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HIV-1 Tat-Based Vaccines: From Basic Science to Clinical Trials

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ABSTRACT

Vaccination against human immunodeficiency virus (HIV)-1 infection requires candidate antigen(s) (Ag) capable of inducing an effective, broad, and long-lasting immune response against HIV-1 despite mutation events leading to differences in virus clades. The HIV-1 Tat protein is more conserved than envelope proteins, is essential in the virus life cycle and is expressed very early upon virus entry. In addition, both humoral and cellular responses to Tat have been reported to correlate with a delayed progression to disease in both humans and monkeys. This suggested that Tat is an optimal target for vaccine development aimed at controlling virus replication and blocking disease onset. Here are reviewed the results of our studies including the effects of the Tat protein on monocyte-derived dendritic cells (MDDCs) that are key antigen-presenting cells (APCs), and the results from vaccination trials with both the Tat protein or *tat* DNA in monkeys. We provide evidence that the HIV-1 Tat protein is very efficiently taken up by MDDCs and promotes T helper (Th)-1 type immune responses against itself as well as other Ag. In addition, a Tat-based vaccine elicits an immune response capable of controlling primary infection of monkeys with the pathogenic SHIV89.6P at its early stages allowing the containment of virus spread. Based on these results and on data of Tat conservation and immune cross-recognition in field isolates from different clades, phase I clinical trials are being initiated in Italy for both preventive and therapeutic vaccination.

INTRODUCTION

HIV INFECTION IS PROGRESSIVELY SPREADING, particularly in the developing countries where the increasing number of deaths due to AIDS strongly and urgently calls for an effective, safe, and inexpensive vaccine against AIDS. Since the characterization of HIV in the early 80s, many attempts have been made to find and develop a candidate vaccine of proven safety and efficacy. However, due to the high variability of the envelope (Env) proteins and to the limited accessibility of relevant cross-neutralizing epitopes, vaccine studies aimed at inducing adequate titers of long-lasting antibodies (Ab) neutralizing different virus strains have substantially failed (Parren *et al.*, 1999). Nevertheless, protection against heterologous pathogenic viruses was achieved in nonhuman primates vaccinated with live-attenuated simian immunodeficiency virus (SIV), pro-

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viding the first evidence that protective immunity can be induced and a valuable tool to better understand the mechanisms and correlates of protection from pathogenic viruses (for a review, see Ensoli and Cafaro, 2001). However, the appearance of revertant pathogenic viruses and the apparent pathogenicity of the attenuated viruses in newborn macaques (Baba *et al.*, 1995; Whatmore *et al.*, 1995; Ezzel, 2002) constitute at the present an insurmountable obstacle to their use for vaccination in humans.

Because Env-based vaccines have failed at inducing a sterilizing immunity against different virus strains, secondary end points are being considered in vaccine strategies, such as the control of viral infection and the block of disease onset. To this goal, different vaccine approaches, based on recombinant proteins, DNA plasmids or expression vectors (mostly in prime-boost regimens) are being tested in animal models or human volunteers. Interest has also recently focused towards those nonstructural viral proteins that have a key role in the virus life cycle and are adequately immunogenic and conserved among several virus subtypes. Among these, the HIV-1 Tat protein fulfills these requirements and, to date, is considered as a potential and promising vaccine candidate (for a review, see Ensoli and Cafaro, 2001). The more attractive features of Tat are: (1) its early expression and critical role in the virus life cycle; (2) the correlation of the anti-Tat immune response with nonprogression to AIDS in infected individuals; (3) its peculiar property to be efficiently taken up by professional antigen presenting cells (APCs) and to be presented in the context of the major histocompatibility complex (MHC) class I; (4) the conservation among geographically distinct isolates; (5) the safety, immunogenicity, and efficacy of Tat vaccination in macaques. These aspects are briefly described.

Role of Tat in the virus life cycle

The Tat protein of HIV is expressed very early after infection before the structural genes (env, gag, and pol), even before viral integration (Wu and Marsh, 2001). Tat is a potent transcriptional transactivator of HIV-1 gene expression (Arya *et al.*, 1985; Fisher *et al.*, 1986), and essential for viral replication, transmission, and disease progression (H.K. Chang *et al.*, 1994, 1995). In the absence of Tat, in fact, extremely poor amounts of structural proteins are expressed with consequent negligible production of infectious virus (Dayton *et al.*, 1986; Fisher *et al.*, 1986). Further, Tat is released by infected T lymphocytes in the extracellular milieu (Ensoli *et al.*, 1990, 1993; H.C. Chang *et al.*, 1997), and can enter both infected cells, in which promotes HIV replication, and uninfected cells in which induces the expression or repression of cellular genes controlling the cell cycle and cell activation (Frankel and Pabo, 1988; Ensoli *et al.*, 1993; H.K. Chang *et al.*, 1994, 1995; Li *et al.*, 1997). In addition, Tat has been recently shown to induce the expression of the chemokine receptors (and HIV-1 coreceptors) CCR5 and CXCR4 (Huang *et al.*, 1998; Secchiero *et al.*, 1999), essential for the infectivity of macrophage- and T-cell-tropic HIV-1 strains, respectively. Of note, because infected cells express Tat very early after infection, they may represent a relevant target for cytotoxic T lymphocytes (CTLs) that may block infection at its early stages. Therefore, an effective immune response to Tat may efficiently inhibit virus replication and dissemination, thus preventing the progression to AIDS.

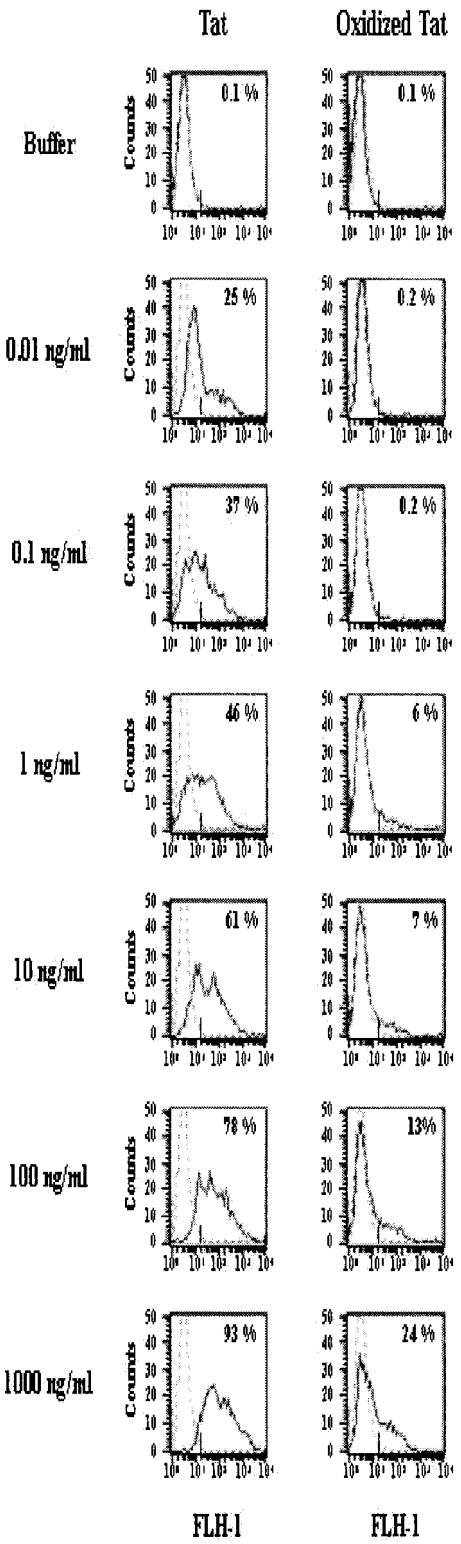
The protective role of the anti-Tat immune response

Several studies support the concept that an immune response to Tat has a protective role, and may control disease progression *in vivo*. First, the protein is immunogenic and well conserved (see the following section), eliciting Ab responses that have been associated with nonprogression to AIDS (Reiss *et al.*, 1990; Rodman *et al.*, 1993; Re *et al.*, 1995; Zagury *et al.*, 1998). Accordingly, by using an algorithm based on two optimized ELISA assays, we have observed a higher prevalence of both anti-Tat IgM and IgG Ab in asymptomatic Italian patients compared to patients in advanced stages of disease (S. Buttò, V. Fiorelli, A. Tripiciano, *et al.*, in preparation). Similarly, CTLs to Tat are frequently detected in asymptomatic HIV-1–infected individuals (Addo *et al.*, 2001), and have been shown to inversely correlate with progression to AIDS (van Baalen *et al.*, 1997). This correlation has been recently confirmed by us in vaccine trials with nonhuman primates (Cafaro *et al.*, 1999, 2000, 2001). Finally, a recent study (Allen *et al.*, 2000) has shown that anti-Tat CTLs are key to control early virus replication during primary SIV infection, exerting an immune pressure leading to selection of less pathogenic escape mutants.

Uptake, presentation, and induction of Th-1 responses by soluble native Tat protein

Extracellular Tat is taken up by cells (Frankel and Pabo, 1988; Ensoli *et al.*, 1993; H.C. Chang *et al.*, 1997; Tyagi *et al.*, 2001), and, unlike most soluble proteins, enters the MHC class I pathway of presentation and elicits CTL activity (Moy *et al.*, 1996; Kim *et al.*, 1997), a feature that Tat shares with very few other soluble proteins called "penetratins" (reviewed by Derossi *et al.*, 1998).

We investigated further these aspects and found that monocyte-derived dendritic cells (MDDCs), among the most potent APCs, take up Tat much more efficiently than other cell types such as T cell blasts and B-lymphoblastoid cell lines and in a time-, dose- and maturation-dependent fashion (Fanales-Belasio *et al.*, 2002). Specifically, Tat is taken up by MDDCs at doses as low as 0.01 ng/ml (Fig. 1) and the uptake peaks after 5–10 min. This process is markedly hampered by the oxidation/inactivation of the protein (Fig. 1), and by low temperature. Of note, the uptake is more efficient upon MDDCs maturation, suggesting the involvement of a receptor-mediated pathway upregulated upon cell maturation, and excluding an involvement of the pino-phagocytic pathways, both diminished in mature MDCCs (Bell *et al.*, 1999). Moreover, Tat also induces MDCCs maturation at relatively high doses (1.2 to 20 μg/ml), as indicated by a dose-dependent increase of both the surface expression of MHC and costimulatory (CD40, CD80, CD86) molecules (Fig. 2A) and of the production of interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) and of the β-chemokines MIP-1α, MIP-1β, and RANTES (Fig. 2B). In contrast, oxidized Tat had no effects. Consistent with these data, MDDCs treated with Tat have an increased capacity to present both allogeneic and recall Ag potentiating



T cell responses against heterologous Ags (Fig. 3) (Fanales-Belasio *et al.*, 2002).

Conservation of Tat among geographically distinct isolates

Studies conducted in Uganda and in South Africa indicate that the Tat sequence, from African individuals infected with A, C, and D subtypes, is conserved in its immunogenic epitopes (Buttò *et al.*, in preparation) and that sera from these individuals recognize and neutralize *in vitro* the activity of a Tat protein derived from a distantly related subtype B isolate (Buttò *et al.*, in preparation). Furthermore, a recent sequence data analysis confirmed the conservation of the immunodominant B cell epitopes of Tat among distantly related HIV-1 subtypes (Goldstein *et al.*, 2001; and our unpublished data). Thus, there is clear evidence that, due to cross-clade recognition, a Tat-based approach may work against infection with different viral clades.

Safety, immunogenicity, and efficacy of anti-Tat vaccine in macaques

Injection of biologically active Tat protein or wild-type *tat* DNA is safe, as indicated by our safety studies conducted in 276 mice, 48 guinea pigs, and 27 monkeys in which no local or systemic toxicity or adverse effects (at the biochemical, hematologic, or immunologic level) were ever observed (Caselli *et al.*, 1999; Cafaro *et al.*, 2001; our unpublished data). Data from others confirmed the absence of toxicity of biologically active Tat or *tat* DNA in mice or monkeys (Hinkula *et al.*, 1997; Osterhaus *et al.*, 1999; Pauza *et al.*, 2000; Nilsson *et al.*, 2001; Allen *et al.*, 2002; Silvera *et al.*, 2002; Caputo *et al.*, 2002, in press). In addition, no enhancement of virus replication nor CD4+ T cell decline was detected in SHIV89.6P-infected monkeys with AIDS that received Tat protein and *tat* DNA in a safety study of the therapeutical approach (our unpublished data). Moreover, vaccination of HIV-1–infected individuals with *tat* DNA confirmed that immunization with Tat is safe, because no induction of virus transcription was observed, despite the evidence of boosting of specific immune responses (Calarota *et al.*, 1998, 1999).

The immunogenicity of the HIV-1 Tat protein or *tat* DNA has also been demonstrated in mice and in monkeys in which vaccination with the Tat protein or *tat* DNA induced both humoral and cellular (including CTL activity) Tat-specific immune responses (Hinkula *et al.*, 1997; Caselli *et al.*, 1999; Ca-

FIG. 1. Native, but not oxidized, Tat is efficiently taken up by MDDCs. Human MDDCs were incubated with serial concentrations (0.01 to 1,000 ng/ml) of the HIV-1 Tat protein, native or oxidized by exposure to light and air for 16 h, or Tat reconstitution buffer. After 10 min cells were washed, fixed, permeabilized, stained with a specific affinity-purified rabbit anti-Tat polyclonal Ab (or isotype control) followed by secondary FITC-conjugated antirabbit Ab, and analyzed by flow cytometry as reported (Fanales-Belasio *et al.*, 2002). The percentage of positive cells (compared to isotype stained samples) is reported in each panel. Data are from one donor representative of the 14 analyzed.

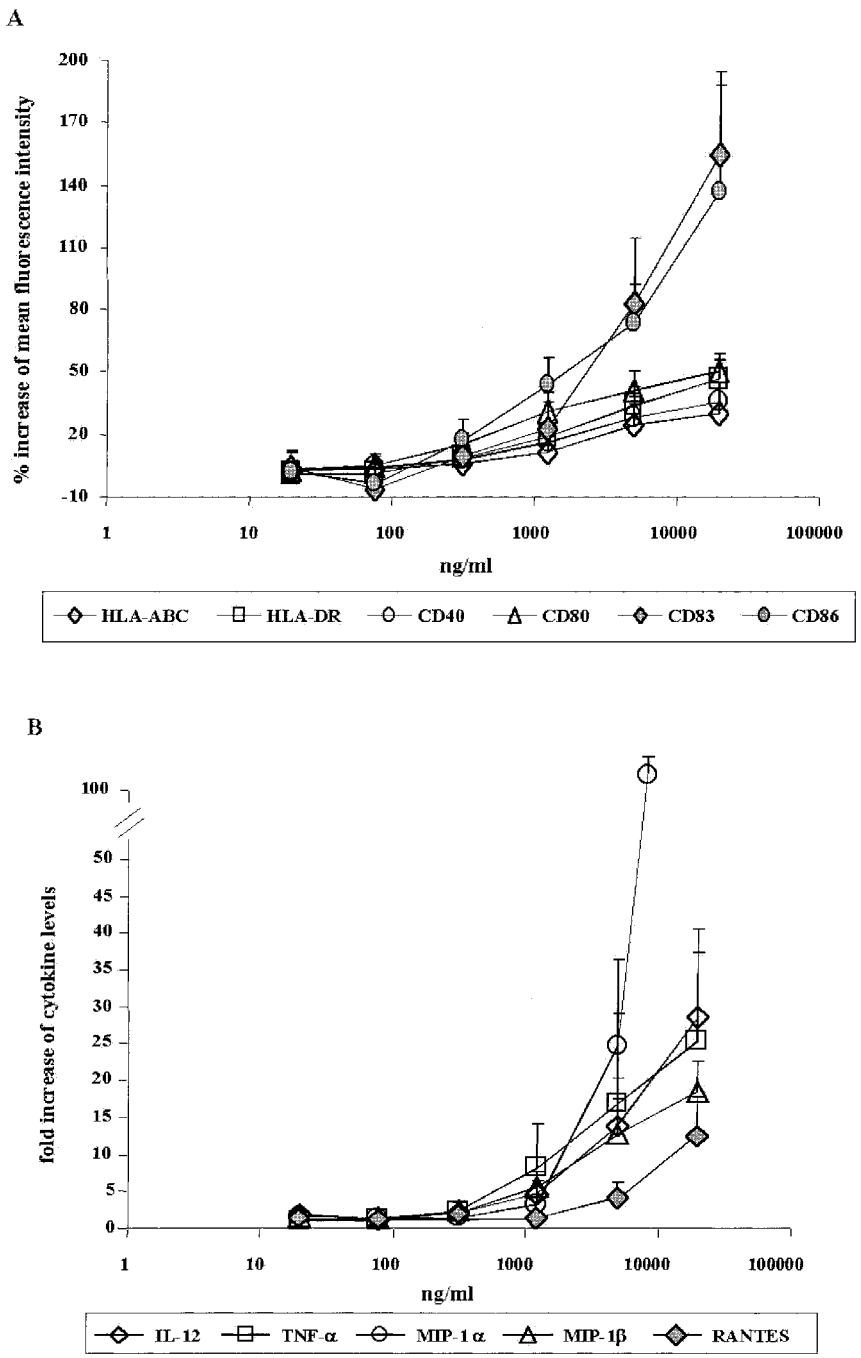


FIG. 2. Tat enhances both the expression of surface HLA and costimulatory molecules and the production of the cytokines IL-12 and TNF-α and of the β-chemokines MIP-1α, MIP-1β, and RANTES in human MDDCs. (**A**) Cells from 10 different donors were exposed for 18 h to native Tat (20 to 20,000 ng/ml) or reconstitution buffer, stained with fluorochrome-conjugated monoclonal Ab, and then analyzed by flow cytometry. The mean (and SEM) percentage increases of the expression (as mean fluorescence intensity) of HLA-ABC (diamonds), HLA-DR (squares), CD40 (circles), CD80 (triangles), CD83 (gray diamonds), and CD86 (gray circles) are reported. (**B**) Supernatants of MDDCs exposed for 18 h to Tat (20 to 20,000 ng/ml) or reconstitution buffer were assayed by ELISA to measure the levels of IL-12 (diamonds), TNF-α (squares), MIP-1α (circles), MIP-1β (triangles), and RANTES (gray diamonds). The mean (and SEM) fold increases of the cytokines levels (pg/ml) induced by Tat compared to buffer are reported. A very poor cytokine or β-chemokine production was induced by oxidized-inactivated Tat protein (E. Fanales-Belasio, S. Moretti, G. Barillari, *et al.*, unpublished data).

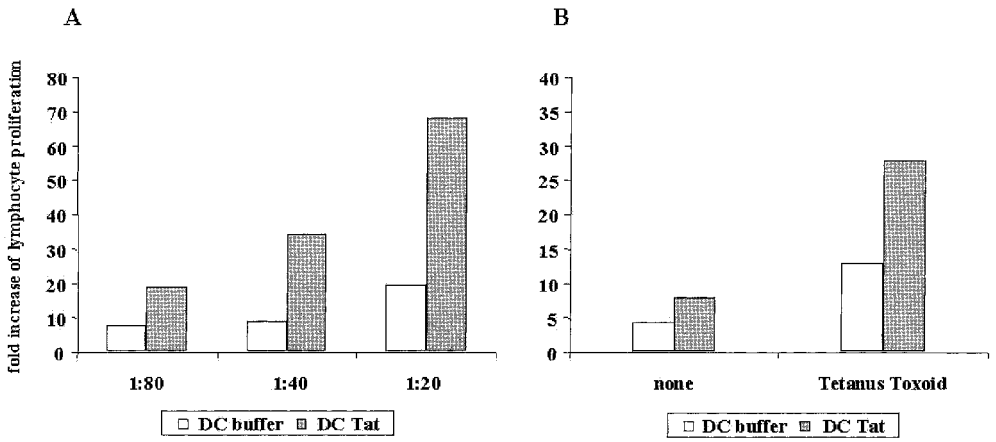


FIG. 3. Tat enhances both allogeneic and recall antigen presentation by MDDCs. (**A**) Cells were exposed for 18 h to native Tat (10,000 ng/ml) (gray columns) or reconstitution buffer (white columns) and cultured together with allogeneic peripheral blood lymphocytes (PBLs) at different cell to cell ratios. ^{3}[H]-thymidine uptake was measured after 6 days of culture to evaluate lymphocyte proliferation. Data from a representative experiment out of four performed with different donors are expressed as the fold increase of lymphocyte proliferation at the MDDCs/PBLs ratios of 1:20, 1:40, and 1:80. (**B**) MDDCs exposed to Tat, as above reported, were cultured in the presence or absence of tetanus toxoid (TT 5 μg/ml) together with syngeneic PBLs (ratio 1:20) from a patient responsive to this recall Ag. Data are expressed as the fold increase of lymphocyte proliferation.

faro *et al.*, 1999, 2000, 2001; and unpublished data; Allen *et al.*, 2002; Silvera *et al.*, 2002; Caputo *et al.*, 2002). Similar results were reported in monkeys immunized with viral vectors expressing SIV Tat and Rev (Osterhaus *et al.*, 1999) or Env, Gag-Pol, Nef, Rev, and Tat (Nilsson *et al.*, 2001). In addition, an increase in humoral and cellular anti-Tat immune responses was detected in HIV-1 infected individuals immunized with HIV-1 *tat* DNA (Calarota *et al.*, 1998, 1999). Finally, our recent data indicate that in mice the mucosal or systemic administration of a biologically active Tat protein alone or combined with an unrelated Ag (Candida, ovalbumin, streptavidin) is safe, and elicits immunity to both (M. Marinaro, A. Riccomi, R. Rappuoli, *et al.*, submitted; S. Bortsutzky, V. Fiorelli, F. Rharbaoui, *et al.*, in preparation; S. Dominici, M.E. Laguardia, G. Serafini, *et al.*, in preparation).

To evaluate the efficacy of a preventive *tat* vaccine in controlling virus replication, preclinical trials with the Tat protein or *tat* DNA were carried out in monkeys as two arms of the same protocol.

In the Tat protein protocol (Cafaro *et al.*, 1999, 2000), 7 *Macaca fascicularis* (cynomolgus monkeys) were immunized with a biologically active Tat protein, as reported in Table 1. Six monkeys were immunized subcutaneously (s.c.) with 10 μg of Tat with RIBI (three monkeys) or Alum (three monkeys) as adjuvant, while one received Tat alone (6 μg) intradermally (i.d.). Two control monkeys were injected s.c. with either RIBI or Alum alone, and two naive monkeys were included at the challenge as additional controls. The six monkeys vaccinated s.c. received eight boosts in 36 weeks, while a ninth final boost was performed at week 42 intramuscularly (i.m.) with Tat associated to immune stimulating complexes (ISCOMs) (Davis *et al.*, 1997). The monkey immunized i.d. was boosted nine times, and did not receive the ISCOMs boost.

TABLE 1. TAT PROTEIN VACCINE PROTOCOL

Monkey	*Immunogen*	*Adjuvant*	*Administration*
54844 54879 54963	Tat protein (10 μg/250 ml)	RIBI (250 μl)	Subcute, 500 μl in one site (dorsal area, close to the neck)
54899 55396 54240	Tat protein (10 μg/250 ml)	Alum (250 μl)	Subcute, 500 μl in one site (dorsal area, close to the neck)
54222	Tat protein (6 μg/ml)	Nil	Intradermic, 150 μl in two sites (upper dorsal area)
55123	Saline buffer (250 μl)	RIBI (250 μl)	Subcute, 500 μl in one site (dorsal area, close to the neck)
55129	Saline buffer (250 μl)	Alum (250 μl)	Subcute, 500 μl in one site (dorsal area, close to the neck)

For further details refer to Cafaro *et al.,* 1999.

No signs or symptoms related to toxicity (acute or chronic, local or systemic) were ever detected in both vaccinated and control animals throughout the immunization period.

The six monkeys inoculated with Tat and RIBI or Alum developed high titers of anti-Tat Ab (up to 1:25,600), capable of neutralizing the activity of Tat to rescue Tat-defective proviruses (Barillari *et al.*, 1992; Ensoli *et al.*, 1993; H.C. Chang *et al.*, 1997). In contrast, the animal given Tat i.d. developed low and transient titers of anti-Tat Ab (1:100) (Table 2).

The monkeys immunized s.c. but not the one vaccinated i.d. developed anti-Tat T cell responses, as indicated by the presence of delayed-type hypersensitivity (DTH) and proliferative responses to Tat (Table 2). Further, a specific anti-Tat CTL activity, barely detectable in the vaccinated animals since week 28 after immunization, at week 36 reached levels above the cut-off in one out of two macaques vaccinated with Tat and RIBI, in two out of three monkeys vaccinated with Tat and Alum, and in the monkey vaccinated i.d. with Tat alone (Table 2). In addition, *in vitro* TNF-α production in response to Tat, a marker for CTLs, was found at week 44 after immunization in vaccinated and protected animals, but not in the infected ones.

At week 50 after immunization (14–18 weeks after the last boost), all the animals were challenged intravenously (i.v.) with the SHIV89.6P, a chimeric virus containing the *env* and *tat* genes of HIV-1 in the backbone of SIVmac239, and highly pathogenic in macaques (Reimann *et al.*, 1996; Karlsson *et al.*, 1997; Cafaro *et al.*, 2000). The virus stock used for the challenge derived from a cynomolgus macaque inoculated with the SHIV89.6P originally derived from rhesus monkeys. Also, this virus stock was highly pathogenic, inducing the death of four out of seven infected monkeys (Cafaro *et al.*, 2000; M.T. Maggiorella, S. Baroncelli, Z. Michelini, *et al.*, submitted). All animals were inoculated with 10 MID_{50} of the virus, except the two naive control monkeys that were challenged with either a threefold lower (2.8 MID_{50}) or higher (28 MID_{50}) doses, respectively (Table 3). Of note, the macaque infected with 28 MID_{50} was euthanized at week 35 after the viral challenge, due to severe worsening of the clinical conditions associated to extremely low CD4+ T cell counts.

After challenge, all the controls but only two out of the seven Tat protein-vaccinated monkeys (one given Tat and RIBI and one Tat and alum) were infected, as indicated by the presence of high plasma levels of p27 Ag (detected by ELISA) and viral RNA [detected by branched DNA (bDNA) and quantitative-competitive RNA-PCR (QT-RNA-PCR) assays], proviral DNA copies, cytoviremia, or positive virus isolation from PBMCs (Table 3). In contrast, all these parameters were always negative for all the other five vaccines up to 2 years postchallenge, although few copies of SIV proviral DNA (<10 copies/μg of DNA) were sporadically detected (Table 3). This, together with the finding of low and transient anti-SIV (or anti-HIV Env) Ab titers in the protected animals, indicated the occurrence of a very limited viral replication.

TABLE 2. TAT PROTEIN VACCINE: IMMUNOLOGICAL RESPONSES AT PRE-CHALLENGE TIME

Monkey	*Vaccination*	*Ab titers*[a]	*Tat neutralization*[b]	*Proliferative response*[c]	*DTH*[d]	*CTL response*[e]	*Tat-induced TNF-α*[f]
54844		++	+	+	−	+	+
54879	RIBI + Tat (10 μg, SC)	++	++	+	+	ND	+
54963		++	++	+++	±	−	ND
54899		+++	+++	+++	++	+	+
55396	Alum + Tat (10 μg, SC)	+++	+++	+	++	−	−
54240		+++	+++	+	±	+	+
54222	Tat protein (6 μg, ID)	±	ND	−	−	+	+
55123	Control RIBI	−	ND	−	ND	−	−
55129	Control Alum	−	ND	−	ND	−	−

[a]Anti-Tat Ab in plasma from vaccinated or control monkeys as determined by ELISA. On the basis of reciprocal of the last positive dilution (cut-off: mean of preimmune sera + 3 SD) the following ranges of positivity are defined: ±, <100, +, 100–1,000; ++, 1,000–20,000; +++, >20,000.

[b]Neutralization of the Tat-mediated (60–500 ng/ml) rescue of *tat*-defective HIV-1 provirus replication in HLM-1 cells by sera from vaccinated or control monkeys. Neutralization was defined as a >50% reduction of p24 values (cut-off = 20 pg/ml) the addition of increasing amounts of Tat compared to controls. The following scoring was defined on the basis of the highest dose of Tat (ng/ml) neutralized; +, 60–120; ++, 240; +++, 500.

[c]Specific immune response to Tat as determined by proliferation assay with monkeys PBMCs. Positivity was defined on the basis of the ratio between Tat-specific and control counts: − <3; +, 3–10; ++, 11–30; +++, >30.

[d]DTH response to the Tat protein in immunized monkeys. Positivity was defined on the basis of evident signs of inflammation (erythema, induration) at the site of inoculation after 48 h: −, erythema <1 mm; ±, erythema ≥1 mm without induration; +, induration with erythema 1–4 mm; ++, induration with erythema ≥5 mm.

[e]Specific anti-Tat CTL response detected with PBMCs after 2 weeks of *in vitro* expansion with Tat. Values above or below the cut-off (5% Tat-specific lysis) were indicated as positive (+) or negative (−), respectively.

[f]Tat-induced TNF-α production by PBMCs, was detected by ELISA: values above or below the cut-off (15.6 pg/ml) were indicated as positive (+) or negative (−), respectively.

For further technical details refer to Cafaro *et al.*, 1999.

TABLE 3. TAT PROTEIN VACCINE: RESULTS AFTER CHALLENGE WITH THE SHIV89.6P, IV

			Postchallenge (up to 2 years)					
Monkey	*Vaccination*	*Challenge dose*	*p27*[a]	*Plasma viremia*[b]	*DNA PCR*[c]	*Cytoviremia or virus isolation*[d]	*Anti-SIV Ab*[e]	*CD4+ T cells*[f]
54844		10 MID_{50}	−	−	− (+)*	−	±	=
54879	RIBI + Tat (10 μg, SC)	10 MID_{50}	−	−	− (+)*	−	±	=
54963		10 MID_{50}	+	+	+	+	+	↓
54899		10 MID_{50}	−	−	− (+)*	−	±	=
55396	Alum + Tat (10 μg, SC)	10 MID_{50}	+	+	+	+	±	↓
54240		10 MID_{50}	−	−	− (+)*	−	±	=
54222	Tat (6 μg, ID)	10 MID_{50}	−	−	− (+)*	−	±	=
55123	Control RIBI	10 MID_{50}	+	+	+	+	+	↓
55129	Control Alum	10 MID_{50}	+	+	+	+	+	↓
12	Nil	2.8 MID_{50}	+	+	+	+	+	↓
2	Nil	2.8 MID_{50}	+	+	+	+	+	↓

[a]p27 in plasma from virus-challenged monkeys as determined by ELISA. Values above or below the cut-off value (20 pg/ml) were expressed as positive (+) or negative (−), respectively.

[b]Plasma-associated virus load (RNA equivalent/ml) as determined by bDNA (cut-off: 1,500 RNA copies/ml) until week 14 post-challenge, by bDNA and QT-RNA-PCR (cutoff: 50 RNA copies/ml) between week 18 and 28 post-challenge, and only by QT-RNA-PCR since week 35. Results above or below the cut-off values were expressed as positive (+) or negative (−), respectively.

[c]Proviral DNA copies/μg of DNA as determined by semiquantitative DNA PCR analysis. +, proviral copy number >1;

[d]SHIV89.6P virus isolation from monkeys PBMCs depleted of CD8+ cells and cocultured with CEMx174. Positively was determined by p27 ELISA (cut-off = 20 pg/ml).

[e]Plasma anti-SIV Ab was determined by ELISA; levels above, equal, or below the cut-off value (mean of negative control plus 3 SD) were expressed as positive (+), border line (±), or negative (−), respectively.

[f]Peripheral blood counts of CD4+ cells as evaluated by flow cytometry. The symbols ↓ and =, respectively, indicate decrease (>50% decline as compared to pre-challenge values) or normality of CD4 T cell numbers.

*(+) positive DNA PCR was detected only sporadically and at low copy number (<10/μg of DNA).

For further technical details refer to Cafaro *et al.*, 1999, 2000.

Plasma levels of anti-SIV or anti-HIV Env Ab in all infected animals were high, correlating with the parameters of viremia (Table 3, and data not shown). Of note, titers in the two infected and vaccinated animals were at least 1 log lower than in the control macaques and delayed, suggesting a possible effect of vaccination also in the infected animals.

Blood CD4+ T cells counts reflected the outcome of the challenge. In the five protected monkeys they remained in the normal range during the 2 years of follow-up, whereas markedly decreased in all the controls and in the two vaccinated and infected animals (Table 3).

Notably, protection from challenge correlated (100%) with the presence, before the challenge, of anti-Tat specific CTL activity and with Tat-induced TNF-α production, but not with the presence of anti-Tat Ab.

In the *tat* DNA vaccination study (Table 4 and Cafaro *et al.*, 2001), five monkeys were immunized with a vector expressing *tat* DNA under the control of the major adenoviral late protein promoter (pCV-*tat*) (Arya *et al.*, 1985; Ensoli *et al.*, 1993), shown to induce a high expression and release of the Tat protein in the absence of cell death (Ensoli *et al.*, 1990, 1993; H.C. Chang *et al.*, 1997). Further, the pCV vector is rich in unmethylated CpG sequences (Cafaro *et al.*, 2001), known to enhance the innate and adaptive immune responses by inducing the maturation of dendritic cells and their production of cytokines driving Th-1 responses (reviewed in Klinman *et al.*, 1999).

As shown in Table 4, four monkeys were immunized i.m. with 1 mg (three animals) or 0.5 mg (one animal) of pCV-*tat*. The fifth monkey was immunized i.d. with 0.2 mg of pCV-*tat*. One monkey was injected i.m. with the DNA vector alone (pCV-0), to evaluate the impact of its immunostimulating effects on nonspecific protection. Vaccines and the control were boosted seven times over 36–42 weeks, and the last boost was performed, except for the one immunized with pCV-0, with the Tat protein in ISCOMs. Neither local nor systemic side effects were observed in all inoculated animals, and all the hematologic parameters (blood cell counts, blood chemistry, and FACS analysis) were always in the normal range throughout the vaccination schedule and up to the time of challenge.

Vaccination with *tat* DNA was immunogenic. Anti-Tat Ab were transient and at low titers in the four animals vaccinated i.m. with pCV-*tat*, while in the monkey inoculated i.d. with 0.2 mg of pCV-*tat* titers could be detected (up to 1:1,600) through-

TABLE 4. *TAT* DNA VACCINE PROTOCOL

Monkey	*Immunogen*	*Adjuvant*	*Administration*
54920 55122 55361	*tat* DNA (1 mg)	Pretreatment (24 h, 1 ml) 0.5% Bupivacaine +0.1% methyl paraben	Intramuscular, 500 μl in 2 sites (femoral quadriceps)
PR2	*tat* DNA (0.5 mg)	Pretreatment (24 h, 1 ml) 0.5% Bupivacaine +0.1% methyl paraben	Intramuscular, 400 μl in 2 sites (femoral quadriceps)
37	*tat* DNA (1 mg)	Nil	Intramuscular, 150 μl in 2 sites (dorsal area)
54219 55361	DNA vector (1 mg)	Pretreatment (24 h, 1 ml) 0.5% Bupivacaine +0.1% methyl paraben	Intramuscular, 500 μl in 2 sites (femoral quadriceps)
55123	Saline buffer (250 μl)	RIBI (250 μl)	Subcute, 500 μl in one site (dorsal area)
55129	Saline buffer (250 μl)	Alum (250 μl)	Subcute, 500 μl in one site (dorsal area)

For further technical details refer to Cafaro *et al*., 2001.

out the follow-up period, with a weak Tat neutralizing activity (Table 5). Anti-Tat Ab were never found in the animal inoculated with pCV-0, or in the control animals.

A proliferative response to Tat was detected in three out of four monkeys inoculated i.m. with pCV-*tat*, and in the one inoculated i.d., which also showed a cutaneous DTH response to the protein (Table 5), but not in the control animals.

Anti-Tat CTL activity was detected in the four monkeys vaccinated i.m. with pCV-*tat* but not in the one vaccinated i.d. or in the control animals (Table 5).

TABLE 5. *TAT* DNA VACCINE: IMMUNOLOGIC RESPONSES AT PRE-CHALLENGE

Monkey	*Vaccination*	*Ab titers*[a]	*Tat neutralization*[b]	*Proliferative response*[c]	*DTH*[d]	*CTL response*[e]
54920		±	−	+	−	+
55122	*tat* DNA (1 mg, IM)	±	ND	+	−	+
55361		−	−	+	−	+
PR2	*tat* DNA (0.5 mg, IM)	±	−	−	−	+
37	*tat* DNA (0.2 mg, ID)	++	+	+	+	−
54219	DNA vector (1 mg, IM)	−	−	−	ND	−
55123	RIBI alone	−	−	−	ND	−
55129	Alum alone	−	−	−	ND	−

[a]Anti-Tat Ab in plasma from vaccinated or control monkeys as determined by ELISA. On the basis of reciprocal of the last positive dilution (cut-off: mean of preimmune sera + 3 SD) the following ranges of positivity are defined: ±, <100, +, 100–1,000; ++, 1,000-20,000; +++, >20,000.

[b]Neutralization of the Tat-mediated (30–500 ng/ml) rescue of *tat*-defective HIV-1 provirus replication in HLM-1 cells by sera from vaccinated or control monkeys. Neutralization was defined as a >50% reduction of p24 values (cut-off = 20 pg/ml) upon the addition of increasing amounts of Tat compared to controls. The following scoring was defined on the basis of the highest dose of Tat (ng/ml) neutralized; +, 30–120; ++, 240; +++, 500.

[c]Specific immune response to Tat as determined by proliferation assay with PBMCs from monkeys. Positivity was defined on the basis of the ratio between Tat-specific and control counts: − <3; +, 3–10; ++, 11–30; +++, >30.

[d]DTH response to the Tat protein in immunized monkeys. Positivity was defined on the basis of evident signs of inflammation (erythema, induration) at the site of inoculation after 48 h: −, erythema <1 mm; ±, erythema ≥1 mm without induration; +, induration with erythema 1–4 mm; ++, induration with erythema ≥5 mm.

[e]Specific anti-Tat CTL response detected with PBMCs after 2 weeks of *in vitro* expansion with the Tat Ag. Values above or below the cut-off (10% Tat-specific lysis) were indicated as positive (+) or negative (−), respectively.

For further technical details refer to Cafaro *et al*., 2001.

All animals, together with those from the Tat protein vaccination protocol, were challenged 14–18 weeks after the last boost with 10 MID_{50} of SHIV89.6P i.v. In the weeks following the challenge and during the entire follow-up, all the macaques vaccinated IM with pCV-*tat* were negative for p27 antigenemia, plasma viremia, and virus isolation (Table 6). SIV proviral DNA was only sporadically detected at very low copy number (range 1–8 copies/μg DNA). In contrast, all the controls and the monkey vaccinated i.d. with 0.2 mg of pCV-*tat* had detectable plasma levels of both p27 and viral RNA, with repeated isolation of infectious virus from the peripheral blood, and the proviral DNA, high early after the challenge, remained detectable for the entire follow-up period (Table 6). Of note, the monkey inoculated with pCV-0 was negative for both antigenemia and plasma viremia. However, virus was isolated 18 weeks after challenge and proviral DNA (55 copies) was detected at week 14 after challenge indicating a partial protection, conceivably conferred by natural immunity.

In the three macaques vaccinated i.m. with 1 mg of pCV-*tat* and in the one injected with pCV-0 the titers of anti-SIV Ab were below the limit of detection, while in the one vaccinated i.m. with pCV-*tat* (0.5 mg) were low (range 1:2–1:100) and transient. Conversely, they were high (over 1:1000) and stable, since week 10 after challenge, in the monkey vaccinated i.d. with pCV-*tat*, and in the controls (Table 6). Anti-SIV Ab in the naive control monkey infected with 28.5 MID_{50} were detected at very low titers (range 1:20–1:50) only at week 29 and 35, a relatively uncommon feature that correlates with fast progression (Dykhuizen *et al.*, 1998), although anti-HIV Ab could be detected since week 10 postchallenge (Cafaro *et al.*, 2001).

In the monkeys injected i.m. with pCV-*tat* or pCV-0, in which viral parameters were mostly negative (Table 6), the number of CD4+ T cells remained in the normal range after the viral challenge and during all the follow-up period. In contrast, in the infected animals, CD4+ T cell number sharply decreased after the challenge, remaining persistently below the baseline values during the follow up. The naive control monkey infected with 28.5 MID_{50} showed a progressive and severe CD4+ T cells decline and had to be euthanized.

Thus, like in the vaccination protocol with the Tat protein, immunization with *tat* DNA was safe, and induced an immune response to the protein, mainly cell mediated, capable of blocking virus replication to undetectable levels, thus preventing the CD4+ T cell decline and disease onset. Protection from virus

TABLE 6. *TAT* DNA VACCINE: RESULTS AFTER CHALLENGE WITH THE SHIV 89.6P, IV

			Postchallenge (up to 2 years)					
Monkey	*Vaccination*	*Challenge (dose)*	*p27*[a]	*Plasma viremia*[b]	*DNA PCR*[c]	*Cytoviremia or virus isolation*[d]	*Anti-SIV Ab*[e]	*CD4+ T cells*[f]
54920		10 MID_{50}	−	−	− (+)*	−	−	=
55122	*tat* DNA (1 mg, IM)	10 MID_{50}	−	−	− (+)*	−	−	=
55361		10 MID_{50}	−	−	− (+)*	−	±	=
PR2	*tat* DNA (0.5 mg, IM)	10 MID_{50}	−	−	− (+)*	−	±	=
37	*tat* DNA (0.2 mg, ID)	10 MID_{50}	+	+	+	+	+	↓
54219	DNA vector (1 mg, IM)	10 MID_{50}	−	−	+	+	−	=
55123	RIBI alone	10 MID_{50}	+	+	+	+	+	↓
55129	Alum alone	10 MID_{50}	+	+	+	+	+	↓
12	Nil	2.8 MID_{50}	+	+	+	+	+	↓
2	Nil	2.8 MID_{50}	+	+	+	+	±	↓

[a]p27 in plasma as determined by ELISA. Values above or below the cut-off value (20 pg/ml) were expressed as positive (+) or negative (−), respectively.

[b]Plasma-associated virus load (RNA equivalent/ml) as determined by bDNA (cut-off: 1,500 RNA copies/ml) until week 14 post-challenge, by bDNA and QT RNA-PCR (cut-off: 50 RNA copies/ml) between week 18 and 28 post-challenge, and only by QT-RNA-PCR since week 35. Results above or below the cutoff values were expressed as positive (+) or negative (−), respectively.

[c]Proviral DNA copies/μg of DNA as determined by semiquantitative DNA PCR analysis. +, proviral copy number >1.

[d]SHIV89.6P virus isolation from monkeys CD8-depleted PBMCs as determined by coculture with CEMx174 and measure of p27 production (cut-off = 20 pg/ml).

[e]Plasma anti-SIV Ab was determined by ELISA; levels over, equal, or below the cut-off value mean of negative controls (plus 3 SD) were expressed as positive (+), border line (±), or negative (−), respectively.

[f]Peripheral blood counts of CD4+ cells as evaluated by flow cytometry. The symbols ↓ and =, respectively, indicate decrease (>50% decline as compared to pre-challenge values) or normality of CD4 T-cell numbers.

*(+) positive DNA PCR was detected only sporadically and at low copy number (<10/μg of DNA).

For further technical details refer to Cafaro *et al.*, 2001.

challenge correlated in all the animals with Tat-specific CTL responses.

Thus, in both arms Tat vaccination was safe and immunogenic, and 9 out of 12 vaccinated monkeys were protected after challenge with the highly pathogenic SHIV89.6P virus (Cafaro *et al.*, 1999, 2000, 2001).

Although not inducing a sterilizing protection, Tat-based vaccines appear to contain virus replication to undetectable levels. Notably, containment almost entirely correlated with the presence of cellular immunity, in particular with anti-Tat CTL activity and Tat-induced TNF-α production also in absence of a relevant humoral response, particularly for DNA immunized animals (Cafaro *et al.*, 1999, 2001). Finally, protection was maintained for the entire follow-up (2 years), and persisted even upon boost of the animals with the recall Ag tetanus toxoid (TT), which is known to rescue virus replication (Maggiorella *et al.*, submitted).

Similar results were reported with a vaccination study in which cynomolgous monkeys immunized with viral vectors (Semliki Forest virus and modified vaccinia Ankara virus) expressing the SIV-Tat and -Rev, were protected upon challenge with a pathogenic SIV (Osterhaus *et al.*, 1999, and personal communication).

In contrast with these results, two recent studies in rhesus macaques indicate that vaccination with Tat protein (Silvera *et al.*, 2002) or *tat* DNA (Allen *et al.*, 2002) was safe and immunogenic, but failed to induce a significant control of viral replication following challenge with SIV or SHIV89.6P pathogenic viruses. However, several differences in the study design including dose and source of Tat (SIV and HIV), monkey species, adjuvant, dose, and schedule of immunization, dose and route of challenge viruses inoculation may account for the different results.

CONCLUDING REMARKS

Taken together, these data indicate that the immunization with Tat may control HIV replication in both vaccinated individuals exposed to the virus and seropositive patients, thus favoring the control of infection by specific immune response. Soon after entry, and prior to initiation of virus integration, the regulatory proteins Tat and Nef are expressed (Wu and Marsh, 2001), and may be recognized and killed by effector lymphocytes, induced by specific vaccination. Suppression of viral replication by CD8-mediated noncytolytic activity may also participate to an effective immune response. Thus, a limited and almost undetectable (as indicated by our monkey studies) viral replication may occur, which, in the absence of pathogenicity, may provide antigenic exposure sufficient to drive an antiviral immune response. Consequently, the anti-HIV immune response would be boosted (against Tat), broadened, and strengthened (against the other viral proteins), inducing protection to further challenges with the same or other virus strains. This hypothesis has recently been confirmed in experiments in which monkeys have been boosted with TT or rechallenged with a high virus dose (Maggiorella *et al.*, submitted, and S. Baroncelli, M.T. Maggiorella, S. Moretti, *et al.*, in preparation). In addition, two new ongoing vaccination protocols in monkeys have confirmed in a larger number of animals the data of safety and immunogenicity already obtained with the Tat protein or DNA (our unpublished data).

All these results indicate that a Tat-based vaccine is a promising candidate for vaccination in humans. Therefore, both preventive and therapeutic phase I clinical trials are currently being organized and will start in Italy within the year 2002. In addition, background (immunologic and virologic) and feasibility studies are ongoing in African countries where subsequent clinical studies (phase II and III) are being planned. Results from these studies indicate that sera from Ugandan and South African patients infected by different HIV strains recognize Tat from clade B at the same extent as individuals infected with A, B, and C clades (Buttò *et al.*, in preparation). Finally, novel approaches with Tat combined with other viral Ags (Gag, Env), delivered by the mucosal or parenteral route in prime-boost regimens, are under evaluation in preclinical studies to develop new and combined vaccine strategies in which also the Tat-adjuvant capabilities are exploited.

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