medium, then used in experiments.

2.2. mAbs

The following mAbs were used: BMA031 (anti-TCR); CH-11 (anti-CD95 IgM, apoptosis inducing), ZB4 (anti-CD95 IgG1, neutralizing) (both from MBL Co., Nagaya, Japan); anti-gp120 (HIV-1) (Intracel, London, UK). Suitable isotype-matched controls (Cymbus Bioscience LTD, Southampton, Hants., UK) were employed.

2.3. Induction of apoptosis

2.3.1. gp120 protocol. 10^7 cells/ml were treated with recombinant HIV-1 gp120 (Intracel, London, UK) (10 μg/ml, 30′ at 37°C), washed once, then treated with mouse anti-gp120 (HIV-1) mAb (10 μg/ml, 30′ at 4°C), washed once and seeded (10^6 cells/w) in 48 well plates coated with anti-TCR mAb (BMA031, 5 μg/ml in PBS, 1 h at 37°C). After 4 h, apoptosis was evaluated by TUNEL [1,6] and confirmed by propidium iodide staining and morphological analysis. Net % gp120-apoptosis was calculated as: % apoptotic cells after induction (gp120/anti-gp120/immobilized anti-TCR)−% apoptotic cells after activation (immobilized anti-TCR only). Positive and negative controls for apoptosis were irradiated cells (10 000 Rad) and cells maintained in IL-2 (180 IU/ml), respectively (not shown).

2.3.2. Anti-CD95 protocol. 10^6 cells/well were seeded in 48 well plates (Costar, Cambridge, MA) and cultured in the presence of different doses of agonistic anti-CD95 mAb CH-11. After 4 h, apoptosis was evaluated as described for gp120-protocol. Net % anti-CD95-apoptosis was calculated as: % apoptotic cells in the presence of CH-11−% apoptotic cells in medium only.

2.4. Inhibition of apoptosis

For inhibition experiments, cells (10^7/ml) were pre-treated for 30′ at 37°C with medium only or with the following agents, at doses previously determined to be non-cytotoxic (not shown): Cyclosporin A (Sandimmun, Sandoz AG, Basel, Switzerland) (50 μg/ml), Genistein (Calbiochem, San Diego, CA) (10 μM), PNU152518 (Pharmacia and Upjohn, Milan, Italy) (10 μM), L-Ascorbic Acid (Sigma Chemical Co, St. Louis, MO) (500 μM) or Glutathione (Tationil, Boehringer Mannheim Italia, Milan, Italy) (500 μg/ml). Apoptosis was then induced following gp120 or anti-CD95 protocol. Inhibitors were maintained (at the same doses used for pre-treatment) throughout all passages of apoptosis induction and culture.

For each experiment, relative % gp120-apoptosis or anti-CD95-apoptosis in the presence of any given inhibitor was calculated as follows: [(% apoptosis with inhibitor/% apoptosis without inhibitor)×100].

2.5. Assay for functional CD95L

Functional CD95L expression was evaluated as previously described [6]. Briefly, clone 103 cells were pre-treated with 1 μg/ml neutralizing anti-CD95 mAb ZB4 (30′ incubation on ice, then extensively washed) to prevent possible CD95L sequestration by CD95 receptors on the surface of the producing cells, then cultured for 1 h following the protocol for induction of gp120-apoptosis. Cells were then collected, washed and used as effectors in 16 h ^{51}Cr release assay against CD95$^+$ Jurkat cells (E/T = 2). The assay was performed in triplicate.

For inhibition experiments, cells were treated with inhibitors (same doses used for apoptosis experiments) 30′ at 37°C prior to CD95L induction.

2.6. Measure of endocellular ROI

DHR (Molecular Probes, Eugene, OR) was used to detect the production of ROI. The fluorescence of this cell-permeable probe is significantly increased after oxidation, particularly by hydroperoxides [28]; the oxidized form, rhodamine-123, is sequestered by active mitochondria and is not subjected to significant leakage from cells maintained at 37°C. A modification of a previously published protocol [29] was used. Cells (10^5/sample in 0.1 ml medium) were loaded with 1 μM DHR (30′ at 37°C), then treated with 1 μg/ml agonistic anti-CD95 mAb CH-11 or with 100 μM H_2O_2, respectively. Samples were harvested at the indicated times and immediately analyzed (5000 cells/sample) by FACScan.

For inhibition experiments, neutralizing anti-CD95 mAb ZB4 (50 ng/ml) or Glutathione (500 μg/ml) were added during DHR loading (30′ at 37°C), then maintained throughout all phases of the assay. Generation of ROI was revealed by an increase in MFI.

3. Results

3.1. Effect of various agents on gp120-apoptosis

A representative experiment showing gp120-apoptosis and apoptosis induced by agonistic anti-CD95 mAb is reported in Fig. 1A and B, respectively. A series of potential cell death inhibitors were screened for in vitro effects on gp120-apoptosis (Fig. 2A). gp120-apoptosis was prevented by the immunosuppressive drug Cyclosporin A, by the PTK inhibitors Genistein and PNU152518, as well as by the anti-oxidant agents Ascorbic Acid and Glutathione. As already reported [6], gp120-apoptosis was also efficiently inhibited by the neutralizing anti-CD95 mAb ZB4, a finding consistent with the involvement of CD95/CD95L interaction.

3.2. Effect of inhibitors of gp120-apoptosis on functional CD95L expression

We then assessed whether agents able to inhibit gp120-apoptosis could block induction of CD95L. Functional CD95L was detected by ^{51}Cr-release assay on CD95$^+$ Jurkat cells (Fig. 2B). Th1 cells treated for gp120-apoptosis had significantly higher lytic activity than cells cultured with anti-TCR only (Fig. 2B) or in medium only (not shown). The lysis was CD95-dependent, as it was inhibited by the neutralizing anti-CD95 mAb ZB4, but not by an irrelevant isotype-matched control ([6] and data not shown); moreover, in the same experiment, no lytic activity was seen against the CD95$^-$ cell line K562 (not shown). A significant decrease of gp120-induced lytic activity was observed in the presence of the immunosuppressive drug Cyclosporin A, known to block CD95L induction in other systems [8,9], as well as with the PTK inhibitors Genistein and PNU152518.

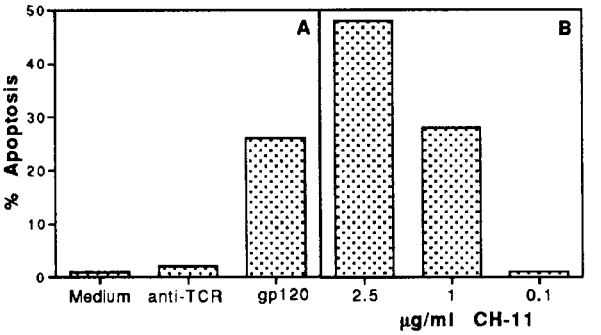

Fig. 1. Apoptosis induced in a Th1 clone by gp120 (A) or by agonistic anti-CD95 mAb CH-11 (B). Clone 103 was cultured in medium, activated with immobilized anti-TCR or treated for gp120-apoptosis (A); alternatively, it was seeded in the presence of the indicated doses of agonistic anti-CD95 mAb CH-11 (B). After 4 h, apoptosis was evaluated by TUNEL. At time 0, apoptotic cells were 2%.

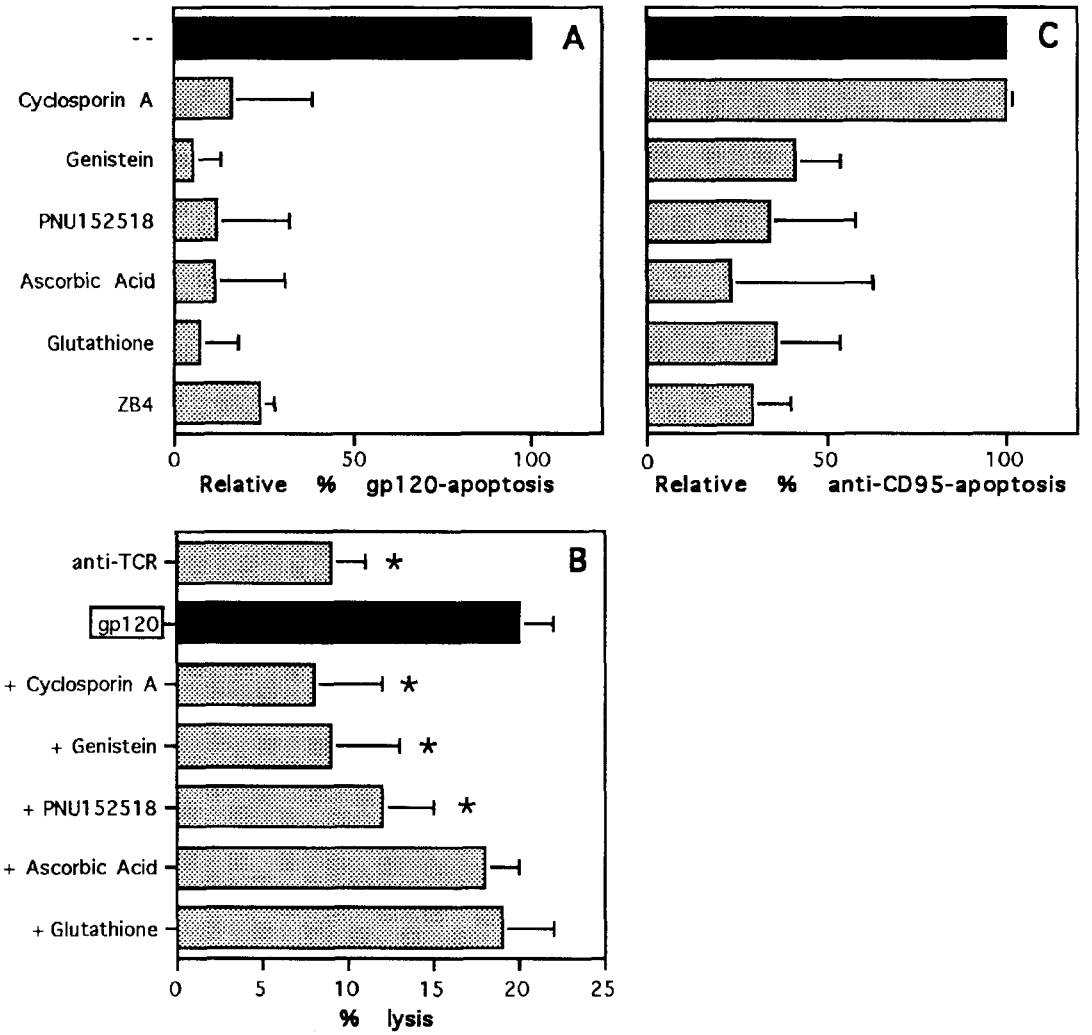

Fig. 2. Effect of various agents on gp120-apoptosis, functional CD95L expression and anti-CD95-apoptosis. (A) Clone 103 was treated for gp120-apoptosis in medium only or in the presence of the indicated agents. After 4 h, apoptosis was evaluated by TUNEL. Data are expressed as relative % gp120-apoptosis (mean ± S.D. from at least three independent experiments). Net gp120-apoptosis in the absence of inhibitors was 17 ± 5% (mean ± S.D. from 7 independent experiments). (B) Th1 clone 103 was activated with anti-TCR only or treated for the induction of gp120-apoptosis in medium only or in the presence of the indicated agents. After 1 h, cells were collected, washed and tested in a 16 h ^{51}Cr-release assay against labelled CD95$^+$ Jurkat cells. * $P < 0.05$ (Student's t-test). (C) Clone 103 was treated with 1 μg/ml agonistic anti-CD95 mAb CH-11 in medium only or in the presence of the indicated agents. After 4 h, apoptosis was evaluated by TUNEL. Data are expressed as relative % anti-CD95-apoptosis (mean ± S.D. from at least three independent experiments). Net anti-CD95-induced apoptosis in the absence of inhibitors was 30 ± 15% (mean ± S.D. from 5 independent experiments).

3.3. *Effect of inhibitors of gp120-apoptosis on cell death triggered by agonistic anti-CD95 mAb*

To test which inhibitors of gp120-apoptosis act downstream CD95L/CD95 interaction, agonistic anti-CD95 mAb was used to induce cell death (Fig. 2C). Apoptosis was blocked by the PTK inhibitors Genistein and PNU152518 as well as by the anti-oxidants Glutathione and Ascorbic Acid but not by Cyclosporin A. As expected [6], the neutralizing anti-CD95 mAb ZB4 also prevented cell death.

In summary, our results indicate that Cyclosporin A inhibits gp120-apoptosis by interfering with CD95L induction, Ascorbic Acid and Glutathione by interfering with the effector phase downstream CD95 triggering; Genistein and PNU152518 act on both steps.

3.4. *Generation of ROI following CD95 triggering*

To directly prove the generation of ROI downstream CD95 cross-linking the cell-permeable probe DHR was used (Fig. 3). An increase in MFI, indicative of ROI generation, was evident after direct triggering of CD95 receptor by agonistic anti-CD95 mAb CH-11. This MFI shift was prevented by either addition of the neutralizing anti-CD95 mAb ZB4 or the anti-oxidant Glutathione. As a positive control for oxidative stress, cells were treated with H_2O_2; as expected, the observed increase in MFI was prevented by Glutathione, but not by ZB4. Similar results were obtained from 3 independent experiments. The MFI shift induced by either CH-11 or H_2O_2 was also inhibited by Ascorbic Acid (not shown). A significant increase in MFI was also seen following treatment with recombinant human CD95L (not shown).

Fig. 4 shows a time-course experiment in which ROI were detected as soon as 90′ after CD95 triggering, peaked at 3 h and then remained elevated up to 20 h. To further support the hypothesis that generation of ROI was linked to cell death,

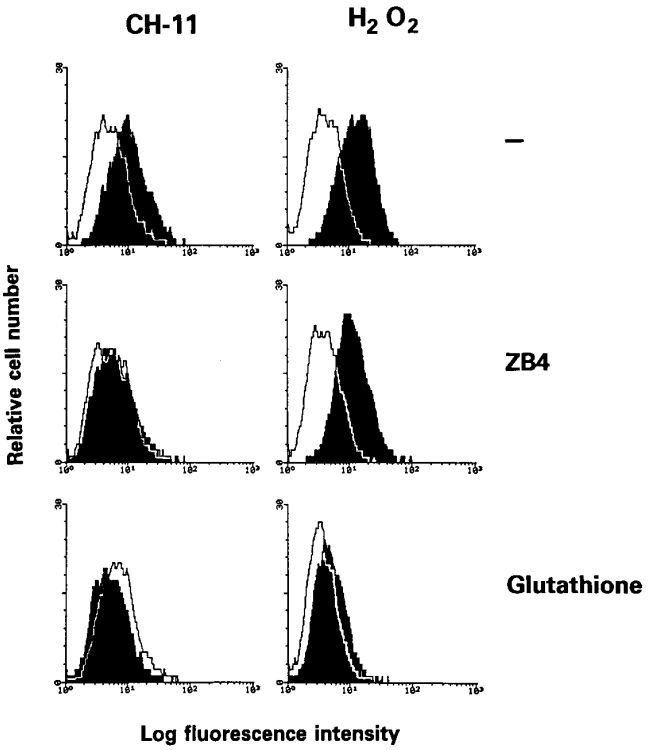

Fig. 3. Endocellular generation of ROI. Cells were loaded with DHR, then treated with 1 μg/ml agonistic anti-CD95 mAb CH-11 (left panels) or 100 μM H_2O_2 (right panels). After 3 h, they were collected and analyzed by FACScan. The experiment was performed in medium only (–) and in the presence of 50 ng/ml neutralizing anti-CD95 mAb ZB4 or 500 μg/ml Glutathione. Cells treated with CH-11 or H_2O_2 (closed areas) were compared with the corresponding untreated cells (open areas).

the presence of apoptotic cells was scored along with DHR fluorescence on the same sample at the indicated time points. Apoptosis was indeed increasing up to 20 h.

4. Discussion

Apoptosis of uninfected $CD4^+$ lymphocytes participates in AIDS progression and a variety of in vitro models have been developed to investigate the potential mechanisms involved in this phenomenon [2]. gp120-apoptosis (resulting from treatment with gp120/anti-gp120 mAb and immobilized anti-TCR or -CD3 mAb) could be detected on isolated $CD4^+$ lymphocytes [2] as well as on $CD4^+$ Th1 clones [1,6] from HIV-seronegative donors. We recently reported [6] that Th1 but not Th2 clones are highly susceptible to gp120-apoptosis and that this differential sensitivity depends on different expression of CD95L, which is upregulated in Th1 but not Th2 cells.

In Th1 clones, gp120-apoptosis can be regarded as a two step phenomenon: treatment with gp120/anti-gp120/anti-TCR induces CD95L expression (step 1); then, interaction between CD95L and its receptor, CD95, triggers cell death (step 2). This model is consistent with the hypothesis that gp120-apoptosis could represent a form of activation-induced cell death

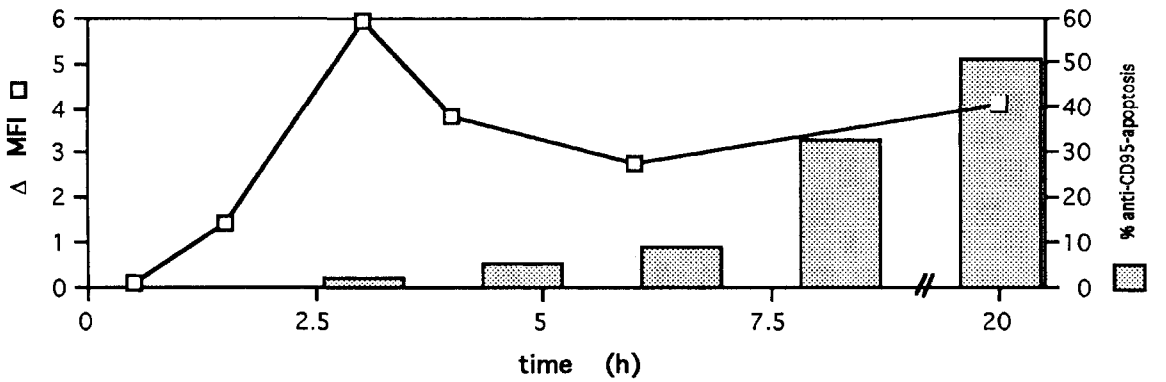

Fig. 4. Kinetics of ROI generation and cell death following CD95 triggering. Cells were loaded with DHR, treated with 1 μg/ml agonistic anti-CD95 mAb CH-11, collected at the indicated times and analyzed by FACScan. Data are expressed as ΔMFI=MFI of treated cells−MFI of untreated cells at the same time point. % anti-CD95-apoptosis was evaluated on the same samples, on the basis of low FSC-H/high SSC-H characteristics of apoptotic cells.

[6], a physiologic phenomenon involved in peripheral cell selection and immune response regulation and clearly dependent on CD95L/CD95 interaction [30].

CD95-mediated apoptosis has been extensively studied in a variety of systems and a detailed model for signal transduction has been proposed, based on the knowledge of adaptor molecules and proteolytic enzymes activated downstream CD95 triggering [30]. However, some aspects, such as the role of protein tyrosine phosphorylation [13–19] as well of oxidative stress [15,21–27], remain controversial. The present work was aimed at studying the effect of PTK inhibitors, anti-oxidants and Cyclosporin A on gp120-apoptosis of Th1 clones.

In our system, Cyclosporin A was able to block gp120-apoptosis (Fig. 2A). The finding that this immunosuppressant interferes with CD95L induction (step 1, Fig. 2B) rather than with the effector phase downstream CD95 triggering (step 2, Fig. 2C) suggested that signal transduction through the calmodulin/calcineurin pathway should be required for gp120-induced CD95L expression. This was in agreement with the observation that Cyclosporin A blocks activation-induced apoptosis by interfering with CD95L expression [8,9]. The efficacy of Cyclosporin A in inhibiting gp120-apoptosis could add a new rationale for its therapeutic use in AIDS, since it has already been proposed on the basis of other observations such as the inhibition of HIV replication in vitro and the delay of AIDS onset in HIV-infected patients treated with Cyclosporin A [31].

The role of PTKs in apoptosis has been suggested in a variety of systems [7]. Here we show that both the broad-spectrum inhibitor Genistein [10] and the more specific agent PNU 152518 [1,11,12] interfere with both steps of gp120-apoptosis. The finding that the activity of one or more PTKs may be required for CD95L induction is consistent with the relevance of tyrosine phosphorylation in TCR signalling [32], whereas the role of PTKs in apoptosis initiated by CD95 triggering is still controversial. Some studies have shown that, in the Jurkat T cell line, CD95-mediated apoptosis is not affected by PTK inhibitors [14,15] and does not imply protein tyrosine phosphorylation [14,16]; moreover, evidence that PTKs such as Abl could have an anti-apoptotic effect has recently been reported [33]. On the contrary, other studies have shown that PTKs participate in CD95-dependent early signalling events [13,17–19]. Our data, obtained in a normal Th1 clone, are clearly in agreement with these last results. Although further work is needed to identify the specific PTKs involved in gp120-apoptosis, the possibility of using PTK inhibitors for AIDS therapy is attractive, since they could reduce $CD4^+$ cell apoptosis as well as interfere with virus replication [34] and its cytopathic effect [35].

Oxidative stress has also a role in AIDS pathogenesis [20]. HIV-infected individuals exhibit systemic Glutathione deficiencies [36–38] and redox balance alterations in their $CD4^+$ lymphocytes [39,40]. Although oxidative stress is a relevant step in many models of apoptosis [20], its role in CD95-mediated apoptosis is still debated. Studies addressing this aspect in monocytes [27] and T cell leukemia cells [25,26] have shown that oxidative injure is part of CD95-dependent apoptosis. On the contrary, studies performed on murine L929 fibroblasts expressing human CD95 [21,22], CD95-sensitive human melanoma [24] and lymphoid cell lines [23,24], presented contrasting evidence.

Here we show that the anti-oxidants Ascorbic Acid and Glutathione efficiently block gp120-apoptosis of our Th1 clone downstream CD95 triggering, a result consistent with the involvement of oxidative stress in this cell death pathway. The possibility that Glutathione may block CD95-mediated cell death by decreasing CD95 expression [41] has been excluded, as no differences were observed between untreated and inhibitor-treated cells (data not shown).

Moreover, we present a direct evidence that CD95-initiated apoptosis in Th1 lymphocytes involves generation of ROI (Figs. 3 and 4), a finding previously reported for monocytes [27] and adult T leukemia cells [26] but not for normal T lymphocytes.

These results should give strength to the rationale for therapeutic use of anti-oxidants in AIDS [42].

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