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# Cytotoxic effect on lymphocytes of *Tat* from human immunodeficiency virus (HIV-1)

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The human immunodeficiency virus type 1 (HIV-1) genome codes for *trans*-activator *Tat*, an 86-residue protein whose expression is critical for viral replication. Full-length *Tat* and *Tat* peptides from HIV-1 were chemically synthesized using optimized solid phase technique. Synthetic $Tat_{2\ 86}$ was found not only to inhibit antigen-induced human peripheral blood lymphocyte (PBL) proliferation in vitro, as described by Viscidi et al. [1989, Science 246, 1606–1608], but also mitogen-induced PBL proliferation, with 50% inhibition obtained at 0.9 and 8 $\mu$M, respectively. To assess the mechanism by which *Tat* exert its inhibitory effect, we analysed its interaction and effect on $CD4^+$-cells. Direct fluorescence and indirect immunofluorescence assays analysed by flow cytometry showed that fluorescein isothiocyanate-labeled and -unlabeled *Tat* interact (>0.2 $\mu$M) with $CD_4$-expressing lymphoid cells (CEM cell line). Experiments of chromium-51 release and Trypan blue exclusion on these tumor cells in vitro have demonstrated the capacity of *Tat* to modify cellular membrane permeability and cell viability, in a dose-dependent manner. The use of *Tat* peptides revealed that those containing the *Tat* basic region from 49 to 57 were able to bind to the cell membrane and to exhibit a cytotoxic activity on lymphocytes. Together, the data suggest that the potential cytotoxicity of *Tat* on lymphocytes could be directly implicated in virus-induced immune dysfunction observed in HIV-1 infected patients.

HIV; *Tat*; Cytotoxicity; Lymphocyte; Synthetic peptide

## 1. INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) genome codes for a potent *trans*-activator protein of 86 amino acid residues, termed *Tat* (Fig. 1), which expression is required for viral replication [1–5]. *Tat* interacts with a *cis*-acting element (TAR) mapped in the R region of the long terminal repeat (LTR) and dramatically increases the steady-state levels of viral messenger RNAs [6–8]. Complex action of *Tat* was previously reported on levels of transcription initiation [7,9,10], anti-attenuation [11], translational efficiency [12], or a combination thereof [13]. This regulatory protein factor seems to be released in a biologically active form in the supernatant of HIV-1-infected cells in vitro [14]. Structure–activity relationships studies using synthetic peptides and site-directed mutagenesis clearly show the existence of several functional domains and importance of the cysteine- and basic residue-rich regions for *Tat* nuclear location and *trans*-activating function [15–23]. In parallel to *trans*-activation, it has been reported some other novel potential molecule activities such as induction and development of Kaposi's sarcoma [14,24] and immunosuppression [25]. In the later case, the recombinant protein seems to selectively inhibit, in a dose-dependent manner, antigen-induced lymphocyte proliferation (tetanus toxoid, candida antigens) while no significant inhibitory effect of *Tat* on cellular proliferation was observed after mitogenic stimulation (phytohemagglutinin P (PHA), pokeweed mitogen). We have previously shown [26] that synthetic *Tat* can act on the central nervous system. This activity corresponds to an interaction and cytotoxic effect of *Tat* on neural cell lines in vitro, as well as a neurotoxicity lethal in mice in vivo. The use of *Tat* peptide derivatives allowed to determine that the conserved basic region 49–57 of *Tat* was necessary and sufficient for cell membrane binding and neurotoxicity. In order to investigate the cellular specificity of *Tat* toxicity and the potential molecule implication in immune dysfunction, we measured the effect of exogenous protein on $CD_4^+$-lymphoid cells (CEM cell line) and on human peripheral blood lymphocyte proliferation in vitro.

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## 2. MATERIALS AND METHODS

### 2.1. *Peptide synthesis*

Chemical synthesis of $Tat_{2-86}$ and *Tat* peptides was performed by the solid-phase method [27,28]. Stepwise elongation of the peptide chains was carried out automatically (synthesizer model 430 A, Applied Biosystems, Foster City, CA) on 4-(oxymethyl)-phenylacet-

Met -Glu -Pro -Val -Asp -Pro-Arg-Leu-Glu-Pro-Trp-Lys-His-Pro-Gly-Ser-Gln-Pro-Lys-Thr-
1 20

Ala -Cys-Thr-Thr-Cys-Tyr-Cys-Lys-Lys-Cys-Cys-Phe-His-Cys-Gln-Val-Cys-Phe-Thr-Thr-
21 40

Lys -Ala -Leu -Gly -Ile -Ser -Tyr -Gly -Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-
41 60

Gly -Ser -Gln -Thr -His -Gln-Val-Ser-Leu-Ser-Lys-Gln-Pro-Thr-Ser-Gln-Pro-Arg-Gly-Asp-
61 80

Pro-Thr-Gly-Pro-Lys-Glu
81 86

Fig. 1. Predicted amino acid sequence of HIV-1 *Tat* protein (Lai isolate).

amidomethyl polystyrene resin (0.5 mmol) using optimized *t*-butyloxycarbonyl (Boc)/benzyl chemistry, as described [29].

2.2. *$CD_4^+$-lymphoid cells*

Cells of the CEM line, clone 13 (American Type Culture Collection, Rockville, MD), were cultured at 37°C in RPMI 1640 medium (Flow Laboratories Inc., Irvine, Scotland) supplemented with 10% fetal calf serum, 1% glutamine, and 1% streptomycin-penicillin antibiotics (Gibco Laboratories, Paisley, Scotland) in a humidified atmosphere with 5% $CO_2$.

2.3. *Human peripheral blood lymphocyte proliferation*

Peripheral blood lymphocytes (PBL) from four healthy HIV-1 seronegative donors were isolated from heparinized blood by the Ficoll-Hypaque technique. Culture medium was RPMI 1640 supplemented with 1% glutamine, 1% antibiotics and 10% heat inactivated fetal calf serum. Cells ($10^5$) in 200 $\mu$l final volume were incubated in microtitre plate wells in the presence of various concentrations of $Tat_{2\text{-}86}$ (0.18 to 35 $\mu$M), or *Tat* peptide derivatives (0.5 to 60 $\mu$M), with or without tuberculin antigen (PPD; 12.5 $\mu$g/ml) or mitogens (T-cell specific phytohemagglutinin P (PHA), B-cell specific pokeweed mitogen). On day 6, cultures were pulsed for 8 h with 1 $\mu$Ci of [$^3$H]thymidine (Amersham Int., Amersham, UK). Cells were harvested and [$^3$H]thymidine incorporation into DNA was counted in a beta counter (Packard, Downers Grove, IL).

2.4 *CEM and PBL cell binding of synthetic* Tat $_{2\text{-}86}$ *and* Tat *peptides*

Cell binding of varying amounts of synthetic $Tat_{2\text{-}86}$ or *Tat* peptides was assessed by either direct fluorescence assay using FITC-labeled $Tat_{2\text{-}86}$ or by indirect immunofluorescence assay, as described previously [26].

2.5. *Chromium-51 release assay from labeled CEM cells*

CEM cells ($2\times10^6$ cells/ml) were radiolabeled for 12 h at 37°C with 100 $\mu$Ci chromium-51 sodium chromate (Amersham Inc., Chicago, IL) in RPMI 1640 containing 1% fetal calf serum. Labeled cells were washed and suspended in medium at $10^6$ cells/ml and dispensed in 100 $\mu$l aliquots into a flat-bottomed 96-well microtitre plate (Corning, Corning, NY). Various amounts of $Tat_{2\text{-}86}$ (0.18–18 $\mu$M) and *Tat* peptide derivatives (0.2–60 $\mu$M) were added to each well. After 4 h incubation at 37°C in 5% $CO_2$ humidified atmosphere, cell-free supernatants were harvested and counted in a gamma counter (Kontron, Zurich, Switzerland). Wells containing labeled cells in RPMI alone or in RPMI plus 1% (v/v) Triton X-100 served as controls for spontaneous and maximal cellular $^{51}$Cr release, respectively.

2.6. *CEM and PBL cell viability*

Viability of CEM or PBL cells ($10^6$) previously incubated (1 h, 37°C) with 50 $\mu$l solutions of $Tat_{2\text{-}86}$ (0.07–35 $\mu$g; 0.14–70 $\mu$M) was monitored by using Trypan blue staining.

## 3. RESULTS

### 3.1. *Inhibition of antigen- and mitogen-induced human lymphocyte (PBL) proliferation in the presence of synthetic* Tat $_{2\text{-}86}$

It was reported that *Tat* from HIV-1 was able to inhibit antigen-induced, but not mitogen-induced, human peripheral blood mononuclear cell (PBL) proliferation [25]. In order to confirm these data and to determine the region responsible for this inhibitory effect, we investigated the effect of synthetic $Tat_{2\text{-}86}$ and *Tat* peptide derivatives on human PBL proliferation. Stimulation of PBL ($10^5$) with PPD antigen in the presence of increased amounts of $Tat_{2\text{-}86}$ (0.18–8.8 $\mu$M), or *Tat* peptides (3.2 to 32 $\mu$M), resulted in a dose-dependent inhibition of PPD-induced lymphocyte proliferation (Fig. 2A). Under our experimental conditions, 50% inhibition was obtained with a concentration of $Tat_{2\text{-}86}$of 0.9 $\mu$M. Peptides $Tat_{21\text{-}86}$ and $Tat_{38\text{-}72}$ also inhibited 60 and 58% of PBL proliferation at concentrations of 2.3 and 3.2 $\mu$M, respectively. The effect of $Tat_{2\text{-}86}$ and *Tat* peptides on mitogen-induced lymphocyte proliferation was further investigated using phytohemagglutinin P (PHA; 1:400) and pokeweed mitogens on PBL samples from four HIV-1 seronegative donors. From the four PBL samples tested, $Tat_{2\text{-}86}$ inhibited 10, 30, 50 and 100% of lymphocyte proliferation at concentrations of about 0.35, 3.5, 8 and 20 $\mu$M, respectively (data not shown). The capacity of various *Tat* peptides to inhibit PHA-induced lymphocyte proliferation is illustrated in Fig. 2B. The results show that peptides $Tat_{21\text{-}86}$, $Tat_{21\text{-}86}$ CM (carboxymethylcysteine derivative), $Tat_{38\text{-}60}$ and

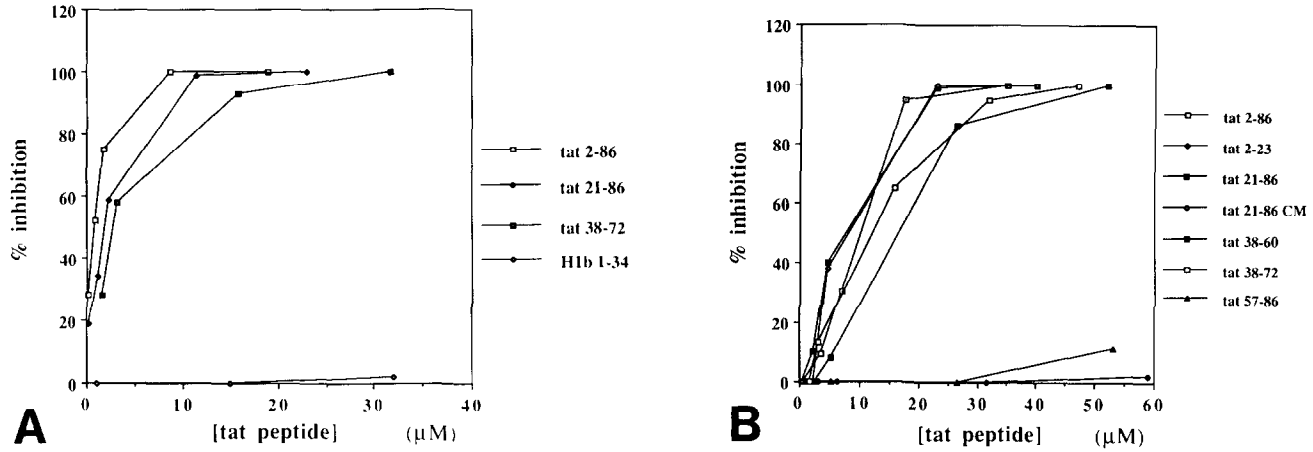


Fig. 2. (A) Inhibition of tuberculin antigen (PPD)-induced human peripheral blood lymphocyte (PBL) proliferation in vitro by *Tat* $_{2-86}$ and *Tat* peptides. (B) Inhibition of PHA-induced lymphocyte (PBL) proliferation in vitro by $Tat_{2-86}$ and *Tat* peptides Percent inhibition of the PPD- and PHA-induced lymphocyte (PBL) proliferation in vitro was calculated as: $100-[(cpm_{experimental}-cpm_{control})/(cpm_{maximal}-cpm_{control})\times 100]$. Spontaneous (cpm $_{control}$) and maximal (cpm $_{maximal}$) PBL proliferations were determined, respectively, in culture medium alone and in medium plus PPD (A), or PHA (B). Spontaneous and maximal PBL proliferations were, respectively, at about 200 and 2,600 cpm (PPD stimulation), 500 and 27,000 cpm (PHA stimulation). The standard deviation (S.D.) of samples was always <10% of the mean. Data are mean counts per minute (cpm) ± 1 S.D. of quadruplicate replicates. Experimental conditions are described in Section 2.

*Tat* $_{38-72}$, which contain the entire basic domain, fully inhibited the PHA-induced proliferative response. These *Tat* peptides induced about 10, 50 and 100% inhibition at concentrations ranging from 2 to 5, 10 to 20, and 20 to 50 μM, respectively. In contrast, $Tat_{2-23}$ and $Tat_{57-86}$, or basic fragment 1–34 of human spleen H1b histone, had negligible inhibitory effect at mM concentration.

To investigate the mechanism by which *Tat* exert its immunosuppressive activity, we analysed both the interaction of synthetic *Tat* and *Tat* peptides with lymphoid cells and their effects on cell permeability and viability.

### 3.2. *Interaction of synthetic* $Tat_{2-86}$ *with* $CD_4^+$*-CEM cell line and PBL cells*

Flow cytometry analysis of CEM cells ($10^6$) previously incubated with increased concentrations of FITC-labeled *Tat* $_{2-86}$ (0.2–20 μM) shows the capacity of labeled $Tat_{2-86}$ to interact with cells below its μM concentration (>0.2 μM), in a dose-dependent manner (Fig. 3). The cell fluorescence could be associated with intracellular labeling due to cellular uptake of FITC-labeled $Tat_{2-86}$ and/or with surface labeling of the cell membrane. This interaction was further analysed by indirect immunofluorescence assay. In agreement with direct binding assay, results obtained shows the dose-dependent *Tat*-cell surface membrane binding stained by rabbit anti-*Tat* antibodies (data not shown). FITC-labeled or -unlabeled *Tat* peptides were used, respectively, in direct (Fig. 4) or indirect (data not shown) binding experiments to delineate the *Tat* site of binding to the cell membrane. Results show that *Tat* peptides (>5 μM) which include the basic domain 49–57 (*Tat* $_{38-60}$ and $Tat_{38-72}$) bound to CEM cells while *Tat* $_{2-23}$ and $Tat_{57-86}$ at about 10-fold higher concentrations were not significantly active for binding. A similar specific binding of *Tat* was obtained when incubation was done with human PBL (data not shown).

### 3.3. *Effect of synthetic* $Tat_{2-86}$ *on the* $CD_4^+$*-CEM cell membrane permeability and cell viability*

Post-binding effect of $Tat_{2-86}$ on cell membrane permeability was investigated by $^{51}Cr$ release assay from $Tat_{2-86}$-treated labeled CEM cells. Incubation (4 h, 37°C) of chromium-51 labeled cells ($10^5$) with increased amounts of *Tat* $_{2-86}$(0.18–μM) clearly shows the protein effect on cell permeability (Fig. 5A,B). A significant cellular $^{51}Cr$ release was observed below μM concentration of $Tat_{2-86}$ (0.2 to 1 μM) and 66% $^{51}Cr$ release was obtained at 18 μM. The synthetic *Tat* peptides that contain the *Tat* basic domain ($Tat_{21-86}$, *Tat* $_{38-60}$, $Tat_{38-72}$ and $Tat_{38-86}$) produced similar effect on these cells (Fig. 5A,B). $Tat_{38-86}$(24 μM) and $Tat_{21-86}$(23 μM) induced 70 and 90% $^{51}Cr$ release, respectively (Fig. 5A). Peptides $Tat_{2-23}$ (60 μM) and *Tat* $_{57-86}$(52 μM) did not elicit significant $^{51}Cr$ release from labeled cells (Fig. 5B), as well as highly basic control fragment 1–34 of human spleen H1b histone [30] at similar concentration (Fig. 5A,B). The effect of *Tat* on cell viability was also investigated by Trypan blue exclusion of CEM cells or human PBL treated with *Tat* (0.1–100 mM). Results (Fig. 6) clearly showed the cytotoxicity of *Tat* with about 40% of cell death at 10 mM *Tat* concentration.

## 4. DISCUSSION

It was reported that *Tat* protein selectively inhibits antigen-induced, but not mitogen-induced, lymphocyte

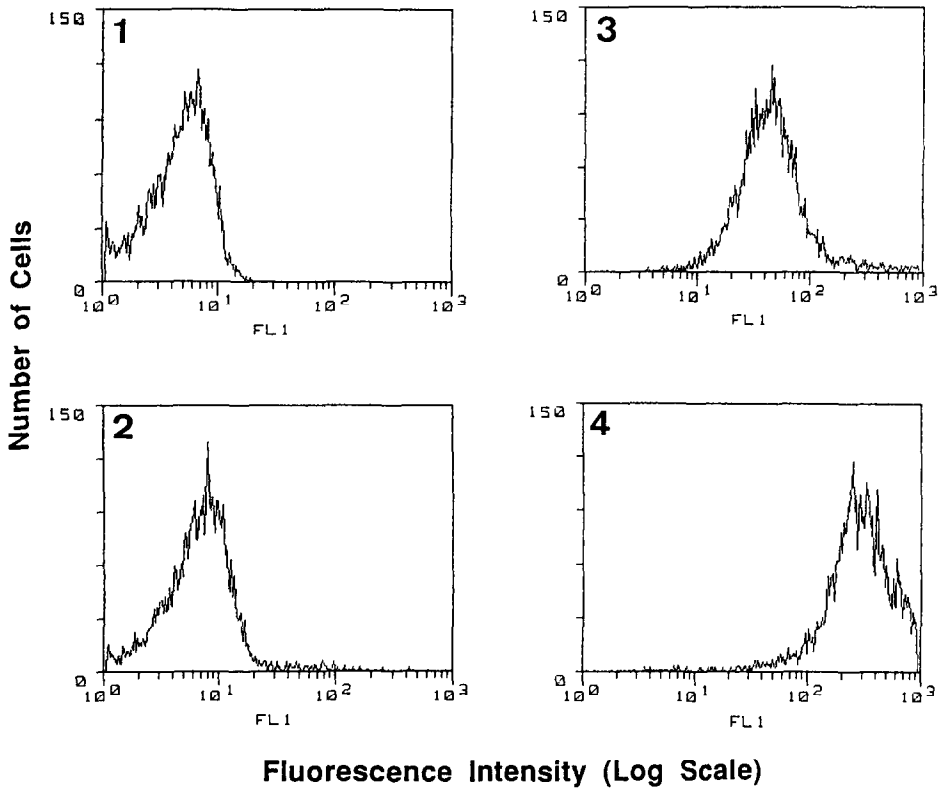


Fig. 3. Binding of synthetic fluorescein isothiocyanate (FITC)-labeled $Tat_{2-86}$ to CEM cells analysed by direct fluorescence assay. Interaction of FITC-labeled $Tat_{2-86}$ with CEM cells ($10^6$) was measured by cell fluorescence intensity using flow cytometry analysis (FACS analyser, Becton-Dickinson). Cell fluorescence in the absence (1) or in the presence of 0.2 $\mu$M (2), 2 $\mu$M (3) and 20 $\mu$M (4) of FITC-labeled $Tat_{2-86}$.

proliferation [25]. We further investigated this immunosuppressive activity by using synthetic $Tat_{2-86}$ and *Tat* peptides. In accordance with previous findings, $Tat_{2-86}$ was found able to inhibit PPD-induced lymphocyte proliferation, in a dose-dependent manner. Similarly to binding assay results, this inhibitory activity was detected at about 0.2 $\mu$M concentration of $Tat_{2-86}$. The 50% inhibition of antigen-induced PBL proliferation was found at 0.9 $\mu$M, while 50% inhibition was previously reported to be at about 50 nM and 0.2 $\mu$M concentrations using respectively recombinant reduced $Tat_{1-72}$ (coding exon 1) and $Tat_{1-86}$ [25]. It was also reported that synthetic $Tat_{1-58}$ caused inhibition, albeit at a concentration 10-fold higher than that of the recombinant protein [25], suggesting together with our results that region 38–58, which contains the entire basic domain, could contribute to the inhibitory activity of the protein. In contrast with this previous report, $Tat_{2-86}$ (>0.35 $\mu$M) was also found able to inhibit PHA-induced lymphocyte activation. Nevertheless, the 50% inhibition of PHA-induced stimulation was observed at $Tat_{2-86}$ concentration 9-fold higher (8 $\mu$M) than that required for 50% inhibition of the PPD-induced stimulation. This concentration of $Tat_{2-86}$ is lower but comparable to those corresponding to 50% of both cell membrane permeability and cell viability (Figs. 5 and 6). *Tat* peptides which contain the basic domain ($Tat_{21-86}$, $Tat_{38-60}$ and $Tat_{38-72}$) were also able to induce comparable inhibition of the proliferative response to PHA (50% inhibition at 10–20 $\mu$M), while $Tat_{2-23}$, $Tat_{57-86}$, or frag-

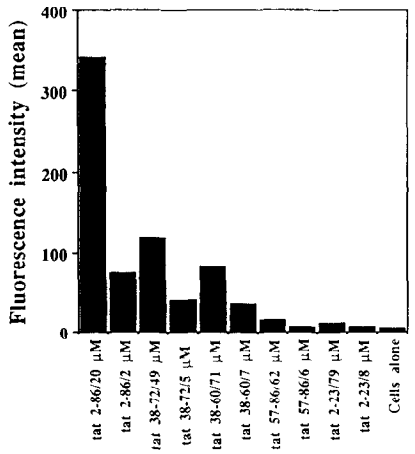


Fig. 4. Binding of fluorescein isothiocyanate (FITC)-labeled *Tat* peptides to CEM cells analysed by direct fluorescence assay. Interaction of various concentrations of FITC-labeled *Tat* peptides with CEM cells was assessed by cell fluorescence intensity using flow cytometry analysis. The background fluorescence of cells alone corresponds to specific autofluorescence of CEM cells.

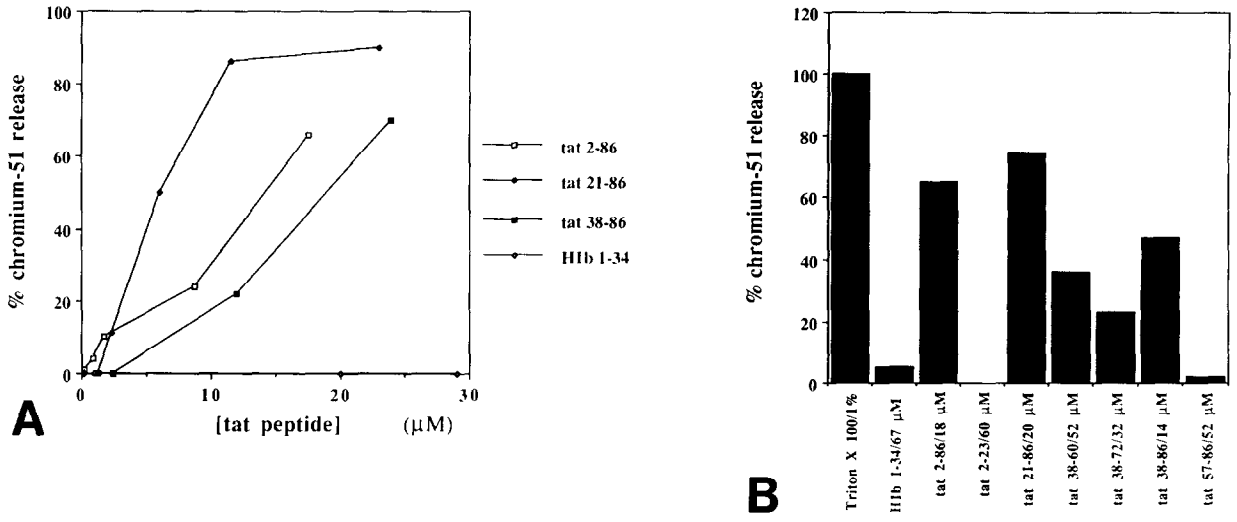


Fig. 5. (A,B) Effect of synthetic $Tat_{2\ 86}$ and Tat peptides on chromium-51 release from labeled CEM cells. $Tat_{2-86}$ (0.18–18 μM) and *Tat* peptides (0.2–60 μM) were incubated with chromated CEM cells ($5\times10^4$). Chromated cells in culture medium alone or in medium plus 1% (v/v) Triton X-100 served as controls for spontaneous (<2,000 cpm) and maximal (13,000 cpm) cellular $^{51}Cr$ release, respectively. The highly basic fragment 1–34 from human spleen H1b histone was used as negative control. Percent chromium-51 release from labeled cells was calculated as: $(cpm_{experimental}-cpm_{control})/(cpm_{maximal}-cpm_{control})\times100$. The standard deviation (S.D.) of samples was always <10% of the mean. Data are mean counts per minute (cpm) ± 1 S.D. of duplicate quadruplicates. Experimental conditions are described in Section 2.

ment 1–34 H1b histone, were found inactive. These results are consistent with the capacity of *Tat* and *Tat* peptides including the basic domain to interact with cell membrane and to modify cell permeability. We have shown by direct and indirect binding assays the capacity of *Tat* to bind to cell membranes via its basic domain which is highly conserved among various HIV-1 isolates. This region (2 lysines and 6 arginines within 9 residues) was previously found to be critical for efficient *Tat trans*-activation and was presumed to be a nuclear targeting signal or a nucleic acid binding site [16,17,21–23, 31]. Post-binding effect of *Tat* and *Tat* peptides on CEM cell membrane permeability was investigated by $^{51}Cr$ release assay of chromium-51 labeled CEM cells. The results obtained have shown that $Tat_{2-86}$ modifies cell membrane permeability below μM concentration (0.2–1 μM), in a dose-dependent manner, with 66% $^{51}Cr$ release obtained at 18 μM of $Tat_{2-86}$. A dose-dependent and specific $^{51}Cr$ release from labeled CEM cells was also observed with *Tat* peptides which contained the basic domain 49–57 ($Tat_{21-86}$, $Tat_{38-60}$, $Tat_{38-72}$ and $Tat_{38-86}$), while $Tat_{2-23}$ and $Tat_{57-86}$, or highly basic fragment 1–34 from human spleen H1b histone [30], were inactive for cellular $^{51}Cr$ release. Binding and $^{51}Cr$ release assays using *Tat* peptides suggest that the basic domain of $Tat_{2-86}$ could be involved in both the interaction and permeabilization of the CEM cell membranes. These results suggest that *Tat*- mediated inhibition of the mitogen-induced lymphocyte proliferation could be directly caused by a cytolytic activity of the protein via its basic domain.

The biological effects are occasionally observed in vitro at relatively high $Tat_{2-86}$ concentration (0.2 to 10 μM), although there is no evidence for free $Tat_{2-86}$ at such a concentration in the plasma of HIV-infected individuals. Nevertheless, it has been reported that *Tat* binds to several cell type at micromolar concentrations and enters these cells by endocytosis [32]. Also, it cannot be ruled out that the high concentration of the protein could be attainable locally within some acutely replicating-HIV cells. In addition, $Tat_{2-86}$ could act synergistically with other viral and/or cellular factors, as it has

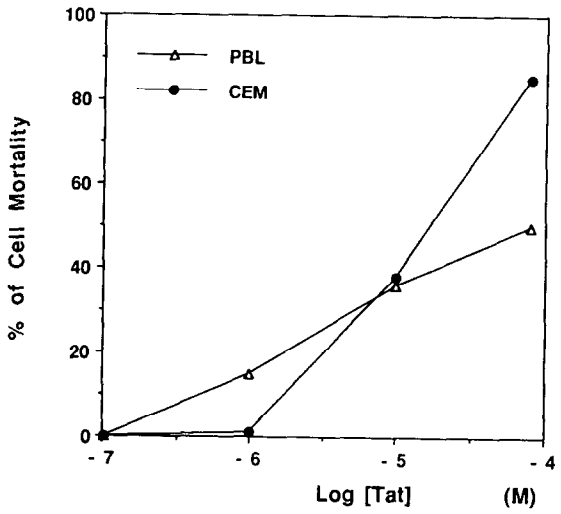


Fig. 6. Dose-dependent effect of synthetic $Tat_{2-86}$ on CEM and PBL cell viability. Viability of CEM cells and human PBL ($10^6$) preincubated (1 h, 37°C) in the absence or in the presence of varying concentrations of synthetic $Tat_{2\ 86}$. The cell viability was monitored by Trypan blue staining.

been shown recently for *Tat trans*-activation [33–35], to bring about increased cell lysis. Such synergistic cytotoxicity has previously been described for human neutrophil defensin, a highly basic peptide exhibiting cytolytic activity at concentrations of 1–50 $\mu$M [36]. Many basic cytotoxic proteins and peptides, implicated in contact dependent antibody-mediated cytotoxicity and antimicrobial activity [37–41], are active at the micromolar concentration. The common mechanism by which all these basic peptides damage target cell membrane is reported to be the pore formation, leading to non-ion-selective membrane permeability, *Tat* $_{2-86}$ may belong to this class of cationic proteins.

In summary, our results show that *Tat* $_{2-86}$ and its peptide derivatives containing *Tat* basic domain are able (i) to bind to CEM and PBL cells, (ii) to modify cell membrane permeability and viability of these cells, and (iii) to inhibit both antigen- and mitogen-induced human lymphocyte (PBL) proliferation in vitro.

Taken together, the results may suggest that *Tat* $_{2-86}$, by acting on the immune system, could be directly involved in the immune dysfunction associated with HIV infection. Thus, the specific inhibition of the *Tat* $_{2-86}$activity could be an effective therapeutic approach to the treatment of AIDS.

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