

Mucosal delivery of the human immunodeficiency virus-1 Tat protein in mice elicits systemic neutralizing antibodies, cytotoxic T lymphocytes and mucosal IgA

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Abstract

Human immunodeficiency virus (HIV)-1 Tat protein induces protection in non-human primates upon systemic vaccination. In view of the design of mucosal vaccines against HIV-1 we studied the immune response to native Tat (aa 1–86) in mice following intranasal delivery of the protein with two mucosal adjuvants, *Escherichia coli* heat-labile enterotoxin (LT) and LT-R72, a non-toxic mutant of LT. Immunization with Tat and the two adjuvants induced in BALB/c but not in C57BL/6 mice high and persistent levels of serum IgG and secretory IgA in vaginal and intestinal fluids. Mice sera neutralized Tat and recognized two epitopes mapping in the regions 1–20 and 46–60. Furthermore, their splenocytes proliferated and secreted IFN- γ and IL-6 in response to Tat. Finally, CTLs were also elicited and they recognized an epitope localized within aa 11–40 of Tat.

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1. Introduction

An ideal vaccine against human immunodeficiency virus (HIV) should elicit immune responses able to prevent tissue invasion at the mucosal level and to neutralize the virus and its pathogenic factors at the systemic level. While parenteral vaccines generally fail to induce mucosal responses, mucosal vaccines are very effective at eliciting high levels of systemic IgG as well as secretory IgA and cell-mediated responses, including CTLs [1]. In this respect, the correlates of protection against HIV infection or disease are still elusive [2]. Studies in non-human primates and in infected or exposed individuals have shown that systemic neutralizing Abs and mucosal Ab responses against HIV are all important [3–8], although they may play a role in different phases of HIV infection. In addition, cell-mediated responses, and particularly CTLs, are presently considered critical for con-

ferring an effective protection against HIV and simian immunodeficiency virus (SIV) infection and disease progression [9–14]. Thus, a mucosal vaccine against HIV has the potential to stimulate the full range of immune responses relevant for protection.

Among the candidate Ags for an anti-HIV vaccine, the regulatory protein Tat has received much attention in the late years [15–17]. Tat is produced very early after infection, prior to expression of the structural genes *env*, *gag*, and *pol* and is essential for viral replication [18]. Furthermore, Tat is released by the infected T lymphocytes in the extracellular milieu [19–21] and enters both infected cells, where it promotes HIV replication, and uninfected cells in which it causes activation or repression of cytokine and cellular genes controlling the cell cycle [18,21–23]. Tat also induces the expression of the chemokine receptors (and HIV-1 co-receptors) CCR5 and CXCR4 [24,25], responsible for the transmission of macrophage- and T-cell-tropic HIV-1 strains, respectively. Thus, extracellular Tat plays a role in the spreading of infection by recruiting new cell targets. The Tat protein also affects the function of different cell types

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[26] and contributes to the pathogenesis of AIDS-associated diseases such as Kaposi's sarcoma [18,27]. Finally, Tat activates the expression of heterologous viral promoters, such as those of herpesviruses and other viruses, favoring the development of opportunistic infections [28].

Because of the large spectrum of activities of Tat, an anti-HIV vaccine should induce immune responses able to inhibit Tat function. Indeed, several studies performed on patients or in non-human primates suggest that an immune response to Tat has a protective role and may control the progression of the disease. Anti-Tat Ab responses and CTLs have been associated with non-progression to AIDS in infected individuals [14,29–33]. Furthermore, the importance of anti-Tat CTLs in the control of infection is supported by a recent study in macaques infected with SIV demonstrating that anti-Tat CTLs are key to control early virus replication [9]. Pre-clinical studies conducted in non-human primates indicate that vaccines based on Tat, either expressed in viral vectors [34] or delivered as a native [11] or chemically inactivated [35] protein or as naked DNA [36] are safe, immunogenic and capable of controlling primary infection with highly pathogenic SIV or SHIV viruses. Finally, human vaccination with an inactivated form of Tat [37], or with naked DNA [38] was safe and elicited good immune responses.

Although these studies strengthened the view of Tat as an important target for an anti-HIV vaccine, little is known on the use of native Tat in mucosal vaccination. Therefore, experiments were conducted in the mouse model to evaluate the immunogenicity of the Tat protein through the mucosal route and to analyze the immune responses induced. Since mucosal delivery of subunit vaccines requires the use of specific adjuvants to overcome mucosal unresponsiveness to soluble Ags [1], we used *Escherichia coli* heat-labile enterotoxin (LT) and its non-toxic mutant LT-R72 [39] as adjuvants for intranasal immunization with the biologically active Tat.

2. Materials and methods

2.1. Reagents

HIV-1 Tat from the human T lymphotropic virus type IIIB isolate (subtype B) was expressed in *E. coli* and purified to homogeneity by heparin-affinity chromatography and high pressure liquid chromatography as described previously [19] according to Good Laboratory Practice rules. The purified Tat protein was biologically active as tested by the rescue assay on the HLM1 cell line [19,21], by the induction of chloramphenicol acetyl transferase (CAT) activity in HL3T-1 cells [40] and by assays on endothelial cells [20,41].

The Tat protein was stored lyophilized at -80°C to prevent oxidation and was reconstituted in degassed phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) immediately prior to use as described [19,21]. To prevent attachment of the protein to surfaces,

plastic tips and vials were previously rinsed in PBS containing 0.1% BSA. In addition, since Tat is also photo- and thermo-sensitive [21], the handling of the protein was performed in the dark and on ice.

LT, LT-R72 and tetanus toxoid (TT) were from Chiron SpA, Siena, Italy. Cholera toxin (CT) was from Calbiochem (La Jolla, CA).

2.2. Mice and immunization protocols

Female BALB/c and C57BL/6 mice were purchased from Charles River (Lecco, Italy). Female BALB.B mice were from Harlan (Milan, Italy). All mice were used between 8 and 12 weeks of age. For intranasal immunizations, groups of five mice were lightly anesthetized with ketamine/xylazine and 15–20 μl (7.5–10 μl per nare) of vaccine containing 10 μg of Tat with or without 1 μg of adjuvant were applied to the nares on days 0, 7, 14 and 21. For subcutaneous immunizations, groups of five mice were injected on days 0 and 14 with 100 μl of vaccine containing 10 μg of Tat either alone or adsorbed to aluminum hydroxide (Chiron) or emulsified in CFA (Sigma, Milan, Italy). In some experiments, mice were immunized subcutaneously with 10 μg of TT with or without aluminum hydroxide. Serum, vaginal washes and fecal samples were collected 1 week after each immunization and were processed as previously described [42]. The samples of four to five immunized mice were analyzed, as specified in legends to figures and tables.

2.3. Analysis of Ab isotypes

Tat-specific Ab titers were measured in sera, saliva, vaginal washes and fecal pellets by endpoint enzyme-linked immunosorbent assay (ELISA). Tat (1 $\mu\text{g}/\text{ml}$ in PBS) was used to coat 96-well plates (Dynex Technologies, Milan, Italy) (100 μl per well) by overnight incubation at 4°C . After washing with PBS 0.05% Tween 20 (PBS-T), the wells were blocked with 200 μl of PBS 0.1% Tween 20. Following overnight incubation at 4°C , serial dilutions of sera and secretions from individual mice were added to the wells and incubated overnight at 4°C . The plates were then washed and biotin-conjugated goat anti-mouse IgG (Sigma) or biotin-conjugated goat anti-mouse IgA (Sigma) diluted 1:1000 in PBS-T was then added to the wells for 2 h at 37°C . The plates were washed before addition of HRP-conjugated streptavidin (Dako, Milan, Italy) diluted 1:2000 in PBS-T for 2 h at 37°C . Finally, after washing with PBS-T, the Ag-Ab reaction was measured by using the TMB substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and measuring absorbance at 450 nm after 5–10 min. Endpoint titers were determined as the reciprocal of the last dilution giving an absorbance of 0.3 units (for IgG) and 0.2 units (for IgA) greater than those of negative controls. To determine the titers of TT-specific Abs an ELISA was performed as previously reported [42].

Anti-LT Abs were measured by coating plates with a PBS solution containing 5 µg/ml of LT and by employing a protocol identical to the one used for measuring anti-Tat Abs.

2.4. Epitope mapping of Tat-specific Abs

Serum samples were tested for IgG Abs to Tat peptides by ELISA. Peptides (synthesized at the Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy) representing different regions of Tat (aa 1–20; aa 21–40, aa 36–50, aa 46–60, aa 56–70, aa 65–80, aa 73–86, aa 83–102), were plated in 96-well flat-bottom plates (Nunc-Immuno™ Plate MaxiSorp™ Surface; Nunc, Kampstrup, Denmark). The wells were coated with 250 ng of synthetic peptides in 200 µl of PBS with calcium and magnesium. After overnight incubation at 4 °C, plates were washed five times with PBS-T and blocked with PBS containing 1% BSA and 0.05% Tween 20 (PBS-BSA-T) for 90 min at 37 °C. Each well was then incubated with 100 µl of serum diluted 1:100 in PBS-BSA-T and incubated at 37 °C for 90 min. Wells were then blocked again with PBS-BSA-T for 15 min at 37 °C. After washings, the same biotin-conjugated goat anti-mouse IgG Abs and HRP-conjugated streptavidin used above were added to the wells and peptide-bound Abs were revealed by the addition of the ABTS substrate (Roche Diagnostic SpA, Lecco, Italy) for 50 min at 37 °C. Absorbance was measured at 405 nm. The cut-off value was defined as 3 standard deviations above the mean A_{405} values of a panel of pre-immune sera.

2.5. Neutralization of Tat activity

The ability of murine sera to block the HIV-1 replication induced by exogenous Tat was evaluated in HLM1 cells. This cell line contains an integrated copy of a HIV-1 *Tat*-defective provirus whose replication is rescued by the addition of exogenous Tat [11,19,21]. Cells (1.5×10^5) were seeded in 24-well plates in 500 µl of Dulbecco's modified essential medium (GIBCO BRL, Life Technologies Italia Srl, Milan, Italy) containing 10% of FBS (GIBCO). After 24 h of culture, medium was replaced with 300 µl of fresh medium containing 2.5 µg/ml of recombinant Tat protein pre-incubated with or without the diluted murine sera, overnight at 4 °C under rotation. Each serum was tested in duplicate. Negative controls were Tat pre-incubated with pre-immune sera. Forty-eight hours later, the rescue of HIV-1 replication was monitored by the measurement of p24 Ag released in the culture supernatants, using an enzyme immunoassay (INNO-GENETICS N.V. Zwijnaarde, Belgium). Results were expressed as the percentage of inhibition of HIV rescue considering 100% the value obtained with Tat alone.

2.6. Proliferation assay and cytokine analysis

Spleens were removed from mice that had been sacrificed by cervical dislocation. Single cell suspensions

from immunized and control mice were obtained by passing organs through a 100 µm nylon cell strainer. After lysis of erythrocytes, the splenocytes were resuspended in complete medium (RPMI-1640 containing 10% FBS [HyClone Lab. Inc., Logan, UT], 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 5.5×10^{-5} M 2-mercaptoethanol, 0.1 mM non-essential amino acids, all from GIBCO), at a final concentration of 2×10^6 cells/ml (24-well plates, Costar, Corning Inc., Corning, NY) and of 5×10^5 cells/well (flat-bottom 96-well plates, Costar) for cytokine analysis and proliferation, respectively. The cell suspensions were stimulated with Tat (0.1 µg/ml) for 5 days in 5% CO₂ at 37 °C. Preliminary experiments were conducted to determine the optimal concentration of Tat to be used for in vitro stimulation. For the assessment of Tat-specific T cell proliferation, each culture condition was assessed in quadruplicate and 0.5 µCi of [³H]thymidine (Amersham Corp., Arlington Heights, IL) was added to each well after 5 days of culture and 12 h prior to harvest. Control wells contained unstimulated cells and the stimulatory index (SI) was calculated as follows: counts per minute (cpm) experimental cultures/cpm control cultures. A SI above 3.0 was considered positive.

For the cytokine analysis, culture supernatants were harvested following 5 days of culture and stored at –70 °C until assayed. Cytokine levels (IFN-γ, IL-4, IL-6, IL-10, IL-12, TNF-α) were measured in culture supernatants by using ELISA assays as previously described [43]. Briefly, 96-well Nunc-Immuno™ Plate MaxiSorp™ Surface (Nunc) were coated with a solution of 1 µg/ml (in 0.1 M Na₂HPO₄, pH 9.0) of monoclonal rat-anti mouse cytokine Ab (PharMingen, San Diego, CA). After overnight incubation at 4 °C, plates were washed with PBS-T and blocked with 200 µl of PBS 1% BSA (Sigma) for 2 h at 37 °C. After washing, serial dilutions of culture supernatants and recombinant mouse cytokines (standards) were added to the wells and incubated overnight at 4 °C. The plates were then washed and appropriate biotin-conjugated rat anti-mouse cytokine Abs (PharMingen) were added to the wells and incubated for 2 h at 37 °C. Finally, after washing and addition of HRP-conjugated streptavidin (Dako) for 2 h at 37 °C, the TMB (Kirkegaard & Perry Laboratories) substrate was added to the wells and absorbance was measured at 450 nm after 10–20 min. Standard curves were generated using murine rIFN-γ, rIL-4, rIL-6, rIL-10, rIL-12, rTNF-α (PharMingen). The ELISA assay were capable of detecting 20 pg/ml of IFN-γ and IL-6, 10 pg/ml of IL-4, 80 pg/ml of IL-10, IL-12 and TNF-α.

2.7. CTL assay

A chromium release assay was employed to measure CTL activity. Effector cells were generated according to Porgador et al. [44]. Briefly, splenocytes were resuspended in complete medium at a cell density of 1×10^7 cells/ml. Cells

were cultured in 24 well plates (750 μ l/well) and 1 ml of media containing the indicated pool of Tat peptides (final concentration of each peptide was 4–5 μ g/ml) was added to the wells. Splenocytes from naive mice were also stimulated to determine whether the in vitro restimulation protocol induced non-specific cytotoxic activity. All cultures were incubated for 7 days in 5% CO₂ at 37 °C. On day 1, human rIL-2 (Chiron, Emeryville, CA) was added to each culture at a final concentration of 20 units/ml. Target cells (p815) were pulsed with 100 μ Ci/ml of Na₂⁵¹CrO₄ with or without the same peptide pools for 3–4 h. Following incubation, effectors and targets were cocultured at the indicated effector to target (E: T) ratios for 4–5 h. The amount of Na₂⁵¹CrO₄ released in the culture supernatants was measured using a Gamma Counter (Wallac Italia, Milan, Italy). The percentage of specific lysis was calculated using the following formula: [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] \times 100, where maximum cpm were obtained with target cells lysed with 1% Triton X-100 and spontaneous cpm with targets cells incubated without effectors.

3. Results

3.1. Mucosal delivery of Tat with LT induces high and persistent serum IgG titers in BALB/c mice

To test the immunogenicity of native Tat through the mucosal route, BALB/c and C57BL/6 mice were immunized intranasally with Tat alone or with Tat and the mucosal adjuvant LT and their anti-Tat serum IgG response was measured. Different results were found in the two mouse strains. In BALB/c, the mice that had been immunized with Tat alone did not develop an IgG response even after repeated doses, whereas the mice immunized with Tat and LT developed Ag-specific IgG Abs (Fig. 1A). Anti-Tat IgG Abs

appeared after the second immunization and progressively increased to reach high levels after four immunizations. Interestingly, the response to Tat was persistent in these mice since 1 year after the last immunization the anti-Tat serum IgG titer had only slightly decreased (Fig. 1B). In contrast to BALB/c mice, the C57BL/6 strain was not responsive to Tat. Neither serum IgG (Fig. 2A, left panel) nor mucosal IgA (data not shown) specific for Tat were found in C57BL/6 mice that had been immunized intranasally with the Ag in the presence of LT or cholera toxin (CT), another potent mucosal adjuvant. On the other hand, these mice developed a serum IgG response to the LT protein that had been co-administered with Tat (Fig. 2A, right panel). To rule out the possibility that the route of administration could influence the immunogenicity of Tat in the C57BL/6 strain, groups of C57BL/6 mice were immunized with native Tat subcutaneously in the presence of two different adjuvants, aluminum hydroxide and CFA. In contrast with BALB/c mice that were responsive to Tat delivered through this route (data not shown), C57BL/6 mice failed to respond to subcutaneously delivered Tat (Fig. 2B, left panel). On the other hand, control C57BL/6 groups immunized subcutaneously with TT and aluminum hydroxide developed an anti-TT IgG response (Fig. 2B, right panel). Finally, to assess whether the major histocompatibility complex (MHC) haplotype of C57BL/6 constrained the response to native Tat, we immunized intranasally BALB.B mice, which are congenic to BALB/c but express the C57BL/6 H-2^b haplotype. Indeed, similarly to C57BL/6 mice, the BALB.B mice were unable to respond to Tat (Fig. 2C, left panel) while they were able to respond to LT (Fig. 2C, right panel). These results indicate that the response to Tat in C57BL/6 mice may be constrained by their MHC haplotype.

3.2. Systemic and mucosal responses adjuvanted by the LT mutant LT-R72

Once assessed that intranasally delivered Tat is highly immunogenic in BALB/c mice if appropriately adjuvanted, we substituted the adjuvant LT with its non-toxic mutant LT-R72 and analyzed the immune response to Tat in serum and in mucosal secretions of BALB/c mice. As shown in Fig. 3, the immunization with Tat alone induced low titers of serum IgG in four mice whereas administration of Tat with LT-R72 induced in all mice 100–1000-fold higher anti-Tat serum IgG titers. Moreover, intranasal immunization with LT-R72 induced Tat-specific IgA in vaginal and intestinal secretions (Fig. 3).

3.3. Epitope mapping and neutralizing activity of anti-Tat serum antibodies

To map the epitopes recognized by the anti-Tat serum Abs in BALB/c mice, sera were tested by ELISA assays for recognition of synthetic peptides spanning the Tat sequence 1–86. Table 1 shows the results obtained with representative

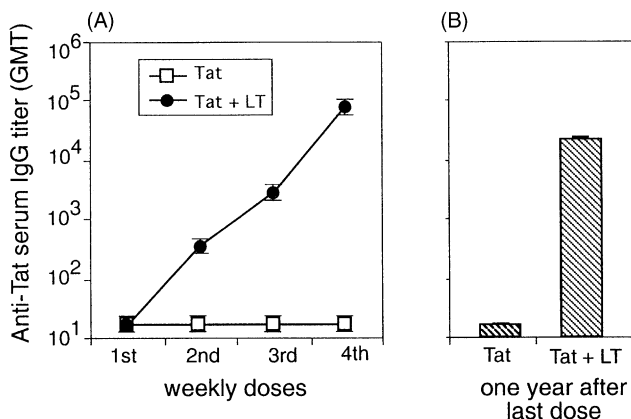


Fig. 1. Serum IgG response to Tat in BALB/c mice. Results are expressed as the IgG geometric mean titer (GMT) \pm S.D. of four individual sera. The anti-Tat titer was determined in sera collected 1 week after each of 4 weekly doses (A) and 12 months after the fourth dose (B).

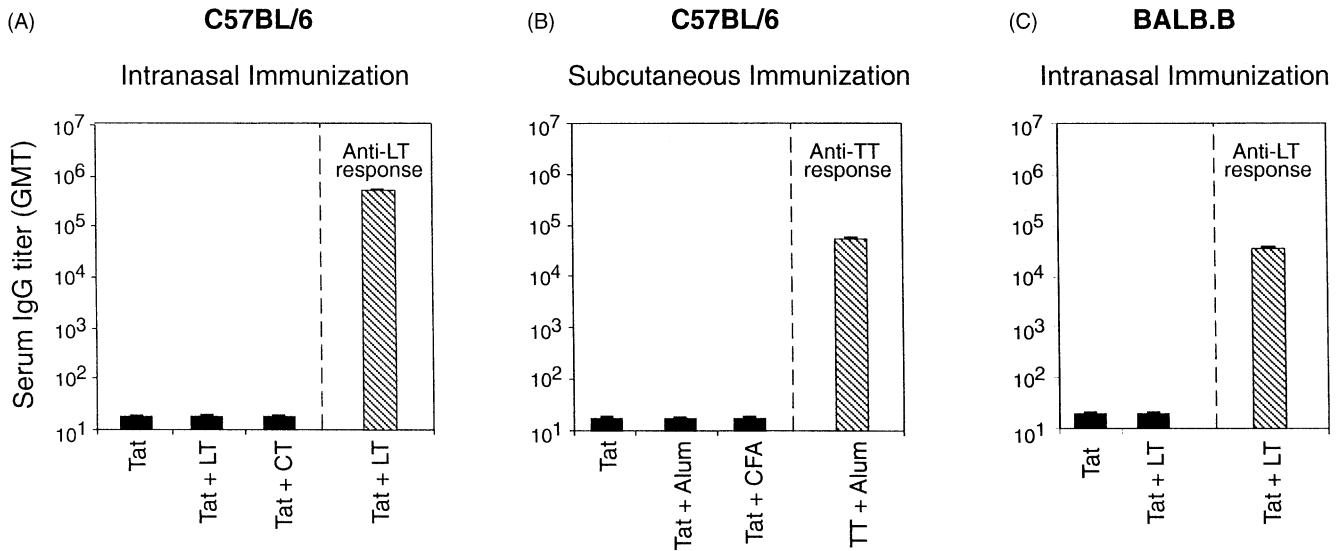


Fig. 2. Lack of serum IgG response to Tat in C57BL/6 and BALB.B mice. C57BL/6 mice received four intranasal doses of Tat alone or Tat + LT or Tat + CT (A) or two subcutaneous doses of Tat alone or Tat + aluminum hydroxide (Alum), Tat + CFA or TT + aluminum hydroxide (B). BALB.B mice received four intranasal doses of Tat alone or Tat + LT (C). The data show the anti-Tat IgG GMT \pm S.D. (left panels) and the anti-LT or the anti-TT IgG GMT \pm S.D. (right panels). The sera were taken 1 week after the last dose. Four individual sera were analyzed for each group of mice.

sera from different immunization groups. All the sera from mice immunized with Tat in the presence of either LT or LT-R72 had a strong reactivity to peptide 1–20. Furthermore, two sera from the mice immunized with Tat alone, which had a low anti-Tat titer, also recognized peptide 1–20. Most of the sera with the highest anti-Tat titers recognized also the peptide 46–60, although the reactivity to this peptide was weaker than that observed for peptide 1–20 (Table 1). Thus, intranasal immunization of BALB/c mice with native Tat (aa 1–86) elicits serum Abs specific for an immunodominant epitope, aa 1–20 and for a second epitope, aa 46–60.

We then asked whether the anti-Tat serum Abs had Tat-neutralizing activity. To this purpose murine sera were tested for the ability to block the replication of a HIV-1 *tat*-defective provirus induced by exogenous Tat in HLM1 cells. Fig. 4 shows the data obtained with pooled sera from mice immunized with Tat and LT-R72. This pool neutralized 30–50% of Tat activity also at high dilutions. Similar data were obtained with sera from animals immunized with the adjuvant LT (data not shown). The fact that no dose–response relationship was observed in the neutralization assay could be due to the high titers of Tat-specific Abs present in these sera.

Table 1
Epitope mapping of serum IgG with synthetic peptides homologous to Tat

Mice were immunized with	Serum (anti-Tat IgG titer) ^a	Response to Tat peptides ^b						
		1–20	21–40	36–50	46–60	56–70	65–80	73–86
Tat	F1 (128)	– ^c	–	–	–	–	–	–
	F2 (1024)	0.3	–	–	–	–	–	–
	F3 (<128)	–	–	–	–	–	–	–
	F4 (512)	0.1	–	–	–	–	–	–
Tat + LT	F13 (131072)	3.0	–	–	–	–	–	–
	F14 (65536)	3.0	–	–	–	–	–	–
	F15 (131072)	3.0	–	–	0.6	–	–	–
	F16 (65536)	2.7	–	–	–	–	–	–
Tat + LT-R72	F5 (131072)	3.0	–	–	0.1	–	–	–
	F6 (131072)	3.0	–	–	2.1	–	–	–
	F7 (131072)	3.0	–	–	0.1	–	–	–
	F8 (131072)	3.0	–	–	0.9	–	–	–

^a The anti-Tat IgG titers reported in parenthesis are those measured against native Tat (see also Figs. 1 and 3).

^b The data are reported as ELISA OD values calculated as described in M&M and obtained with sera diluted 1:100. Pre-immune sera and the sera of mice immunized with LT alone did not show any reactivity to Tat peptides.

^c ‘–’ indicates ELISA OD values equal to background.

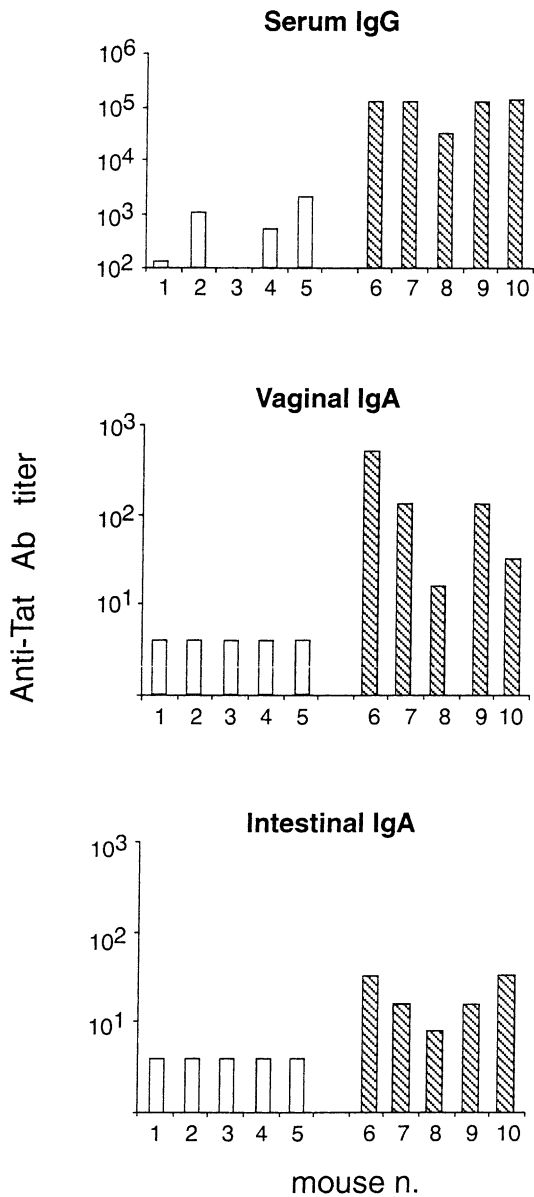


Fig. 3. Systemic and mucosal Ab responses to Tat in BALB/c mice immunized intranasally with Tat alone (mice 1–5) or with Tat + LT-R72 (mice 6–10). Samples were collected 1 week after the fourth dose.

3.4. Proliferative responses and cytokine secretion in response to Tat

To analyze the cell-mediated responses induced by intranasal immunization with Tat, splenocytes of animals immunized with Tat alone or with Tat and LT or Tat and LT-R72 were stimulated in vitro with native Tat. In Table 2, the T cell proliferative responses and the cytokine secretion of two representative mice for each immunization group are shown. The splenocytes of animals immunized with Tat and the adjuvants LT or LT-R72 proliferated vigorously to Tat, whereas those of the animals immunized with Tat alone showed either weak or no proliferative responses. In the same cultures,

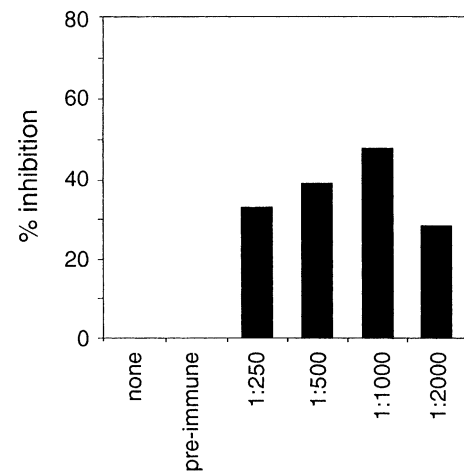


Fig. 4. Tat neutralization activity of sera from BALB/c mice immunized intranasally with Tat + LT-R72. The sera of four animals were collected before (pre-immune) and after four immunizations, were pooled and tested in the neutralization assay. The data represent the inhibition of the p24 release in the culture supernatant of HLM1 cells, as described in the M&M section.

we tested the secretion of the cytokines IFN- γ , IL-4, IL-6, IL-10, IL-12 and TNF- α and found that the mice that had received Tat and LT or LT-R72 produced IFN- γ and IL-6 in response to Tat, whereas the mice immunized with Tat alone did not produce detectable amount of any of these cytokines. On the other hand, IL-4, IL-10, IL-12 and TNF- α were never found in the culture supernatants (data not shown).

3.5. CTL response to Tat

CTL responses to HIV and to Tat have been shown to confer protection [9–14], thus we tested whether intranasal immunization induced CTL responses specific for Tat. The splenocytes of BALB/c mice immunized with Tat and LT were stimulated in vitro with pools of overlapping nonamer peptides (pools A–D in Fig. 5A) and with a pool of two

Table 2
Proliferation and cytokine secretion in response to Tat

Mice immunized with	Proliferation (SI) ^a	Cytokines (pg/ml)	
		IFN- γ	IL-6
Tat	3.4	– ^b	–
Tat	2.8	–	–
Tat + LT	13.8	411	67
Tat + LT	15.6	506	75
Tat + LT-R72	13.3	607	70
Tat + LT-R72	16.2	393	86

The data presented are from two representative mice for each immunization group. Splenocytes were stimulated in vitro with 0.1 μ g/ml of native Tat. Proliferative responses were measured after 6 days and cytokines were determined in the supernatants of parallel cultures after 5 days of stimulation.

^a Stimulatory indices (SI) >3 were considered positive. Background proliferation of unstimulated cultures ranged from 400 to 1200 cpm.

^b ‘–’ means undetectable.

(A)

Pool A	1 - 9	MEPVDPRLE	Pool C	46 - 54	SYGRKKRRQ
	6 - 14	PRLEPWKHP		51 - 59	KRRQRRRPP
	11 - 19	WKHPGSQPK		56 - 64	RRPPQGSQT
	16 - 24	SQPKTACTN		61 - 69	GSQTHQASL
	21 - 29	ACTNCYCKK			
Pool B	26 - 34	YCKKCCFHC	Pool D	66 - 74	QASLSKQPT
	31 - 39	CFHCQVCFI		71 - 79	KQPTSQSRG
	36 - 44	VCFITKALG		76 - 84	QSRGDPTGP
	41 - 49	KALGISYGR		81 - 89	PTGPKQK
Pool E	11 - 24	WKHPGSQPKTACTN			
	21 - 40	ACTNCYCKKCCFHCQVCFIT			

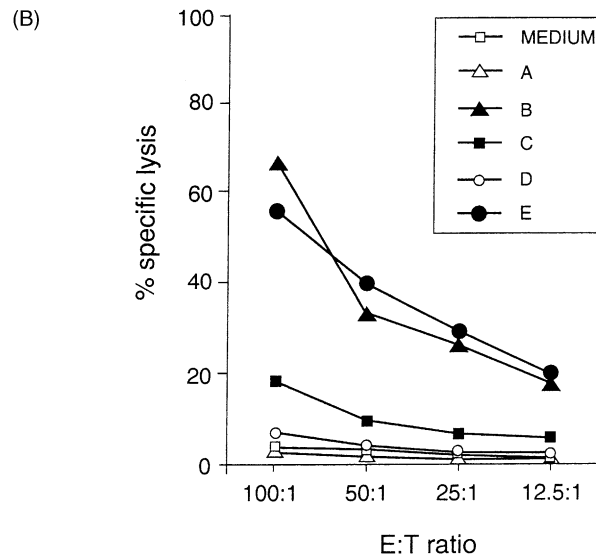


Fig. 5. Cytotoxic response of splenocytes to Tat peptides. (A) Peptide pools used in the CTL assay. (B) CTL activity in response to the indicated peptide pools. The levels of specific chromium release from peptide-stimulated splenocyte cultures added to p815 cells not pulsed with peptides were 0–10% as were the results from naive splenocytes.

longer peptides (pool E). The effector cultures were then tested for killing of target cells pulsed with the same peptide pools. Fig. 5B shows that two pools, pool B and pool E, stimulated high and comparable cytotoxic activities. This indicates that a CTL epitope is localized within aa 11–40 of Tat.

4. Discussion

We have demonstrated that mucosal delivery of native Tat (aa 1–86) with the adjuvants LT or LT-R72 elicits Tat-specific neutralizing Abs in the serum, mucosal IgA in vagina and intestine, Th and CTL responses. Tat was highly immunogenic in BALB/c mice upon intranasal administration whereas it did not elicit a response in C57BL/6 mice upon either intranasal or subcutaneous immunization. The unresponsiveness of this mouse strain to Tat is very likely due to MHC constrain, since BALB.B mice, which

are congenic to BALB/c but express the C57BL/6 haplotype H-2^b, were also unresponsive to Tat. However, the MHC constrain observed in C57BL/6 may not be a factor limiting the response to Tat in outbred animals as well as in man. Indeed, Tat has been shown to be immunogenic in non-human primates and in humans [11,12,34,35,37,38].

In the BALB/c strain, the response to intranasally delivered Tat was high, effective and persistent. High titers of IgG Abs were induced in the serum after three to four immunizations and persisted up to 1 year. Furthermore, the sera of mice immunized with LT or LT-R72 were all able to neutralize the activity of exogenous Tat on HIV replication in vitro. By using synthetic peptides homologous to Tat we identified the sequences 1–20 and 46–60 as the epitopes recognized by the BALB/c sera. Among these, the epitope 1–20 is immunodominant since it was recognized by all the sera tested, including those of animals immunized with Tat alone which had low anti-Tat titers. On the other hand, peptide 46–60 was recognized only by the sera with

the highest anti-Tat titers and to a lesser degree than peptide 1–20.

Interestingly, the proline-rich N-terminal region of Tat has been found to be immunodominant in BALB/c mice also following systemic immunization with native HIV-1 Tat or naked DNA [45,46]. Furthermore, the epitope 1–20 is immunodominant in non-human primates vaccinated with native HIV-1 Tat protein (our unpublished data) and the N-terminal region has been described as one of the major Ab binding site also in HIV-infected individuals [47].

The epitope 46–60 coincides with the basic domain of Tat, a highly conserved region that binds to heparan-sulfate proteoglycans and that mediates important functions of Tat, including entry into cells and nuclear localization of the protein [18,41]. Interestingly, mAbs directed against this region as well as the N-terminal domain of Tat have been shown to inhibit Tat-mediated transactivation of HIV-1 [45]. Furthermore, in HIV-infected individuals the presence of serum Abs specific for these two regions was found to inversely correlate with peripheral blood viral load [30]. Moreover, the sequences of Tat corresponding to the B cell epitopes described here are well conserved across the different viral clades [48]. It is thus noteworthy that the mucosal delivery of Tat not only preserves these regions but also stimulates a neutralizing response to them.

In addition to inducing anti-Tat neutralizing systemic Abs, mucosal immunization with Tat and LT-R72 elicited specific IgA responses in vagina and intestine, two mucosal districts that are relevant for HIV transmission. Antigen-specific IgA, which are released in the lamina propria of epithelia [1], and systemic IgG, which are known to transudate from the serum to the mucosal tissue [1], may cooperate to block the activity of the locally released Tat by HIV-infected cells and may therefore prevent viral spreading. In this regard, systemic anti-HIV neutralizing Abs have been shown to protect against a mucosal HIV challenge in two studies performed in non-human primates [3,7]. Moreover, protection of macaques immunized with SIV proteins in the iliac lymph nodes and rectally challenged with SIV was associated with the increase of Ag-specific IgA secreting cells [5]. Finally, the presence of anti-HIV IgA in the sera of multiple exposed uninfected individuals, suggests a possible protective role for IgA in humans [8].

T-helper responses have been assessed *in vitro* by stimulating splenocytes with sub-mitogenic doses of native Tat. Indeed, concentrations of Tat higher than 1 $\mu\text{g}/\text{ml}$ induced non-specific proliferation in splenocytes from naive animals (data not shown). On the other hand, when Tat was used at the concentration of 0.1 $\mu\text{g}/\text{ml}$, the splenocytes of mice immunized with Tat and the adjuvant but not those of animals immunized with Tat alone proliferated and secreted IFN- γ and IL-6. The cytokine profile observed reflects the stimulation of a mixed Th1/Th2 population, which is the hallmark of mucosal immunization with LT and its derivatives [49,50]. In addition, the stimulation of such a mixed Th1/Th2 popu-

lation was confirmed by the presence of IgG1, IgG2a in the sera of these mice (data not shown).

Of importance, mucosal immunization with Tat and LT induced anti-Tat CTL responses. Two peptide pools, one including overlapping nonamer peptides spanning the sequence 26–49 and a second containing the peptides 11–24 and 21–40, stimulated similar cytolytic activity. This suggests that a CTL epitope may be localized within aa 21–40 and that it is efficiently presented to CTLs when target cells are pulsed with this 20-mer peptide. However, we cannot exclude that peptide 11–24 in pool E is also responsible for CTL induction. Indeed, Morris et al. [51] have recently shown that mucosal immunization with synthetic peptides of Tat stimulates CTLs specific for the sequence 17–25, which is in part contained in our peptide 11–24.

The induction of CTLs *in vivo* upon immunization with Tat may be due to the intrinsic ability of native Tat to enter the cell cytosol and to reach the class-I pathway of Ag presentation [52,53]. In addition to this property of Tat, the route of immunization and the adjuvant used may have favored the stimulation of a CTL response. Indeed, for proteins that normally lack the capacity to enter the cell cytosol, CTL induction has been demonstrated following intranasal administration with LT or LT-R72 [44,54].

The induction of anti-Tat CTLs upon mucosal immunization is particularly relevant because of their protective role. Indeed, in monkeys immunized systemically with native HIV-1 Tat or with Tat DNA and challenged with a highly pathogenic virus the control of infection strongly correlated with the presence of anti-Tat CTL response prior to challenge [11,12,36]. Similarly, in the primary infection of monkeys with SIV a lower viral burden clearly correlated with the early appearance of anti-Tat CTLs [9]. Finally, in humans, CTLs anti-Tat and Rev Ags have been reported to be present in the early phases of HIV infection and to be maintained in long-term non-progressors [14,55].

The mutant LT-R72 was comparable to LT for efficiency and type of immune responses induced against Tat. This LT mutant differs from LT for one amino acid substitution and has reduced toxicity compared to LT [39]. Because of its safety and efficient adjuvanticity LT-R72 is a candidate molecule for the development of mucosal vaccines for human use [56].

The development of mucosal vaccines against HIV is desirable and we have shown that mucosal vaccination with Tat and the adjuvants LT or LT-R72 is feasible and induces an entire spectrum of immune responses that could be important for protection against HIV. Although a mucosal vaccine based solely on Tat may not prevent viral entry at the mucosal level, Tat may be included in a pool of HIV Ags that may provide protective responses useful during different phases of viral infection. Indeed, we are currently testing in mice the possibility to deliver multicomponent intranasal vaccines based on Tat. The control of HIV replication and dissemination by anti-Tat immunity may result in abortive infection with no disease and no virus transmission

in seronegative healthy subjects, whereas in infected individuals it may lower viral load and block disease progression and HIV transmission [15]. Thus, Tat-based strategies could be used for both preventive and therapeutic vaccination.

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