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HIV-1 gp120-dependent induction of apoptosis in antigen-specific human T cell clones is characterized by 'tissue' transglutaminase expression and prevented by cyclosporin A

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Abstract

We investigated the effect of cyclosporin (CsA) on HIV-gp120-dependent induction of cell death by apoptosis of human T cell clones specific for influenza virus haemagglutinin and restricted by HLA-DR1. Preincubation of the clones with gp120 induced a large inhibition of their proliferation which was paralleled by the induction of apoptosis. Exposure to the specific antigen alone was able to trigger apoptosis in a significant fraction of cells, this effect was potentiated by pretreatment with gp120. Apoptosis was characterized by the typical morphological changes and by the expression of 'tissue' Transglutaminase (tTG), one of the few characterized effector elements of programmed cell death. Interestingly, the tTG protein induction was detectable within the first 24 hours following the gp120 treatment and preceded the appearance of the typical apoptotic phenotype. Noteworthy, CsA treatment prevented the gp120-dependent induction of apoptosis by blocking the activation of the Ca^{2+}-dependent effector elements such as tTG.

Key words: AIDS; Protein cross-linking; Immune suppresive agent

1. Introduction

The cellular and molecular mechanisms at the basis of the dramatic immune cell loss caused by HIV in patients suffering acquired immunodeficent syndrome (AIDS) are not yet established [1,2]. One of the unresolved question about the pathology of HIV infection concerns the functional disappearence of T cells specific for 'recall' microbial antigen at early stages of the disease [1]. This suggests that some indirect mechanism, other than virus-induced cytopathogenicity, may also be operative [3]. It has been reported recently that lymphocytes from seropositive individuals (stage II), and from ARC and AIDS patients undergo programmed cell death or apoptosis when cultured in vitro in the absence of growth factors or when stimulated with bacterial superantigen [4–9].

Apoptosis is the silent (not associated with inflammation) physiological process of cell death leading to the controlled elimination of single cells from tissues [10–12]. Independent from their origin, cells undergoing apoptosis display common morphological features, thus suggesting the existence of a general gene-regulated pathway of physiological death [10–12]. The Ca^{2+}-dependent enzymes 'tissue' transglutaminase (tTG) and endonuclease are among the genes recently identified as effector elements of the apoptotic cell death program [10–12]. The activation of tTG in apoptotic cells leads to the formation of highly cross-linked intracellular protein polymers which play a fundamental role in the induction of the irreversible structural changes featuring cells dying by apoptosis [13,14].

Apoptosis plays a key role in the homeostasis of the immune system, not only is it involved in the elimination of self-recognizing T lymphocytes in the thymus [15,16], but this death mechanism also seems to be activated in mature T cells by the separate ligation of CD4 and the T cell receptor for antigen [17]. It has been suggested that AIDS can be considered an activation-induced disease and immunosuppressors such as CsA, which prevent activation, has already been introduced in its therapy [18]. In an attempt to get further insight into the molecular mechanisms involved in the onset of apoptosis in T-cells we investigated the role of HIV gp120 and CsA at the clonal level. To this aim, well characterized influenza-specific $CD4^+$ T cell clones have been analysed for their proliferative response and for apoptosis induction in different culture conditions in the presence of gp120 and/or CsA.

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2. Materials and methods

2.1. Influenza virus antigen and HIV gp120 preparation

The antigen used in this study was the synthetic peptide 306–324 derived from haemoagglutinin (HA). The peptide was prepared by solid phase synthesis using standard t-Boc chemistry in 'T bags' using commercially available amino acids (Novabiochem, Laufelfingen). The peptide was cleaved from the resin by treatment with hydrogen fluoride. Purity was determined by high pressure liquid chromatography and amino acid analysis.

HIV-1 recombinant gp120 produced in the bacolavirus system was purchased from American Biotechnology (USA).

2.2. Antibodies

The anti-gp120 polyclonal rabbit antibodies were purchased from American Biotechnology (USA); the mouse anti-human CD4 mAb OKT4 from Ortho (USA); the mouse anti-human CD3 mAb OKT3 from Ortho (USA); the affinity-purified monospecific rabbit IgG raised against soluble 'tissue transglutaminase' of human red blood cells was a kind gift from L. Fesus (Department of Biochemistry, University Medical School of Debrecen, Debrecen, Hungary). Rabbit antiserum specific for cow glial fibrillary acidic protein were obtained from Dakopatts (Hagersten).

2.3. Cell lines

The DR1-expressing murine L-cells (0.5–3.1) [19] were maintained in culture in DMEM supplemented with 10% FCS in 25 cm^2 tissue culture flasks and subcultured, after trypsin treatment, at 1:10 dilution twice weekly.

EBV-transformed lymphoblastoid B-cell lines (B-LCLs), from the 10th International Histocompatibility Workshop, were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin at 50 units/ml, and streptomycin at 50 μg/ml in 25 cm^2 flasks and were regularly passaged.

2.4. T cell clones

T cell clones HC4 and HC6 were generated from a DR1,4 individual by stimulating PBMC with purified influenza HA (5 μg/ml), and cloned by limited dilution in Terasaki plates at 1 cell/well with 10^4 irradiated autologous PBMC, 1 μg/ml HA306–324 peptide and 20 U/ml recombinant IL-2 (rIL-2) (Boeringher Mannheim, Germany). The clones were maintained in culture by weekly stimulation with autologous PBMC, HA306–324 peptide and rIL-2 [16].

2.5. gp120 and CsA treatment

T cell clones were plated out in 24-well plates ($3–5 \times 10^5$ cells/well) in the presence of gp120 and rIL-2 (20 U/ml), in a total volume of 500 μl. After 24–48 h incubation, the cells were harvested, washed and left to 'rest' for a further 24–48 h in the absence of IL-2. T cells were analyzed at both times for their proliferation as well as apoptosis. In some experiments, T cell clones were also cultured with HA306–324 peptide- pulsed DR1-expressing L cells during the last 24 h in the presence or in the absence of various concentration of CsA (Sandoz, Switzerland; as indicated in the figure legends).

2.6. T cell proliferation assay

T cell clones (10^4 cells/well) were cultured in the presence of Mitomycin-C (65 μg/ml) (Sigma, St. Louis, MO) treated B-LCL's or DR1-expressing L cells transfectants (2×10^4 cells/well) in flat-bottom microtiter plates, in a total volume of 200 μl. For antigen-specific responses, the antigen presenting cells were pre-pulsed with antigen. Wells were pulsed with 1 μCi of tritiated thymidine ([^{3}H]TdR) (Amersham International, UK) after 48 h and the cultures harvested onto glass fiber filters 18 h later. Proliferation was measured as [^{3}H]TdR incorporation by liquid scintillation spectroscopy (Optifluor was from Packard, Zurich). The results are expressed as mean cpm for triplicate cultures. Standard errors were routinely less than 10%.

2.7. Immunocytochemistry

Immunocytochemical staining of human T cells was performed using as primary antibody an affinity-purified monospecific IgG raised in rabbits against human red blood cell soluble tTG (1:100) [12]. Incubations with the primary antibody were carried out in a wet chamber overnight at 4°C. A biotinylated goat anti-rabbit IgG, as second antibody, was used followed by a preformed avidin–horseradish peroxidase complex (Immunon, MI, USA). The reaction was developed using aminoethylcarbazole (AEC), (CRL, USA) as chromogen substrate and 0.01% H_2O_2. Cells were counterstained in Mayer's haemalum. Endogenous peroxidase activity was blocked by methanol–H_2O_2.

2.8. Evaluation of the apoptotic index

The percentage of apoptotic cells was evaluated by counting the tTG positive cells showing the typical apoptotic morphology (shrunken cells with condensed chromatin; Fig. 3D, arrows) as well as the apoptotic ghosts (Fig. 3E,F, arrow heads) scored at the light microscopy (Laborlux D; Leitz, Wetzlar, Germany) over 1,000 total cells (including the apoptotic ones). In order to avoid subjective bias, the counts were carried out by two different workers and the results pooled.

3. Results

The human T cell clones HC4, and HC6 restricted by HLA-DR1 and raised against influenza heamagglutinin (specific for the HA peptide (306–324); [19]) were cultured for 48 h with gp120 and IL-2, followed by 48 h in tissue culture medium (without any further addition of

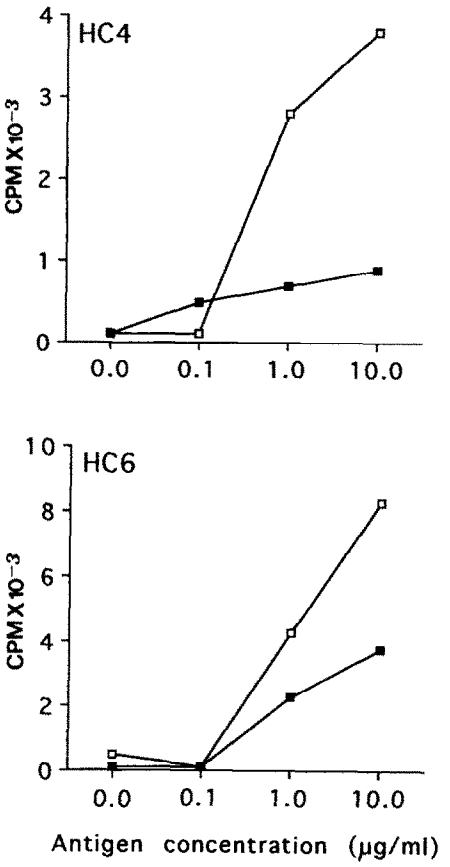


Fig. 1. Functional inhibition induced by gp120 on T cell clones specific for HA306–324 antigen. The human T cell clones HC4 and HC6 were exposed to gp120 (10 μg/ml) in the presence of 20 U/ml of rIL-2 for 48 h. After two extensive washes, the T cells were left to rest for a further 48 h and then their proliferative capacity to antigen pulsed DR1-expressing B-LCL was tested. Symbols: (□) rIL-2 (20 U/ml); (■) rIL-2 + gp120 (10 μg/ml). The results are expressed as mean cpm for triplicate cultures. Standard errors were routinely less than 10%. The results represent a typical experiment out of 5.

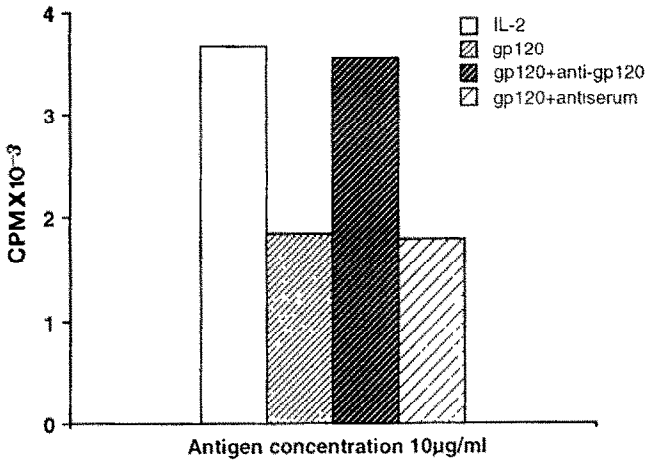


Fig. 2. Competitive effect of an anti-gp120 antibody on the inhibition of proliferative response of human T cell clone HC4. The cell clone HC4 was treated either with gp120 (10 μg/ml) for 48 h or with the gp120 pretreated with its specific antibody. As control, an unrelated antiserum was used. After two extensive washes, the HC4 proliferative capacity was tested against DR1-expressing B-LCL pulsed with HA306–324 peptide. The results are the mean of triplicate measurements in two different experiments. The results are expressed as mean cpm for triplicate cultures. Standard errors were less than 10%.

IL-2). The T cells were then harvested, washed and transferred to a proliferation assay in the presence of DR1-expressing B-LCL-pulsed with different concentrations of HA306–324 peptide. Fig. 1 shows that pre-incubation with 10 μg/ml of gp120 induces a reduction of subsequent proliferation between 30–70%, depending on the clone used. The effect of gp120 on the proliferative response of the T cell clone to DR1-expressing L cells pulsed with HA306–324 peptide (1 μg/ml) was dose-dependent (data not shown) and its specificity was demonstrated by protecting from inhibition by gp120 preincubation with an anti-gp120 antiserum before addition to T cell clones (Fig. 2). CsA treatment (at 200 ng/ml) completely abolished the residual proliferative capacity shown by both HC4 and HC6 clones after gp120 treatment (data not shown). FACS analysis on the HC4 clone showed that 24 h of gp120 treatment induced a marked decrease of CD4 expression which was reversed during an additional 48 h culture in fresh medium in the presence of IL-2; no modulation of CD3 expression was caused by gp120 under the same culture conditions described (data not shown).

To analyse whether the interaction between the HIV protein gp120 and the CD4 receptor can cause apoptosis in uninfected T cells, we have investigated, beside morphology, the tTG protein expression by immunocytochemistry using an affinity-purified rabbit antibody raised against human red blood cell tTG which does not cross-react with other TGs as previously described [12,13,20]. Fig. 3 shows that tTG protein is induced in the clone HC4 during the first 24 h exposure to gp120 (Fig. 3C). In fact, the tTG protein is not detectable in untreated cells (Fig. 3B) while the number of tTG-positive cells reached a value close to 30% following 24 h treatment with gp120 (Fig. 3C). The tTG protein level was further increased after exposure for additional 24 h to the specific antigen and, at this time, its expression was revealed in T-cells characterized by typical apoptotic phenotype (shrunken cell containing condensed chromatin; Fig. 3D). In culture, apoptotic cells undergo secondary necrosis [10–12]. This event is highlighted in cultures incubated with gp120 for 48 h by less defined positivity with the tTG antibody and loss of intracellular organelles (Fig. 3E,F). The remaining cell ghosts (Fig. 3E,F) are identical to the highly resistant cross-linked scaffold assembled through the action of tTG in apoptotic cells (Fig. 3A). By counting the tTG positive cells showing the apoptotic morphology in addition to the cross-linked apoptotic remnants we could quantify the apoptotic index in our cultures. Data indicate that the inhibition of proliferation observed after exposure to gp120 (Fig. 1) was paralleled by the induction of apoptosis in both clones, in the presence or absence of HA306–324-pulsed DR1-expressing L cells (Fig. 4). Although, some differences were observed between the clones, apoptosis was induced in 50–60% of cells after gp120 pretreatment and subsequent exposure to HA306–324-pulsed DR1-expressing L cells (Fig. 4). Noteworthy, exposure to HA306–324-pulsed DR1-expressing L cells triggered apoptosis per se in a significant proportion of T cells (Fig. 4). The specificity of the effect exerted by the gp120 was demonstrated by protecting from the induction of apoptosis in T cell clones by gp120 preincubation with an anti-gp120 antiserum. In fact, the data reported in Fig. 5 clearly indicate that preincubation of gp120 with anti-gp120 antibody prevented the inhibition of the proliferative capacity (Fig. 1) by blocking apoptosis. In Fig. 6 the effect of CsA on T-cells apoptosis induced by gp120 followed by exposure to the specific antigen is shown. Data clearly indicate that CsA completely prevents the induction of apoptosis subsequent to the exposure to gp120 and HA306–324-pulsed DR1-expressing L cells (Fig. 6A). However, the block of apoptosis is not associated to the down-regulation of the specific effector elements expressed in the late stages of the apoptotic pathway. In fact, CsA treatment leads to the accumulation of an increased number of cells expressing the tTG protein (Fig. 6B).

4. Discussion

Previous reports have indicated that T cells, either polyclonal or monoclonal, after preincubation with gp120 in vitro, become refractory to the stimulation through the T cell receptor [21]. Several mechanisms could account for gp120-induced inhibition. It is possible

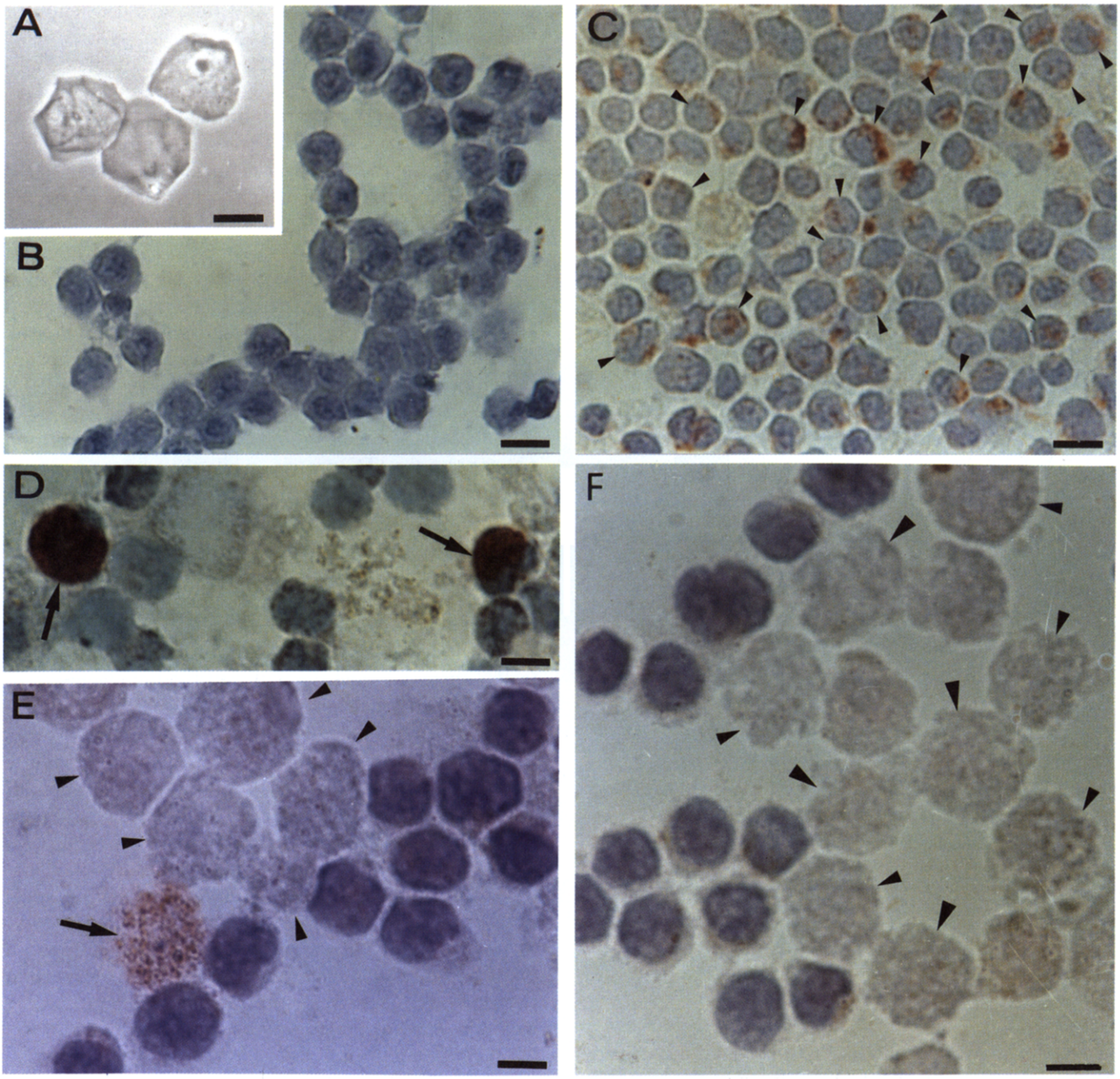


Fig. 3. 'Tissue' transglutaminase expression in gp120 treated human T cells. T cells (clone HC4) were smeared on slides, fixed in 2.5% paraformaldehyde and, after immunostaining with the monospecific anti-tTG antibody, counterstained with Mayer's haemalum, as described in section 2. Panel A shows the morphological features of highly cross-linked apoptotic bodies isolated from human T cell clone HC4 upon preincubation with gp120 and exposition to the HA306–324-pulsed DR1-expressing l cells as previously described [19] (phase contrast; bar = 10 μm). Panel B shows the untreated T cells grown in presence of rIL-2; note the absence of positive reaction to the tTG antibody (bar = 12 μm). Panel C shows the clone HC4 pretreated with gp120 for 24 h; about 30% of the T cells present an intense tTG expression in the cytoplasm (arrow heads; bar = 12 μm). Panels D–F show the clone HC4 pretreated with gp120 for 24 h and then exposed for other 24- (C) or 48- (D–F) h to the HA306–324 pulsed DR1-expressing l cells. In these panels, the T cells were heavily stained with the anti-tTg antibody and showed the typical apoptotic morphology (shrunken cytoplasm and marginated condensed chromatin; D, arrows; bar = 10 μm). After 48 h in the presence of HA306–324-pulsed DR1-expressing L cells (E–F), apoptotic cells underwent secondary necrosis progressively losing their organelles (note the absence of the nuclear staining E–F, arrows heads) including positivity to the tTG antibody (E, arrow; bar = 3 μm), thus indicating different stages of degradation in the absence of phagocytosis (E–F, arrow heads; F, bar = 8 μm).

that occupancy of the CD4 molecule with gp120 could reduce availability of CD4 for interaction with MHC class II molecules on the APC (antigen presenting cells) [22]. Alternatively, in models dealing with T cell stimulation by cross-linked monoclonal antibodies specific for CD3 or TCR in APC-free systems, the gp120-dependent

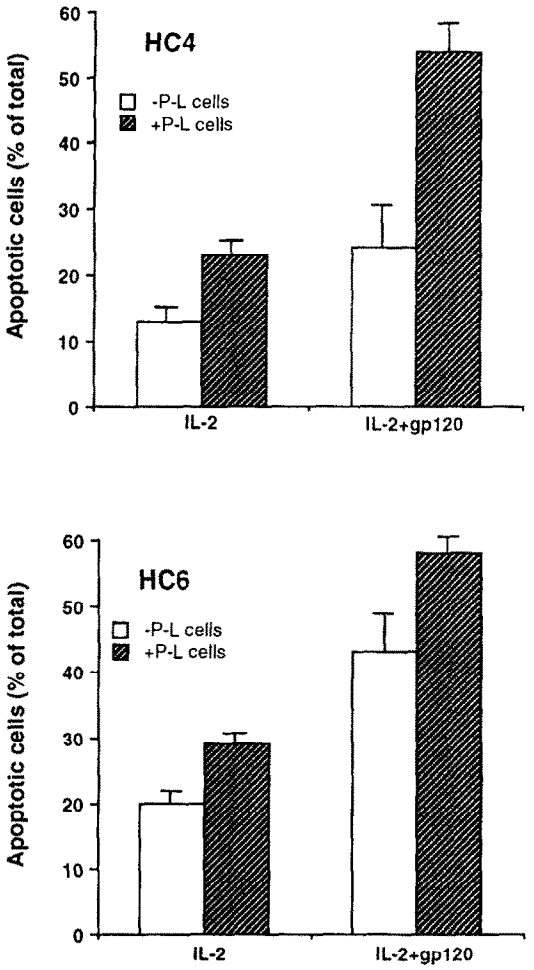


Fig. 4. Apoptosis induced by gp120 in T cell clones specific for HA306–324 peptide. The human T cell clones HC4 and HC6 were exposed to either rIL-2 (20 U/ml) alone or rIL-2 and gp120 (10 μg/ml) for 48 h. After two extensive washes, T cells were cultured in the presence (hatched bars) or absence (open bars) of HA306–324 peptide-pulsed DR1-expressing L cells for 48 h. Apoptosis was quantified as described in section 2. Results represent the mean + S.D. of five different experiments.

suppression of T cell proliferation can be mainly ascribed to the inhibition of the CD3/TCR phospholipase C transduction pathway [23]. In the majority of the studies dealing with the inhibition of T cell proliferation by gp120, the final effect of gp120-CD4 interaction is described as functional deletion, anergy, or temporary suppression [21,23]. In fact, the proliferative response to IL-2 is conserved, and removal of gp120 in the presence of IL-2 could reverse the inhibition [24].

The results presented here support a third possible mechanism of gp120-induced T cell inhibition, namely the induction of apoptosis. It has been recently reported that when preceded by ligation of CD4, signalling through TCR results in T cell unresponsiveness which elicits cell death of mature T cells by apoptosis [7,15]. The interest in such a phenomenon is enhanced by recent reports showing that T cells from AIDS patients are 'primed' for apoptosis [5,7] and that clonal deletion of particular TCR Vβ regions is present in AIDS patients [24]. Against this background, we addressed the possibility that incubation with gp120 causes apoptosis in $CD4^+$ T cells either before or after stimulation through TCR occupancy. In particular: (a) presentation of the specific antigen itself induces apoptosis in the T cell clones; (b) gp120 induces apoptosis which is further enhanced by stimulation with the antigen; (c) CsA prevents the antigen-dependent induced T-cell apoptosis.

The induction of apoptosis in the T-cells was characterized by the appearance of the morphologically typical apoptotic phenotype (condensed cells with pycknotic chromatin; Fig. 3D) DNA fragmentation (data not shown) and by the expression of one of the few characterized effector elements of the apoptotic pathway, tTG [12,26–28]. It is interesting to note that untreated cells do not express the tTG protein, while the enzyme is markedly induced upon the binding of gp120 to the CD4 receptor and/or after stimulation with the antigen (Figs. 3C and 6B). This tTG protein expression in the absence of the extreme apoptotic phenotype could highlight a pre-apoptotic stage during which the T cells are 'primed' for apoptosis which is then affected if the primed cell receives additional CD3-transduced signals. According to the current knowledge [10–12], only a sustained increase in the intracellular Ca^{2+} level triggers the irreversible commitment to death by activating the effector genes (tTG, endonuclease) which in turn modify the structure of the cells toward the typical morphology of apoptosis [12]. In fact, tTG is a Ca^{2+} dependent enzyme that is not active at the Ca^{2+} levels normally detected in viable cells [12]. However, the raise in the intracellular Ca^{2+} concentration reported in cells undergoing apoptosis is sufficient to activate the enzyme [19,26–28]. In keeping with these findings is important to mention that CsA is an immunosuppressive agent that blocks T-cells activation by preventing lymphokine production and by interfering with the TCR-mediated Ca^{2+} signal transduction [30]. On such a basis it very likely that CsA, by preventing the increase of intracellular free Ca^{2+}, inhibits the activation of the effector 'killer' elements which elicit the irreversible final events of the apoptotic pathway. In line with this hypothesis is the accumulation, in the presence of a reduced apoptosis, of 'primed' tTG positive cells upon CsA treatment (Fig. 6B).

The mechanism through which gp120-CD4 interaction primes cell for apoptosis is not clear. Steric perturbation of CD4-TCR interaction may be responsible for the T cell anergy induction [21]. Inappropriated kinase activity could also be involved [21]. As in immature thymocytes [15,31], mature murine T cells [17] and hybridomas [17], the induction of apoptosis is not an obligatory response to TCR stimulation, but is the consequence of incomplete signal transduction [29]. This suggestion is consistent with the observation that gp120 can stimulate

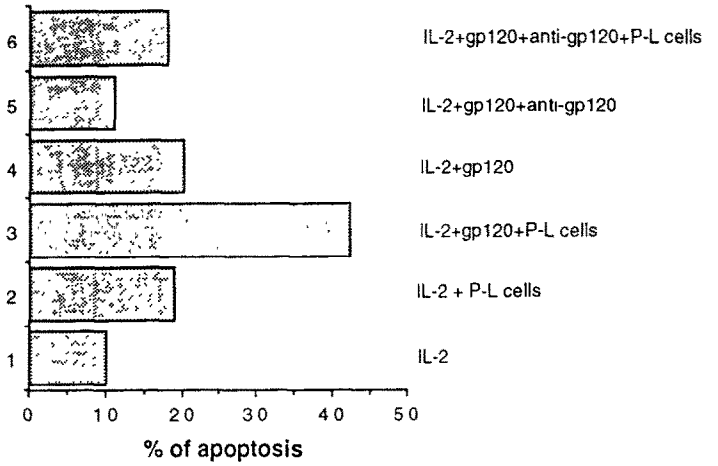
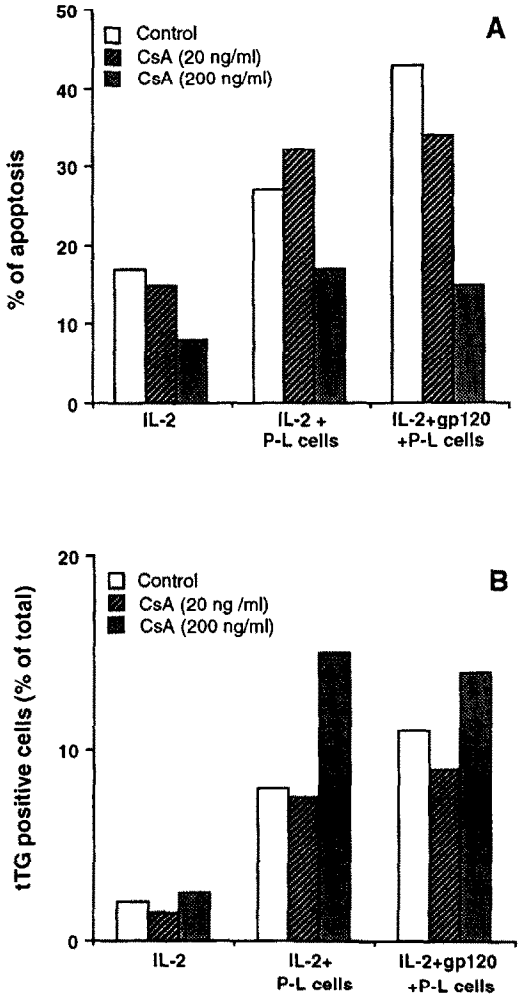


Fig. 5. Competitive effect of an anti-gp120 antibody on the induction of apoptosis of human T cell clone HC4. The T cell clone HC4 was treated either with gp120 (10 μg/ml) for 48 h (3–4) or with the gp120 pretreated with its specific antibody (34 μg/ml) (5–6). Pretreated T cells were exposed for additional 48 h to HA306–324 peptide-pulsed DR1-expressing L cells (2–3, 6). The results are the mean of duplicate measurements carried out in two different experiments and are expressed as% of apoptosis (see section 2).



IL-2 receptor expression [32] and IFN production [33], but it does not appear to increase tyrosine protein kinase signalling [34].

Although the cytopathic effect of HIV has been shown to be associated with apoptosis [4–9,35,36] and the inhibition of cell proliferation of T cells from AIDS patients due to an active T cell death process, no biochemical events associated to this 'primed stage' induced by gp120 [4–7,35,36] has been reported. We show here that the binding of gp120 to the CD4 molecules is able to induce the expression and the subsequent activation of effector elements of cell death program such as tTG.

In conclusion, we show here $CD4^+$ T cells from established T cell clones undergo apoptosis after either exposure to gp120 and/or stimulation with nominal antigen. Furthermore, the finding that CsA seems to prevent the gp120 induced apoptosis by blocking the activation of the enzyme/s catalyzing the final irreversible events of the apoptotic pathway its worthnoting in view of the use of specific immunosuppressive drugs in AIDS therapy.

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Fig. 6. Effect of CsA on (A) gp120 and/or antigen presentation induced apoptosis of human T cell clone HC4, and on (B) tTG expression. The human T cell clone HC4 was exposed to gp120 (10 μg/ml) in the presence of 20 U/ml of rIL-2 for 24 h. After two extensive washes, cells were exposed to DR1-expressing B-LCL pulsed with HA306–324 peptide in the presence and absence of different concentrations of CsA. (A) The results are the mean of duplicate measurements in two different experiments and are expressed as% of apoptosis (see section 2); (B) the results are the mean of triplicate measurements in two different experiments and are expressed as % of tTG positive cells (see section 2).

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