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Resistance of Chimpanzee T Cells to Human Immunodeficiency Virus Type 1 Tat-Enhanced Oxidative Stress and Apoptosis

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CD4$^+$ T-cell depletion in AIDS patients involves induction of apoptosis in human immunodeficiency virus (HIV)-infected and noninfected T cells. The HIV type 1 (HIV-1)-transactivating protein Tat enhances apoptosis and activation-induced cell death (AICD) of human T cells. This effect is mediated by the CD95 (APO-1/Fas) receptor-CD95 ligand (CD95L) system and may be linked to the induction of oxidative stress by Tat. Here we show that HIV-1 Tat-induced oxidative stress is necessary for sensitized AICD in T cells caused by CD95L expression. Tat-enhanced apoptosis and CD95L expression in T cells are inhibited by neutralizing anti-Tat antibodies, antioxidants, and the Tat inhibitor Ro24-7429. Chimpanzees infected with HIV-1 show viral replication resembling early infection in humans but do not show T-cell depletion or progression towards AIDS. The cause for this discrepancy is unknown. Here we show that unlike Tat-treated T cells in humans, Tat-treated chimpanzee T cells do not show downregulation of manganese superoxide dismutase or signs of oxidative stress. Chimpanzee T cells are also resistant to Tat-enhanced apoptosis, AICD, and CD95L upregulation.

Depletion of CD4$^+$ T-helper lymphocytes in AIDS patients involves induction of apoptosis in human immunodeficiency virus (HIV)-infected (24) and noninfected (8) T cells. We (25) and others (19) have shown that HIV type 1 (HIV-1) Tat and gp120 (1, 25) enhance apoptosis and anti-CD3-, staphylococcal enterotoxin B (SEB)-, or anti-T-cell receptor-mediated activation-induced cell death (AICD) of human T cells. This effect is inhibited by reagents that block the function of CD95 or its ligand, CD95L [e.g., F(ab′)$_2$ anti-APO-1 antibody fragments and soluble CD95 decoys, respectively] (6). Therefore, the CD95-CD95L system (6) plays an essential role in gp120- and Tat-mediated T-cell apoptosis. Since Tat induces oxidative stress and downregulation of manganese superoxide dismutase (Mn-SOD) expression in human T cells (26), Tat-enhanced AICD may operate, at least in part, via these mechanisms. HIV-1-infected chimpanzees show productive viral infection resembling early infection in humans (10). Chimpanzees, however, do not develop chronic T-cell depletion or progress toward AIDS (14). The reason for this discrepancy is still unknown.

Tat-enhanced AICD in human T cells is linked to Tat-mediated oxidative stress. To determine whether Tat-enhanced AICD is linked to Tat-mediated oxidative stress, we tried to inhibit this stress with the reducing reagents *N*-acetylcysteine and 2-mercaptoethanol and with a specific anti-Tat antibody (2E12; generated by M. O. Westendorp) and the Tat inhibitor Ro24-7429. We pretreated 5×10^5 Jurkat T cells with synthetic Tat (1 μg ml^{-1}) for 12 h, transferred the cells into 96-well flat-bottom plates coated with monoclonal anti-CD3 antibody (OKT3; 10 μg ml^{-1}), and cultured them for a further 24 h at 37°C. Thus, the cells were cultured for a total of 36 h in the presence of the above-mentioned reagents. Cell death was measured by fluorescence-activated cell sorter (FACS; Becton Dickinson, Mountain View, Calif.) analysis and propidium iodide (PI) uptake (dye exclusion; PI, 2.5 μg ml^{-1}). As shown in Fig. 1a, Tat-enhanced AICD was inhibited by neutralizing anti-Tat antibodies. The same inhibitory effect was seen with the Tat inhibitor Ro24-7429, previously shown to inhibit HIV-1 replication in vitro (Fig. 1b) (14, 16). Tat enhances apoptosis by influencing oxidative stress within cells (25, 26). Therefore, reagents that diminish oxidative stress should be able to inhibit this effect. Figure 1c shows that this was the case. Both 2-mercaptoethanol and *N*-acetylcysteine inhibited Tat-enhanced AICD in a dose-dependent manner. We have previously shown that Tat-enhanced AICD is caused by increased expression of death-inducing CD95L (25). Thus, we tested whether *N*-acetylcysteine and Ro24-7429 also decrease expression of CD95L mRNA. Jurkat T cells (10^6) were pretreated for 12 h with synthetic soluble Tat (1 μg ml^{-1}), transferred to 24-well plates coated with anti-CD3 antibody (30 or 100 μg ml^{-1}), and cultured for a further 24 h at 37°C. Cells were grown in the presence of *N*-acetylcysteine or Ro24-7429 as indicated in Fig. 1b to d. After incubation, the cells were washed once with 1.5 ml of Tris-buffered saline and subsequently used for preparation of total RNA. Semiquantitative reverse transcription (RT)-PCR was performed as described previously (21). To control for equal amounts of total RNA being used in the RT-PCR, we amplified β-actin cDNA with specific human β-actin primers (Stratagene, La Jolla, Ca-

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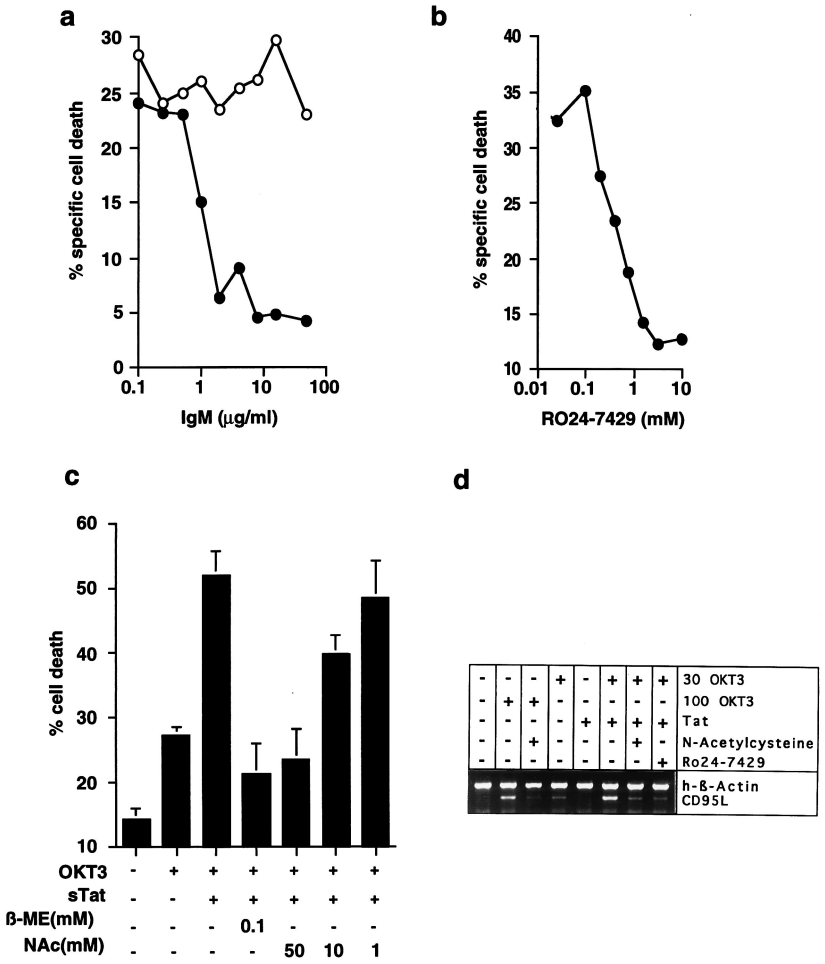

FIG. 1. Inhibition of HIV-1 Tat-enhanced AICD (a to c) and CD95L mRNA expression (d) in anti-CD3-stimulated Jurkat T cells treated with a neutralizing anti-Tat antibody (a), the Tat inhibitor Ro24-7429 (kindly provided by Hoffmann-La Roche) (3, 16) (b), or 2-mercaptoethanol and *N*-acetylcysteine (c). (a) Dose-dependent inhibition of Tat-enhanced AICD with increasing concentrations of a neutralizing anti-Tat antibody (filled circles) versus a control antibody (open circles). Cells were treated, and specific cell death was measured. The percentages of specific cell death were calculated as follows: 100 × [experimental PI uptake (%) − spontaneous PI uptake of cells in medium (%)]/[100% − spontaneous PI uptake (%)]. (b) Dose-dependent inhibition of Tat-enhanced AICD with increasing concentrations of the Tat inhibitor Ro24-7429. (c) Inhibition of Tat-enhanced AICD with 0.1 mM 2-mercaptoethanol (β-ME; Merck, Hohenbrunn, Germany) or with increasing concentrations of *N*-acetylcysteine (NAc; Sigma, Munich, Germany). (d) Inhibition of Tat-enhanced CD95L mRNA expression in anti-CD3-stimulated (30 or 100 μg ml^{-1}) Jurkat T cells by *N*-acetylcysteine and Ro24-7429. CD95L mRNA expression was examined by RT-PCR (12). All experiments were done three times except that shown in panel d, which was done twice. h-β-Actin, human β-actin; OKT3, anti-CD3 antibodies; sTat, synthetic Tat.

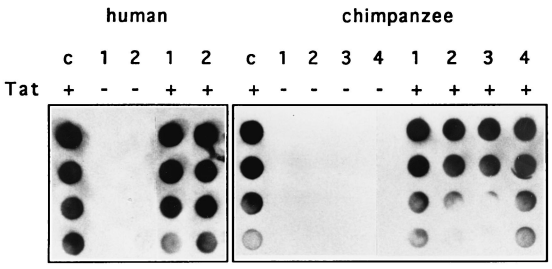

FIG. 2. Uptake of extracellular synthetic Tat by chimpanzee and human PBMC. Cells (10^6) of two human donors (humans 1 and 2) and four chimpanzees (chimpanzees 1 to 4) were incubated at 37°C for 12 h with (+) or without (−) 6 μg of synthetic Tat (amino acids 1 to 86) (18, 26) in 1 ml of medium. Cells were subsequently washed four times with 2 ml of PBS. By FACS analysis cell surface staining with a specific anti-Tat antibody (made by M. O. Westendorp) showed that no Tat was bound to the cell surfaces (data not shown). Levels of intracellular Tat taken up by human and chimpanzee PBMC were detected in cell lysates, at a dilution factor of 2, by enhanced chemiluminescence dot blot analysis as described previously (26). As a positive control (lanes c), 1 μg of synthetic Tat in 100 ml of buffer was blotted onto the membrane.

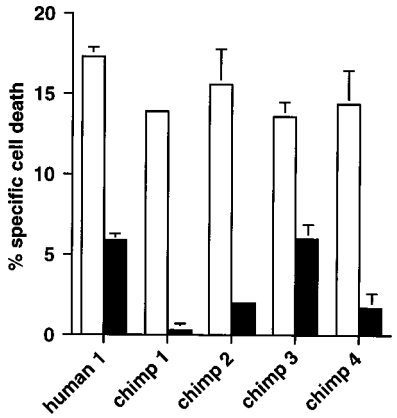

FIG. 3. SEB-induced apoptosis in human and chimpanzee T cells by the CD95-CD95L system. SEB-induced apoptosis (open bars) in T cells in PBMC from four healthy chimpanzees (chimpanzees 1 to 4) and one healthy human donor (human 1) was inhibited by $F(ab')_2$ anti-APO-1 antibody fragments (6) (filled bars).

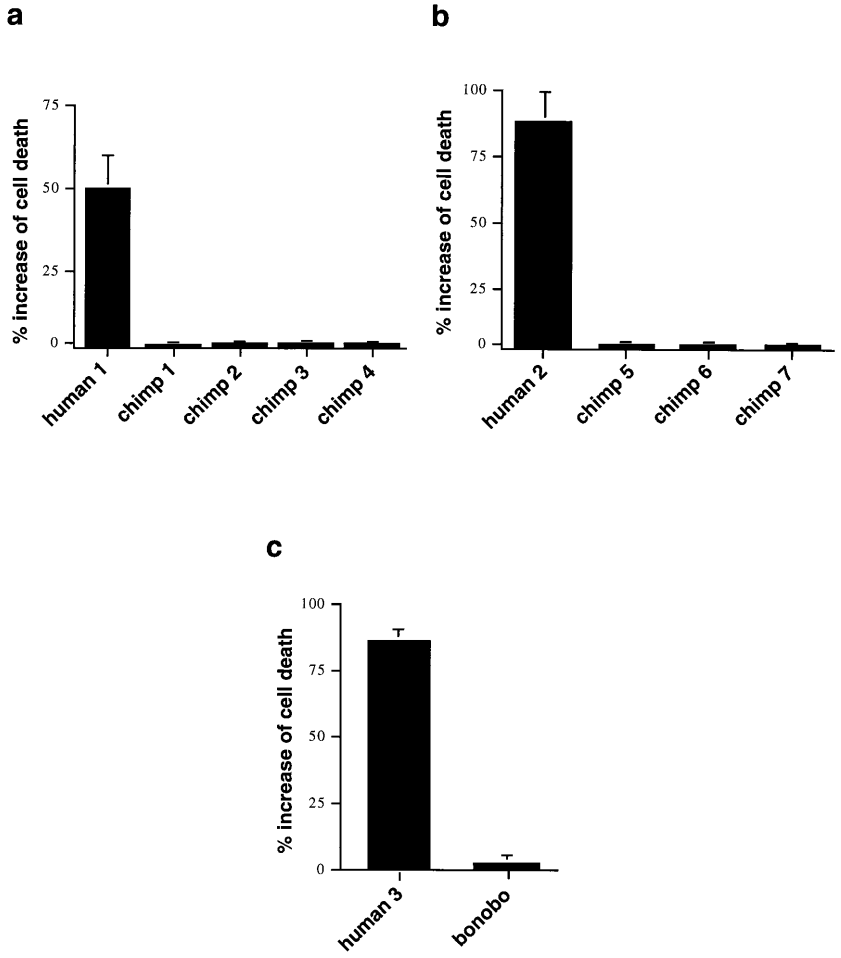

FIG. 4. Tat does not increase SEB-induced apoptosis in chimpanzee (a and b) and bonobo (c) T cells. Tat also does not alter Mn-SOD expression (d) or the level of GSH (e) in chimpanzee PBMC compared with those in human PBMC. The experiments whose results are shown in panels a and b were done separately with PBMC from different donors. (c) Tat does not enhance SEB-induced apoptosis in bonobo T cells. This experiment was done like those whose results are shown in panels a and b with bonobo PBMC incubated in plates coated with anti-CD3 antibody (OKT3; 20 $\mu g\ ml^{-1}$). The percent increase in cell death was calculated as follows: 100 × [PI uptake in the presence of Tat (%) − PI uptake in the absence of Tat (%)]/PI uptake in the absence of Tat (%). Values for SEB-induced absolute (background was not subtracted) cell death of human T cells were 15.5% (a), 14% (b), and 19.7% (c), and those of chimpanzee T cells were 12.9% (chimpanzee 1), 13.3% (chimpanzee 2), 10.8% (chimpanzee 3), 9.4% (chimpanzee 4), 10.1% (chimpanzee 5), 13.8% (chimpanzee 6), 14.1% (chimpanzee 7), and 24.7% (bonobo). Values for SEB-induced and Tat-mediated absolute cell death of human T cells were 23% (a), 27.1% (b), and 36.8% (c), and those of chimpanzee T cells were 13.1% (chimpanzee 1), 13.5% (chimpanzee 2), 10.9% (chimpanzee 3), 9.4% (chimpanzee 4), 10.1% (chimpanzee 5), 14.0% (chimpanzee 6), 14.1% (chimpanzee 7), and 25.4% (bonobo). (d) Mn-SOD expression in human and chimpanzee (chimpanzees 1 to 3) PBMC treated (filled circles) or untreated (open circles) with extracellular synthetic Tat measured via dot blot analysis (26). Total cell lysates (10^6 cells and equal amounts of protein) were blotted onto a Hybond nitrocellulose membrane (Amersham) with a dilution factor of 2. The membrane was washed six times with PBS, incubated with a mouse monoclonal antibody against human Mn-SOD, washed six times as described above, incubated for 90 min at room temperature with peroxidase-labeled goat anti-mouse antibody diluted 1:4,000 (Dianova, Hamburg, Germany), and washed again as described above. Signals were developed by performing the enhanced chemiluminescence reaction as described by the manufacturer (Amersham); autoradiographs were quantified densitometrically. The densities of the dots are shown in arbitrary units (AU). Mn-SOD expression in non-Tat-treated PBMC expressed in arbitrary units was 25,321 (human cells, 1/1 dilution). (e) Measurements of intracellular GSH levels as described previously (20, 26). Levels of GSH were estimated by FACS analysis of monochlorobimane-stained cells (26). Cells kept in culture medium were loaded with 20 μM monochlorobimane (Molecular Probes Inc., Eugene, Oreg.) for 10 min at 37°C. The reaction was stopped by the addition of ice-cold medium. Then cells were washed three times with cold PBS; FACS measurements were performed immediately after staining with a FACS Vantage cell sorter (Becton Dickinson) with the excitation level set at 351 to 364 nm and a mission filter (Omega 450 DF-65) at a wave length of 450 nm. Dead cells were excluded by forward- and side-scatter gating; mean values from duplicated samples were considered for data analysis. GSH levels (mean percentage of control levels) in PBMC from three human and three chimpanzee donors treated with synthetic Tat (controls used untreated cells) are shown.

lif.). The result of this RT-PCR (Fig. 1d) shows that both reagents abolished increased levels of expression of CD95L mRNA induced by Tat. Therefore, Tat-enhanced AICD is directly linked to enhanced CD95L expression mediated by Tat-induced oxidative stress.

Resistance of chimpanzee T cells to HIV-1 Tat-enhanced oxidative stress and apoptosis. Deregulation of the CD95-CD95L system may contribute to increased and accelerated apoptosis and $CD4^+$ T-cell depletion in AIDS patients (25). This hypothesis is strengthened by our results, which show increased CD95-CD95L expression in $CD4^+$ lymphocytes (2, 5) and elevated levels of anti-CD95 autoantibodies in HIV-positive individuals (23). Such autoantibodies may facilitate CD95-mediated apoptosis. Furthermore, it was shown that $CD4^+$ and $CD8^+$ T cells from HIV-positive individuals are more susceptible to anti-CD95-induced apoptosis

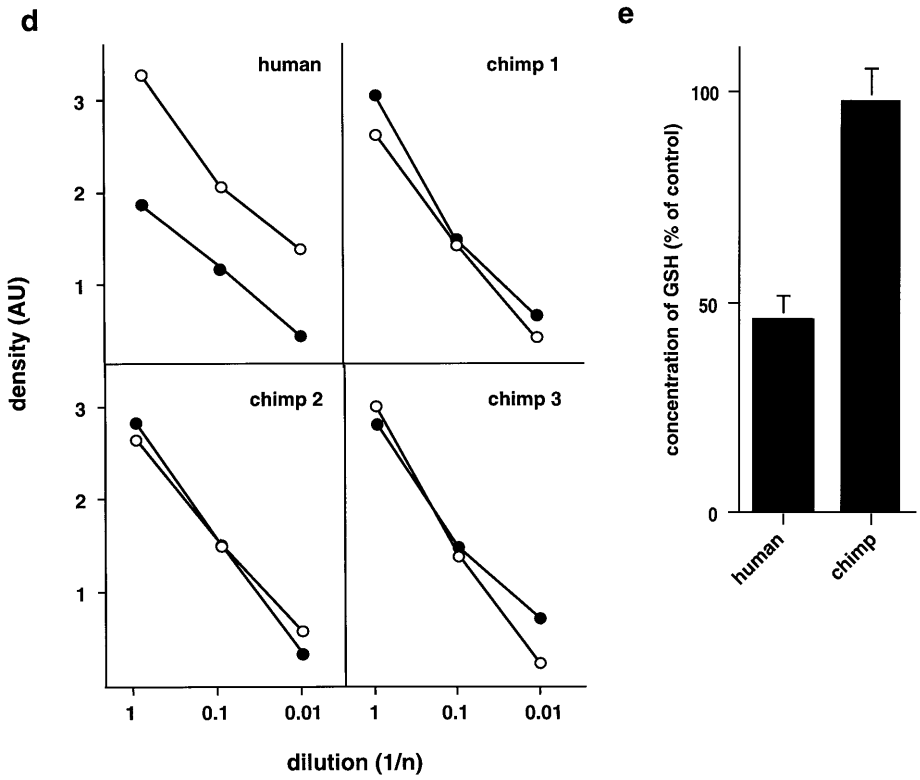

FIG. 4—*Continued.*

than cells from HIV-negative individuals (17). Chimpanzees chronically infected with HIV do not show an increase in the level of apoptosis (12, 22) and do not develop AIDS (13, 14). We speculated, therefore, that chimpanzee T cells might show a reactivity to Tat different from that of human T cells. To investigate this possibility, we tested the effect of Tat on T cells in the peripheral blood mononuclear cells (PBMC) of seven common chimpanzees (*Pan troglodytes*) and one bonobo (*Pan paniscus*; also called a pygmy chimpanzee) and compared the effect with that on T cells in PBMC from HIV-negative human individuals. First, we investigated the uptake of synthetic Tat (18, 26) in PBMC over a 12-h period. Figure 2 shows that we found no difference in Tat uptake levels in chimpanzee versus human PBMC. Next, we tested whether AICD in chimpanzee T cells involves the CD95-CD95L system as in humans (6). We stimulated 2.5×10^5 PBMC each from a healthy human donor and four chimpanzees at 37°C for 24 h with SEB (100 ng ml^{-1}) in the presence or absence of blocking $F(ab')_2$ anti-APO-1 antibody fragments. Subsequently, cell death was measured as described above. Figure 3 shows that SEB-induced AICD in chimpanzee T cells was inhibited by $F(ab')_2$ anti-APO-1 antibody fragments (6) to an extent similar to that in human T cells. Thus, the mechanism of AICD in chimpanzees is the same as in humans and uses, at least in part, the CD95-CD95L system. Surprisingly, however, the Tat-mediated increase in the level of apoptosis in SEB-stimulated T cells pretreated with synthetic Tat (1 μg ml^{-1}) for 12 h seen in human T cells (25) was not seen in common chimpanzee or in bonobo T cells (Fig. 4a to c). The lack of Tat effects applied even to Tat concentrations of up to 20 μg ml^{-1} (data not shown). In addition, Tat-mediated downregulation of Mn-SOD (9, 26) and the reduced glutathione (GSH) levels seen in SEB-activated human T cells (26) were also not observed in chimpanzee T cells (Fig. 4d and e). This is consistent with recent findings with HIV-positive chimpanzees which do not demonstrate a significant alteration in cystine and glutamate levels in plasma in comparison with HIV-positive human individuals whose decreased cystine and elevated glutamate levels correlate with decreased $CD4^+$ T-cell numbers (7). Finally and most significantly, unlike in human T cells, Tat induced no upregulation of CD95L mRNA expression in SEB-stimulated chimpanzee T cells (Fig. 5a and b). This was shown by a quantitative CD95L-PCR described briefly as follows. PBMC (10^7) were incubated in the presence or absence of Tat for 12 h. Subsequently, the cells were stimulated with phorbol myristate acetate (5 ng ml^{-1}) plus ionomycin (2 μM) or SEB (100 ng ml^{-1}) for 3 h. The cells were then harvested and washed twice with phosphate-buffered saline (PBS). Total RNA was prepared from PBMC as described elsewhere (4), reverse transcribed, and amplified by PCR as described in reference 15. Taken together, our results show that, despite comparable levels of uptake of Tat, the reactivity of chimpanzee T cells to Tat is entirely different from that of human T cells. Thus, our results point toward a fundamental difference between Tat activities on human genes and its activities on chimpanzee genes that influence AICD in T cells. These genes may include regulators of the cellular redox state, such as GSH-reductase or Mn-SOD and the CD95L gene.

The lack of increased levels of reactivity of chimpanzee T cells toward Tat may help to explain why enhanced apoptosis and progressive T-cell depletion are not observed in HIV-infected chimpanzees. These findings may advance our understanding of why chimpanzees do not develop AIDS (13, 14) and may stimulate new therapeutic approaches to HIV infection in humans (25).

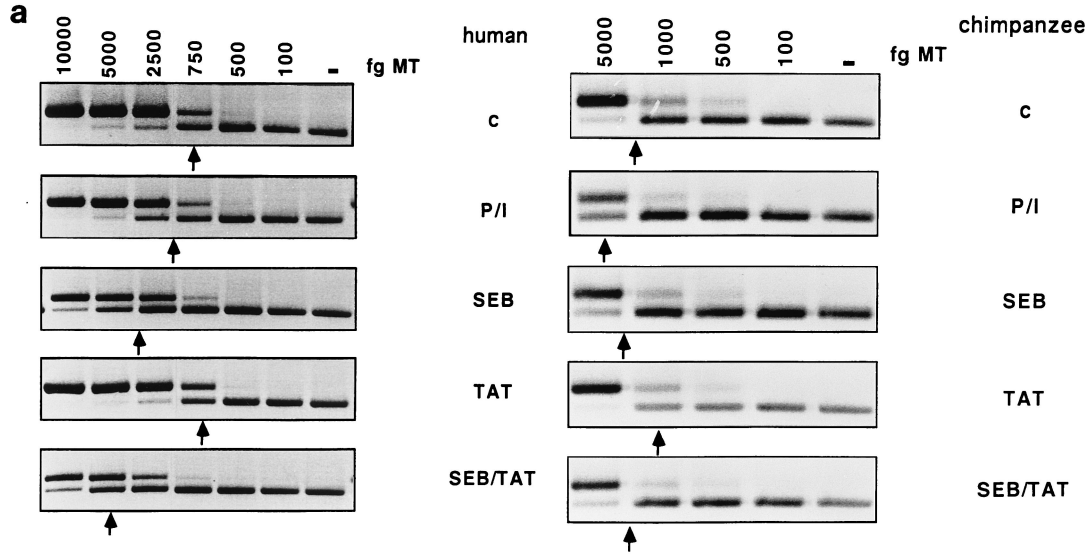

FIG. 5. Tat enhances CD95L mRNA expression in human but not in chimpanzee T cells. (a) Results of quantitative CD95L PCR (15) with PBMC of one representative human and one chimpanzee donor treated or untreated with phorbol myristate acetate (5 ng ml^{-1}) plus ionomycin (2 μM) (P/I), SEB (100 ng ml^{-1}), Tat (1 μg ml^{-1}), or SEB (100 ng ml^{-1}) plus Tat (1 μg ml^{-1}). In the PCR, 250 ng of total cDNA (lower bands) was coamplified with increasing amounts of a mutant CD95L cDNA fragment (upper bands, with concentrations indicated at the top) in the presence of specific CD95L primers. Titration points are indicated by arrows. (b) Percent increases of CD95L mRNA expression in T cells of two humans and four chimpanzees treated with SEB plus Tat, calculated with the results from the quantitative CD95L PCR as follows: {100 × [(CD95L cDNA from PBMC treated with SEB and Tat) − CD95L cDNA from PBMC treated only with SEB] (in femtograms)}/CD95L cDNA from untreated PBMC (in femtograms). The percent increases in CD95L mRNA expression of T cells treated with SEB only were 350% (human 1), 250% (human 2), 50% (chimpanzee 1), 100% (chimpanzee 2), 66.6% (chimpanzee 3), and 50% (chimpanzee 4). Tat alone had no influence on CD95L mRNA expression.

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