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Apoptosis Enhancement by the HIV-1 Nef Protein[1]

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The HIV-1 *nef* gene, essential for AIDS pathogenesis, encodes a 27-kDa protein (Nef) whose biochemical and biological functions are unclear. It has been suggested that Nef expression contributes to the T cell depletion observed during the disease by promoting their apoptosis. We report that in $CD4^+$ human lymphoblastoid cell lines transfected with the *nef* cDNA obtained from three different HIV-1 strains, expression of the Nef protein enhances and accelerates the response to four unrelated apoptotic agents (staurosporine, anisomycin, camptothecin, and etoposide) but not to an anti-Fas agonist Ab. Nef reduces the expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L and induces a striking enhancement of apoptotic hallmarks, including mitochondrial depolarization, exposure of phosphatidylserine on the cell surface, activation of caspase-3, and cleavage of the caspase target poly(ADP-ribose) polymerase. Interestingly, the peptide Z-Val-Ala-DL-Asp-fluoromethylketone (a broad-spectrum caspase inhibitor) reduces, but does not abolish, phosphatidylserine exposure, suggesting that Nef also activates a caspase-independent apoptotic pathway. Surprisingly, Nef expression increases DNA degradation but without causing oligonucleosomal fragmentation. An increased apoptotic response and down-modulation of Bcl-2/Bcl-X_L following Nef expression are observed also in NIH-3T3 fibroblasts. These data show that Nef enhances programmed cell death in different cell types by affecting multiple critical components of the apoptotic machinery independently from the Fas pathway. ***The Journal of Immunology,*** **2001, 166: 81–88.**

The HIV-1 protein Nef is an essential modulator of AIDS pathogenesis, endowed with still elusive biochemical and biological properties (1). Indeed, some long-term nonprogressing AIDS patients are infected with a *nef*-deleted HIV-1 virus (2), and an intact *nef* gene is necessary for high titer viral replication in animal models (3). Transgenic *nef* expression in mouse $CD4^+$ T cells causes the development of an immune syndrome closely resembling human AIDS (4). Furthermore, in T lymphocytes Nef determines the internalization of the CD4 receptor and MHC class I molecules (5–7). Nef interacts with tyrosine kinases of the Src family (8, 9), with serine/threonine kinases (10, 11), and with the nucleotide exchange factor Vav (12), thus altering their function (13–15). By interfering with the signal transduction machinery, Nef can activate T cells in a variety of experimental models (4, 16–20). Activated T cells become highly susceptible to apoptosis in a process called activation-induced cell death, physiologically relevant for a correct balance of the immune response (21). Accordingly, prolonged expression of an activating CD8-Nef chimera in Jurkat T cells leads to their apoptotic death (22).

The dysregulation of the apoptotic process contributes to the pathogenesis of a wide variety of human diseases, including viral infections (23). Several investigators have proposed that, during the course of HIV-1 infection, apoptotic cell death plays a central role in the dramatic depletion of T cells characteristic of AIDS (24–29). Interestingly, macaques infected with a *nef*-deleted SIV do not develop AIDS-like symptoms, due to a dramatic reduction in the apoptotic death of CTL and $CD4^+$ cells (30).

In this context, we have investigated whether Nef expression can alter the cellular response to different apoptotic stimuli. Apoptotic pathways can be triggered either through the activation of death receptors of the TNF receptor superfamily, such as Fas, or by several receptor-independent stress stimuli (31). The engagement of the Fas signaling cascade directly activates the caspase proteases, which irreversibly dismantle the cell by cleaving specific protein substrates (32, 33). Alternatively, when death receptor-independent apoptosis occurs, mitochondrial alterations are mandatory for caspase activation and the execution of the programmed cell death (PCD)[3] program (31, 34, 35). The control of mitochondrial function is the result of the interplay among the Bcl-2 protein family members, some of which promote cell survival, such as Bcl-X_L and Bcl-2 itself, whereas others promote apoptosis (36, 37).

In this work, we show that Nef increases the apoptotic response to several unrelated stimuli in different cellular models. Interestingly, Nef quenches Bcl-2 and Bcl-X_L expression, and it enhances and accelerates alterations of the mitochondrial function. Moreover, in lymphoblastoid T cells, Nef increases caspase-mediated degradative events, activates additional caspase-independent processes, and interferes with the degradation of DNA.

Materials and Methods

Cell culture and apoptosis induction

Human T lymphoblastoid CEM cells were grown in suspension in RPMI 1640 culture medium supplemented with 5% FBS (Life Technologies, Rockville, MD) and 2 mM L-glutamine in a humidified 5% CO_2 incubator at 37°C. They were positive for the surface markers CD4 and CD45 and negative for the markers CD3, CD8, CD14, and CD19 (38). These CEM cells were used to stably express the HIV-1 Nef protein, and they were selected in the presence of G418 (1 mg/ml), as described previously (39). In this work, Nef-expressing CEM cells are referred to as CEM/Nef cells. Unless otherwise stated, *nef* was from the HIV-1_{A01} strain. However, CEM cells expressing the Nef protein of the HIV-1_{SF2} or HIV-1_{LAI} strains were

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[3] Abbreviations used in this paper: PCD, programmed cell death; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CMXRos, chloromethyl X-rosamine; DEVD-pNA, Asp-Glu-Val-Asp-*p*-nitroanilide; $\Delta\psi_m$, mitochondrial inner membrane electrochemical potential; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PS, phosphatidylserine; STS, staurosporine; Z.VAD-fmk, Z-Val-Ala-DL-Asp-fluoromethylketone; Anis, anisomycin; MAP, mitogen-activated protein.

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used to confirm all experiments. NIH-3T3 fibroblasts stably expressing the HIV-1 Nef_{BRU} protein were obtained as previously reported (40) and selected in the presence of G418 (0.7 mg/ml). Experiments were performed on a pool of clones expressing the viral protein, referred to as pool 3, and as a control on a pool of clones transfected with an empty vector (pool 2) and on wild-type NIH-3T3 fibroblasts. In all cell lines, Nef expression was assessed as described previously (38, 39, 40).

To trigger apoptosis, native and CEM/Nef cells were incubated for 5 h with the different agonists in multiwell tissue culture plates at the seeding density of 10^6 cells/ml. NIH-3T3 fibroblasts were seeded onto 60-mm petri dishes; once cells became subconfluent, they were incubated in apoptotic conditions for 7 h. Caspase inhibitors were preincubated for 30 min before addition of the proapoptotic compounds. Control experiments were performed to exclude for solvent (DMSO) nonspecific effects on apoptosis induction.

Flow cytometric analysis of mitochondrial inner membrane electrochemical potential ($\Delta\psi_m$) and phosphatidylserine (PS) exposure

Cytometric recordings of $\Delta\psi_m$ and cell surface exposure of PS were performed simultaneously on CEM cells as described elsewhere (41). Briefly, after induction of apoptosis, 10^6 cells were resuspended in HEPES buffer (10 mM HEPES, 150 mM NaCl, and 5 mM $CaCl_2$). Cells were then incubated for 15 min at 37°C in FITC-conjugated annexin V, chloromethyl X-rosamine (CMXRos, 200 nM), and propidium iodide (PI; 1 μg/ml). Samples were analyzed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Data acquisition was performed using a CellQuest software and data analysis with a WinMDI software. We used forward and side scatters to eliminate debris, and FITC-annexin V (FL1), CMXRos (FL2), and PI (FL3) fluorescent signals were then showed as density plot diagrams. Cells that did not display plasma membrane integrity, as assessed by PI exclusion, were not considered for further analysis. Data are shown as arbitrary units of fluorescence on a logarithmic scale. A quadrant was set on the diagrams experiment-by-experiment, and it was kept constant in all of the conditions of each experiment to point out the different cell populations. In NIH-3T3 fibroblasts, as PS exposure was hardly measurable once cells were put in suspension, apoptosis was determined by contemporarily measuring $\Delta\psi_m$ breakdown and cell shrinkage, recorded as a forward scatter parameter reduction (42). PI-positive cells and debris were excluded as above. Statistical analyses were performed by applying Student's *t* test; data are presented as means ± SD. In control experiments, cells were incubated in the presence of the mitochondrial uncoupling agent carbonyl cyanide *m*-chlorophenyl-hydrazone to verify the loss of $\Delta\psi_m$.

DNA fragmentation assays

DNA fragmentation was analyzed by electrophoresis on agarose gel and by using the cytofluorometric TUNEL technique. In the former case, after induction of apoptosis, 3×10^6 cells were washed in PBS and lysed in a buffer containing 10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100 (pH 8.0). Samples were then incubated in 100 μg/ml RNase A (30 min, 37°C) and 100 μg/ml proteinase K (10 min, 56°C). DNAs were precipitated in 0.5 M NaCl-isopropanol, washed in 70% ethanol, and loaded on a 1.5% agarose gel. The TUNEL technique was applied on 10^6 cells by use of the in situ cell death detection kit (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's instructions. Data are presented as cytofluorometric recordings of fluorescence intensity on a logarithmic scale vs number of recorded events.

Western immunoblot analysis

Cytosolic extracts were prepared by lysing CEM cells at 4°C in a buffer composed by 135 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 1% Nonidet P-40, in the presence of phosphatase and protease inhibitors (1 mM vanadate, 1 μg/ml leupeptin, 1 μM pepstatin, 1 mM PMSF, and 100 μg/ml soybean trypsin inhibitor). Cell lysates were then loaded on SDS-polyacrylamide gels and proteins were blotted onto Hybond-C Extra membranes (Amersham, Little Chalfont, U.K.) following standard methods. Nonspecific binding was blocked by a 1-h incubation in TBS with the addition of 5% BSA and 0.1% Tween 20 (pH 7.4). Abs were incubated for 2 h at room temperature, and HRP-conjugated secondary Abs were added for 1 h. Proteins were visualized by enhanced chemiluminescence (Amersham).

Caspase activity measurements

Caspase activity was measured as cleavage of the chromophore-conjugated substrate Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA; DEVDase activity) by using the ApoAlert caspase-3 assay kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Each experiment was performed in duplicate, and protease activity was measured by a colorimetric assay at 405 nm on an Elx 800 microplate absorbance reader (Bio-Tek Instruments, Winooski, VT).

Chemicals and Abs

Staurosporine (STS), anisomycin (Anis), camptothecin, etoposide, PI, and carbonyl cyanide *m*-chlorophenyl-hydrazone were purchased from Sigma (St. Louis, MO). FITC-conjugated annexin V was obtained from Boehringer Mannheim and CMXRos was purchased from Molecular Probes (Eugene, OR). The caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (Z.VAD-fmk) was obtained from Bachem (Subendorf, Switzerland). CH-11 anti-Fas mAb was purchased from Upstate Biotechnology (Lake Placid, NY), anti-poly(ADP-ribose) polymerase (PARP) and anti-caspase-7 mAbs were obtained from PharMingen (San Diego, CA); anti-Bcl-X_L polyclonal Ab, anti-Bcl-2, and anti-caspase-3 mAbs were from Transduction Laboratories (Lexington, KY); and anti-α-actin polyclonal Ab was from Santa Cruz Biotechnology (Santa Cruz, CA).

Results

Mitochondrial depolarization and phosphatidylserine exposure following apoptosis induction

To evaluate PCD induction in T lymphoblastoid CEM cells, we have contemporaneously measured two different apoptotic parameters, the loss of the inner $\Delta\psi_m$ and the exposure of PS on the external leaflet of plasma membrane. Cells have been incubated with the protein kinase inhibitor STS, with the protein synthesis inhibitor and mitogen-activated protein (MAP) kinase activator Anis or with the CH-11 anti-Fas agonist Ab. As shown in the cytofluorometric experiment of Fig. 1*A*, treatment of CEM cells with STS or Anis induces the accumulation of a cell population exhibiting both a breakdown of $\Delta\psi_m$ and flipping of PS through plasma membrane (*lower right quarter* of each diagram). Remarkably, these apoptotic cell populations are highly increased when three different *nef* alleles (LAI, A01, and SF2) are expressed (Fig. 1*A*). As exemplified in Fig. 1*B*, the enhancement of these mitochondrial and plasma membrane changes is observed in CEM/Nef cells also after treatment with two other unrelated compounds, camptothecin and etoposide, which respectively inhibit DNA topoisomerase I and II. On the contrary, Fas receptor triggering with the anti-Fas agonist Ab induces a comparable response between native and Nef-expressing CEM cells (Fig. 1*A*).

We have observed a dose-dependent loss of $\Delta\psi_m$ and PS flipping across plasma membrane by using a wide range of agonist concentrations. Nef expression highly increases the effect of STS or Anis for every tested concentration (a representative experiment with Anis is shown in Fig. 2*A*), while it never alters the response to the anti Fas Ab (data not shown). The Nef-mediated enhancement of apoptotic induction after STS or Anis treatment, but not after Fas receptor engagement, is statistically significant, as confirmed by the Student's *t* test analysis displayed in Table I. Moreover, apoptosis induction is accelerated in Nef-expressing CEM cells. As depicted in Fig. 2*B*, after 1 h of incubation with Anis, CEM/Nef cells markedly display both $\Delta\psi_m$ breakdown and PS exposure, whereas in native CEM cells these apoptotic changes are recorded only after 2 h. This kinetic effect is also observed by treating CEM cells with STS, but not with the anti-Fas Ab (data not shown).

Decrease of the expression of the Bcl-2 and Bcl-X_L proteins

Since $\Delta\psi_m$ homeostasis and early apoptotic changes are regulated by the Bcl-2 protein family, we have investigated the expression of two prominent antiapoptotic components of this family, Bcl-2 and Bcl-X_L. Nef markedly reduces the expression levels of both these proteins, as assessed by Western blot assay (Fig. 3, *A* and *B*). Apoptosis induction does not change the basal expression level of

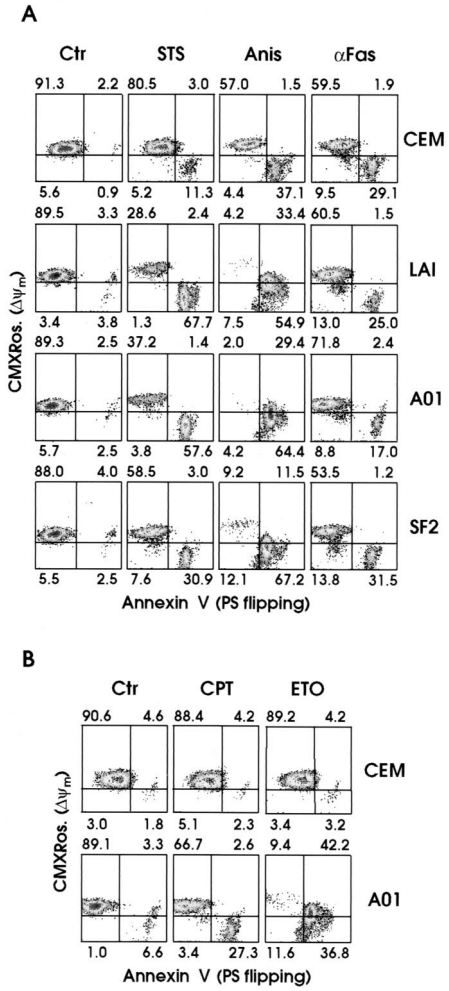


FIGURE 1. Nef expression enhances plasma membrane PS flipping and $\Delta\psi_m$ dissipation. CEM cells have been exposed for 5 h to: *A*, 50 ng/ml STS, 1 μg/ml Anis, 150 ng/ml CH-11 anti-Fas agonist Ab (αFas) and *B*, 1 μM camptothecin (CPT) and 100 μM etoposide (ETO). The experiment has been performed in *A* on native CEM cells and on CEM cells expressing three different *nef* alleles (LAI, A01, and SF2) and in *B* on native CEM cells and on CEM/Nef$_{A01}$ cells. Staining with CMXRos is shown on the vertical axis and with FITC-annexin V on the horizontal axis. Cells exposing PS on the cell surface are in the *right quarters* of each diagram, while cells displaying loss of $\Delta\psi_m$ are in the *lower quarters*. Percentage of each cell population is indicated. Ctr, Control.

Bcl-2/Bcl-X$_L$ in any of our experimental conditions (data not shown). Interestingly, the expression level of caspase-3 is not different between native and Nef-expressing CEM cells (Fig. 3*C*).

Enhancement of caspase activation

Treatment of native CEM cells with a broad-range caspase inhibitor, the peptide Z.VAD-fmk, before apoptosis triggering with STS or Anis shows that caspase activation is required for cell surface PS exposure, but not for $\Delta\psi_m$ dissipation (compare the *lower quarters* of the cytofluorometric diagrams in Fig. 4*A*). However, in CEM/Nef cells Z.VAD-fmk only partially inhibits PS exposure on the cell surface, while it reduces $\Delta\psi_m$ breakdown (Fig. 4*B*). In contrast, the appearance of both the apoptotic parameters is completely abolished by the caspase inhibitor when cells are incubated with the anti-Fas agonist Ab, independently of the expression of Nef (Fig. 4).

Caspase activation has been investigated by studying the cleavage of caspase-3, the main effector protease of this family. As shown in Fig. 5*A*, all three proapoptotic compounds cause the generation of the cleaved, i.e., activated, p17 form of caspase-3. Consistently with the cytofluorometric measurements, this effect is dramatic in the case of STS and Anis on CEM/Nef cells (see also the reduction of the uncleaved caspase-3 in the *upper part* of Fig. 5*A*). Pretreatment with the caspase inhibitor Z.VAD-fmk before apoptosis induction completely blocks the cleavage of caspase-3, independently of the apoptotic agonist and of Nef expression (Fig. 5*A*).

To directly assess caspase activity, we have measured with a colorimetric assay the cleavage of the chromophore-conjugated DEVD-pNA peptide (DEVDase activity), which is a substrate of caspase-3-like proteases. Nef expression significantly increases the DEVDase activity elicited by STS and Anis, but not by the anti-Fas Ab, and this enzymatic activity is completely abolished by pretreatment with Z.VAD-fmk (Fig. 5*B*).

Furthermore, we have investigated the cleavage of one nuclear caspase substrate, the PARP. PARP is cleaved into the expected 85-kDa fragment in all our apoptotic conditions (Fig. 6*A*). Consistently with cytofluorometric data, Nef significantly enhances the processing of PARP after STS or Anis treatment. Fig. 6*B* depicts the ratio between cleaved and intact PARP in the different conditions. As reported in Fig. 6*A*, the caspase inhibitor causes a complete prevention of the proteolysis of PARP in all of our experimental conditions, independently of Nef expression.

DNA degradation

One of the final steps of the apoptotic process, the degradation of DNA, has been also investigated. By using the cytofluorometric TUNEL technique, we have detected DNA double-stranded breaks in all conditions of apoptosis triggering. As depicted in Fig. 7, both STS and Anis cause a stronger DNA degradation in Nef-expressing than in native CEM cells. Instead, the effect of the anti-Fas Ab is comparable between the two cell types. In all conditions tested, a complete inhibition of DNA degradation has been observed following pretreatment with Z.VAD-fmk. DNA cleavage has been also studied with agarose gel electrophoresis. Surprisingly, the caspase-dependent DNA laddering induced by STS, Anis, or the anti-Fas Ab in CEM cells is abolished by Nef expression, even though the appearance of a smeared signal confirms the presence of a non-oligonucleosomal DNA degradation (Fig. 8). This lack of DNA degradation is independent of the duration of the apoptosis induction (from 1 to 8 h; data not shown), thus excluding the possibility of losing either early or late DNA fragments.

Apoptotic response in NIH-3T3 fibroblasts

The effect of Nef on the apoptotic process was measured in an unrelated cell type, NIH-3T3 fibroblasts stably transfected with the viral protein (40). As reported in Fig. 9*A*, in Nef-expressing cells (indicated as pool 3 in the figure), the protein level of Bcl-2 and Bcl-X$_L$ is reduced, whereas the expression of caspase-7 is unaffected. Caspase-3 was not tested because our Ab was not reactive on these cells. Apoptosis induction was assessed by quantifying $\Delta\psi_m$ breakdown and cell volume reduction, an intermediate step during the course of the apoptotic pathway (42). A typical experiment is displayed in Fig. 9*B*. Three unrelated proapoptotic compounds caused a marked mitochondrial depolarization (vertical axis of the diagrams) and cell shrinkage (horizontal axis of the diagrams) only in Nef-expressing cells.

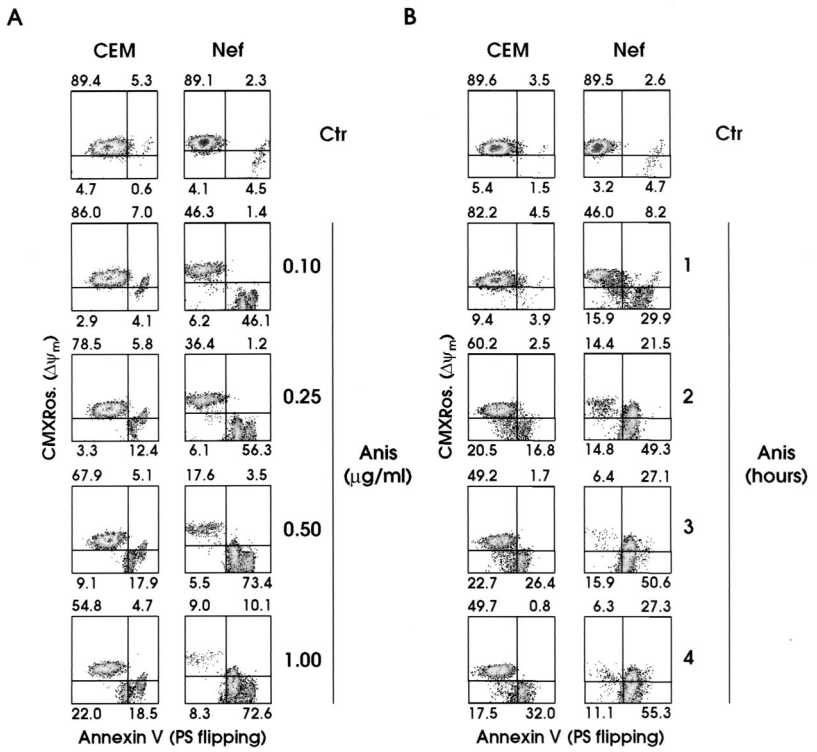


FIGURE 2. Dose-response and kinetics analysis of apoptosis induction. In *A*, CEM and CEM/Nef cells have been exposed for 5 h to the indicated concentrations of Anis, while in *B*, cells have been triggered with 1 μg/ml Anis for the reported times. Diagrams are labeled as in Fig. 1, and the percentage of each cell population is indicated. Ctr, Control.

Discussion

Depletion of functional immune cells is a hallmark of AIDS. This can be achieved either through a direct cytopathic effect on infected cells (43, 44) or through the activation of apoptotic programs (45). T cell apoptosis has been proposed as a mechanism involved both in the early steps of HIV-1 infection and in the massive T cell depletion which leads to immune suppression (28). The HIV-1-Nef protein is a possible mediator of this apoptosis induction. In fact, Nef promotes the transcription of Fas ligand and Fas in T cells, increasing their sensitivity to Fas-mediated apoptosis (46, 47), and in a soluble form it triggers cytolysis in several hematopoietic cells by binding to their cell surface (48). Therefore, it seems likely that Nef can induce apoptosis by interacting with different transduction pathways, although the precise mechanisms of its interferences with the apoptotic cascades remain to be clarified.

In the present study, we show that the HIV-1 Nef protein enhances the apoptotic process induced by several unrelated agents in different cell types. In fact, when three different alleles of *nef* are expressed in a $CD4^+$ T cell line, CEM cells, and these are incubated with staurosporine or Anis, CEM/Nef cells show a higher degree of loss of $\Delta\psi_m$, PS exposure on the cell surface, caspase activation, PARP cleavage, and DNA degradation with respect to native CEM cells. The increase in the apoptotic response has been also observed by using other proapoptotic compounds such as camptothecin and etoposide. Moreover, the apoptotic effect of these agents is accelerated in CEM/Nef cells. Consistently, apoptosis induction is increased when Nef is transfected in a different cell line, NIH-3T3 fibroblasts, as measured by $\Delta\psi_m$ breakdown and cell shrinkage.

The marked enhancement in $\Delta\psi_m$ breakdown caused by Nef in these cell lines suggests that its expression alters an early and common step of the apoptotic program (37). The homeostasis of $\Delta\psi_m$ is under the tight control of the Bcl-2 family proteins (35, 37, 49). Interestingly, we have observed that both CEM/Nef cells and

Table I. *Percentage of cells displaying loss of $\Delta\psi_m$ and PS exposure on the cell surface*[a]

Cell Lines	Control (n = 9)	STS (50 ng/ml, n = 9)	Anis (1 μg/ml, n = 6)	Anti-Fas Ab (150 ng/ml, n = 4)
CEM cells	7.7 ± 1.5%	23.4 ± 4.4%	49.2 ± 10.1%	42.0 ± 6.3%
CEM/Nef cells	7.5 ± 1.1%	76.9 ± 9.2%	92.4 ± 4.4%	36.8 ± 7.3%
p	>0.05	<0.001	<0.001	>0.05

[a] Cell populations showing at least one of these two parameters are considered as apoptotic. Experimental conditions are as in Fig 1; n, Number of experiments. A Student's t test analysis has been performed over mean ± SD values to assess for significant differences ($p < 0.01$).

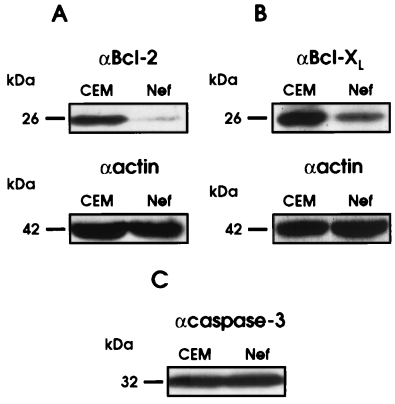


FIGURE 3. Nef reduces the expression level of Bcl-2 and Bcl-X_L, but not of caspase-3. Western immunoblot assays have been performed on native and Nef-expressing CEM cells to assess the expression level of Bcl-2 (*A*), Bcl-X_L (*B*), and caspase-3 (*C*). To verify the amount of protein load, blots have been rehybridized with an anti-α-actin Ab (*A* and *B*). In *C*, the same blot of *A* has been used.

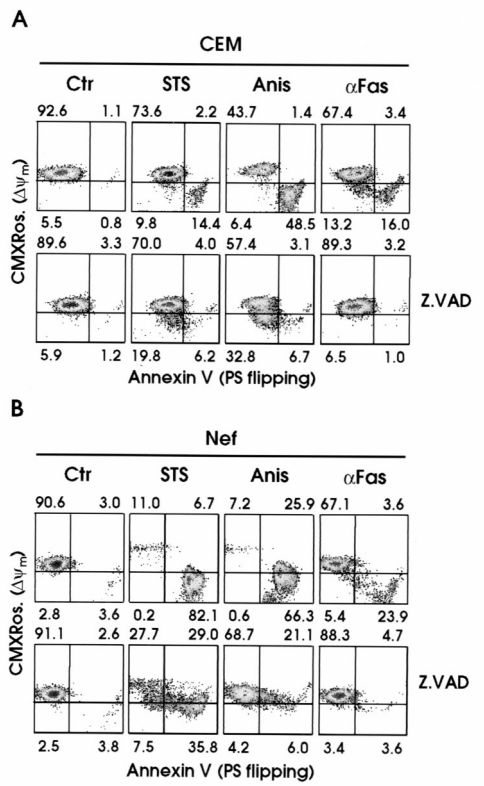


FIGURE 4. Caspase inhibition affects PS exposure on the cell surface and $\Delta\psi_m$ breakdown. CEM cells (*A*) and CEM/Nef cells (*B*) have been incubated for 30 min with or without the caspase inhibitor Z.VAD-fmk (Z.VAD, 100 μM) before a 5-h treatment with 50 ng/ml STS, 1 μg/ml Anis, or 150 ng/ml CH-11 anti-Fas agonist Ab (αFas). Diagrams are labeled as in Fig. 1, and the percentage of each cell population is indicated. Ctr, Control.

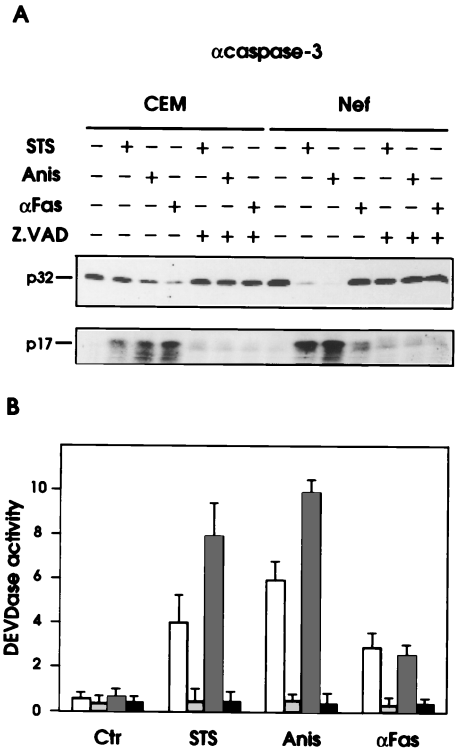


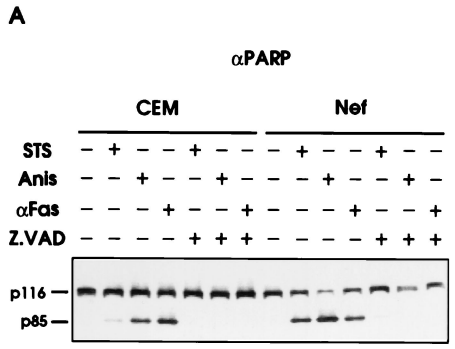
FIGURE 5. Nef expression increases caspase activation. CEM cells have been treated for 5 h with 50 ng/ml STS, 1 μg/ml Anis, or 150 ng/ml CH-11 anti-Fas agonist Ab (αFas). *A*, Western immunoblot assay displaying cleavage of the p32 form of the caspase-3 to the active p17 fragment. Preincubation with the caspase inhibitor Z.VAD-fmk (Z.VAD, 100 μM) abolishes the processing of caspase-3. *B*, Colorimetric measurement of the enzymatic activity of caspase-3-like proteins. The cleavage of the substrate peptide DEVD-pNA (DEVDase activity) is indicated on the vertical axis as arbitrary units of enzymatic activity, and it is completely prevented by preincubating cells with Z.VAD-fmk (Z.VAD, 100 μM). □, ▨: CEM cells without and with Z.VAD-fmk, respectively; ▨, ■: CEM/Nef cells without and with Z.VAD-fmk, respectively. Vertical bars over each column represent SDs; the effect of STS and Anis is significantly different between CEM and CEM/Nef ($p < 0.01$ with a Student's *t* test analysis). Ctr, Control.

Nef-expressing NIH-3T3 fibroblasts display a dramatic reduction in the expression level of the anti-apoptotic proteins Bcl-2 and Bcl-X_L. These two proteins inhibit the release of caspases and caspase-activating factors from apoptotic mitochondria (37), and contribute to the maintenance of the proton gradient responsible for $\Delta\psi_m$ by inducing a proton efflux from mitochondria (35, 50). Consistently, we have measured a lower apoptosis induction in native than in Nef-expressing CEM cells following treatment with the uncoupling agent carbonyl cyanide *m*-chlorophenyl-hydrazone, which dissipates the proton gradient (A.R., unpublished observations). Moreover, Bcl-2 regulates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) homeostasis by increasing the buffering capacity of the endoplasmic reticulum and mitochondria (51). A reduced expression of Bcl-2 could be at least partially responsible for the higher $[Ca^{2+}]_i$ that we previously observed in CEM/Nef cells, both in basal conditions and after discharge of intracellular pools (38). An excessive Ca^{2+} release from intracellular stores and a dysregulation of $[Ca^{2+}]_i$ homeostasis may facilitate the execution phase of PCD (52), and this could explain the observed enhancement of apoptosis in Nef-expressing CEM cells.

Nef could down-regulate Bcl-2/Bcl-X_L expression by modulating their transcription. In fact, the activity of several components of signal transduction cascades, such as Src-like kinases, the p21-activated kinase, and some MAP kinases, is affected by Nef (1, 4, 12–15). Furthermore, Nef could control the early steps of several apoptotic pathways by interacting with p53 (53), the phosphatidylinositol 3-kinase (40) or the MAP kinase cascades. By tuning some of these signaling pathways, Nef would enhance the apoptotic response even upstream of the modulation of Bcl-2/Bcl-X_L.

In the Fas apoptotic pathway, when the caspase activation cascade occurs immediately downstream of Fas receptor engagement (type I cells; Ref. 37), apoptosis induction cannot be altered by Bcl-2/Bcl-X_L (31). Accordingly, we have measured a comparable response to the triggering of the Fas receptor between native and Nef-expressing cells. In apparent discrepancy with our results, Zauli et al. (47) showed a positive correlation between Nef expression and the increase in Fas-mediated T cell death. However, these authors quantified a different apoptotic parameter, i.e., subdiploid DNA, which could partially explain this discrepancy. In addition, in our hands, CEM cells were much more responsive to the cross-linking of Fas than the Jurkat cells used by Zauli et al. (47). Thus, possible differences between native and Nef-expressing CEM cells could be overwhelmed by the high apoptotic effect of the anti-Fas Ab. Nonetheless, our results suggest that an increase in the apoptosis induction through the triggering of the Fas system is not common to all cases of Nef expression in T cells.

Remarkably, the broad-range caspase inhibitor Z.VAD-fmk only partially reduces PS exposure on the cell surface of CEM/Nef cells treated with STS or Anis. This is surprising, because several



B

Cell lines	Ctr	STS	Anis	αFas
CEM	1.4±1.9%	16.9±6.8%	27.8±10.9%	30.7±1.8%
CEM/Nef	1.3±0.8%	47.8±10.5%	86.5±9.5%	34.7±9.6%
p	>0.05	<0.01	<0.01	>0.05

FIGURE 6. Nef expression enhances the caspase-dependent cleavage of PARP in CEM cells. Cells have been treated for 5 h with 50 ng/ml STS, 1 μg/ml Anis, or 150 ng/ml CH-11 anti-Fas agonist Ab (αFas). In *A*, detection of the cleavage of p116 PARP to the expected p85 fragment has been performed by Western immunoblot assay. The cutting of PARP is inhibited when cells are preincubated with Z.VAD-fmk (Z.VAD, 100 μM). In *B*, the ratio between the p85 and the p116 forms of PARP has been measured with a densitometric analysis after Western immunoblot experiments, and a Student's *t* test analysis has been performed over mean ± SD values to assess for significant differences ($p < 0.01$; $n = 4$). Ctr, Control.

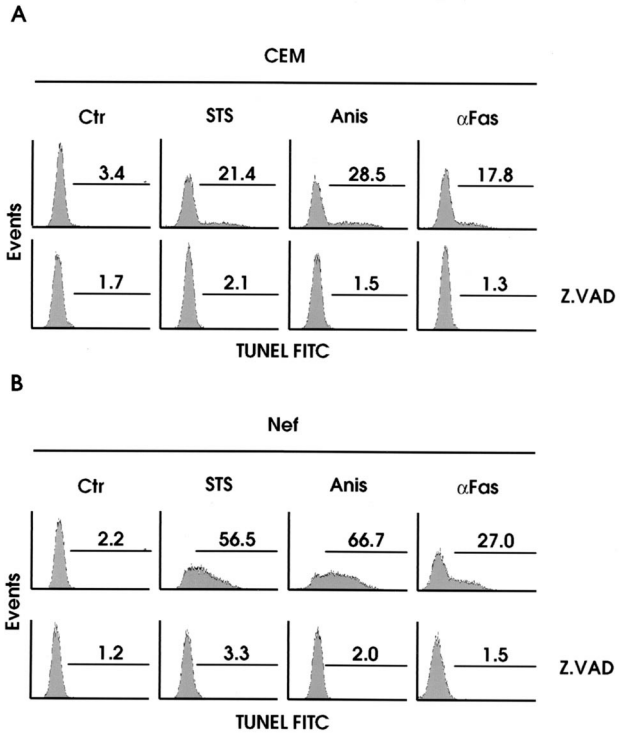


FIGURE 7. Nef expression enhances DNA degradation in CEM cells. Cells have been treated for 5 h with 50 ng/ml STS, 1 μg/ml Anis, or 150 ng/ml CH-11 anti-Fas agonist Ab (αFas). DNA cleavage has been assessed with the cytofluorometric TUNEL technique in native and in Nef-expressing CEM cells (*A* and *B*, respectively). The peak on the left represents cells with intact DNA, that on the right apoptotic cells, whose percentage is indicated over the horizontal line. On the horizontal axis is reported the intensity of the fluorescent signal, on the vertical axis the number of recorded events. Preincubation with the caspase inhibitor Z.VAD-fmk (Z.VAD, 100 μM) completely abolishes DNA degradation. Ctr, Control.

reports describe PS externalization as a caspase-dependent phenomenon (41, 54, 55). Neither caspase-3 or PARP cleavage nor DEVDase activity were detectable in CEM/Nef cells incubated with Z.VAD-fmk, thus indicating that caspase inhibition is complete. However, activation of a Z.VAD-fmk-insensitive caspase in CEM/Nef cells cannot be formally ruled out, and this might be responsible for PS flipping across plasma membrane. Alternatively, STS or Anis could trigger in CEM/Nef cells, but not in wild-type CEM cells, a caspase-independent PCD program in addition to the normal apoptotic pathway, or they could kill a fraction of Nef-expressing CEM cells by necrosis. Phosphatidylserine flipping onto the external leaflet of the plasma membrane has been recently recorded in both of these types of cell death (56, 57).

Moreover, we never observe DNA laddering, which is a caspase-dependent process (58), in CEM/Nef cells, even though in our conditions these cells display an equal or higher degree of caspase activation with respect to native CEM cells. A lack of sensitivity of the agarose gel technique is possible. However, our results are highly reproducible both on native and Nef-expressing CEM cells ($n>10$), independently of the degree of caspase activity (compare Figs. 5*B* and Fig. 8). Therefore, some additional step beyond caspase activation could be abrogated by Nef. We have recently proposed that a Cl^- efflux across the plasma membrane or a coupled K^+ efflux intervenes in the activation process of the endonuclease responsible for nucleosomal DNA fragmentation (41). Because we have observed that CEM/Nef cells lack a Ca^{2+}-dependent K^+ conductance (38), a correlation between alterations in plasma membrane ion fluxes and DNA laddering during apoptosis can be envisaged, and this possibility is under current investigation. Interestingly, CEM cells show a caspase-dependent DNA degradation if measured by the TUNEL technique. This ap-

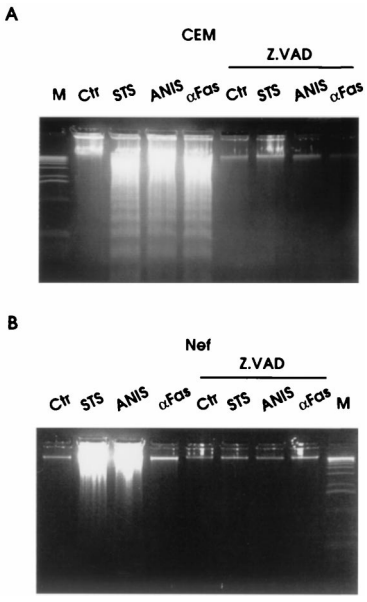


FIGURE 8. Nef expression abolishes oligonucleosomal DNA fragmentation in CEM cells. Native and Nef-expressing CEM cells (*A* and *B*, respectively) have been treated for 5 h with 50 ng/ml STS, 1 μg/ml Anis, or 150 ng/ml CH-11 anti-Fas agonist Ab (αFas). Oligonucleosomal DNA cleavage has been investigated on agarose gel electrophoresis (M, 1-kb m.w. marker; Promega, Madison, WI). Preincubation with the caspase inhibitor Z.VAD-fmk (Z.VAD, 100 μM) abrogates DNA laddering in native CEM cells. Ctr, Control.

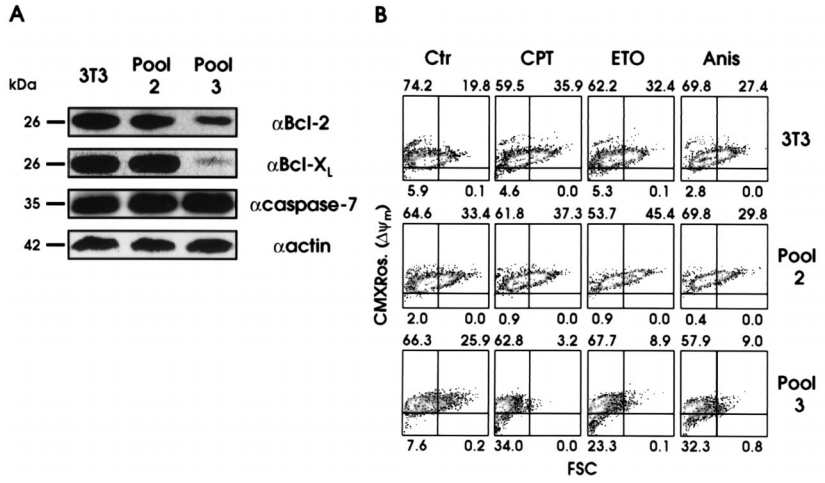


FIGURE 9. In NIH-3T3 fibroblasts, Nef reduces the expression level of Bcl-2 and Bcl-X_L, but not of caspase-7, and it enhances cell volume reduction and $\Delta\psi_m$ dissipation. The experiments have been performed on wild-type cells (3T3), on cells transfected with an empty vector (pool 2), and with a vector containing the *nef* gene (pool 3). In *A*, a Western immunoblot assay has been performed. The blot has been tested with anti-Bcl-2, anti Bcl-X_L, and anti-caspase-7 Abs. To verify the amount of protein load, the same blot has been rehybridized with an anti-α-actin Ab. In *B*, cells have been exposed for 7 h to 1 μM camptothecin (CPT), 100 μM etoposide (ETO), or 1 μg/ml Anis. Staining with CMXRos is shown on the vertical axis, while the horizontal axis displays the forward scatter (FSC) parameter, which is directly proportional to the cell volume. Cells in the *right quarters* of each diagram are smaller, and those in the *lower quarters* display loss of $\Delta\psi_m$. Percentage of each cell population is indicated. Ctr, Control.

optotic feature is increased in CEM/Nef cells, consistently with their higher caspase activity. This apparent discrepancy with the agarose gel data could be explained if a high molecular weight DNA degradation, detectable by the TUNEL technique, was not followed in CEM/Nef cells by oligonucleosomal DNA fragmentation.

In AIDS patients, the number of dying T cells exceeds the number of HIV-infected cells, and the apoptotic loss of T lymphocytes during HIV-1 infection can be due to multiple mechanisms (45). Here, we demonstrate for the first time that, when expressed in different cell types, Nef increases the sensitivity to death-receptor-independent apoptosis. The detailed molecular mechanism by which the viral protein controls this phenomenon remains to be established, even though the down-modulation of Bcl-2 and Bcl-X_L suggests an involvement of Nef in the early phases of the apoptotic cascade. Other groups have shown that Nef up-regulates Fas ligand expression, potentially triggering Fas signaling in bystander cells (47), and that it induces cytolysis in its soluble form (48). By these means, Nef would be able to kill noninfected cells during the course of the disease.

Therefore, Nef could play a central role in HIV-dependent T cell depletion through its many effects on various apoptotic cascades, both on infected and bystander cells.

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