Cell-Surface-Expressed HIV-1 Envelope Induces the Death of CD4 T Cells during GP41-Mediated Hemifusion-like Events

Julià Blanco,* 1 Jordi Barretina,* Karine F. Ferri,† Etienne Jacotot,‡ Arantxa Gutiérrez,* Mercedes Armand-Ugón,* Cecilia Cabrera,* Guido Kroemer,† Bonaventura Clotet,* and José A. Esté*  

*Laboratori de Retrovirologia, Fundació irsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Catalonia, Spain; †CNRS-UMR1599, Institut Gustave Roussy, 94805 Villejuif, France; and ‡Theraptosis Research Laboratory, Theraptosis S.A., Pasteur-Biotop, 75724 Paris, France

Received June 20, 2002; returned to author for revision July 23, 2002; accepted September 13, 2002

Cells expressing the HIV-1 envelope glycoprotein complex (gp120/gp41, Env) induce the death of target cells either after cell-to-cell fusion or after cell-to-cell contact in a fusion-independent fashion. Here, we demonstrate that Env-induced death of single cells (including primary CD4 T cells) required gp120 and gp41 function. The gp41 peptide C34, which blocked syncytium formation, completely inhibited the death of single target cells by specifically acting on gp41 function. Moreover, Env-induced single cell death was exclusively observed in CD4 cells and was associated with specific gp41-mediated transfer of lipids from the membrane of Env-expressing cells to the target cell but not with detectable cytoplasm mixing (complete fusion). We conclude that after gp120 function, gp41 mediates close cell-to-cell contacts, thereby triggering cell death in single uninfected cells in the absence of detectable cell-to-cell fusion. © 2003 Elsevier Science (USA)

**Key Words:** HIV; envelope; gp41; cell death; fusion.

**INTRODUCTION**

Human immunodeficiency virus (HIV) kills CD4 T cells either by direct infection (Bolton et al., 2002; Gandhi et al., 1998; Lenardo et al., 2002) or by inducing the death of uninfected bystander cells (Finkel et al., 1995; Miura et al., 2001). The HIV envelope (Env) glycoprotein complex (gp120/gp41)n is one of the viral factors involved in the death of both infected (LaBonte et al., 2000) and bystander cells (Blanco et al., 1999; Laurent-Crawford et al., 1993). On the surface of HIV particles, Env forces the viral and the cellular membranes to fuse in a multistep process that requires the interaction of gp120 with its receptors, CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984), and a member of the chemokine receptor family, mainly CXCR4 or CCR5 (Moser, 1998). These interactions of gp120 are thought to trigger the exposure of the transmembrane subunit of Env (gp41) and the insertion into the cellular membrane of a hydrophobic peptide (the fusion peptide), followed by the rearrangement of gp41 into a six-helix hairpin structure that tethers together viral and cellular membranes, causing them to mix and fuse (Munoz-Barroso et al., 1998).

When expressed on the surface of infected cells, Env drives cell-to-cell fusion with CD4 T cells (Sodroski et al., 1986) and potently induces apoptosis in these cells (Laurent-Crawford et al., 1993). While cell-to-cell fusion leads to the death of syncytia (Ferri et al., 2000), single (unfused) bystander cells die after the interaction of gp120 with CD4 and CXCR4 receptors (Blanco et al., 1999; Ferri et al., 2000; Maldarelli et al., 1995), suggesting that gp41-mediated irreversible fusion is not required for apoptosis. This fact and the ability of gp120 to kill uninfected CD4 T cells (Berndt et al., 1998; Cicala et al., 2000) led to the general belief that Env-mediated death of single bystander cells was a gp120 effect. However, the classical signaling pathways elicited by CD4 and CXCR4 receptors appear not to be directly involved in Env-mediated apoptosis (Biard-Piechaczek et al., 2000; Blanco et al., 1999; Moutouh et al., 1998).

In this work, we have evaluated the mechanism of cell-surface-expressed Env-induced cell death in single cells. Our data show that gp41 actively participates in the molecular events leading to single cell death, which is associated with membrane hemifusion, suggesting a new role of gp41 in fusion-independent pathogenic Env effects.

**RESULTS**

Cell-surface-expressed Env induces gp41-mediated single cell death

To develop a quantitative assay of HIV Env-induced death, chronically infected H9/IIIB cells were cocultured with CEM-8D6 cells, which express GFP under the control of HIV-LTR, at a ratio of 1:10 infected-uninfected cells. After 24 h, cocultures showed massive cell-to-cell fusion (syncytium formation) and cell death, which was clearly

---

1 To whom correspondence and reprint requests should be addressed. Fax: 34-93-4653968. E-mail: jblanco@ns.hugtip.scs.es.
observed by nuclear condensation and fragmentation in syncytium-forming cells (Fig. 1A) and by morphological changes in single cells (Fig. 1B). In such cocultures, we have quantified two cell-death parameters (total nuclear apoptosis, single cell death) and cell-to-cell fusion (GFP expression) as a control of HIV Env function. In cell-death measurements, we assumed that the low ratio of infected cells used (9% of total cells) had a weak impact on the final effect. In fact, when we analyzed the DNA degradation characteristic of apoptosis in isolated nuclei, we observed that the level of hypodiploid nuclei reached 50% (Fig. 1C), which was consistent with the increased degradation described in DNA of target cells (Jacotot et al., 1996). Similarly when we analyzed the morphology of single cells in a forward vs side scatter plot, we observed increased percentages of cell death (Fig. 1B). Dead cells were identified as CEM, because of its expression of CD4 (H9/IIIB cells lack CD4 expression). Analysis of single cells was also used to evaluate the extent of HIV infection (measured by GFP expression) in living and dead cells. Single cells remaining after 24 h of coculture showed relatively low levels of GFP expression (7–15% of positive cells). Moreover, the percentage of dead cells evaluated in a forward vs side scatter plot was similar in gated GFP+ and GFP− cells (Fig. 1D), suggesting that productive HIV infection is not a prerequisite for target cells to die after Env presentation. To characterize single cell death by comparison to total hypodiploidy and cell-to-cell fusion, we have studied the inhibitory profiles of different drugs. Agents blocking gp120/CD4 interaction, such as the anti-CD4 mAb Leu3a and the anti-gp120 mAb IgGb12, or agents blocking gp120/CXCR4 interaction, such as SDF-1α, AMD3100, and TW70, inhibited death and fusion (Table 1). In contrast, cell-to-cell fusion and death parameters (nuclear apoptosis and single cell death) were insensitive to the RT inhibitor AZT at the concentration tested (Table 1). As expected, C34, an HIV inhibitor peptide derived from the C region of the extracellular domain of gp41, blocked cell-to-cell fusion and total cell death (IC50 0.6 ± 0.2 and 1.5 ± 0.7 ng/ml, respectively, Table 1). Surprisingly, C34 protected CEM-8D6 from Env-induced single cell death with a similar IC50 (16 ± 0.6 ng/ml). Thus, syncytial (fusion-dependent) and single-cell (fusion-independent)
Inhibition of Env-Induced Apoptosis and Cell-to-Cell Fusion

<table>
<thead>
<tr>
<th></th>
<th>H9/IIIB + CEM8D6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (µg/ml)*</td>
</tr>
<tr>
<td></td>
<td>Total apoptosis</td>
</tr>
<tr>
<td>Leu3a</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>mAb IgGb12</td>
<td>0.6 ± 0.04</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>AMD3100</td>
<td>0.022 ± 0.009</td>
</tr>
<tr>
<td>TW70</td>
<td>0.62 ± 0.08</td>
</tr>
<tr>
<td>C34</td>
<td>0.0016 ± 0.0007</td>
</tr>
<tr>
<td>AZT</td>
<td>&gt;25</td>
</tr>
</tbody>
</table>

* CEM8D6 and H9/IIIB were cocultured at a ratio 10:1 for 24 h in the presence of increasing concentrations of the indicated drugs. Cocultures were analyzed as indicated under Materials and Methods. All data were fitted by nonlinear regression. Values are mean ± SD given by the fitting program.

** Determined by propidium iodide staining of isolated nuclei.

† Determined by measuring GFP expression in a plate fluorometer.

‡ Determined by changes in cell morphology in target cells.

ND, not determined.

Gp41-dependent cell death requires gp120-mediated cell-to-cell contacts

To explore the possible implication of cell-to-cell contacts or contact-independent mechanisms in single cell death, we cultured primary CD4 T cells with chronically infected H9/IIIB cells or with a cell-free concentrated virus preparation coming from H9/IIIB cells. We normalized the amounts of effector cells and virus to have similar amounts of Env protein as measured in a sCD4-based ELISA. Supernatants from H9/IIIB cells contained 38 ng of particle-associated gp120/ml and where highly infectious as observed after titration in MT-4 cells (>10^6 infectious viral particles/ml), while chronically infected cells contained 8 ng gp120/10^6 cells. After 24 h of incubation with cell-surface- or virus-presented Env, only cell-surface Env presentation was able to induce the death of primary cells (Fig. 4). No signs of cell death were observed even after 24 h treatment of primary cells with high concentrations of cell-free viral preparations.

Death of primary CD4 T cells was specifically induced by Env-expressing cells and was completely inhibited by blocking the interaction of gp120 with CD4 or CXCR4 with the antibody Leu3a or the CXCR4 antagonist TW70, respectively. Cell death was abolished by blocking gp41 function (Fig. 5A) but was insensitive to the RT inhibitor AZT, suggesting that HIV replication is not involved in gp41-mediated cell death. To completely rule out any effect of viral particles or soluble viral proteins in cell death, we cocultured primary CD4 T cells with HeLa cells expressing HIV Env (from the isolate IIIB) or Tat. HeLa-Env but not HeLa-Tat cells induced cell death in CD4 T cells, confirming that Env expression in effector cells is sufficient to kill single CD4 T cells. In all experiments, C34 was as effective as the gp120 blocking agents in abolishing the morphological changes observed in target cells (Fig. 5B). This inhibition was specific for gp41 because C34 failed to block FAS-induced apoptosis in CD4 T cells (Fig. 5B) or gp120-dependent HIV binding or HIV-induced chemotaxis (data not shown).

We have also compared the effect of chronically HIV-1-infected cells on primary CD4 and CD8 T cells. Only the combination of Env-expressing effector cells and...
CD4 T cells increased the level of cell death. No signs of cell death were observed in cocultures of CD4 T cells with control cells (Fig. 5A) or in cocultures of CD8 T cells with HIV-1-infected or control cells (Fig. 5C), suggesting that specific Env-mediated CD4-dependent cell-to-cell contacts are required for single cell death. The requirement of gp120/CD4 interactions was confirmed by the lack of single cell death observed in cocultures of envelope-expressing cells (H9/11002) with several CD4+/H11002 cells such as the B cell line SKW6.4 or the CD4+ CEM clone A2.01 (data not shown). These data suggest that Env induces a CD4-specific and gp41-dependent form of death that exclusively occurs during the contact of HIV infected with uninfected cells.

Cell death is associated with gp41-mediated lipid transfer

To evaluate the mechanism by which gp41 participates in Env-induced cell death, we labeled HeLa-Env cells with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) or CMFDA, two dyes that label either the plasma membrane via lipophilic interactions or the cellular content via derivatization of thiol moieties. Then, DiO- or CMFDA-labeled HeLa-Env cells were incubated with primary CD4 T cells, and the transfer of lipids or cytoplasm from HeLa-Env cells to CD4 T cells was assessed by flow cytometry analysis of recovered CD4 T cells. Cocultures of CD4 T cells with Env-negative HeLa cells served as negative controls for the assessment of nonspecific dye transfer. Irrespective of the loaded probe, HeLa control cells did not induce significant modifications in CD4 T cell viability (Fig. 6) or major changes in fluorescence staining (Fig. 6). In contrast, labeled HeLa-Env cells induced the emergence of a dead cell population with low forward scatter (mean 31 ± 3%, Fig. 6A). Dead cells showed high fluorescence staining when cocultured with DiO-labeled HeLa-Env cells but not when cocultured with CMFDA-labeled HeLa-Env cells (Fig. 6B), suggesting that dead CD4 T cells mixed their membranes but not their cytoplasm with Env-expressing cells. To confirm that the uptake of DiO by dying cells was a direct consequence of Env-mediated cell-to-cell contacts, we
cocultured anti-Fas pretreated CD4 T lymphocytes (4 h) with control HeLa cells. Cells dying after FAS treatment did not show enhanced DiO uptake compared to control cells (Fig. 6A). The direct association of lipid mixing and cell death was confirmed by analyzing the viability of hemifused and non-hemifused cells after gating CD4 T cells as DiO+/H11001 (hemifused) or DIO-/H11002 (non-hemifused). The death of hemifused cells was significantly higher than that of non-hemifused cells (60 ± 6% vs 19 ± 4%, Fig. 6C), suggesting a link between lipid mixing and cell death events.

Quantification of lipid and cytoplasm transfer was performed after gating living and dead CD4 T cells (as in Fig. 6). Cells displaying fluorescence values above those of untreated cells were considered as positive for lipid or cytoplasmic mixing. Quantification of lipid transfer in morphologically living cells showed that 26 ± 14% of these cells had contacted and taken up lipids from HeLa-Env cells, a value significantly different from the control coculture 7 ± 3% (Fig. 7). The quantification of positive cells in the dead population showed that 72 ± 12% of cells dying after Env presentation had specifically taken up the membrane probe vs a 17 ± 1% in control HeLa cocultures. Conversely, the cytoplasmic probe was not incorporated into living or dying cells above the control values (9 ± 6 or 11 ± 7%, respectively, Fig. 7). To further confirm these data, we used calcein-labeled effector cells. Calcein is a low molecular weight fluorescent probe that can be more easily exchangeable during cell-to-cell contacts. Despite this, C34-sensitive probe transfer from effector cells to living or dead CD4 T cells was not detected when the percentage of positive cells was evaluated (Fig. 8A). Similarly, when probe transfer was evaluated using mean fluorescence intensity values in parallel in cocultures of DiO-, CMFDA-, or calcein-labeled HeLa Env cells, only DiO-labeled HeLa-Env cells induced significant and C34-sensitive probe transfer, while CMFDA- or calcein-labeled HeLa Env cells failed to specifically increase fluorescence levels in target cells (Fig. 8B).

The transfer of lipids and the induction of cell death were specific functions of gp41 since both were blocked...
by the gp41 inhibitor C34 (Figs. 5 and 7). The extent of this inhibition was similar to that observed in the presence of the CXCR4 antagonist AMD3100, which was used as a positive control of inhibition of Env function (Fig. 7). All these data suggest that CD4 T cells die after contacting Env/H11001 cells by a mechanism related to gp41-mediated membrane mixing in the absence of detectable cytoplasm transfer.

**DISCUSSION**

Death of target cells interacting with Env-expressing cells occurs via two major mechanisms: death of syncytium-forming cells and death of single cells. Syncytium formation results in a type of cell death that involves a primary alteration of mitochondrial function leading to secondary nuclear apoptosis (Ferri et al., 2000) that was inhibited by all agents blocking cell-to-cell fusion, whether they target gp120 or gp41 (Table 1). Here, we show that unfused, single target cells died via a gp41-dependent mechanism that required close cell-to-cell contact and was not an indirect consequence of soluble factors. Supernatants from infected cultures containing similar or even higher amounts of envelope glycoproteins than those presented by Env-expressing cells did not induce short-term (24 h) apoptotic changes in target cells (Fig. 4). Env presentation was sufficient to induce a C34-sensitive death of single target cells (Figs. 2 and 5). Single dead cells contact Env-expressing cells close enough to specifically take up their membrane lipids in a hemi-fusion-like process (Figs. 6 and 7) that is a specific function of gp41 (Munoz-Barroso et al., 1998). Taken together, these data support the idea that gp41 actively participates in the death of bystander target cells.

The role of Env in HIV-induced cell death has been recently contested, because high viral inputs are not able to induce cell death (Bolton et al., 2002; Lenardo et al., 2002). However, although high amounts of virion-presented Env did not induce cell death, cell-surface presentation efficiently did (Fig. 4). The reasons for such a difference could be found in the number or in the polarization of Env–receptor contacts (Wei et al., 1999). The role of cell-surface-expressed Env in the death of single bystander cells has been described by several authors (Blanco et al., 1999; Ferri et al., 2000; Kolesnitchenko et al., 1995; Maldarelli et al., 1995; Vlahakis et al., 2001). This effect, which bypasses death receptors (Ohnimus et al., 1997; Roggero et al., 2001), was assumed to be a gp120-dependent phenomenon. However, our data demonstrate that cell death occurs by a gp41-dependent mechanism, in which gp41 promotes intimate membrane contacts that unexpectedly lead to cell death instead of fusion. To explain such a “kiss and run” model of gp41-dependent lethal signaling, one can assume that hemifusion mediated by HIV Env occurs before fusion pore formation as in the case of influenza virus hemagglutinin (Melikyan et al., 2000). Possibly, only a few hemifusion events progress to become expanding fusion pores (Leikina and Chernomordik, 2000), probably because irreversible fusion requires higher levels of organization than hemifusion, either at the Env or at the membrane level. Therefore, the outcome of the contacts between effector (Env+) and target (CD4+) cells might be determined by a hypothetical transition time from hemifusion to fusion. Short transition times would favor cell-to-cell fusion and syncytial death, whereas long transition times might favor the induction of killing signals.
CD4 T cell death is associated with gp41-mediated membrane mixing. Purified CD4 T cells (untreated or pretreated with anti-Fas mAb CH11) were cultured with DiO- or CMFDA-labeled HeLa or HeLa-Env cells as indicated. After 24 h, the morphology of CD4 T cells was evaluated to assess cell death (the percentage of dead CD4 T cells is given for each coculture). The transfer of probes occurring in cocultures of CD4 T cells with DiO- (A) and CMFDA-labeled (B) HeLa-Env or HeLa cells was quantified in CD4 T cells after gating cells as living or dead cells, as shown in Fig. 3. (C) Analysis of cell death in hemifused and non-hemifused cells. Cocultures of primary CD4 T cells with DiO-labeled HeLa-Env cells were analyzed after gating CD4 T cells as hemifused (DiO+) or non-hemifused (DiO−). Cell death was assessed by gating morphologically dead cells.
before fusion progression, as in the case of U937 cells (Fig. 2). Alternatively, receptor density (Doms, 2000) or membrane composition (Saez-Cirion et al., 2002) may regulate Env function and therefore determine the outcome of cell-to-cell contacts.

The role of Env subunits in generating the death of target cells is another intriguing issue. The extensively described gp120-induced apoptosis (Berndt et al., 1998; Cicala et al., 2000; Roggero et al., 2001) suggests that in native Env heterotrimers, gp120 might induce cell death in a gp41-dependent manner. Gp41 could coordinate CD4/CXCR4 oligomerization and hemifusion. Thus, the killing signal would occur at synchrony with but not as a consequence of hemifusion. Consistent with this model, the cooperation between gp41 and gp120 into Env heterotrimers and the existence of monomeric gp120/CD4 contacts have been described or suggested (Salzwedel and Berger, 2000; Staropoli et al., 2000). On the other hand, gp41 may interact with the cell surface (Cladera et al., 2001; Demaria and Bushkin, 1996) and then induce intracellular signals (Speth et al., 2000). In our model, CD4 and CXCR4 were required for gp41-dependent death to occur. Agents blocking one of these interactions completely blocked gp41-dependent death (Table 1) and no evidence for cell death was observed when CD4− cells such as CD8 T cells (Fig. 5C) or the B cell line SKW6.4 (data not shown) were cocultured with Env+ cells. Moreover, Env point mutations that affect CD4 binding, V3 integrity, or the fusion process abrogate pro-apoptotic effects of Env (Laurent-Crawford et al., 1995). These data may suggest that gp120 allows for the proper presentation of gp41 to the surface of the target cell. However, we have been unable to observe cytotoxic activity of recombinant gp41 (data not shown), suggesting that if it exists, this activity of gp41 in single cells should be specific for the native form of this protein. In different experimental models, native gp41 has been shown to specifically kill Env-expressing cells by toxic effects of gp41 intracellular peptides (Chernomordik et al., 1994) or by Env-mediated intracellular fusion events (Cao et al., 1996; LaBonte et al., 2000). Similarly, gp41-dependent cell-to-cell fusion has been described as a mechanism of bystander cell lysis (Stock et al., 2000). In our model, complete fusion does not seem required for gp41 to participate in cell death. It is possible that a high number of hemi-fusion-like events during cell-to-cell contacts perturb plasma membrane and induce an apoptotic signal that either is sufficient to kill cells or can complement gp120-dependent signals to induce cell death. In this regard, it has been recently shown that native but not monomeric gp120 presentation to CD4 T cells destabilized plasma membrane in the virus-to-cell contact microdomains (Kozak et al., 2002). However, incubation of CD4 and coreceptor-bearing target cells with an excess of HIV Env-expressing cells did not result in measurable permeabilization of the target cell membrane (Dimitrov et al., 2001), suggesting that target cell death is not the consequence of sudden loss of plasma membrane function but of more subtle Env-mediated membrane perturbations. A detailed kinetic study of hemifusion, fusion, and cell death parameters (membrane permeability, mitochondrial function) will help to understand the causal relationships between gp41 functions and cell death.

Unstimulated primary CD4 T cells are markedly sensitive to gp41-mediated single cell death, maybe as a consequence of its reduced fusogenic capacity as compared to CEM-8D6 cells. Therefore, gp41-mediated single cell death could contribute to HIV pathogenesis in vivo, especially in lymphoid tissues, where the contact of

FIG. 7. Quantification of membrane and cytoplasm mixing. The transfer of probes occurring in cocultures of DiO- and CMFDA-labeled HeLa-Env and HeLa cells was quantified in CD4 T cells. Cells were gated as living or dead according to their forward and side scatter values, and green fluorescence was analyzed. Data are mean ± SD of three experiments. The percentage of positive cells in the dead and living populations was defined as (DiO−(or CMFDA−) dead CD4 cells/total dead CD4 cells × 100) and (DiO+ (or CMFDA+) living CD4 cells/total living CD4 cells).
infected and uninfected CD4 T cells is more likely to occur. It has been reported that HIV-infected lymphoid tissues may show increased apoptosis in uninfected cells (Finkel et al., 1995; Miura et al., 2001). Moreover, the correlation of Env function and AIDS progression in a monkey model (Etemad-Moghadam et al., 2001) and the apparent involvement of Env function in the upregulation of early markers of apoptosis in HIV disease (Castedo et al., 2001) suggest a role for Env in HIV pathogenesis in vivo. The current clinical use of anti-HIV drugs targeting gp41 function, such as T-20 (Kilby et al., 1998), which in our experimental model is able to block cell-to-cell fusion and single cell death (Barretina et al., unpublished results), will help to evaluate the extent and the relevance of Env-induced cell death in HIV-infected individuals. The protection of uninfected CD4 T cells from Env-induced death may be an additional benefit in anti-HIV therapy.

**MATERIALS AND METHODS**

**Reagents**

Neutralizing mAb against the CD4 binding site (IgGb12) was obtained from Dr. D. R. Burton and Dr. P. W. Parren through the Medical Research Council. The anti-CD4 mAb Leu3a was from BD (San Jose, CA). The CXCR4 agonist SDF-1α was from Peprotech (London, U.K.). The synthetic CXCR4 antagonist AMD3100 has been previously described (Bridger et al., 1995; Schols et al., 1997) and its inhibitory activity against Env-induced apoptosis has been also characterized (Blanco et al., 2000). The CXCR4 antagonists TW70, a cyclic peptide derived from T-22, has been also described (Xu et al., 1999). The gp41-derived peptide C34 covering the sequence 628–661 in the second extracellular helical region of gp41 (Eckert et al., 1999; Kliger et al., 2001) was kindly provided by Dr. P. Kim (MIT, Cambridge, MA).

**Cells**

H9/IIIB (Popovic et al., 1984), CEM-GFP (Gervaix et al., 1997), HeLa-Env, and HeLa-Tat cells were obtained through the Medical Research Council, from Drs. R. C. Gallo, J. Corbeil, and R. M. Ruprecht, respectively. U937 cells were obtained from the ATCC (Rockville, MD). The B cell line SKW6.4 was obtained from Dr. R. Franco. Adherent and lymphoid cell lines were cultured in DMEM and RPMI, respectively, both supplemented with 10% heat-inactivated fetal calf serum (GIBCO). Peripheral blood mononuclear cells (PBMC) from healthy donors were purified by Ficoll–Hypaque sedimentation. CD4 and CD8 T cells were purified from freshly isolated PBMC by immunomagnetic negative selection (Stem Cell, Vancouver, Canada) as indicated by the manufacturer and were used without previous stimulation. Preparations were >96% positive for CD3 staining and >94% positive for the corresponding CD4 or CD8 marker as assessed by flow cytometry.

**Quantitative assays for HIV cytopathic effects**

Since the ability of CEM-GFP cells to fuse with H9/IIIB was relatively low, a more sensitive subclone (8D6) of this cell line was selected. This clone will be referred to as CEM-8D6. H9/IIIB cells (2–4 × 10⁴ cells) were cocultured with CEM-8D6 (2 × 10⁵ cells) for 24 h in 96-well plates. CEM-8D6 clone, as the parental CEM-GFP cell line, expresses green fluorescent protein under the control of the HIV-1 LTR (Gervaix et al., 1997). H9/IIIB cells produce infectious viral particles and therefore express HIV-1 Env and Tat. Therefore, syncytia formed after fusion of H9/IIIB and CEM-8D6 cells produce GFP due to the transactivator activity of Tat. Total GFP production can be easily measured as a surrogate of total cell-to-cell fusion events. Green fluorescence was measured in a Fluoroscan plate fluorometer (Labsystems, Helsinki, Finland), excitation 485 nm, emission 513 nm. Fluores-
cence of blank wells containing only target cells was subtracted to all measures.

Total cell death was defined as the number of hypodiploid nuclei and was assessed by propidium iodide staining of isolated nuclei. Cells were washed and re-suspended in hypotonic labeling solution as described (Nicoletti et al., 1991), incubated for 1 h, and analyzed for DNA content in a FACSCalibur flow cytometer (BD). Hypotonic buffer containing 3.4 mM sodium citrate, 0.05 mg/ml propidium iodide, 0.1 mM EDTA, 1 mM Tris pH 8, and 0.1% Triton X-100 destroys plasma membranes, breaks syncyta, and releases nuclei from single cells and syncytium forming cells, allowing for the quantification of all apoptotic nuclei (Maldarelli et al., 1995).

The death occurring in single cells was quantified by flow cytometry in a forward vs side scatter plot. Dead cells were identified by increased side and reduced forward scatter values (Berndt et al., 1998). More than 90% of dead cells were identified as CEM-8D6 target cells by its CD4 expression (data not shown). Productively infected target cells were identified by increased GFP expression. To calculate IC50 values, target and effector cells were cocultured with serial dilutions of drugs in triplicate wells and data were fitted by nonlinear regression.

For the simultaneous assessment of mitochondrial and nuclear features of apoptosis, cells were stained with the potentiometric dye JC-1 (Molecular Probes, Leiden, The Netherlands), as well as Hoechst 33342 (Sigma, Madrid, Spain), 2 μM each (30 min, 37°C) in complete medium (Ferri et al., 2000). Cells exhibiting a low mitochondrial membrane potential were identified by the red-green shift in the emission spectrum of JC-1, using a Leica microscope equipped with a CCD camera.

**Cocultures of Env+ cells with primary T cells**

H9/IIIB cells (effector cells, 2–4 × 10⁵ cells) were also used in cocultures with primary CD4 T cells (target cells, 2 × 10⁶ cells) for 24 h in 96-well plates. Cocultures involving HeLa cells were performed in 24-well plates, by seeding 2 × 10⁶ target and 2 × 10⁵ effector cells. Cell death occurring in single cells was quantified by flow cytometry in a forward vs side scatter plot or by studying phosphatidylserine externalization (Annexin V staining kit, Roche). Annexin V staining was evaluated in total or in gated CD4 T cells. Primary CD4 T cells are easily identified by morphological parameters; they show lower forward and side scatter values than H9/IIIB or detached HeLa cells (data not shown). To confirm the nature of dying cells, CD4 staining was performed before cell death analysis in gated CD4 T cells. Dead cells were identified as a separate CD4 T cell population showing reduced forward and increased side scatter values (Berndt et al., 1998). For the assessment of absolute cell number, analysis was performed in TruCount tubes (BD). The total number of CD4 T cells was calculated in control cultures, while cocultures allowed for the quantification of remaining single living and dead cells. The difference between the total number of cells in control cultures or in C34-treated cocultures and the total number of cells in untreated cocultures was the number of lost cells. Both syncytium formation and disintegration of dead cells may contribute to the loss of cells from the culture.

**Viral stocks**

In some experiments, parallel cultures with cell-free virus preparations or coculture supernatants were performed. HIV-1 IIIB particles were obtained from H9/IIIB cultures as described (Blanco et al., 2002). Briefly, cell-free supernatants from overnight cultures of infected cells were obtained by serial centrifugation (1500 rpm for 5 min and 3000 rpm for 10 min, respectively). Supernatants were filtered through 0.45-μm filters to eliminate small membrane vesicles and virus aggregates. Filtered supernatants were then ultracentrifuged (50,000 g for 30 min) and viral pellets stored at −80°C until use. The level of p24 in final preparations was measured by ELISA (Innogenetics, Madrid, Spain). The level of gp120 was also measured by ELISA in lysates (NP-40 0.5% in PBS) of viral or cellular pellets corresponding to 15 ml of supernatant or 100 × 10⁶ H9/IIIB cells. Lysates were clarified (10,000 rpm, 10 min) before use. Briefly, Maxisorb Nunc plates were incubated overnight with sCD4 (Progenics, AIDS Reagent Program, 600 ng/well), washed twice with wash buffer (PBS 0.05% NP-40), saturated with BSA 3% in PBS for 3 h, and washed again before loading serially diluted (PBS BSA 1%) samples. Standards were also prepared in PBS BSA 1% using rsgp120 (AIDS Reagent Program). Samples were incubated for 1 h, washed five times, and incubated with anti-gp120 mAb 17b (AIDS Reagent program). Bound antibodies were revealed using a peroxidase-labeled goat anti-human antibody (Jackson ImmunoResearch, dilution 1/1000) and TMB as substrate. The titer of viral stocks was determined in MT-4 cells as described (Blanco et al., 2002), showing more than 10⁶ infectious particles/ml.

**Anti-FAS treatment**

Purified CD4 T cells were treated with the agonistic anti-FAS antibody CH11 (Immunotech, Marseilles, France) for 4 h at 37°C in the absence or the presence of C34. After this incubation cells were extensively washed to remove the apoptotic stimulus, cultured at a density of 10⁶ cells/ml, and analyzed after 24 h for cell morphology. This treatment induces morphological changes consistent with apoptosis in 25–35% of CD4 T cells. Anti-FAS-treated CD4 T cells were also used in coculture experiments as a control for nonspecific lipid and cytoplasm mixing.
Lipid and cytoplasm mixing assays

HeLa-Env or HeLa cells were incubated with the fluorescent membrane probe DiO (Molecular Probes) in a 1:0.6 RPMI:Diluent C (Sigma) mixture (34 μM, 15 min, 37°C) or with the cytoplasmic fluorescent probes of 5-chloromethylfluorescein diacetate (CMFDA), or calcein (Molecular Probes; 5 μM, 15 min, 37°C in RPMI medium). After extensive washes, labeled cells were cocultured with target cells for 24 h. At this time, HeLa and HeLa-Env cells were mostly attached to the plastic, whereas target CD4 cells remain bound or fused to the cell monolayer. Unfused cells were recovered by aspirating culture medium and analyzed by flow cytometry (FACSCalibur, BD) to evaluate the transfer of fluorescent probes from effector cells to unfused CD4 T cells. Lymphocytes were gated as living or dead in a forward vs side scatter plot and the fluorescence of each population was assessed. The percentage of positive cells was determined after setting markers with untreated cells; the mean fluorescence intensity (MFI) of target cells was also calculated setting markers with untreated cells; the mean fluorescence of each population was assessed.

Acknowledgments

This work was supported by the Fundación irsiCaixa, the Spanish Fondo de Investigaciones Sanitarias (FIS), Projects 00/0893 and 01/1116, and the Acción Integrada/Picasso Program HP99-71. J. Blanco is a researcher of the Fundació per a la Recerca Biomèdica Germans Trias i Pujol; FIS 98/3047. J. Barretina holds a FIS predoctoral scholarship (99/9226).

References


