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HIV-1 Tat Protects CD4$^+$ Jurkat T Lymphoblastoid Cells from Apoptosis Mediated by TNF-Related Apoptosis-Inducing Ligand

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We have here investigated the effect of TNF-related apoptosis-inducing ligand (TRAIL), a new member of the TNF cytokine superfamily, on the survival of Jurkat lymphoblastoid cell lines stably transfected with plasmids expressing the wild-type or mutated (Cys22) human immunodeficiency virus type 1 (HIV-1) *tat* gene. Jurkat cells transfected with wild-type *tat* were resistant to TRAIL-mediated apoptosis, while Jurkat cells mock-transfected with the control plasmid or with a mutated nonfunctional *tat* cDNA were highly susceptible to TRAIL-mediated apoptosis. Also, pretreatment with low concentrations (10–100 ng/ml) of extracellular synthetic Tat protein partially protected Jurkat cells from TRAIL-mediated apoptosis. Taken together, these results demonstrated that endogenously expressed *tat* and, to a lesser extent, extracellular Tat block TRAIL-mediated apoptosis. Since it has been shown that primary lymphoid T cells purified from HIV-1-infected individuals are more susceptible than those purified from normal individuals to TRAIL-mediated apoptosis, our findings underscore a potentially important role of Tat in protecting HIV-1-infected cells from TRAIL-mediated apoptosis. © 2001 Academic Press

Key Words: **HIV-1; Tat; TRAIL; apoptosis.**

INTRODUCTION

The human immunodeficiency virus-type 1 (HIV-1) Tat protein is pivotal for HIV-1 transcription and replication (1–3). The coding sequences of the *tat* gene are located in two exons, the first within the central region of the viral genome and the second overlapping the envelope gene. The spliced *tat* mRNA is translated into an 86- to 101-amino-acid Tat protein, which transactivates HIV-1 gene expression mainly acting on a stable RNA hairpin (the transactivation response element or TAR) that forms the 5′-untranslated leader of nascent viral transcripts (4–5). Tat can be divided into five distinct domains called N-terminal, cysteine-rich, core, basic, and C-terminal sequences (6). The cysteine-rich region is responsible for the formation of intramolecular disulfide bonds (7), while the basic region contains nuclear localization signals (8) and the binding site to TAR.

A number of studies have also clearly demonstrated that Tat protein plays a crucial role in the regulation of survival and activation of infected and uninfected lymphoid T cells, modulating the transcription of genes involved in cell survival/proliferation (9–21). It has also been shown that Tat can be actively released by HIV-1-infected cells (9) and that extracellular Tat shows opposite effects on the survival/death of lymphoid T cells. Interestingly, Tat protein can be taken up by bystander infected and uninfected cells (22), thus inducing biological effects through an autocrine/paracrine loop (23).

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily of cytokines (24). One peculiarity of TRAIL, with respect to TNF-α and CD95 ligand, other well-known death-inducing ligands (DIL), is that it is expressed in a wide variety of normal tissues, thus showing a much less restricted tissue distribution with respect to the other DIL. At present, the *in vivo* role of the TRAIL/TRAIL receptor system is not known, but *in vitro* studies have demonstrated that normal tissues are resistant to TRAIL-induced death, whereas several tumoral cell lines are clearly sensitive to the cytotoxic effects of TRAIL. In fact, TRAIL elicits apoptosis in a wide array of transformed cell lines by interacting with two main receptors, namely, TRAIL-R1 and TRAIL-R2 (25, 26), which engage a caspase-dependent apoptotic pathway. Interestingly, TRAIL-mediated apoptosis can be regulated negatively through the expression of decoy receptors, such as TRAIL-R3 or TRAIL-R4, which cannot activate the death pathway and rather activate NF-κB (in the case of TRAIL-R4) (27).

Although the biological effect of TRAIL on primary lymphoid cells is still not completely understood, peripheral blood mononuclear cells (PBMC) purified from

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HIV-1 seropositive patients are more susceptible to the cytotoxic activity of TRAIL with respect to cells purified from healthy donors (28, 29). In this context, we have here investigated whether and how *tat* expression and extracellular Tat protein affect the ability of TRAIL to modulate apoptosis in lymphoid T cells.

MATERIALS AND METHODS

Reagents

Both *r*His6-tagged TRAIL and *r*His6-tag control peptides were produced in bacteria and purified by affinity chromatography on Ni^{2+} affinity resin, as previously described (30).

Synthetic (Technogen, Caserta, Italy) HIV-1 Tat and recombinant HIV-1 p24 (Intracell, Cambridge, MA) proteins were dissolved in PBS containing 0.1% bovine serum albumin (BSA) and aliquoted at −70°C before use.

Cell Lines, Plasmids, and Treatments

The parental Jurkat $CD4^+$ lymphoblastoid T cell line was obtained from American Type Culture Collection (Rockville, MD) and kept in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, GIBCO) at an optimal cell density of $0.8–1 \times 10^6$.

Stably *tat*-transfected Jurkat cell clones were obtained, as previously described (31), by transfecting Jurkat cells with the following plasmid constructs: (i) pRPneoSL3*tat*, containing HIV-1 *tat* cDNA cloned in sense under the control of the SL3 murine leukemia virus long terminal repeat region, (ii) pRPneoSL3, representing the backbone control plasmid, (iii) pRPneoSL3*tat*22, containing the HIV-1*tat* clone, mutated in a single nucleotide resulting from substitution of cysteine 22 which is replaced by glycine (*tat*22) (31). The pLTRCAT plasmid represents a pCAT vector, where the long terminal repeat region of HIV-1 was cloned in front of the bacterial chloramphenicol acetyltransferase (CAT) gene. All stably transfected Jurkat cell clones were grown in RPMI plus 10% FCS and kept under continuous selective pressure by adding 800 μg/ml of the G418 antibiotic (Sigma Chemicals, St. Louis, MO). In all the experiments, parental and all stably transfected Jurkat cell clones were kept in RPMI 1640 plus 1% FCS for 12 h and then treated with increasing concentrations of TRAIL (0–1000 ng/ml) or equimolar concentrations of *r*His6-tag irrelevant peptide. In order to determine the interference of extracellular Tat on TRAIL-induced apoptosis, parental Jurkat cells were kept in RPMI 1640 plus 1% FCS for 12 h and pretreated with synthetic Tat protein at different concentrations (0–2000 ng/ml) for 30 min before adding 100 ng/ml of TRAIL.

In all experiments the analysis of apoptotic cells was performed at 12, 24, 48, and 72 h after TRAIL treatment.

CAT Assay

Transient transfection experiments were carried out using the DEAE–dextran method, as previously described (17). Briefly, 10×10^6 of pRPneoSL3/*tat*, pRPneoSL3, pRPneoSL3/*tat22*, and parental Jurkat cells were transfected with 5 μg of LTR-CAT plasmid and 500 μg DEAE–dextran for 60 min in 2 ml of medium. After transfection, Jurkat cells were seeded in 10 ml of RPMI plus 10% FCS for 24 h. Cells were then lysed and the clarified lysates were assayed for CAT activity using volumes of extracts corresponding to equal amounts of proteins, as determined by the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA).

Intracellular Tat Protein Analysis

The intracellular Tat content was determined by flow cytometry, as described previously (32). Briefly, transfected pRPneo*tat*, pRPneo*tat22*, pRPneo, and parental Jurkat cells were fixed in 0.5 ml of 70% ethanol (EtOH) for 30 min at 4°C. Samples were washed twice in PBS, treated with 200 μl of guinea pig normal serum (10% v/v in PBS, Dako, Copenhagen, Denmark) for 15 min at room temperature to saturate unspecific binding sites, and then incubated for 2 h at 37°C with 200 μl of anti-Tat polyclonal serum (Intracell, diluted 1:100) in 2% BSA/PBS. After two washings in PBS, samples were treated with a fluorescein-conjugated swine anti-rabbit (FITC-SAR, Dako) IgG diluted (1:200) in PBS/2% BSA. After several washes in PBS, samples were resuspended in 300 μl PBS and analyzed by flow cytometry. Negative controls consisted of normal rabbit or anti-HIV-1 p24 polyclonal serum (Intracell) followed by identical second layer labeling as above.

Western Blot Analysis

Western blot assays were performed as described (12). Briefly, after electrophoresis on 12% acrylamide gels and blotting onto nitrocellulose membrane, blotted filters were treated overnight with an anti-TRAIL monoclonal antibody (1/500 in PBS/1% BSA; Pharmingen, San Diego, CA). After overnight incubation, filters were washed, treated with a peroxidase-labeled anti-mouse IgG (1/1000 in PBS/1% BSA; Dako), and detected with a colorimetric reaction, as previously described (32).

Analysis of the DNA Content by Propidium Iodide Staining and Flow Cytometry

Analysis of the cell cycle and of apoptosis was performed by labeling EtOH-fixed cells with propidium

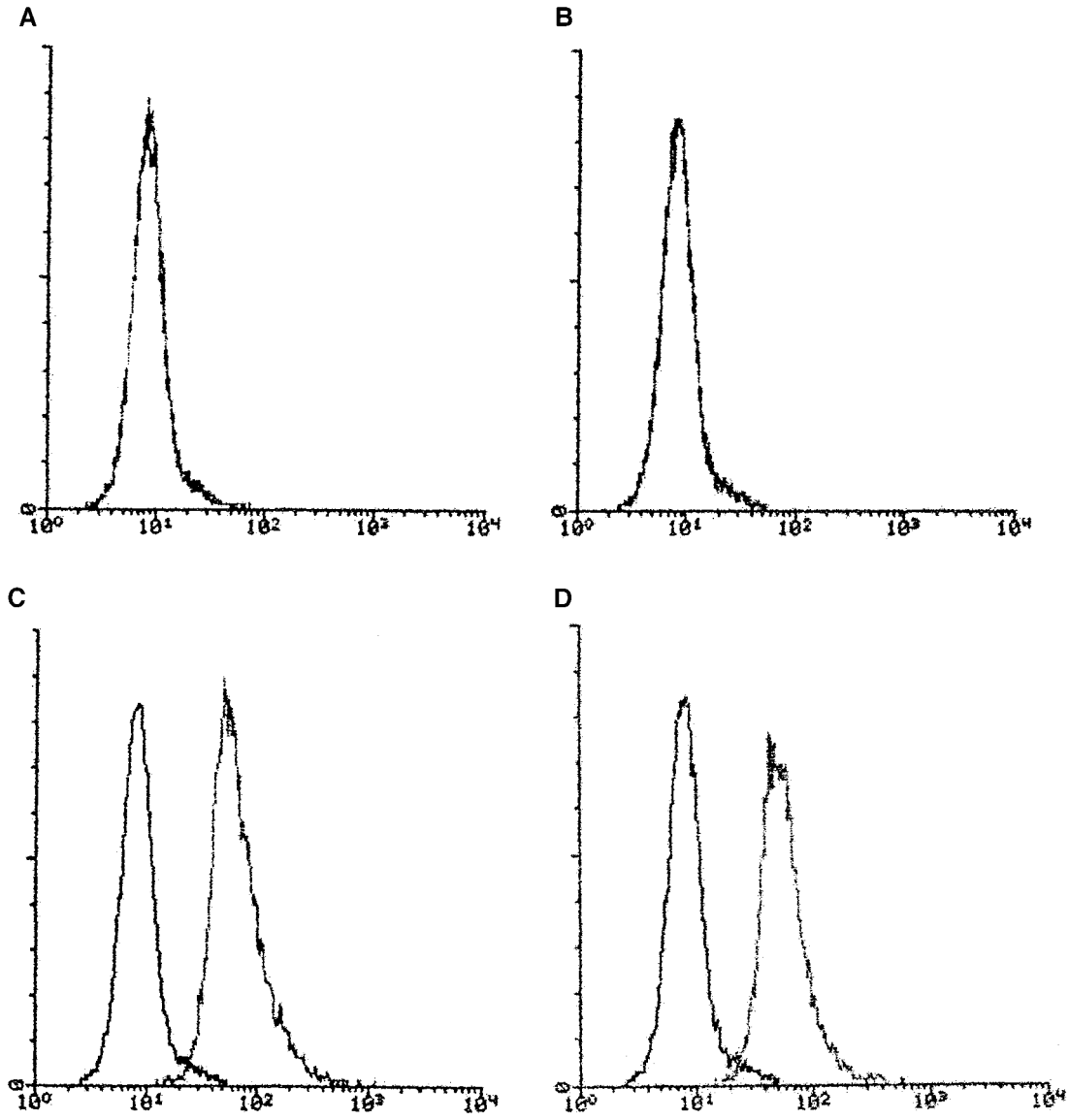

FIG. 1. Flow cytometric detection of intracellular Tat in wild-type (A), pRPneoSL3 (B), pRPneoSL3*tat* (C), and pRPneoSL3*tat*22 (D) Jurkat cells. In A and B fluorescence areas representing anti-Tat polyclonal antibody-stained cells and anti-p24 polyclonal antibody-stained control cells were overlapped. In C and D, the right fluorescence peak represents anti-Tat polyclonal-stained cells, whereas the left fluorescent peak represents anti-p24 polyclonal antibody-stained control cells; *X* axis, fluorescence intensity (logarithmic scale); *Y* axis, relative number of cells. A representative of three separate experiments is shown.

iodide (PI), as previously described (10). pRPneoSL3, pRPneoSL3*tat,* pRPneoSL3*tat22,* and parental Jurkat cells were recovered after 12, 24, 48, and 72 h of TRAIL treatment. The samples were washed twice in phosphate-buffered saline (PBS) and fixed by 70% (v/v) EtOH for 60 min at 4°C. Cells were treated with RNase A (0.5 mg/ml in PBS; Sigma) for 15 min at 37°C. The cells were then stained with propidium iodide (PI; 40 μg/ml in PBS; Sigma) for 15 min at 4°C. The PI fluorescence of the labeled cells was analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) equipped with an argon ion laser tuned at 488 nm. PI fluorescence was analyzed in logarithmic scale to allow a better identification of apoptotic cells as a subdiploid cell peak (8). To evaluate the apoptotic cell number, the threshold was triggered on the red fluorescence where a clear-cut distinction between cell debris and apoptotic cells can always be identified. At least 5000 to 10000 events for each sample were collected. The data were analyzed using Lysis II analysis software (Beckton-Dickinson).

TUNEL Assay

The TUNEL assay was performed by using the TUNEL Boehringer kit (Boehringer) following the

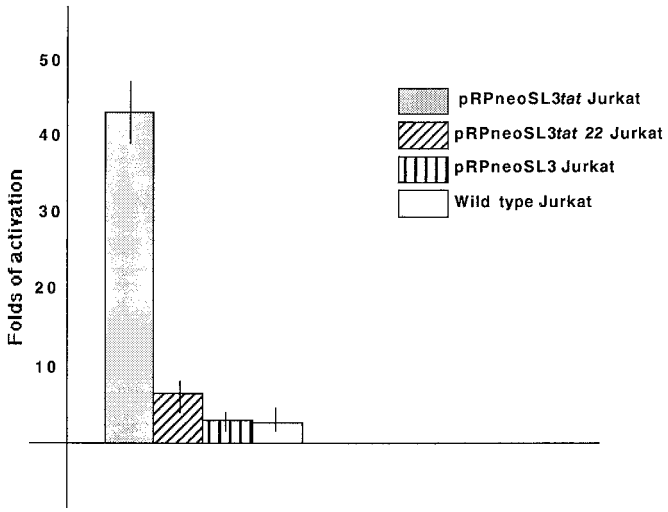

FIG. 2. CAT assay of transient transfection with the pLTR-CAT construct. Reported values represent the ratio between the acetylation values achieved in different cell lines with respect to the value achieved in wild-type Jurkat cells. A representative of three separate experiments is shown.

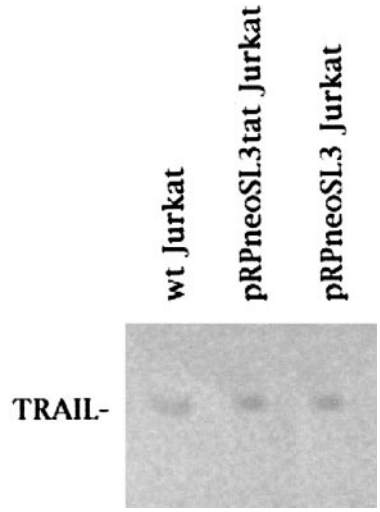

FIG. 3. Western blot assay of TRAIL protein content in the tested cell lines.

manufacturer's instructions. Briefly, the cells were fixed in 4% paraformaldheyde diluted in PBS for 15 min at room temperature. The samples were washed twice in PBS and permeabilized for 2 min in ice with PBS/0.5% Triton X-100. After extensive washings, the cells were treated with a reaction solution containing terminal deoxynucleotide transferase (TdT) enzyme and fluorescein-conjugated deoxyuridine triphosphate (FITC-dUTP) following the manufacturer's instructions. The samples were incubated for 60 min at 37°C and then washed extensively with PBS. The number of apoptotic TUNEL-positive cells was quantified by flow cytometry.

Gel Electrophoresis

To evaluate the presence of DNA fragmentation, cellular DNA was extracted from pRPneoSL3, pRPneoSL3*tat*, pRPneoSL3*tat22*, and parental Jurkat cells by using an apoptotic gel fragmentation kit (Boehringer), following the manufacturer's instructions. The samples were then analyzed by means of 2% agarose gel electrophoresis and stained with ethidium bromide.

Phenotypic Analysis of Surface TRAIL Receptors

Parental, pRPneoSL3, pRPneoSL3*tat*, and pRPneoSL3*tat*22 Jurkat cell clones were analyzed for the surface expression of TRAIL receptor (R)-1, TRAIL-

TABLE 1

Apoptotic Cell Percentage in Flow Cytometry Analysis of Propidium Iodide Staining at Different Times

	Culture time (h)			
	12	24	48	72
Jurkat without treatment	6.3 ± 0.9	10.3 ± 2.4	15.4 ± 2.1	24.7 ± 2.7
Jurkat + 100 ng/ml BSA	6.7 ± 0.6	10.0 ± 2.2	15.9 ± 2.0	24.3 ± 3.4
Jurkat + 10 ng/ml TRAIL	9.4 ± 1.8	15.7 ± 3.0	39.6 ± 2.7	50.1 ± 5.8
Jurkat + 100 ng/ml TRAIL	13.2 ± 2.2	35.9 ± 3.5	53.4 ± 3.9	78.6 ± 6.7
Jurkat + 500 ng/ml TRAIL	14.6 ± 1.7	38.3 ± 3.9	52.7 ± 4.5	76.9 ± 6.4
Jurkat + 1000 ng/ml TRAIL	17.3 ± 2.1	41.2 ± 4.1	60.8 ± 5.9	83.4 ± 7.3
pSL3 Jurkat w/o treatment	6.1 ± 0.7	9.6 ± 2.0	14.3 ± 1.9	23.4 ± 2.6
pSL3 Jurkat + 100 ng/ml BSA	6.5 ± 0.4	9.8 ± 2.4	16.5 ± 2.2	22.6 ± 3.0
pSL3 Jurkat + 10 ng/ml TRAIL	8.8 ± 1.6	14.5 ± 2.7	36.6 ± 3.1	52.1 ± 4.5
pSL3 Jurkat + 100 ng/ml TRAIL	14.0 ± 1.8	37.3 ± 3.2	55.8 ± 4.8	76.0 ± 7.5
pSL3 Jurkat + 500 ng/ml TRAIL	15.0 ± 1.5	38.5 ± 3.6	56.0 ± 5.2	74.1 ± 7.3
pSL3 Jurkat + 1000 ng/ml TRAIL	17.9 ± 2.5	40.0 ± 4.4	58.8 ± 4.6	80.7 ± 7.1

Note. Wild-type Jurkat and pRPneoSL3 Jurkat cells were treated with different concentrations of TRAIL protein. Data are expressed as means ±SD of four separate experiments performed in duplicate.

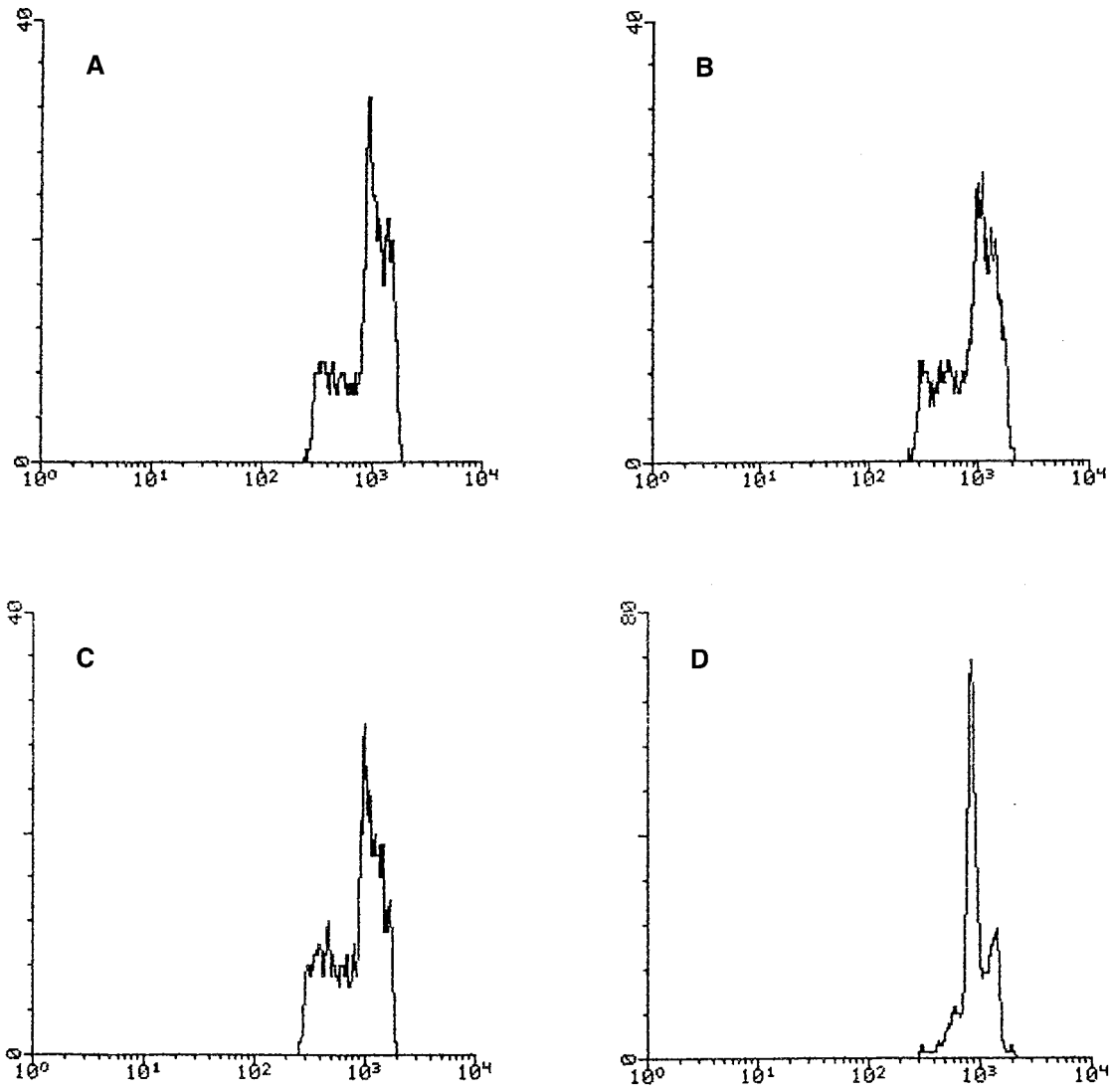

FIG. 4. Propidium iodide staining revealed by flow cytometry procedure. Wild-type Jurkat cells (A), pRPneoSL3 Jurkat cells (B), pRPneoSL3*tat*22 Jurkat cells (C), and pRPneoSL3*tat* Jurkat cells (D) were stained with propidium iodide 24 h after TRAIL treatment. Apoptosis is revealed by subdiploid peak; *X* axis, fluorescence intensity (logarithmic scale); *Y* axis, relative number of cells. A representative of four separate experiments is shown.

R2, TRAIL-R3, and TRAIL-R4 by indirect staining with primary goat anti-human TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 IgG (all from R&D Systems, Oxon, UK) followed by phycoerythrin (PE)-conjugated rabbit anti-goat IgG secondary antibody (Ab, Sigma). Briefly, staining was performed on 5×10^5 cells in 200 μl of PBS containing 1% FCS and 5 μl of each primary antibody on ice for 30 min. Cells were washed twice, supplemented with 3 μl of PE-conjugated rabbit anti-goat IgG on ice for 30 min, washed twice with PBS, and then analyzed by FACScan. Aspecific fluorescence was assessed by using normal goat IgG followed by a second layer as above. Samples were assayed in duplicate and gates containing viable cells were used to collect 10,000 events.

Statistical Analysis

Statistical analysis was performed using the two-tailed Student's *t* test.

RESULTS AND DISCUSSION

Evaluation of Tat Protein Expression in Jurkat Cell Lines

In the first set of experiments we evaluated the levels of Tat protein produced by *tat*-transfected cell lines, using a flow cytometric procedure. As shown in Figs. 1A–1D, the staining of the pRPneoSL3*tat* and pRPneoSL3*tat*22 Jurkat cells with a rabbit polyclonal anti-Tat antibody showed a clear shift in the fluores-

TABLE 2

Apoptotic Cell Percentage in Flow Cytometry Analysis of Propidium Iodide Staining at Different Times

	Culture time (h)			
	12	24	48	72
Jurkat + *r*His-tag	6.9 ± 0.7	10.2 ± 2.1	15.3 ± 1.8	24.8 ± 3.1
Jurkat + TRAIL	13.6 ± 2.4	35.4 ± 4.2	54.6 ± 3.6	74.2 ± 6.3
pRPneoSL3 Jurkat + *r*His-tag	6.5 ± 0.4	9.8 ± 2.4	16.5 ± 2.2	22.6 ± 3.0
pRPneoSL3 Jurkat + TRAIL	14.0 ± 1.8	37.3 ± 3.2	55.8 ± 4.8	76.0 ± 7.5
pRPneoSL3*tat*22 Jurkat + *r*His-tag	6.4 ± 0.5	9.6 ± 2.4	16.2 ± 3.2	25.3 ± 4.1
pRPneoSL3*tat*22 Jurkat + TRAIL	13.9 ± 2.1	36.0 ± 4.0	59.5 ± 4.7	80.1 ± 7.4
pRPneoSL3*tat* Jurkat + *r*His-tag	2.2 ± 0.4	3.0 ± 1.1	6.8 ± 1.6	9.7 ± 1.7
pRPneoSL3*tat* Jurkat + TRAIL	3.5 ± 0.5	5.4 ± 1.3	9.1 ± 1.5	13.4 ± 2.5

Wild-type Jurkat, pRPneoSL3 Jurkat, pRPneoSL3*tat*22 Jurkat, and pRPneoSL3*tat* Jurkat cells were treated with 100 ng/ml of TRAIL protein or with *r*His-tag at equimolar concentrations. Data are expressed as means ±SD of four separate experiments performed in duplicate.

cence intensity of the whole cell population. On the other hand, no detectable shift was observed either in pRPneoSL3 or in parental Jurkat cells in comparison with the background fluorescence levels observed in cells stained with anti-HIV-1 p24 polyclonal antibody. These experiments demonstrate that pRPneoSL3*tat* and pRPneoSL3*tat*22 Jurkat cells produce significant amounts of Tat protein.

To ascertain whether Tat protein produced by pRPneoSL3*tat* Jurkat cells was biologically active, we carried out transient transfection experiments by using the pLTR-CAT plasmid, in which the long terminal

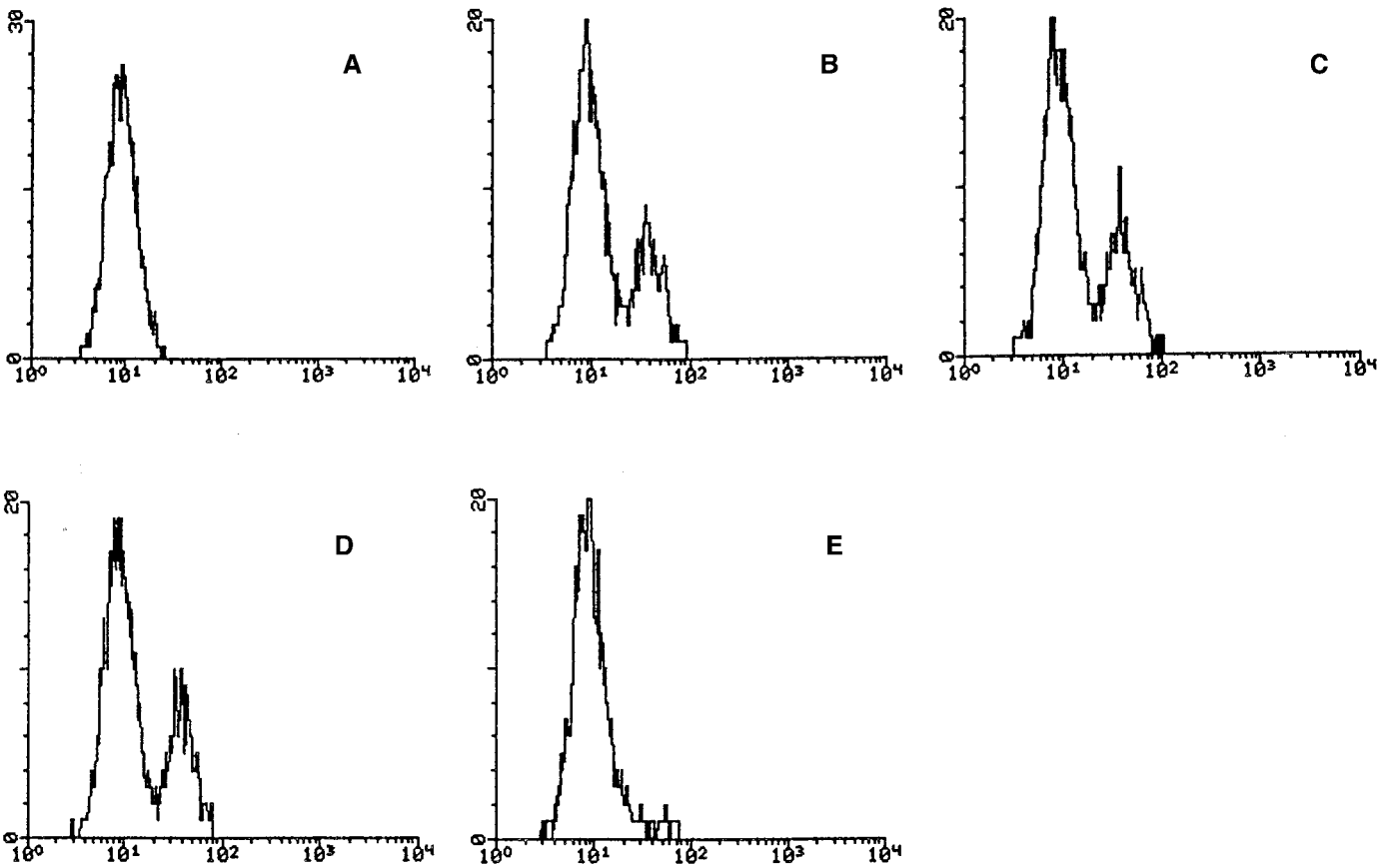

FIG. 5. TUNEL analysis by flow cytometry. Background fluorescence represented by overlaid peaks obtained with each cell line treated without TdT (A), wild-type Jurkat cells (B), pRPneoSL3 Jurkat cells (C), pRPneoSL3*tat*22 Jurkat cells (D), and pRPneoSL3*tat* Jurkat cells (E) after 24 h of TRAIL treatment. The apoptotic cells are represented by the right fluorescence peak in the histogram; *X* axis, fluorescence intensity (logarithmic scale); *Y* axis, relative number of cells. A representative of four separate experiments is shown.

TABLE 3

Apoptotic Cell Percentage in Flow Cytometry Analysis of the TUNEL Technique at Different Times

	Culture time (h)			
	12	24	48	72
Jurkat + *r*His6-tag	6.3 ± 0.5	9.2 ± 2.0	14.7 ± 1.7	25.8 ± 3.2
Jurkat + TRAIL	13.5 ± 2.1	32.7 ± 3.1	50.4 ± 3.6	74.3 ± 6.0
pRPneoSL3 Jurkat + *r*His6-tag	6.7 ± 0.6	8.9 ± 1.9	15.7 ± 2.0	25.6 ± 3.5
pRPneoSL3 Jurkat + TRAIL	15.2 ± 1.5	35.8 ± 3.5	58.6 ± 4.3	74.2 ± 7.3
pRPneoSL3*tat*22 Jurkat + *r*His6-tag	6.9 ± 0.9	10.3 ± 1.2	13.3 ± 2.7	24.3 ± 5.8
pRPneoSL3*tat*22 Jurkat + TRAIL	12.7 ± 1.6	35.5 ± 5.2	61.5 ± 8.7	82.1 ± 9.4
pRPneoSL3*tat* Jurkat + *r*His6-tag	2.1 ± 0.2	3.2 ± 0.9	6.4 ± 1.5	9.6 ± 1.9
pRPneoSL3*tat* Jurkat + TRAIL	3.2 ± 0.5	5.0 ± 1.1	8.8 ± 1.8	12.6 ± 3.1

Wild-type Jurkat, pRPneoSL3 Jurkat, pRPneoSL3*tat*22 Jurkat, and pRPneoSL3*tat* Jurkat cells were treated with 100 ng/ml of TRAIL protein or with *r*His-tag at equimolar concentrations. Data are expressed as means ±SD of four separate experiments performed in duplicate.

repeat of HIV-1 is cloned in front of a bacterial CAT gene reporter. The CAT assay (Fig. 2) demonstrated that pRPneoSL3*tat* Jurkat cells translate a biologically active Tat protein. As expected (31), pRPneoSL3*tat22* Jurkat cells were unable to significantly activate HIV-1 LTR, as the mutation in cysteine 22 of Tat lead to the translation of a Tat protein which is inactive on the LTR promoter.

Taking into account that TRAIL is produced by different cell types, including T cells (33), we next evaluated its expression in different transfected and wild-type Jurkat cell lysates. Western blot analysis performed with an anti-TRAIL monoclonal antibody demonstrated that Jurkat cells expressed TRAIL and that the amount of TRAIL produced was equivalent in the different Jurkat cell clones (Fig. 3). Thus, *tat* overexpression did not significantly affect the amount of TRAIL produced by Jurkat cells.

Stably tat-Transfected Jurkat Lymphoblastoid CD4$^+$ T Cells Are Resistant to TRAIL-Induced Apoptosis

We next investigated whether endogenous *tat* expression was able to interfere with TRAIL-mediated cytotoxicity. In order to select the optimal concentration of TRAIL protein inducing apoptosis of Jurkat cells, we first treated wild-type and pRPneoSL3 Jurkat cells with increasing concentrations of reccombinant TRAIL (0–1000 ng/ml). The percentage of apoptosis dose-dependently increased upon TRAIL addition, reaching a plateau at concentrations of 100 ng/ml or higher (Table 1). Moreover, maximal levels of apoptosis were already achieved after 24 h of culture in wild-type, pRPneoSL3, and pRPneoSL3*tat*22 Jurkat cells (Fig. 4). In sharp contrast, pRPneoSL3*tat* Jurkat cells were resistant to apoptosis induced by TRAIL ($P < 0.001$) at all time points examined (up to 72 h) (Table 2). These data demonstrate that endogenous *tat* gene expression is able to counteract the ability of TRAIL to induce apoptosis. Of note, a single mutation in the Cys22 residue, which abrogated the transactivating activity of *tat*, also abolished its ability to protect Jurkat cells from apoptosis.

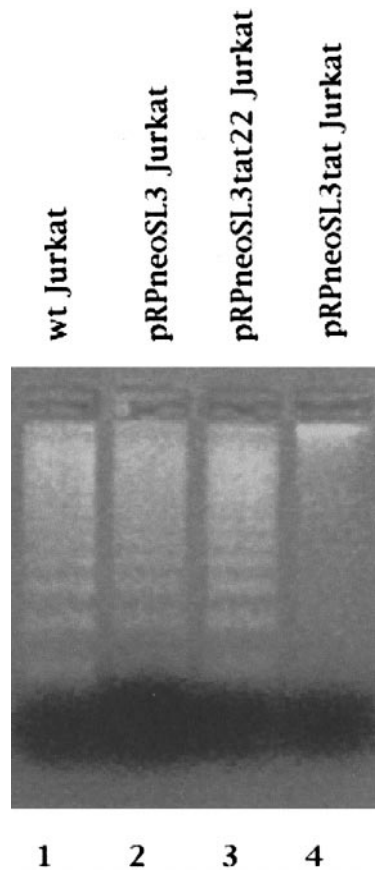

FIG. 6. Gel electrophoresis apoptosis analysis. The apoptosis ladder in DNA extracted from wild-type Jurkat cells, pRPneoSL3 Jurkat cells, pRPneoSL3*tat*22 Jurkat cells but not in DNA of pRPneoSL3*tat* Jurkat cells is clearly shown at 24 h after TRAIL treatment. A representative experiment is shown.

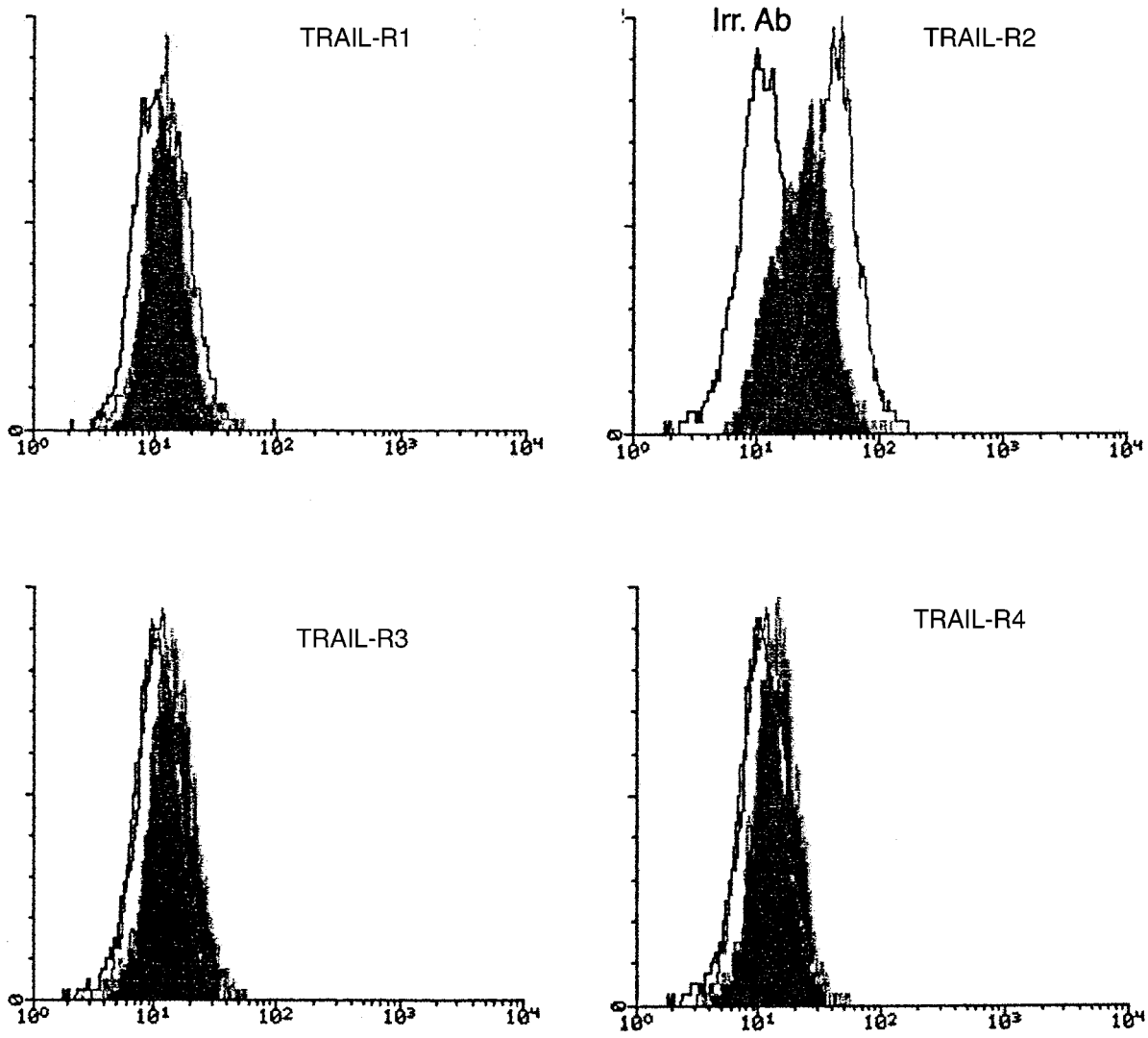

FIG. 7. Evaluation of the surface expression of TRAIL-R1, -R2, -R3, and -R4 performed in pRPneoSL3*tat* and pRPneoSL3 Jurkat cells. Shadowed areas represent pRPneoSL3*tat* Jurkat cells, while unshadowed areas represent pRPneoSL3 (control) Jurkat cells. Negative controls are represented by cells stained with an isotype-matched irrelevant IgG (Irr. Ab); *X* axis, fluorescence intensity (logarithmic scale); *Y* axis, relative number of cells. A representative of four separate experiments is shown.

To confirm these results with a complementary approach, parallel experiments were carried out by using the TUNEL technique. Flow cytometry analysis of these samples (Fig. 5) confirmed the anti-apoptotic activity of endogenously expressed *tat*. The number of apoptotic cells revealed by TUNEL was comparable with that obtained by PI staining and flow cytometry (Table 3). Consistently with the data obtained by PI staining and by TUNEL, TRAIL-treated pRPneoSL3*tat* Jurkat cells do not display any detectable ladder after 24 h of TRAIL treatment (Fig. 6). On the other hand, the internucleosomic degradation responsible for the ladder was observed in wild-type Jurkat, pRPneoSL3 Jurkat, and pRPneoSL3*tat*22 Jurkat cells upon TRAIL treatment.

Expression of Endogenous tat Does Not Substantially Modify the Surface Expression of TRAIL Receptors in Jurkat Lymphoblastoid CD4⁺ T Cells

The only TRAIL receptor clearly detectable on the surface of parental wild-type Jurkat cells was TRAIL-R2 (DR5) (data not shown). Similarly, pRPneoSL3*tat*, pRPneoSL3*tat*22, and pRPneoSL3 Jurkat cell lines expressed surface TRAIL-R2, but not the other TRAIL receptors (Fig. 7). In four separate experiments, the mean fluorescence intensity of TRAIL-R2 did not show significant differences among the various Jurkat cell lines examined.

TABLE 4
Apoptotic Cell Percentage in Flow Cytometry Analysis of Propidium Iodide Staining

	Culture time (h)			
	12	24	48	72
Jurkat without treatment	14.3 ± 1.8	36.3 ± 3.4	51.4 ± 3.1	76.7 ± 3.7
Jurkat + 1 ng/ml Tat	7.7 ± 0.5	15.4 ± 2.3	24.7 ± 2.7	31.2 ± 4.0
Jurkat + 10 ng/ml Tat	4.5 ± 1.2	11.3 ± 2.2	14.6 ± 2.0	24.1 ± 4.9
Jurkat + 100 ng/ml Tat	4.1 ± 1.5	8.2 ± 1.6	14.4 ± 1.5	21.4 ± 4.2
Jurkat + 1000 ng/ml Tat	13.9 ± 3.0	48.4 ± 4.1	60.9 ± 3.9	79.8 ± 7.4
Jurkat + 2000 ng/ml Tat	15.1 ± 3.2	49.6 ± 3.9	61.6 ± 7.3	78.5 ± 6.7
Jurkat + 1 ng/ml p24	15.0 ± 1.9	36.4 ± 3.1	50.8 ± 4.7	75.8 ± 4.0
Jurkat + 10 ng/ml p24	14.5 ± 2.4	35.3 ± 2.7	52.5 ± 5.0	74.1 ± 4.9
Jurkat + 100 ng/ml p24	14.7 ± 2.5	36.2 ± 3.6	54.4 ± 4.5	75.4 ± 4.2
Jurkat + 1000 ng/ml p24	14.9 ± 2.0	37.4 ± 3.1	52.8 ± 3.9	74.8 ± 6.4
Jurkat + 2000 ng/ml p24	13.9 ± 2.2	35.6 ± 3.8	53.6 ± 4.3	73.5 ± 4.7

Several Tat or p24 concentrations were added to Jurkat cells cultures before TRAIL treatment (100 ng/ml). Data are expressed as means ±SD of four separate experiments performed in duplicate.

Low Concentrations of Extracellular Tat Counteract the TRAIL-Mediated Apoptosis of Jurkat Cells

In the next series of experiments, Jurkat cells were pretreated with different concentrations of extracellular Tat (0–2000 ng/ml) and the degree of apoptosis was analyzed by PI staining and flow cytometric analysis at various time points (0–72 h) after TRAIL treatment (100 ng/ml). The results shown in Table 4 and Fig. 8 demonstrated that pretreatment with exogenous Tat (at 10–100 ng/ml) significantly ($P < 0.05$) counteracted the induction of apoptosis mediated by TRAIL, even though to a lesser extent than that with endogenously expressed *tat*. On the other hand, at 1000–2000 ng/ml, this survival effect was no longer observed. In parallel experiments, cell cultures were treated with increasing concentrations of HIV-1 p24, which did not affect TRAIL-mediated apoptosis. Interestingly, in order to observe the protective effect of extracellular Tat, the order by which Tat and TRAIL are added to Jurkat cells appears crucial. In fact, when the cells were treated with Tat and TRAIL together or with TRAIL 30–60 min before Tat protein, Tat was unable to protect from TRAIL-mediated apoptosis (data not shown).

It has been shown that Tat upregulates the transcription of both viral and cellular promoters affecting the survival, growth, and function of T cells (34). In this respect, we and other groups of investigators have previously demonstrated that Tat protein modulates the survival and proliferation of different cell types, including T cells (10–21). In particular, while endogenously expressed *tat* and low concentrations of exogenously added Tat protein increase the survival and activation of quiescent T cells (16–18, 21), high concentrations of extracellular Tat induce apoptosis of preactivated T cells as well as of Jurkat cells (11–14, 16, 21). We and other authors have previously shown that this dual role of extracellular Tat on lymphoid T cell survival/growth (16) mainly depends on the imbalance among survival/activation signals, represented by the PI-3 kinase (35) and ERK/MAPK (36, 37) pathways, both of which are maximally stimulated by low (pM) concentrations of extracellular Tat, and the JNK pathway, which is maximally activated by high (μM) concentrations of Tat (38, 39). The results of the present study indicate that Tat protein effectively abolished TRAIL-mediated apoptosis in $CD4^+$ lymphoblastoid T cells. Of note, mutation in a single residue (Cys22) completely suppressed Tat ability to protect Jurkat cells from apoptosis, clearly indicating that a conserved Tat conformation was essential for the anti-apoptotic activity of Tat.

The molecular mechanisms by which Tat inhibits TRAIL-mediated apoptosis remain to be fully elucidated, since apparently they do not involve gross changes in the surface expression of TRAIL receptors mediating a death signal. However, we have previously demonstrated that endogenously expressed *tat* can promote the transcription of Bcl-2 (17), via the Ser133 phosphorylation of CREB transcription factor (37). Our findings are particularly noteworthy in light of previous findings, which have recently demonstrated that peripheral blood mononuclear cells obtained from HIV-1-seropositive patients are more sensitive to TRAIL-mediated apoptosis than those purified from healthy individuals (28, 29). The ability of Tat to protect HIV-1-infected cells from apoptosis induced by TRAIL or other DIL likely represents a part of a viral strategy aimed to protect infected cells during a critical phase of the viral life cycle. The resulting scenario is that Tat, a dominant factor in the early phase of the viral life cycle, can promote the survival of infected cells in order to suppress apoptosis mediated by a factor such as TRAIL that could impair complete viral maturation. Our findings also imply that the $CD4^+$ T cells killed by

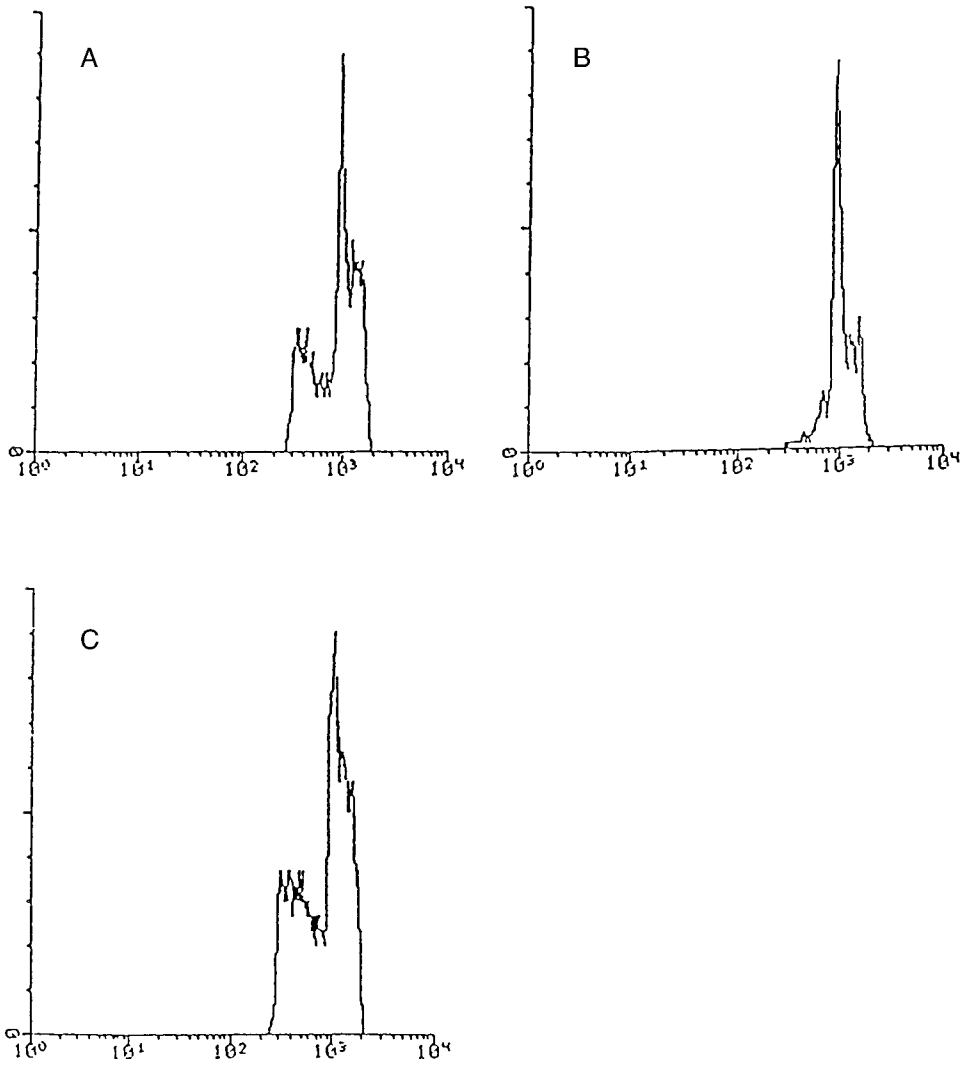

FIG. 8. Propidium iodide staining of Jurkat cells treated with different concentrations of Tat and subsequently with 100 ng/ml of TRAIL protein revealed by flow cytometry 24 h after TRAIL treatment. Wild-type Jurkat cells without Tat treatment (A), with 100 ng/ml of Tat (B), and 1000 ng/ml (C). Apoptosis is revealed by subdiploid peak; *X* axis, fluorescence intensity (logarithmic scale); *Y* axis, relative number of cells. A representative of four separate experiments is shown.

TRAIL in HIV-1-infected individuals are likely to be the uninfected ones.

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