DNA Immunization with HIV-1 *tat* Mutated in the *trans* Activation Domain Induces Humoral and Cellular Immune Responses Against Wild-Type Tat[1]

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Intramuscular immunization of mice with plasmids encoding two transdominant negative mutants of the HIV-1 Tat protein (Tat_{22} and $Tat_{22/37}$) elicited a humoral response to wild-type Tat that is comparable to that induced by inoculation of wild-type *tat* DNA or Tat protein. The percentage of the responders and the Ab titers continued to increase after three additional DNA boosts and pretreatment with bupivacaine at the site of inoculation, without a significant difference ($p > 0.05$) among the three groups of mice immunized with mutant and wild-type *tat* genes. By utilizing synthetic peptides representing the amino acid sequence of Tat, one major B cell epitope was defined within the cysteine-rich domain of Tat. Anti-Tat IgG Abs directed against this epitope were found in mice immunized with all *tat* DNA constructs, whereas different Tat epitopes were detected in mice immunized with the Tat protein. Similarly, IgG2a was the predominant isotype in DNA-immunized mice, with both mutants and wild-type *tat* genes, as compared with protein immunization, which induced mostly IgG1 and IgG3. Sera from most immunized mice neutralized the effect of extracellular Tat in activating HIV-1 replication. A cellular response was also elicited as indicated by the proliferation of splenocytes when stimulated with wild-type Tat. These results indicate that the wild-type Tat Ag is recognized by Abs and T cells induced by DNA immunization with mutated *tat* genes, suggesting the possible use of these Tat transdominant mutants, lacking viral *trans* activation activity and capable of blocking wild-type Tat activity, in the development of an anti-HIV-1 vaccine. *The Journal of Immunology,* 1999, 162: 5631–5638.

S everal studies in HIV-1-infected patients and in nonhuman primates vaccinated with live attenuated viruses suggest that the presence of an early and broad immune response, both cellular and humoral, against the structural and nonstructural proteins of HIV-1 inversely correlates with progression to AIDS (1–8). Genetic immunization through inoculation of specific viral genes (9, 10) offers encouraging results for future vaccine trials because the viral proteins can be expressed with MHC molecules on the surface of the host cells. This mimics the use of a live attenuated virus (11–15), without the risks of generating pathogenic viruses, it can induce both arms of the immune system and it may protect against infection (16, 17).

The HIV-1-regulatory proteins Tat and Rev, as well as the accessory proteins Nef, Vif, Vpr, and Vpu, are considered attractive targets for the development of a multicomponent vaccine against HIV-1 infection. These proteins are well conserved among different isolates and thus may be less susceptible to mutations, as compared with structural genes, leading to production of escape virus variants (18, 19). In addition, they play a critical role in virus gene expression and replication and in the pathogenesis of AIDS and are immunogenic (1, 20–22). Among the regulatory genes, *tat* appears to be one of the most interesting targets.

The Tat protein is synthesized early during infection, and it is essential for virus replication (23). Tat is released by HIV-1-infected cells in the absence of cell death both in vitro and in vivo (24–28). Extracellular Tat can be taken up by HIV-1-infected cells, migrates to the nucleus, and *trans* activates the expression of the HIV-1 genome through autocrine and paracrine loops (25, 28–35). In addition, extracellular Tat induces expression of the HIV-1 coreceptors on the target cells promoting virus spreading (36, 37) and exerts several effects on different types of uninfected cells (38), thus contributing to the pathogenesis of several AIDS-associated diseases including a severe form of Kaposi's sarcoma, the most frequent tumor arising in AIDS patients, and lymphoproliferative and neurological disorders (24–27, 39–45).

Tat is a 86–102-aa protein encoded by two exons. The first exon is conserved among different viral isolates and contains four functional domains including the amino-terminal (aa 1–21), the cysteine-rich (aa 22–37), the core (aa 38–48), and the basic (aa 49–72) domains. The cysteine-rich region represents the Tat *trans* activation domain. The basic region contains the nuclear localization signals and the binding sequences to the Tat-responsive element (23). In addition, the basic region and the RGD sequence of the second exon are required for the interaction of extracellular Tat with cell surface molecules (28, 32) and for Tat uptake by the target cells.

Abs against Tat functional domains as well as a CTL response specific to Tat have been detected in humans (8, 46–48). Ab titers and early CTL anti-Tat were shown to correlate with nonprogression to AIDS (8, 48, 49). In addition, in vitro studies have demonstrated that anti-Tat Abs neutralize the activity of extracellular Tat on HIV-1 replication, cell functions, angiogenesis, tumor promotion, and formation of metastases (24–26, 48, 50, 51). Altogether these observations suggest that the induction of humoral and cellular immune responses against Tat would likely induce, in addition to a prophylactic effect, an immunotherapeutic activity in HIV-1-infected patients, possibly extending the asymptomatic period of the disease.

The ability of the wild-type *tat* gene, as well as of the Tat protein, to evoke a broad immune response has been recently shown in mice (52, 53) and in humans (54, 55). However, the long term effects of the constitutive expression of a viral gene following genetic immunization are presently unknown. Therefore, in view of the possible use of the *tat* gene as a component of an anti-HIV-1 vaccine (prophylactic and/or therapeutic) and considering the possibility that the long term expression and release of wild-type Tat in vivo may determine reactivation of HIV-1 infection and other pathogenic effects in seropositive individuals, we have investigated two *tat* genes mutated in the *trans* activation domain (tat_{22} and $tat_{22/37}$) as to their capability of eliciting an immune response against the wild-type Tat protein in the mouse model. The tat_{22} and $tat_{22/37}$ genes were chosen because they lack HIV-1 *trans* activation activity and display a transdominant negative phenotype (56, 57). Therefore, the mutated protein products can also block the *trans*-activating activity of wild-type Tat protein which is produced in infected individuals.

Materials and Methods

DNA immunogens

The pC vector containing the hCMV IE[3] promoter was derived from the plasmid pGEM7Zf(−) (Promega, Madison, WI). The 825-bp fragment, containing the hCMV IE promoter, was obtained by *Bam*HI-*Cla*I digestion of the LNCX vector (58) and cloned into the *Bam*HI-*Cla*I sites of pGEM7Zf(−). Plasmids pC-*tat*, pC-*tat*$_{22}$, and pC-*tat*$_{22/37}$ were constructed following insertion of the HIV-1 *tat*, *tat*$_{22}$ (Cys^{22} to Gly), and *tat*$_{22/37}$ (Cys^{22} to Gly, Cys^{37} to Ser) cDNAs into the pC vector. Briefly, the *Sal*I-*Sma*I fragments of 360 bp containing the full-length cDNAs of HIV-1 (HXBC2 clone) *tat*, *tat*$_{22}$, or *tat*$_{22/37}$ were derived from the plasmids pTZ18U-*tat*, -*tat*$_{22}$ or -*tat*$_{22/37}$ previously described (56), filled in with Klenow enzyme, and inserted into the *Sma*I site of the polylinker region downstream to the hCMV promoter in the pC vector. The orientation of the cDNAs was determined following digestion with *Bam*HI which cuts at nucleotide 356 in the *Sal*I-*Sma*I fragments containing the *tat* genes. The reporter plasmid pU3RCAT, where expression of the CAT gene is driven by the HIV-1 LTR promoter, has been previously described (56). Plasmid DNAs were purified on CsCl gradients, according to standard procedures (59), and resuspended in sterile PBS, without calcium and magnesium.

Cell cultures and transfections

Jurkat T cells were grown in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% heat-inactivated FBS (Life Technologies). Monolayer cultures of murine BALB/c 3T3 fibroblasts were grown in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS. The Superfect reagent provided by Qiagen (Hilden, Germany) was used for transient transfection of 5×10^5 BALB/c 3T3 fibroblasts, according to the manufacturer's instructions. Cells were transfected with the pU3RCAT plasmid (1 μg) alone or in combination with pC-*tat* (1 μg). To assay for functionality of the *tat* mutant vectors, cells were cotransfected with pU3RCAT (1 μg), pC-*tat* (1 μg), and pC-*tat*$_{22}$ (10 μg) or pC-*tat*$_{22/37}$ (10 μg) vectors. The DEAE-dextran technique was used for transient transfections of 10^7

Jurkat cells (59). CAT activity was measured 48 h after transfection with amounts of cell extracts normalized to total protein content (59).

DNA immunization

Six-week-old female BALB/c mice (Nossan, Milan, Italy) were immunized with the plasmids, containing the tat_{22} or the $tat_{22/37}$ gene. Animals were then tested for the development of humoral and cellular responses to wild-type Tat protein. Control animals included mice injected with the pC-*tat* or the empty vector pC. Each experimental group contained 10 animals. The dose of 100 μg of plasmid DNA in 100 μl was given to each mouse by bilateral i.m. injections in the quadriceps muscles of the posterior legs (day 0). The same dose of DNA was administered at days 15 and 30. Two blood samplings were taken at days 45 and 75 (bleedings 1 and 2), respectively. Mice were boosted three more times (at days 90, 120, and 150) with 50 μg/50 μl/animal of the plasmids injected i.m. in the quadriceps muscle of the right posterior leg. One week before the last two DNA immunizations, 100 μl of 0.5% bupivacaine hydrochloride (Sigma, St. Louis, MI) in isotonic NaCl were administered i.m. into the site of DNA inoculation. Blood drawings were taken 2 wk after each DNA injection at days 105, 135, and 165 (bleedings 3–5), respectively. Blood samples were withdrawn by endocardiac puncture from mice anesthetized with a mixture containing 30 μl of Ketavet-100 (Gellini Farmaceutici, Aprilia, Italy), 30 μl of Rompun (Bayer, Milan, Italy), and 30 μl of isotonic solution. Sera were prepared by centrifugation of coagulated blood at 8000 rpm for 5 min and stored at −70°C.

Tat protein expression and purification

The Tat protein was expressed in *Escherichia coli* and isolated by successive rounds of high pressure chromatography and ion-exchange chromatography as previously described (25, 28). The purified Tat protein is >95% pure as tested by SDS-PAGE and has full biological activity as tested by the rescue assay described below. The Tat protein was stored lyophilized at −70°C and resuspended in degassed PBS containing 0.1% BSA and 0.1 mM DTT before use. The plasticware was previously rinsed in PBS-BSA buffer for each procedure involving the use of Tat.

Tat protein immunization

Two 8-wk-old female BALB/c mice were immunized i.p. with wild-type Tat protein (10 μg/mouse) that was mixed with an equal volume of CFA (Sigma). Ten days later, each animal was boosted with 5 μg of protein mixed with an equal volume of in CFA. Two control mice were given the adjuvant alone. Ten days after the second immunization, the animals were sacrificed to collect blood and spleens.

Anti-Tat serology

The presence of anti-Tat-specific IgG in the sera of the vaccinated animals was determined by ELISA. The Ag was a recombinant maltose-binding protein (MBP) fused to Tat (MBP-Tat). The MBP-Tat fusion protein was expressed in *E. coli* by means of the pMal-c2 expression vector (New England Biolabs, Beverly, MA), containing the full-length wild-type HIV-1 (HXBC2) tat cDNA, and purified according to manufacturer's instructions. The purity and integrity of MBP-Tat was controlled by SDS-PAGE followed by Coomassie blue staining. In the fusion protein, Tat corresponds to ~25% of the total protein. Control Ag represented by MBP alone was also used. The concentration of Ags were 5 μg/ml for MBP-Tat and 3 μg/ml for MBP diluted in 0.1 M carbonate buffer (pH 9.6). Ninety-six-well immunoplates (Nunc, Naperville, IL) were coated with 100 μl/ well of each Ag, sealed, and incubated for 16 h at 4°C. Plates were washed three times with PBS (pH 7.4) containing 0.05% Tween 20 (Sigma). Sera were diluted in carbonate buffer and tested in duplicate. Fifty-microliter aliquots were added to each well and incubated for 90 min at 37°C. Plates were washed three times with PBS/Tween 20 buffer before addition of 100 μl of horseradish peroxidase-labeled goat anti-mouse IgG (Bio-Rad, Richmond, CA) diluted 1/1000 in PBS without calcium and magnesium and containing 0.1% Tween 20 and 1% BSA, and incubated for 90 min at room temperature. Following three washes with PBS/Tween 20, 100 μl of ABTS substrate (Boehringer Mannheim, Mannheim, Germany) were added for 45 min at room temperature. The absorbance was measured at 405 nm. The cutoff value for DNA and protein immunization corresponds to the mean absorbance value (+2 SD) of all the control sera derived from animals immunized with the empty pC vector or the adjuvant alone. Absorbance values higher than the cutoff were considered positive.

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[3] Abbreviations used in this paper: hCMV IE, human cytomegalovirus immediate early promoter; CAT, chloramphenicol acetyltransferase; SI, stimulation index; LTR, long terminal repeat; MBP, maltose-binding protein.

FIGURE 1. *A,* Functional analysis of wild-type and mutated *tat* expression vectors. BALB/c 3T3 cells were cotransfected with pC-*tat* (1 μg) and the reporter plasmid pU3RCAT (1 μg) alone (*lane 1*) or in the presence of pC-*tat*$_{22}$ (10 μg) (*lane 2*) or pC-*tat*$_{22/37}$ (10 μg) (*lane 3*). In *lanes 2* and *3,* 84% and 60% reduction of Tat *trans* activation activity on HIV-1 LTR-CAT was induced by *tat*$_{22}$ and *tat*$_{22/37}$ gene products, respectively, as compared with HIV-1 LTR-CAT *trans* activation induced by pC-*tat* alone (*lane 1*) that was given a 100% value. *B,* Schematic representation of the immunization schedule. Boosts 5° and 6° were preceded by bupivacaine (B) treatment. At the fifth bleeding, mice were sacrificed, and the spleens were collected for analysis of the cell-mediated immune response.
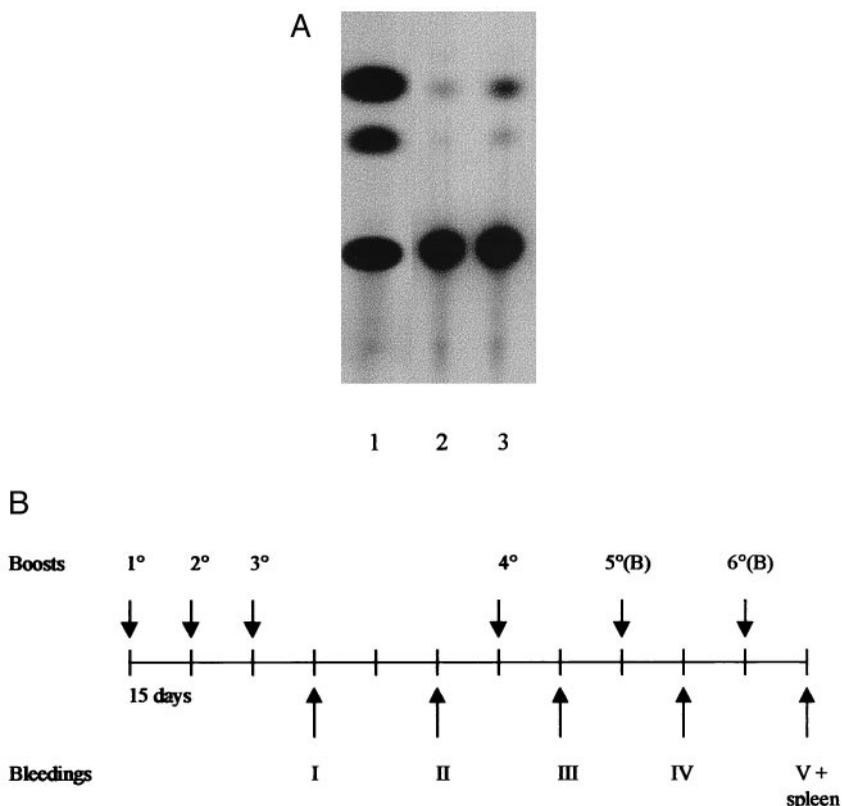
Epitope mapping of anti-Tat Abs and Ab isotyping

For epitope mapping, eight synthetic peptides (synthesized at the Department of Pharmaceutical Science, University of Ferrara, Ferrara, Italy), representing different regions of Tat, were used at the concentration of 10 μg/ml. After coating with 100 μl/well, plates were blocked with 100 μl/well of PBS with 3% BSA for 2 h at 37°C, washed three times, and incubated with 50 μl/well of each serum, diluted in PBS with 3% BSA. The assays were then performed as described above.

For isotyping, plates were coated with MBP-Tat and incubated with the mice sera as described above. After washing, 100 μl of goat anti-mouse IgG1, IgG2, IgG2a, IgG2b, or IgG3 (Sigma) were added to the wells and incubated for 90 min at room temperature. Immunocomplexes were detected with a horseradish peroxidase-labeled rabbit anti-goat (Sigma) and ABTS substrate as described above.

Neutralization assays

For neutralization assays, HLM1 cells, a HeLa-CD4$^+$ cell line containing an integrated copy of a *tat*-defective provirus, were seeded in 24-well plates at a density of 6×10^5 cells/well in DMEM supplemented with 10% inactivated FBS. After 24 h, cells were washed with PBS, and Tat protein (30 ng/ml) was added alone or after prior incubation with an equal volume of the mouse serum, as previously described (25, 28). After 48 h, the rescue of HIV-1 replication was monitored in the culture supernatants by using a p24 Ag capture assay (NEN-DuPont, Stevenage, U.K.).

Tat-specific T cell proliferation

Mononuclear cells were purified from spleens of sacrificed animals by Ficoll-Histopaque 1083 (Sigma) density gradients, washed twice with PBS, and resuspended at 2×10^6/ml in RPMI 1640 supplemented with 10% heat-inactivated FBS. Cells were counted by trypan blue exclusion dye and cultured at 2×10^5/well in 200 μl in the presence of affinity-purified Tat protein (0.1, 1, or 5 μg/ml) or Con A (10 μg/ml, Sigma) for 5 days. [*methyl*-^{3}H]Thymidine (2.0 Ci/mmol, NEN-DuPont) was added to each well (0.5 μCi), and cells were incubated for 16 h. [^{3}H]Thymidine incorporation was measured with a β-counter. The SI was calculated by dividing the mean counts/min of six wells of Ag-stimulated cells by the mean counts/min of six wells of the same cells grown in the absence of the Ag. A value >2.3-fold SI that corresponds to the mean SI of the control mice immunized with the pC vector (+2 SD) was considered positive.

Statistical analysis

Student's t and χ^2 tests were performed as described (60).

Results

Vectors containing the full-length cDNA of the HIV-1 wild-type *tat* gene (pC-*tat*) or the two *tat* transdominant negative mutants pC-*tat*$_{22}$ and pC-*tat*$_{22/37}$ under the transcriptional control of the hCMV IE promoter were constructed as described in *Materials and Methods.* As shown in Fig. 1*A,* pC-*tat* expressed a functional Tat protein after transfection into murine BALB/c 3T3 fibroblasts, as detected by the activation of the reporter plasmid pU3RCAT, where expression of the CAT gene is driven by the HIV-1 LTR (Fig. 1*A, lane 1*). Similarly, the plasmids pC-*tat*$_{22}$ and pC-*tat*$_{22/37}$ expressed transdominant negative mutants capable of competing wild-type Tat activity on the HIV-1 LTR-CAT vector after transfection of pC-*tat* with a 10-fold molar excess of each mutant plasmid (Fig. 1*A, lanes 2* and *3*). Similar results were obtained in human Jurkat T cell lines (data not shown).

To determine whether *tat*$_{22}$ and *tat*$_{22/37}$ genes can elicit specific humoral and cellular immune responses, mice were immunized with each plasmid (Fig. 1*B*). Control mice were immunized with pC-*tat* or the empty pC vectors. All animals in each group were injected three times (days 0, 15, and 30) with 100 μg of each plasmid DNA. Two weeks after the third immunization (day 45), a humoral response to wild-type Tat was detected in 20 and 30% of mice injected with pC-*tat*$_{22}$ or pC-*tat*$_{22/37}$, respectively, as well as in 60% of control animals vaccinated with pC-*tat.* However, a slight decrease in the percentage of positive animals was observed in almost all groups 4 wk later (day 75) (Table I). There was no significant difference ($p > 0.05$) between the percentage of positive animals in the groups immunized with *tat* mutants and wild-type *tat.* All mice were then boosted three more times with 50 μg of the plasmid DNAs (days 90, 120, and 150). In addition, since

Table I. *Humoral immune response to wild-type Tat protein after DNA immunization with wild-type or mutated* tat *genes[a]*

	Bleedings				
Plasmid	1	2	3	4	5
pC	0/9	0/7	0/4	0/5	0/4
	(0.17 ± 0.05)	(0.13 ± 0.06)	(0.26 ± 0.02)	(0.24 ± 0.03)	(0.13 ± 0.02)
pC-*tat*	6/10	3/9	7/9	7/8	8/8
	(0.33 ± 0.02)	(0.30 ± 0.002)	(0.52 ± 0.20)	(1.01 ± 0.80)	(0.69 ± 0.41)
pC-*tat*$_{22}$	2/10	1/8	5/7	5/7	7/7
	(1.00 ± 0.70)	(1.54 ± 0.0)	(0.56 ± 0.20)	(1.09 ± 0.70)	(1.10 ± 0.88)
pC-*tat*$_{22/37}$	3/10	4/10	5/8	5/7	6/7
	(0.48 ± 0.09)	(0.66 ± 0.50)	(0.42 ± 0.09)	(0.59 ± 0.30)	(0.65 ± 0.77)

[a] Results are expressed as the number of responder animals vs the total immunized mice. The Ab response was assayed on murine sera (diluted 1/25) by ELISA using MBP-Tat as the Ag. In each group of mice immunized with wild-type or mutated *tat* genes, mean OD$_{405 nm}$ values ± SD of the responders (positive sera) are reported in parentheses; values that were below the cutoff were not included in the mean. In the control group receiving the pC plasmid, mean OD$_{405 nm}$ values ± SD of all animals are reported in parentheses. Cutoff values are represented by the mean OD$_{405 nm}$ value plus 2 SD of the control group and corresponded to 0.27, 0.25, 0.30, 0.30, and 0.17 from blood drawings 1 to 5, respectively. The loss of animals from the first blood drawing to the fifth was the result of their accidental death caused by the endocardic puncture. From the estimate of χ^2, the percentage of responders in the group of mice injected with pC-*tat*$_{22}$ or pC-*tat*$_{22/37}$ is not significantly different from that of mice immunized with pC-*tat* ($p > 0.05$).

bupivacaine has been shown to increase the uptake of DNA from muscle cells and enhance the response to DNA immunization (16), 1 wk before the last two immunizations the animals were given bupivacaine at the site of inoculation. Following these additional boosts and the bupivacaine treatment, the percentage of responder animals increased to 100% in the group immunized with pC-*tat*$_{22}$ as well as in the control mice receiving pC-*tat*, and to 85% in the group boosted with pC-*tat*$_{22/37}$ (bleeding 5 at day 165) (Table I). Ab titers increased from a mean value of 1/50, detected at the first bleeding, up to 1/200–1/3200 at the fifth bleeding (Table II). All anti-Tat-positive sera did not bind the bacterial MBP Ag alone, confirming that the reactivity to MBP-Tat used as Ag was specific to Tat (Table II). To compare the results of DNA immunization with protein immunization, two mice were immunized with the Tat protein. They were injected twice (days 0 and 10) with the Ag and sacrificed 10 days after the second immunization. In both animals, anti-Tat-specific Abs were detected with titers of 1/6400 (Table II).

The epitope reactivity of the sera was analyzed by using eight synthetic peptides representing different amino acid sequences of Tat. The epitope reactivity of the Abs was broad with one major epitopic site mapped at residues 21–40 in mice immunized with all *tat* constructs (Fig. 2A). A second reactive epitope was identified at residues 73–86 in sera of pC-*tat*$_{22}$ and pC-*tat* immunized animals, and at amino acids 52–72 in pC-*tat*$_{22/37}$-immunized mice (Fig. 2A). Epitopic sites mapping at amino acids 1–20 and 21–40 were instead recognized by mice injected with the Tat protein (Fig. 2B). The isotype analysis of the IgG subclasses indicated a prevalence of IgG2a in mice vaccinated with both the mutated and the wild-type *tat* genes (Fig. 3), whereas IgG1 and IgG3 isotypes were detected in animals immunized with the Tat protein (data not shown).

The ability of the murine sera to block HIV-1 replication induced by extracellular Tat was then evaluated in HLM1 cells that contain an integrated copy of an HIV-1 *tat*-defective provirus the replication of which is activated by the addition of exogenous protein. To this purpose, cells were incubated with wild-type Tat in the presence of the animal sera, and virus rescue was measured 48 h later by determining the p24 Ag released in the culture supernatants. The results of these experiments showed that anti-Tat Abs of mice immunized with pC-*tat*$_{22}$ and pC-*tat*$_{22/37}$ reduced extracellular Tat activity, resulting in average decreases of 22.4 and 38%, respectively, of Tat-induced HIV replication. A similar neutralization activity ($p > 0.05$) was exhibited by sera of mice immunized with pC-*tat* with an average inhibition of 49%. Animals immunized with the Tat protein almost completely prevented the

rescue of Tat-induced HIV replication with an average inhibition of 85% (Table II).

A proliferative response to Tat was evaluated on animals splenocytes in the presence of Tat. A specific response was exhibited by animals inoculated with pC-*tat*$_{22}$ or pC-*tat*$_{22/37}$ plasmids, as well as by the control mice treated with pC-*tat* DNA or the Tat protein (Table III). Splenocytes from several mice responded to as

Table II. *Ab response to HIV-1 wild-type Tat protein*

Immunogen	No. of Mice	MBP-Tat[a]	MBP[a]	% Neutralizing Activity[c]
pC	2	0.112	0.081	0
	3	0.144	0.054	0
	6	0.160	0.065	0
	8	0.137	0.031	0
pC-*tat*	11	0.347 (1/200)[b]	0.083	ND
	12	1.066 (1/400)	0.098	ND
	13	0.460 (1/200)	0.068	54
	15	0.503 (1/200)	0.080	ND
	16	0.208 (1/100)	0.054	ND
	17	0.988 (1/400)	0.044	0
	19	1.427 (1/400)	0.102	61
	20	0.510 (1/400)	0.065	81
pC-*tat*$_{22}$	21	3.0 (1/3200)	0.225	47
	22	0.703 (1/400)	0.095	34
	23	0.658 (1/400)	0.087	0
	24	0.701 (1/400)	0.065	31
	25	0.744 (1/400)	0.086	ND
	27	0.473 (1/200)	0.055	0
	28	1.426 (1/1600)	0.120	ND
pC-*tat*$_{22/37}$	33	0.147	0.038	ND
	34	2.370 (1/3200)	0.200	45
	35	0.193 (1/100)	0.056	ND
	36	0.696 (1/200)	0.073	69
	37	0.452 (1/200)	0.048	0
	38	0.295 (1/200)	0.052	ND
	40	0.378 (1/200)	0.061	ND
Tat protein	T1	2.459 (1/6400)	0.105	93
	T2	2.611 (1/6400)	0.098	78

[a] Animal sera (fifth blood drawing, 1/25) were assayed by ELISA, using MBP-Tat or MBP as the Ag. The cutoff values were 0.17 and 0.090 for MBP-Tat and MBP, respectively. Results are expressed as OD$_{405 nm}$.
[b] In parentheses are listed the end point ELISA titers.
[c] Murine sera were tested for their capability to neutralize the rescue of an HIV-1 *tat*-defective provirus induced by extracellular wild-type Tat. Values represent the percentage of inhibition of extracellular wild-type Tat activity relative to the mean value of HIV-1 rescue obtained with Tat that was preincubated in the presence of pC control murine sera, which was given a 100% value. From estimate of Student's *t* test, the difference in neutralization activity values exhibited by anti-Tat Abs of mice immunized with mutated or wild-type *tat* DNA is not significant ($p > 0.05$).
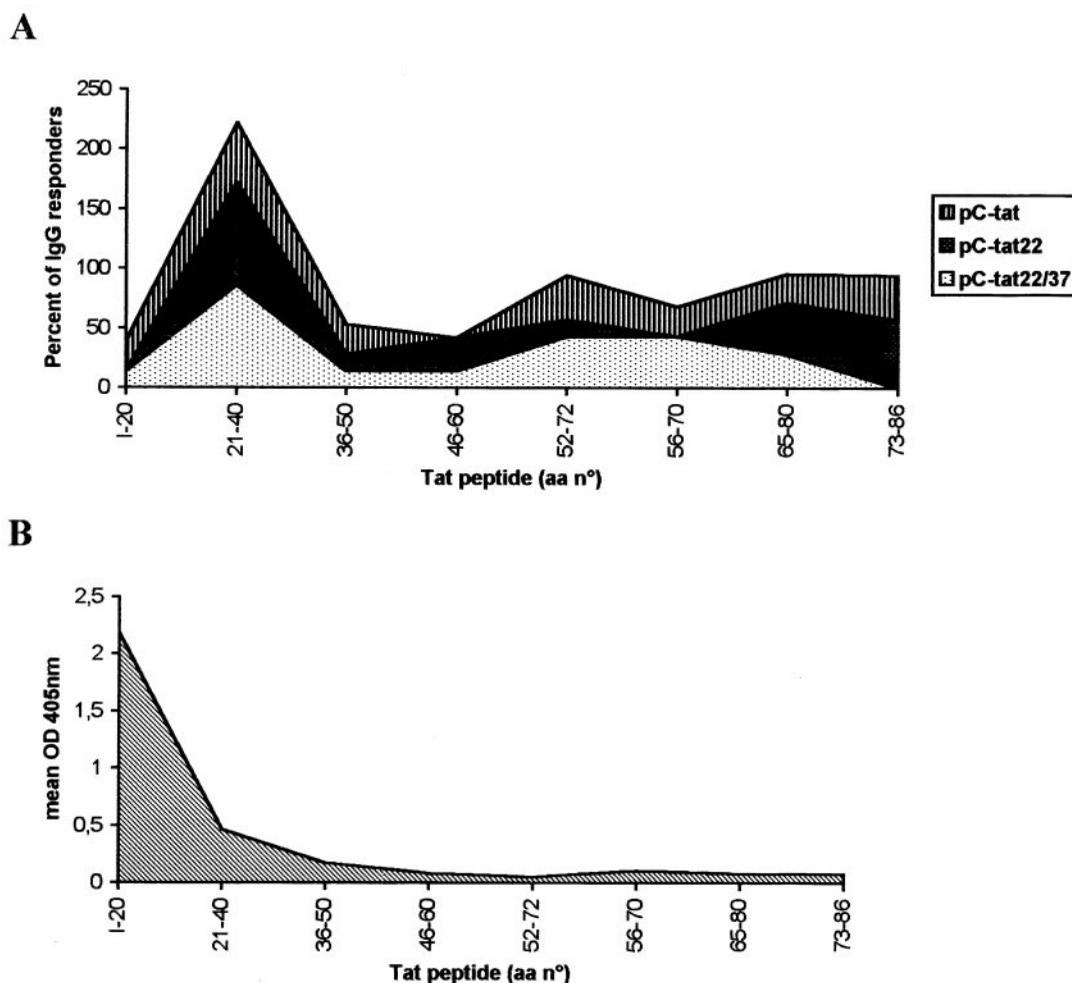
FIGURE 2. Epitope mapping of anti-Tat Abs from immunized animals. *A*, DNA-immunized mice. The results are expressed as the percentage of responder animals on the total vaccinated animals. Cumulative values are shown. *B*, Tat protein-immunized mice. The results are expressed as the mean $OD_{405\,nm}$ of the two immunized animals. Cutoff values, corresponding to the mean $OD_{405\,nm}$ value plus 2 SD of the controls, were 0.012, 0.020, 0.076, 0.054, 0.019, 0.047, 0.056, and 0.033 for each peptide, respectively.

little as 0.1 μg/ml recombinant Tat (data not shown); however, the highest responses were seen after stimulation with 1 and 5 μg/ml of Tat. Although the highest frequency (87%) of animals showing a proliferative responses to Tat was observed in the group of mice immunized with wild-type *tat* DNA, a similar ($p > 0.05$) T cell response specific to Tat was elicited, in the same range of SI, in 50 and 71% of mice immunized with the tat_{22} and $tat_{22/37}$ plasmids, respectively (Table III).

Discussion

Previous studies indicated that the control of viral replication at the onset of the HIV-1 infection is likely to have a dramatic impact on disease progression in an infected individual (3–8, 61). Tat is released early after infection and plays a key role in the beginning of HIV-1 replication, in virus spreading, and in AIDS pathogenesis. In addition, Tat is conserved among virus isolates; therefore, it represents an important candidate for the development of a vaccine against HIV (62, 63). DNA vaccination with wild-type *tat* was recently shown to induce humoral and cellular immune responses in mice and in humans (52–55).

In the current study, we have considered that constitutive expression of the *tat* gene following genetic immunization may induce HIV-1 replication and eventually exerts pathogenic effects

when administered to humans. Thus, we have elicited and analyzed the immune response toward the wild-type Tat protein that is evoked by tat_{22} and $tat_{22/37}$ genes. These genes are mutated in the *trans* activation domain of Tat and therefore lack Tat activity. In addition, they block the effect of wild-type Tat on the HIV-1 LTR.

Our results demonstrate that these *tat* mutant genes induce humoral and cellular immune responses specific to the wild-type Ag. The humoral response was readily detected after the third immunization with each gene. The mean titers and the number of responder mice increased following three additional boosts and treatment with bupivacaine that was previously shown to facilitate the uptake of plasmid DNA by the muscle tissue (16). The Ab titers, the IgG isotypes, the Tat epitopes recognized by the Abs, the capability of animal sera to neutralize the rescue of HIV-1 replication by exogenous Tat, and the T-cell proliferative responses were comparable ($p > 0.05$) in the three groups of mice immunized with the mutant or the wild-type *tat* genes. In addition, after vaccination with the *tat* genes, Abs reacted with a broad spectrum of linear Tat epitopes, mapping at residues 21–40, 52–72, and 73–86, and the prevalence of IgG2a subclass was suggestive of a Th1 profile.

A broad immune response, both humoral and cellular, was also detected after protein immunization. In these animals, however,
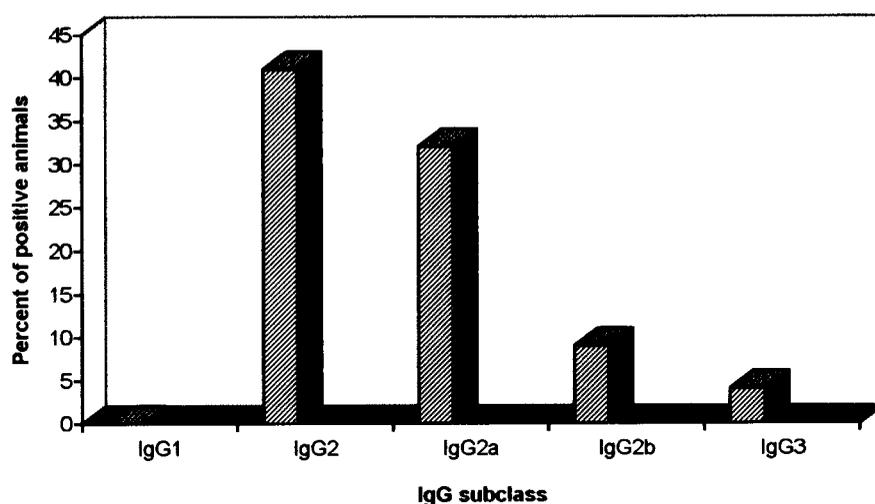
FIGURE 3. Isotype analysis of anti-Tat IgG from DNA-immunized mice. Results are expressed for each IgG subclass as the cumulative percentage of the responders on the total animals immunized with wild-type and mutated *tat* genes.

anti-Tat Ab titers were higher, and consequently the rescue of Tat-induced HIV-1 replication was blocked more effectively than by sera from DNA-immunized mice. Finally, in these mice the epitope reactivities were more restricted, and the presence of IgG1 and IgG3 isotypes was suggestive of a Th2-like response. These differences between genetic vs protein immunization with *tat* may be explained by a diverse conformation of the native Tat protein expressed in vivo, as compared with the exogenous recombinant protein injected into the animal. Indeed, because of its high content in cysteine residues, the Tat protein is very labile when exposed to air and light, and it is sensitive to temperature (28). In addition, Ags produced by plasmid DNA administered i.m. might be presented less efficiently to the immune system by MHC class I molecules on myocytes, whereas a soluble Ag injected by the i.p. route may be readily presented to T cells in the spleen or in the local lymph nodes. Another reason for the differences observed is that most of the Tat molecules, released by the injected myocytes, may be locally retained and hardly reach the immune compartment.

The results of this study demonstrate that both arms of the immune system can be primed by tat_{22} and $tat_{22/37}$ genes that induce a broad response specific to the wild-type Tat protein and similar to that elicited by the wild-type *tat* DNA. These results show that genetic vaccination with tat_{22} and $tat_{22/37}$ induces a Th1-like response similarly to that found with wild-type *tat*, by us in this study and by others previously (53). The data presented also indicate that neutralizing anti-Tat Abs raised by *tat*-mutated genes can block the effect of the extracellular Tat protein on virus replication. These results are encouraging for the development of an anti-HIV-1 vaccine based on Tat. In fact, Tat-neutralizing Abs may control virus replication and the spreading of infection (8, 48–50). In addition, a Th1 response may be the most effective in controlling the progression of HIV-1 infection to AIDS (64–67).

Several questions, however, remain to be settled and represent the matter for further studies. For instance, the cysteine substitutions in tat_{22} and $tat_{22/37}$ gene mutants fall just within the aa 21–40 epitope which is immunodominant in DNA immunization and in HIV-1 natural infection (22, 46, 47). It would be interesting, therefore, to compare the immune response to the Tat protein and to Tat 21–40 peptide mutated in the same cysteine residues, although the results may not be directly comparable because the immunodominant epitope in protein immunization is at aa 1–20. Also, it is not clear at present whether a genetic or a protein immunization to Tat would be more protective in natural HIV-1 infection. From the results of this

study, a DNA immunization seems to be preferable, due to the presence of a cellular response with the characteristics of a Th1 reaction. Moreover, it would be now of great interest to perform an alternate prime-and-boost experiment, where mice will be primed with either *tat* DNA or Tat protein and then boosted with the other. In this study, we have intentionally avoided boosting with the Tat protein the animals immunized with the *tat* genes, because our aim was to characterize the response of mice to a pure *tat* DNA immunization. Comparing the results of these experiments to the results obtained by immunization with

Table III. *Lymphoproliferative response to wild-type Tat protein*

Immunogen	No. of Mice	Tat[a]		Con A[a]
		1 μg/ml	5 μg/ml	
pC	2	1.5	2	24
	3	1.2	1.7	88
	6	1.3	1.5	23
	8	2	2.1	20
pC-*tat*	11	2.5	4.7	14
	12	2.8	4.0	45
	13	1.4	1.6	20
	15	2.2	3.2	12.5
	16	1.9	2.7	45
	17	14	21	98
	19	13	12	276
	20	1.8	3.9	28
pC-tat_{22}	21	1.2	2.6	28
	22	ND	ND	ND
	23	1.2	1.8	20
	24	3.1	6.9	28
	25	3.5	8.0	24
	27	1.3	2.3	17.8
	28	1.0	1.7	12.7
pC-$tat_{22/37}$	33	1.0	1.9	28
	34	2.0	1.7	28
	35	2.3	7.3	47
	36	6.3	30	133
	37	3.5	8.5	47
	38	1.8	3.8	17
	40	4.4	19	123
Tat protein	T1	2.5	4	9.7
	T2	2.7	6.9	4.5

[a] Cells were stimulated with affinity-purified recombinant Tat protein or Con A. Values represent the SI of murine splenocytes after wild-type Tat or Con A activation. A SI higher than 2.3 was considered positive. From estimate of χ^2, the difference in the percentage of responder mice immunized with mutated or wild-type *tat* DNA is not significant ($p > 0.05$).

only *tat* DNA or Tat protein would provide useful information and suggestions to establish an effective program for Tat immunization (genetic and/or protein) in natural HIV-1 infection. In addition, the extension of these studies to the animal model of nonhuman primates which are susceptible to virus infection will determine the effect of vaccination with Tat on protection from infection and from disease progression.

Acknowledgments

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