HIV/gp120 and PMA/ionomycin induced apoptosis but not activation induced cell death require PKC for Fas-L upregulation

Paola Accornero[a], Marina Radrizzani[a], Alessandra Carè[b], Gianfranco Mattia[b], Claudia Chiodoni[a], Roland Kurrle[c], Mario P. Colombo[a,*]

[a] *Division of Experimental Oncology D, Istituto Nazionale Tumori, via Venezian 1, 20133 Milan, Italy*
[b] *Istituto Superiore di Sanità, Rome, Italy*
[c] *Behringwerke AG, Marburg, Germany*

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Abstract HIV protein gp120 in combination with T cell antigen receptor (TCR) triggering induces apoptosis (gp120-apoptosis) in Th1 cells. Gp120-apoptosis occurs by induction of Fas-L and subsequent triggering of the Fas apoptotic pathway. Here, through the use of several compounds inhibiting induction of Fas-L, we show that, in a Th1 clone, a protein kinase C (PKC) independent pathway activated by TCR stimulation is distinguishible from a PKC dependent pathway activated by either phorbol 12-myristate 13-acetate (PMA)/ionomycin or asynchronous stimulation of TCR and CD4 as occurs in gp120-apoptosis.

Key words: Human immunodeficiency virus; gp120; Apoptosis; Protein kinase C; Fas; $CD4^+$ Th1 clone

1. Introduction

It has been suggested that depletion of $CD4^+$ T lymphocytes during HIV-1 infection results mostly from indirect killing of uninfected cells [1]. Accordingly, $CD4^+$ T cells from healthy donors undergo apoptosis after HIV-gp120/CD4 cross-linking (CD4XL) followed by CD3/T cell antigen receptor (TCR) activation (gp120-apoptosis) [2–4].

The hypothesis that gp120-apoptosis could be an exacerbation of activation induced cell death (AICD) proposed by Westendorp et al. [5], has been confirmed by us in $CD4^+$ Th1 clones [4]. During gp120-apoptosis two phases actually occur: (i) the induction phase, in which Fas(APO-1/CD95)-L is upregulated, and (ii) the effector phase, consisting of apoptotic cell death after Fas/Fas-L interaction [4].

The Fas/Fas-L apoptosis has a role in tissue homeostasis, embryogenesis, immune development and homeostatic regulation of normal immune responses [6]. Fas/Fas-L is likely involved in transplants rejection and graft versus host disease (GVHD) [7]. Alterations of the Fas/Fas-L system have been described in immune disorders such as autoimmune diseases [8] and AIDS [9]. Upregulation of Fas expression and increased sensitivity to Fas-agonist mediated apoptosis have been observed in lymphocytes from HIV^+ patients [9]. HIV-1 infection is, indeed, responsible for Fas-L production by monocytes [10,11] and soluble Fas-L is higher in serum from HIV^+ patients than from healthy donors [9]. These data suggest a role for Fas/Fas-L induced apoptosis in AIDS disease and are strengthened by in vitro studies showing that HIV proteins may induce Fas-L expression in uninfected T cells [4,5,12].

Although the events downstream Fas triggering have been studied in detail [6,13,14], little is known about the pathway leading to Fas-L induction. In T lymphocytes Fas-L upregulation occurs in conditions of hyper-activation (such as in AICD) [15] or following the treatment with phorbol 12-myristate 13-acetate (PMA)/ionomycin (PMA/io) [5,16]. Fas-L can also be induced by CD4 cross-linking (CD4XL) in PBMC [17] and in Th1 clones following TCR stimulation [4].

In our system, several compounds are able to prevent gp120-apoptosis acting either on Fas-L induction or downstream Fas/Fas-L interaction [18]. They include two protein tyrosine kinase (PTK) inhibitors: genistein (with a broad spectrum of activity) and PNU152518 (likely targeting Met or Abl [18]), the immunosuppressant cyclosporin A (known to affect the calmodulin/calcineurin pathway) and the anti-oxidants reduced glutathione and ascorbic acid. PTK inhibitors affect both phases of gp120-apoptosis, whereas cyclosporin A and anti-oxidants impair Fas-L induction and Fas/Fas-L donwstream events, respectively.

Here we evaluated whether gp120-apoptosis induces Fas-L expression through a pathway common to that of PMA/io or that of TCR hyperactivation. Our aim was to inhibit the induction of Fas-L that follows the sequential triggering of CD4 and TCR (as in gp120-apoptosis) but not that induced by TCR triggering only (as in Ag-induced apoptosis). We focused on the role of protein kinase C (PKC) that has been shown necessary for Fas-L induction by PMA/io but not by TCR hyperactivation (AICD) in $CD8^+$ T cells [19]. We tested whether PKC inhibition could impair gp120-apoptosis by regulating Fas-L. Moreover, we evaluated which inhibitors of gp120-apoptosis [18] are able to prevent Fas-L induction by the PKC dependent PMA/io.

2. Materials and methods

2.1. Cells

Clone 103, a human $CD3^+$ $CD4^+$ Th1 clone, has been previously described [3,4].

2.2. Induction of apoptosis

Cells were seeded at 10^6 cells/ml in 48-well plates in the presence or absence of 50 μg/ml precoated (1 h at 37°C) anti-TCR mAb (BMA031) [20] for AICD, 1 μg/ml anti-Fas mAb (CH-11) (MBL, Nagoya, Japan) for anti-Fas-apoptosis, or 10 ng/ml PMA (Sigma,

*Corresponding author. Fax: (39) (2) 2362692.
E-mail: mcolombo@istitutotumori.mi.it

Abbreviations: AICD, activation induced cell death; CD4XL, CD4 cross-linking; GVHD, graft versus host disease; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTK, protein tyrosine kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

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St. Louis, MO, USA) and 500 ng/ml ionomycin (Calbiochem, Novabiochem, La Jolla, CA, USA) for PMA/io-apoptosis. Gp120-apoptosis was induced by treatment with recombinant HIV-1 gp120 (Intracel, London, UK), anti-gp120 and anti-TCR, as described [4]. Cell death was evaluated at 4 h by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) [4].

To represent data from several experiments, % apoptotic cells is expressed as '% relative apoptosis' calculated considering the net value of apoptotic cells in each experiment as 100%. Net % of apoptosis was calculated subtracting % apoptosis in the presence of anti-TCR alone for gp120-apoptosis (12.55 ± 6.55 in 11 independent experiments), or % apoptosis in medium only for PMA/io-apoptosis (14.75 ± 8.6 in 8 independent experiments), AICD (13.67 ± 1.5 in 3 independent experiments) and anti-Fas apoptosis (30 ± 15 in 3 independent experiments).

2.3. Inhibition of apoptosis

For inhibition experiments, cells (10^7/ml) were pre-treated for 30 min 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, Basel, Switzerland) (50 μg/ml), genistein (Calbiochem) (10 μM), PNU152518 (Pharmacia and Upjohn) (10 μM), L-ascorbic acid (Sigma) (500 μM) or reduced glutathione (Tationil, Boehringer Mannheim Italia, Milan, Italy) (500 μg/ml), calphostin C (Sigma) (5 nM), H-7 (Calbiochem) (5 μM). Apoptosis was then induced following the already described protocols. Inhibitors were maintained, at the same doses used for pre-treatment, throughout all passages of apoptosis induction and culture. To test inhibition with calphostin C, samples were kept exposed to light during all passages as calphostin C is not active in the dark [21]. Light exposure, per se, did not affect our results (not shown).

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

2.4. Assay for functional Fas-L

Functional Fas-L expression was evaluated as previously described, by analyzing the ability of clone 103 to lyse Fas^+ Jurkat cells in a Fas-dependent manner [4]. For inhibition experiments, clone 103 cells were treated with inhibitors (same doses and conditions used for apoptosis experiments) 30 min at 37°C prior to and during the incubation for Fas-L induction.

For each experiment, relative % lysis in the presence of any inhibitor was calculated as follows: [(% lysis with inhibitor/% lysis without inhibitor) × 100].

2.5. Immunocytochemical analysis

Cytospins (2×10^5 cells/each) were prepared 1–4 h after induction of gp120- or PMA/io-apoptosis, respectively, with or without inhibitors. The slides were dried and stained within 24 h. Samples were fixed with PFA 4% in PBS, then permeabilized with 0.1% Triton X-100. To neutralize endogenous peroxidases they were treated for 5 min with 3% H_2O_2 in Met-OH. After blocking with 5% pooled human AB heat-inactivated serum, samples were incubated with anti-Fas Ligand clone 33 (Transduction Laboratories, Lexington, KY, USA) 1 h at room temperature, then washed and incubated with a biotinylated anti-mouse secondary antibody (Sigma, St Louis, MO, USA) 30 min at room temperature. To amplify the staining, the TSA-direct Kit (NEN Life Science Products, Brussels, Belgium) was employed according to the manufacturer's indications. Briefly, after blocking, samples were incubated with streptavidin-conjugated HRP for 30 min, then washed and incubated with FITC-conjugated Tyramide for 7 min. Cells were then washed and counterstained 10 min with 25 μg/ml PI (Sigma). Slides were observed under a confocal microscope TCS4D Leica (Leica, Heidelberg, Germany). Negative and positive controls were cells stained with isotype-matched irrelevant mAb (Cymbus Bioscience, Southampton, UK) and anti-MHC class I mAb, respectively (not shown).

2.6. RNA extraction and RT-PCR

Semiquantitative reverse transcription-polymerase chain reaction analysis was performed according to standard procedures. Briefly, total RNA was extracted by the standard guanidium thiocyanate-CsCl technique. Total RNA obtained from 10^5 cells was reverse transcribed with oligo-dT as the primer. After S26 normalization (25 PCR cycles), an aliquot of RT-RNA was amplified. Fas-L RT-RNA amplification within the linear range was obtained by 35 PCR cycles (i.e. this cycle number allowed a linear RT-RNA dose response). PCR products were analyzed by Southern blotting with an internal oligomer as probe. The sequences of the S26 primers are: forward 5′-GCCTCCAAGATGACAAAG-3′ (nucleotides (nt) 15–32) and reverse 5′-CCAGAGAATAGCCTGTCT-3′ (nt 395–412); probe GAGCGTCTTCGATGCCTATGTGCTTCCCAA (from nt 191 to nt 220) [22]. PCR conditions: 95°C, 45 s; 56°C, 45 s; 72°C, 45 s. The sequences of Fas-L primers are: forward 5′-CAAGTCCAACTCAAGGTCCAT-3′ (nt 641–661) and reverse 5′-AACGTATCTGAGCTCTCTCTG-3′ (nt 966–986); probe 5′-ACCTATGGAATTGTCCTGCTTTCTGGAGTG-3′ (nt 681–710) [23]. PCR conditions: 95°C, 45 s; 55°C, 45 s; 72°C, 45 s. The Fas-L expression level, normalized to S26, was analyzed by a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

3.1. PKC role in Fas-L induction during AICD, gp120- or PMA/io-apoptosis

To evaluate whether stimulation of gp120-apoptosis may induce Fas-L through a pathway common to that activated by either PMA/io or hyperactivation of TCR, we tested the role of PKC. Fas-L upregulation was evaluated by its ability to kill, in a Fas-dependent fashion, a sensitive target such as the Jurkat cell line. Cells of $CD4^+$ clone 103 were stimulated to induce AICD, gp120-apoptosis or were stimulated with PMA/io in the presence or absence of the specific PKC inhibitor calphostin C (Fig. 1A).

PKC inhibition could not prevent Fas-L expression during AICD, confirming the recent evidence obtained by Anel et al. in $CD8^+$ T cells [19]. On the contrary calphostin C inhibits Fas-L induction by gp120 and PMA/io (Fig. 1A). It is worth noting that this inhibition takes place only when experiments are performed in the light (Fig. 1A) but not in the dark (not shown), a condition in which calphostin C is inactive [21]. These initial data suggest that PMA/io and gp120, but not AICD, require PKC for Fas-L induction. This hypothesis was confirmed using a different PKC inhibitor, H-7 (not shown).

When the effect of calphostin C was tested on apoptosis, we found that not only gp120-apoptosis and PMA/io-apoptosis

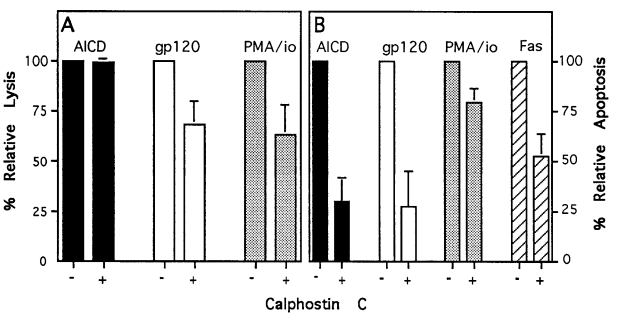

Fig. 1. PKC role in Fas-L (A) and apoptosis induction (B) by gp120, PMA/io and AICD. A: Functional Fas-L expression (cytotoxicity on Jurkat cells) by clone 103 cells stimulated for AICD (closed bars), gp120-apoptosis (open bars) or with PMA/io (shaded bars) were evaluated, in the presence or absence of 5 nM calphostin C under light exposure, 4 h after induction. (Mean ± S.D. of three independent experiments.) B: AICD (closed bars), gp120-apoptosis (open bars), PMA/io-apoptosis (shaded bars) or anti-Fas-apoptosis (dashed bars) in the presence or absence of 5 nM calphostin C under light exposure. (Mean ± S.D. from 2 independent experiments is shown.)

but also AICD were inhibited (Fig. 1B). This finding suggests that, although differentially required for Fas-L induction, PKC is part of the pathway downstream Fas triggering, leading to apoptosis. In fact calphostin C blocks apoptosis directly induced by anti-Fas agonistic mAb (Fig. 1B).

3.2. *A common pathway of Fas-L induction by gp120 or PMA/io stimulation*

PKC was required for Fas-L induction during both gp120- and PMA/io-apoptosis suggesting the activation of a common pathway. Thus we tested whether other compounds already found to block gp120-apoptosis [18] may inhibit PMA/io-apoptosis.

We evaluated induction of apoptosis (Fig. 2A) and functional Fas-L (Fig. 2B) by PMA/io in the presence of the inhibitory compounds. PTK inhibitors PNU152518 and genistein, as well as the Ca^{2+}-dependent calcineurin inhibitor, cyclosporin A, were able to inhibit both apoptosis and Fas-L induction (Fas-L-dependent lytic activity) (Fig. 2). Furthermore, anti-oxidants ascorbic acid (Fig. 2A) and glutathione (not shown) acting downstream Fas/Fas-L interaction only inhibited PMA/io induced apoptosis.

These results suggest that PMA/io and gp120 activate a common signalling pathway inducing Fas-L that involves calmodulin/calcineurin, one or more PTK as well as PKC.

3.3. *Immunocytochemical analysis of Fas-L expression*

In Fig. 2B, Fas-L expression was measured indirectly by its ability to lyse Fas susceptible Jurkat cells. To directly test the effect of inhibitory compounds on Fas-L expression induced by gp120 or PMA/io, immunocytochemistry was assessed.

To increase the detection of Fas-L an amplification kit (MM) was used on cytospins. Fig. 3 shows microphotographs from one of three independent experiments in which clone 103 cells were treated for gp120- (left panel) or PMA/io-apoptosis (right panel) in the presence or absence of the indicated inhibitors. Basal expression of Fas-L is shown by cells cultured with anti-TCR mAb (A), or in medium only (B). Confirming our previous data (here and [18]), Fas-L expression was upregulated by both gp120- and PMA/io-treatment (Fig. 3C and D, respectively). Fas-L upregulation by both treatments was inhibited by PNU152518 (E and F), genistein (not shown), calphostin C (G and H) and CsA (K and L). Ascorbic acid (I and J) and GSH (not shown) had no effect.

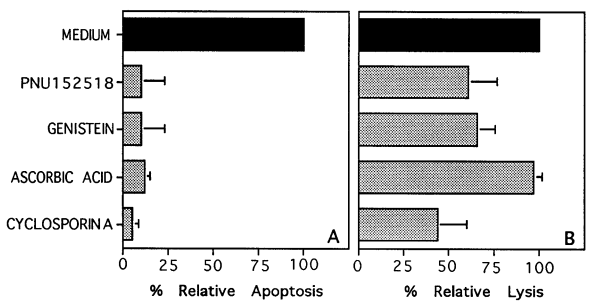

Fig. 2. Activity of inhibitory compounds on apoptosis (A) and functional Fas-L expression (B) induced by PMA/io stimulation. Clone 103 cells were cultured for 4 h with PMA/io in the presence or absence of the indicated agents. Apoptosis (A) and functional Fas-L expression (B) were then evaluated by TUNEL and cytotoxic activity against Fas^+ Jurkat cells, respectively. (Mean ± S.D. of 4 independent experiments is shown.)

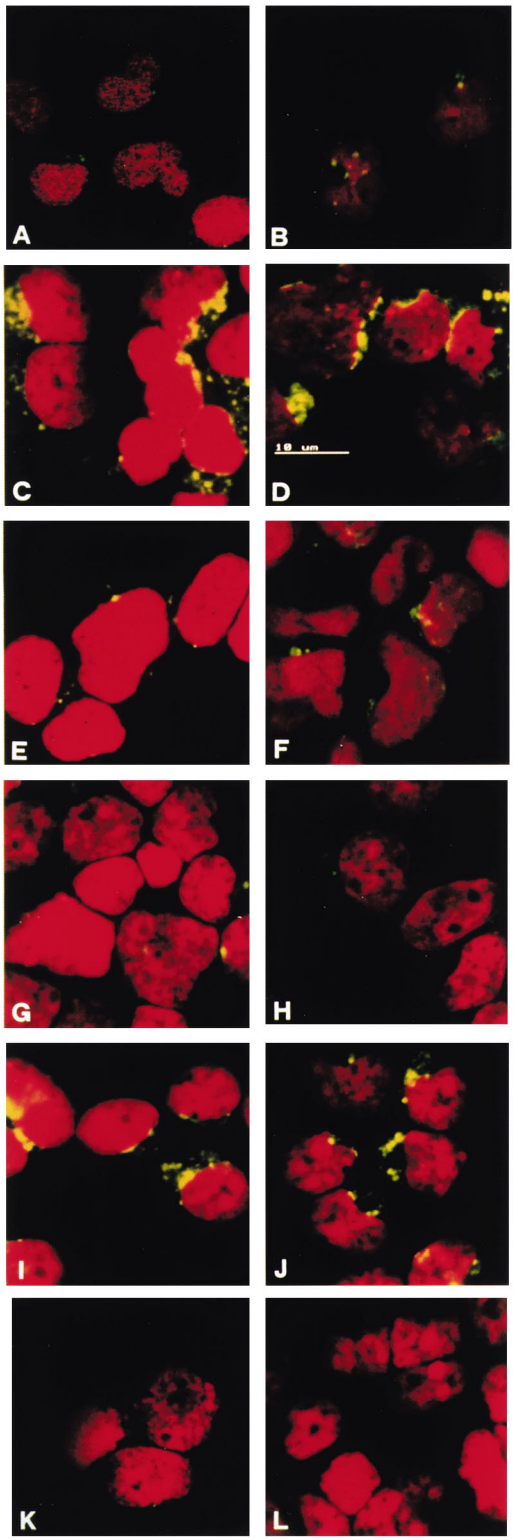

Fig. 3. Modulation of Fas-L expression by several inhibitors in the gp120 or PMA/io models. Immunocytochemical analysis of Fas-L expression in gp120-apoptosis or PMA/io treated cells. The left side of the panel shows samples 1 h after induction of gp120-apoptosis, the right side of the panel shows cells cultured for 4 h. with PMA/io. Basal Fas-L expression is indicated by anti-TCR stimulated cells (A) and cells cultured in medium only (B). Cells were stimulated for gp120-apoptosis (C) or PMA/io (D) in medium only, or in the presence of 10 μM PNU152518 (E–F), 5 nM calphostin C (G–H), 500 μg/ml ascorbic acid (I–J) or 50 μg/ml cyclosporin A (K–L).

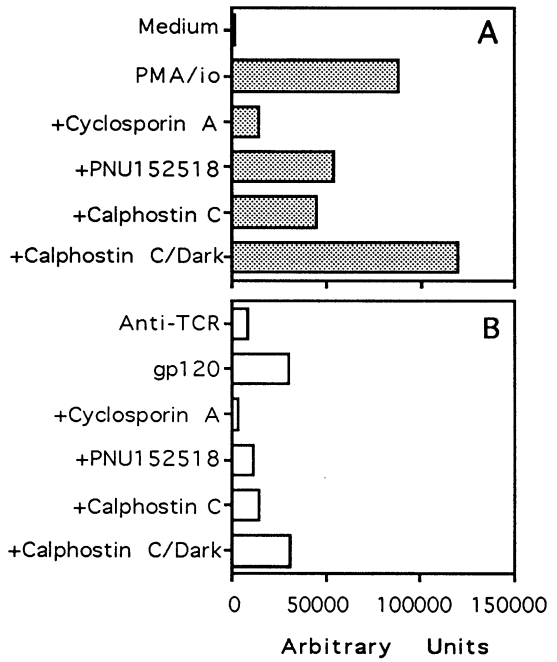

Fig. 4. Effect of the inhibitory compounds on Fas-L mRNA induction. Phosphorimager analysis of PMA/io (B) or gp120 (C) treated cells 30 min after induction in the presence or absence of the indicated compounds (S26 mRNA was used as a control). One of two independent experiments is shown.

This immunocytochemical analysis confirms the previous data indicating the existence of a common pathway for Fas-L induction during gp120- and PMA/io-apoptosis.

3.4. Fas-L regulation at mRNA level

Functional data and the immunocytochemical staining showed that some compounds can inhibit Fas-L induction by gp120 or PMA/io. To test whether such inhibition occurs at transcriptional level, RT-PCR analysis was performed.

Kinetic experiments showed that Fas-L mRNA could be detected as early as 30 min after gp120- or PMA/io-stimulation, but not after TCR activation (not shown). Thus the analysis of Fas-L induction in the presence of the inhibitory compounds was performed after 30 min of stimulation. Phosphorimager analysis of PMA/io and gp120 treated cells is represented in Fig. 4 (A and B, respectively). CsA, PNU152518, and calphostin C (used under light) impaired upregulation of Fas-L mRNA.

Semiquantitative analysis indicates that PMA/io stimulation induces about three times more Fas-L than gp120-apoptosis (Fig. 4). This was in agreement with the functional data, in fact Jurkat cell lysis by clone 103 stimulated with PMA/io or gp120 was $38 \pm 5\%$ and $15 \pm 3\%$, respectively.

4. Discussion

To explain the immunodeficiency occurring after HIV infection two major mechanisms have been hypothesized: a direct cytopathic effect of the virus on the infected lymphocytes [24] and the induction of apoptotic death of uninfected $CD4^+$ T cells [10,25]. In vitro models and clinical studies suggest that both mechanisms are likely to be responsible of $CD4^+$ T cells depletion. Indeed some viral proteins can induce cell death of uninfected lymphocytes either directly [26,27] or in association with TCR stimulation [5]. In particular, gp120, a protein of the viral envelope that binds CD4, determines apoptotic cell death of $CD4^+$ lymphocytes when TCR is also triggered [2,28]. We recently demonstrated that gp120-apoptosis occurs in Th1 but not in Th2 clones and that it is mediated by induction of Fas-L and triggering of the Fas apoptotic pathway [4]. This is likely a sensitization to AICD, in fact gp120-apoptosis follows standard TCR activation (we use a dose of anti-TCR mAb that alone induces proliferation and cytokine production) [4]. In the present paper we compared the pathway leading to Fas-L expression induced during gp120-apoptosis, AICD and PMA/io stimulation.

Anel et al. [19] recently showed that, in $CD8^+$ T cells, PKC is necessary for Fas-L induction by PMA/io but not by hyperactivation of TCR such as in AICD. We confirm these data in a $CD4^+$ Th1 clone and further observe that PKC is necessary for inducing Fas-L when CD4XL precedes TCR triggering. Thus it is possible to distinguish a 'physiologic' PKC independent pathway that is activated by antigenic stimulation of TCR (as supposed for controlling the expansion of activated clones in vivo [29]) and a 'pathologic' PKC dependent pathway that is activated when TCR and CD4 are asynchronously stimulated. This may contrast with the general idea that CD4 is an accessory receptor potentiating the TCR stimulation, an event that occurs when the two receptors are contemporaneously activated [30]. These data are apparently in disagreement with a recent study demonstrating that when CD4XL precedes TCR activation Fas-L is not induced and tyrosine phosphorylation is prevented [31]. In fact a difference was reported between short and long term cultured clones, suggesting that the PKC-dependent Fas-L induction is restricted to long term stimulated clones, such as our clone 103. We underline that long term activated $CD4^+$ T cell clones can be considered a valuable model to study T cell depletion in HIV infection since they allow to reproduce results obtained by others using bulk cultures [2,32]. These clones could mimic the in vivo setting where HIV infection determines a strong and prolonged stimulation of the immune system [33]. It is likely that the chronically activated T lymphocytes undergo apoptosis [34].

In our model asynchronous activation of CD4 and TCR induces Fas-L via PKC. In addition to Fas-L induction, PKC acts downstream Fas/Fas-L interaction. Whether PKC participate to Fas-apoptosis has been debated [35–37] but not directly studied. Our data strongly support a PKC role in anti-Fas induced apoptosis in $CD4^+$ T cell clones; whether PKC operates toward the activation of caspases [38] or GD3 [14] remains to be studied.

PMA/io stimulation and gp120-apoptosis activate a common pathway for Fas-L induction. This pathway involves PKC, one or more PTK and the Ca^{2+}-dependent calcineurin. Evidence from other authors indicates that CsA can prevent Fas-L mRNA transcription induced by PMA/io stimulation [19], and that a CsA sensitive transcriptional regulation of Fas-L induced by either PMA/io or TCR/CD3 activation may involve NFκB [39], NF-ATp [40] and/or Egr activation [41].

Our data contribute to the knowledge of events leading to Fas-L expression in $CD4^+$ T lymphocytes. The importance of understanding this mechanism is based on the possibility to interfere with the pathologies characterized by altered expres-

sion of Fas-L, including AIDS and several autoimmune diseases (LES, autoimmune diabetes, Ashimoto's thyroiditis) [8]. Although further studies are necessary to better define the pathway regulating Fas-L expression, our data indicate the possibility of preventing its induction after an asynchronous triggering of CD4 and TCR (such as in gp120-apoptosis) without affecting the more physiologic Ag-mediated AICD. We suggest that, following TCR triggering, activation of PKC may redirect a positive response (proliferation and cytokine production) to a negative one (cell death). An aspecific stimulation, like PMA/io, can activate the PKC dependent pathway in the absence of CD4 and TCR triggering; we do not know whether PMA/io activates other cytoplasmic components, besides PKC, to induce Fas-L.

These findings suggest the possibility for a new pharmacological approach for pathologies involving an unregulated expression of Fas-L such as AIDS. At the moment treatment of AIDS is directed to control T lymphocyte infection and virus replication. Encouraging new therapies are waiting for a long follow-up validation in the light of the high virus variability and possible existence of silent virus reservoirs in the host. The possibility of controlling Fas-L expression and thus of preventing death of uninfected T lymphocytes, may help to preserve the immune system.

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