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Mini Review

HIV-1 Tat vaccines

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The inexorable spreading of the HIV pandemic and the increasing deaths for AIDS in the developing countries underscore the urgency for an effective, safe and inexpensive vaccine against AIDS. Although many attempts have been made, a candidate vaccine of proven efficacy and safety in non-human primate models is not yet available. This is mostly due to HIV envelope (Env) variability and to the difficulty of eliciting high titres of long-lasting neutralising antibodies capable of blocking the entry of different virus strains. Nevertheless, studies in non-human primates vaccinated with live attenuated SIV viruses and protected from heterologous challenges with highly pathogenic strains provide the most compelling evidence that it is possible to generate a protective immune response against different HIV strains (for a review, Ensoli and Cafaro, 2000). However, ethical concerns and the appearance of revertant pathogenic viruses in vaccinees (Whatmore et al., 1995; Baba et al., 1995; Ezzell, 1997) presently hamper their use in humans. Nevertheless, this approach provides a model to study the still elusive correlates and mechanisms of protection.

Sterilising immunity against different virus strains has not yet been achieved with Env-based vaccines and secondary end-points in HIV vaccine development are being considered, leading to the concept that control of viral infection and block of disease onset may be at present a more achievable goal of AIDS vaccine strategies.

For this type of approach is required a viral product or products which have particular features: they have to play a key role in the virus life cycle; must be immunogenic; must be conserved among several virus subtypes.

The Tat protein of HIV-1 fulfils these requirements making Tat an attractive vaccine candidate (for a review, Ensoli and Cafaro, 2000). The rationale is based on: (i) the early expression of Tat and the critical role in the virus life cycle; (ii) the correlation of the *anti*-Tat immune response with non-progression in infected individuals; (iii) the unique property of Tat to be efficiently taken up by antigen presenting cells (APCs) and be presented in the context of the MHC class-I; (iv) the conservation among geographically distinct isolates; (v) the safety, immunogenicity, and efficacy in macaques. These aspects are briefly described.

1. Role of Tat in the virus life cycle

The Tat protein of HIV is a potent transcriptional transactivator of *HIV*-1 gene expression that is produced very early after infection and prior to the expression of the structural genes *env*,

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gag, and *pol* (Arya et al., 1985; Fisher et al., 1986) Tat is essential for viral replication, transmission and disease progression (Chang et al., 1994). In fact, in the absence of Tat no or negligible amounts of structural proteins are expressed and therefore no infectious virus is made (Fisher et al., 1986; Dayton et al., 1986). Further, during acute infection and prior to cell death, Tat is released by the infected T lymphocytes in the extracellular milieu (Ensoli et al., 1990, 1993 Chang et al., 1997) and enters both infected cells, in which promotes HIV replication, and uninfected cells in which it causes activation or repression of cytokines and cellular genes controlling the cell cycle (Frankel and Pabo, 1988; Ensoli et al., 1993; Chang et al., 1994). More recently it has been shown that Tat also induces the expression of the chemokine receptors (and HIV-1 co-receptors) CCR5 and CXCR4 (Huang et al., 1998; Secchiero et al., 1999), responsible for the transmission of macrophage- and T cell-tropic HIV-1 strains, respectively. Thus, extracellular Tat plays also a role in the spreading of infection by recruiting new cell targets. Of note, since infected cells express Tat very early after infection, they may represent key cytotoxic T lymphocyte (CTL) target to block infection at its early stages. Therefore, an effective immune response to Tat may inhibit its function or abolish its production. As a result, virus replication and dissemination may be substantially contained and progression to disease prevented.

2. The protective role of the *anti*-Tat immune response

Several studies suggest that an immune response to Tat has a protective role and may control disease progression in vivo. The Tat protein is both well-conserved and immunogenic (see Section 4) eliciting antibody responses which have been associated with non-progression to AIDS (Reiss et al., 1990; Rodman et al., 1993; Re et al., 1995; Zagury et al., 1998). Accordingly, using two highly reliable and optimised Elisa assays, we have observed a higher prevalence of both *anti*-Tat IgM and IgG antibodies in asymptomatic Italian patients as compared to patients with more advanced stages of disease (Buttò et al., in preparation).

CTLs have also been shown to inversely correlate with progression to AIDS (van Baalen et al., 1997). Our results with the Tat vaccine has confirmed such a correlation in the non-human primate model vaccine (Cafaro et al., 1999, 2001, Section 5). Finally, the protective role of *anti*-Tat CTLs is also supported by a more recent study (Allen et al., 2000), indicating that *anti*-Tat CTLs are key to control early virus replication after primary infection in the SIV model and exert a selective immune pressure on the virus leading to the appearance of slowly replicating and apparently less pathogenic escape mutants. Very recently a high frequency of CTLs against Tat and Rev in HIV-1 infected individuals has been reported (Addo et al., 2001). Notably, *anti*-Tat CTLs were detected in 10% of recent seroconverters, 20% of patients in the chronic phase, and 80% of controllers (individuals who contained their HIV-1 plasma viraemia below 1000 RNA copies per millilitres in the absence of anti-retroviral therapy), further supporting the correlation of *anti*-Tat CTLs and non-progression.

3. MHC class-I presentation of soluble Tat protein

Extracellular Tat is taken up by cells (Frankel and Pabo, 1988; Cornali et al., 1996; Chang et al., 1994), it is processed and presented in the context of the major histocompatibility complex (MHC) class I molecules (Moy et al., 1996; Kim et al., 1997), leading to elicitation of CTLs, a feature that Tat shares with very few other soluble proteins called 'penetratins' (reviewed by Derossi et al., 1998).

Our data indicate that APCs uptake Tat at a much higher efficiency than other cell types. However, only biologically active Tat protein (and not its oxidised or inactivated form) is taken up by APCs, in particular dendritic cells (Fanales-Belasio et al., in preparation), enters the class-I pathway and elicits CTLs responses. In addition, biologically active Tat retains the native confor-

mation required for the induction of neutralising antibodies (Buttò et al., in preparation).

4. Conservation among geographically distinct isolates

Studies conducted in Uganda and in South Africa indicate that the Tat antigenic sequence, obtained from African individuals infected with A, C and D subtypes is conserved in its functional and immunogenic epitopes (Buttò et al., in preparation) and that sera from these individuals are able to recognise the Tat protein used as vaccine, which derives from a distantly related subtype B isolate and to neutralise its activity in inhibition assays (Buttò et al., unpublished data). Together with recent sequence data analysis showing conservation of the immunodominant B cell epitopes of Tat among distantly related HIV-1 subtypes (Goldstein et al., 2001) these data provide strong evidence that a Tat-based approach may work as a universal vaccine against HIV/AIDS.

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

The biologically active Tat protein or wild type Tat DNA are 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, hematological or immunological level) were ever observed (Cafaro et al., 1999, 2000, 2001; Caselli et al., 1999; our unpublished data). This is in agreement with data from others showing that vaccination of mice and of rhesus or cynomolgus macaques with biologically active Tat or with *tat* DNA was safe (Hinkula et al., 1997; Osterhaus et al., 1999; Pauza et al., 2000). Similarly, we did not detect any enhancement of virus replication or of $CD4^+$ T cell decline in SHIV89.6P infected monkeys that received 20 μg of Tat protein and 1 mg of *tat* DNA the same day for five times over 12 weeks. Of note, vaccination of HIV-1^+ individuals with *tat* DNA confirmed that immunisation with Tat is safe in that no activation of virus transcription, the most potent of the Tat effects, was detected in these individuals, in which the Tat protein was expressed as indicated by the boosting of *anti*-Tat immune responses (Calarota et al., 1998, 1999).

The biologically active Tat protein or Tat DNA are immunogenic, as indicated by studies in mice (Hinkula et al., 1997; Caselli et al., 1999) and by our studies in monkeys in which immunisation with the Tat protein or *tat* DNA induced both humoral and cellular (including CTLs) Tat-specific immune responses (Table 2; Cafaro et al., 1999, 2000, 2001 and Cafaro et al., unpublished data). Similar results were reported in an *anti*-SIV Tat vaccine study in which monkeys were immunised with viral vectors (Semliki Forest virus and modified vaccinia virus Ankara) expressing the SIV-Tat and -Rev (Osterhaus et al., 1999). Notably, an increase in humoral and cellular *anti*-Tat immune response was also detected in HIV-1^+ individuals immunised with HIV-1 *tat* DNA (Calarota et al., 1998, 1999). Further, our very recent data indicate that in mice mucosal or systemic co-immunisation with the Tat protein and an unrelated antigen (*Candida* or ovalbumin) is safe and elicits immunity to both (Marinaro et al., in preparation). Taken together, these results are clearly at variance with the reported immune suppression in the course of Tat vaccination (Cohen et al., 1999).

To evaluate the efficacy of vaccination with Tat (either protein or DNA) in controlling virus replication in a preventive approach, pre-clinical trials with the Tat protein or *tat* DNA were carried out in Mks. The first protocol included different arms and evaluated the safety, immunogenicity and efficacy of both the Tat protein (three arms) and *tat* DNA (two arms). As mentioned above, Tat vaccination was safe and immunogenic in all arms and nine out of 12 monkeys were protected after challenge with the highly pathogenic SHIV89.6P virus (Cafaro et al., 1999, 2000, 2001).

Given the complexity of the experimental design, results from the Tat protein and from the *tat* DNA approaches are discussed separately.

In the Tat protein protocol (Cafaro et al., 1999), seven *Macaca fascicularis* (cynomolgus

monkeys) were immunised with a biologically active Tat protein (Chang et al., 1997), as reported in Table 1, panel A. Six monkeys were immunised subcutaneously (SC) with 10 μg of Tat with RIBI (three monkeys) or Alum (three monkeys) adjuvant. One macaque was immunised intradermally (ID) with Tat alone (6 μg). Two control monkeys were injected SC with either RIBI or Alum alone. Two naive monkeys were included in the protocol at the time of challenge as additional controls. During the following 36 weeks the six monkeys vaccinated SC received eight boosts. The last boost for was performed intramuscularly (IM) with Tat associated to immune stimulating complexes (ISCOMs) (Davis et al., 1997). The monkey immunised ID was boosted nine times and did not receive the ISCOMs boost.

No toxicity (acute or chronic, local or systemic) was ever detected in the vaccinated animals throughout the immunisation period.

The six monkeys inoculated with Tat and RIBI or alum developed high titres of *anti*-Tat antibodies (1:12,800 and 1:25,600, respectively) that were capable of neutralising the activity of Tat to rescue Tat-defective proviruses (Ensoli et al., 1993; Chang et al., 1997; Barillari et al., 1992). In

Table 1
Protocol and schedule of immunisation of cynomolgus monkeys (*M. fascicularis*) with the active Tat protein or *tat* DNA

	Monkey	Immunogen	Adjuvant	Administration	Vaccination schedule (weeks)
(A) Tat protein vaccine	54844, 54879, 54963	Tat protein (10 μg/250 μl)	RIBI (250 μl)	SC, 500 μl in one site (dorsal area: neck)	0, 2, 6, 11, 15, 21, 28, 32, 36, 42 (last boost with ISCOMS)
	54899, 55396, 54240	Tat protein (10 μg/250 μl)	ALUM (250 μl)	SC, 500 μl in one site (dorsal area: neck)	0, 2, 6, 11, 15, 21, 28, 32, 36, 42 (last boost with ISCOMS)
	54222	Tat protein (6 μg/300 μl)	Nihil	ID, 150 μl in two sites (upper dorsal area)	0, 5, 12, 17, 22, 27, 32, 38, 42, 48
(B) *Tat* DNA Vaccine	54920, 55122, 55361	pCV-*tat*[a] (1 mg)	Pre-treatment (5 days, 1 ml) 0.5% Bupivacaine 0.1% methylparaben Saline	IM, 500 μl in two sites (femoral quadriceps)	0, 2, 6, 11, 15, 21, 31 (last boost with ISCOMS)
	PR2	pCV-*tat*[a] (0.5 mg)		IM, 400 μl in two sites (femoral quadriceps)	0, 5, 10, 22, 27, 32, 37, 42 (last boost with ISCOMS)
	37	pCV-*tat*[a] (0.2 mg)		ID, 150 μl in one site (dorsal area)	0, 5, 10, 22, 27, 32, 37, 42 (last boost with ISCOMS)
	54129	pCV-0[a] (1 mg)		IM, 500 μl in two sites (femoral quadriceps)	0, 5, 10, 22, 27, 32, 37, 42
(C) Controls	55123	Buffer (250 μl)	RIBI (250 μl)	SC, 500 μl in one site (dorsal area: neck)	0, 2, 6, 11, 15, 21, 28, 32, 36, 42 (last boost with ISCOMS)
	55129	Buffer (250 μl)	ALUM (250 μl)	SC, 500μl in one site (dorsal area: neck)	0, 2, 6, 11, 15, 21, 28, 32, 36, 42 (last boost with ISCOMS)
	12	Nihil	Nihil		
	2	Nihil	Nihil		

[a] The vector contains 12 unmethylated CpG sequences.

Table 2
Tat protein and DNA vaccine: immunological responses

Group	Monkey	Ab titres[a]	Tat Neutralization[b]	SHIV Neutralization[c]	Proliferative response[d]	DTH[e]	CTL[f]	Tat-induced TNFα[g]
(A) RIBI+Tat	54844	++	+	ND	+	−	+	+
(10 μg, SC)	54879	++	++	ND	+	+	ND	+
	54963	++	++	+	+	±	−	ND
Alum+Tat	54899	+++	+++	+	+	++	+	+
(10 μg, SC)	55396	+++	+++	+	+	++	−	−
	55240	+++	+++	ND	+	±	+	+
Tat (6 μg, ID)	54222	±	ND	ND	−	−	+	+
(B) *tat* DNA	54920	±	−	ND	+	−	+	+
(0.5 or 1 mg, IM)	55122	±	ND	ND	+	−	+	+
	55361	−	−	ND	+	−	+	ND
	PR2	−	−	−	−	−	+	ND
tat DNA (0.2 mg, ID)	37	++	+	−	+	+	−	ND
Vector-CpG (1 mg, IM)	54219	−	−	ND	−	ND	−	−
(C) Control RIBI	55123	−	ND	ND	−	ND	−	−
Control alum	55129	−	ND	ND	−	ND	−	−
Naïve	2	ND	ND	ND	ND	ND	ND	ND
Naïve	12	ND	ND	ND	ND	ND	ND	ND

[a] Reciprocal of the last dilution positive by specific ELISA test (cut off: mean of pre-immune sera+3 SD).
[b] Neutralising index (N.I.) measured as the capability of sera to block (95%) the rescue of *tat*-defective provirus by the addition of serial concentrations of Tat protein: ++++, 120 μg/ml; +++, 90 μg/ml; ++, 60 μg/ml;+, 30 μg/ml; 0, no neutralisation.
[c] Neutralisation by immune as compared to pre-immune plasma of SHIV89.6P replication upon acute infection of CEM×174 cells.
[d] Stimulation index (ratio between the specific and the control proliferative response); +, >3.
[e] Skin test performed on the back of the animal and ø measured at 48 h: ++, 5 mm;+, 1–4 mm; 0, <1 mm.
[f] Specific killing measured after 2 week in vitro expansion of *anti*-Tat CTLs: +, >5%.
[g] TNFα production in PBMCs, $CD8^+$ and $CD8^-$ T cells upon Tat stimulation: +, >15.6 pg/ml (ELISA test).

contrast, the animal given Tat ID developed low and transient titres of *anti*-Tat antibodies (1:100) (Table 2, panel A).

The *anti*-Tat vaccine also elicited a T cell response, as indicated by the development of delayed-type hypersensitivity (DTH) and proliferative response to Tat. Again, these responses were detected in the monkeys immunised SC but not in the one vaccinated ID (Table 2, panel A). Further, a specific *anti*-Tat CTL activity began to be detectable in the vaccinated animals at week 28 after immunisation but only at week 36 it reached levels above the cut-off in 2/3 macaques vaccinated with Tat and RIBI, in 2/3 monkeys vaccinated with Tat and Alum, and in the monkey vaccinated ID with Tat alone (Table 2, panel A).

At week 50 after immunisation (14–18 weeks after the last boost), all the animals were challenged intravenously (IV) with the SHIV89.6P, a chimaeric virus that contains the *tat* gene of HIV-1 and is highly pathogenic in macaques (Reimann et al., 1996; Karlsson et al., 1997; Cafaro et al., 2000). The virus stock used for the challenge was derived from a cynomolgus macaque inoculated with the original SHIV89.6P from rhesus mon-

keys (Cafaro et al., 2000). All animals were inoculated with 10 MID_{50} of the virus, except the two naive control monkeys that were challenged with either a three-fold lower (2.8 MID_{50}) or higher (28 MID_{50}) doses, respectively, as additional controls of the virus inoculum. Of note, the macaque infected with 28 MID_{50} was euthanised at week 35 after the viral challenge, due to very low $CD4^+$ T cell counts and severe worsening of the clinical conditions.

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 levels of p27 antigen (detected by ELISA) and viral RNA (detected by branched-DNA and Quantitative–Competitive RNA-PCR) in plasma, proviral DNA copies, cytoviraemia or positive virus isolation (Table 3, panel A and C). In contrast, all these parameters were always negative for all the other five vaccinees up to 2 years post-challenge, with the exception of SIV proviral DNA that was sporadically and barely detected (< 10 copies/μg of DNA) in a few animals (Table 3, panel A). This and the presence of low and transient *anti*-SIV (or *anti*-HIV Env) antibody titres in these protected animals indicated that infection occurred but it was blocked by the immune response induced by the vaccination.

Of note, *anti*-SIV or *anti*-HIV Env antibodies correlated with the level of infection. They were very high in all the control monkeys, followed by the two infected and vaccinated animals that had at least 1 log lower titres and a delayed increase as compared to the control macaques, whereas they were very low and transient in the five protected monkeys (Table 3, panel A, and data not shown). Consistent with the virological data, the number of $CD4^+$ T cells remained in the normal range after the viral challenge and during all the follow-up period in the five protected monkeys, whereas it decreased considerably in all the controls and in the two vaccinated and infected macaques (Table 3, panel A and C). Notably, protection correlated (100%) with the presence prior to challenge of *anti*-Tat specific CTLs and with TNFα production by $CD8^+$ T cells upon Tat stimulation, but not with the presence of *anti*-Tat antibodies. Of interest, a potent and stable CD8-mediated non-cytolitic antiviral (CD8-NCA) activity was detected early after challenge in all the vaccinated and protected macaques, whereas enhancement of infection was detected in four of the five infected animals (Table 3, panel A and C). In contrast, no correlation of protection was observed with the production of β-chemokines at post-challenge time (RANTES, MIP-1α, MIP-1β) (Goletti et al., in preparation).

In the DNA arms of the study (Cafaro et al., 2001), five monkeys were immunised 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). The plasmid pCV-0 and pCV-*tat* were chosen since pCV-*tat* is capable of high expression and release of the Tat protein in the absence of cell death (Ensoli et al., 1990, 1993; Chang et al., 1997). Further, the pCV vector is rich in unmethylated CpG sequences (Cafaro et al., 2001) known to boost the innate immunity and enhance adaptive immune responses by inducing the maturation of dendritic cells and long-lasting Th-1 and CTL immune responses (reviewed in Klinman et al., 1999). Of note, the natural immune response induced by the sole CpG sequences can protect against intracellular bacterial infections in murine systems underscoring their potency and importance in protection against pathogens and in vaccine strategies (Klinman et al., 1999).

As shown in Table 1, panel B, four monkeys were immunised IM, three with 1 mg and one with 0.5 mg of pCV-*tat*, respectively. The fifth monkey was immunised ID with 0.2 mg of pCV-*tat*. One monkey was injected IM with the empty vector (pCV-0), to evaluate the impact of the immunostimulating effects of the CpG motifs on protection. Vaccinees were boosted eight times over 37–42 weeks. The last boost was IM with the Tat protein in ISCOMs for all monkeys with the exception of the one immunised with pCV-0 that was not boosted. At the time of vaccination and during all the follow-up period, neither local nor systemic side effects were observed in all inoculated animals and all the haematological parameters (blood cell counts, blood chemistry and FACS analysis) were always in the normal range.

Table 3
Preventive Tat protein and DNA vaccines: results from challenge and correlates of protection.

Monkey	Vaccination	Challenge dose (SHIV89.6 P, IV)	Post-challenge results						Immune correlates of protection		
			P27[a]	Plasma viraemia[b]	DNA-PCR[c]	Cytoviraemia or virus isolation[d]	*Anti*-SIV[e]	$CD4^+$ T cells decline[f]	CTL[g]	Tat-induced TNFα[h]	CD8-NCA[i]
(A) 54844	RIBI + Tat (10 μg, SC)	10 MID_{50}	−	−	− (+)*	−	+ (−)	−	+	+	+
54879		10 MID_{50}	−	−	− (+)	−	+ (−)	−	ND	+	+
54963		10 MID_{50}	+	+	+	+	+	+	−	ND	− (E)
54899	Alum + Tat (10 μg, SC)	10 MID_{50}	−	−	− (+)	−	+ (−)	−	+	+	+
55396		10 MID_{50}	+	+	+	+	+	+	−	−	− (E)
54240		10 MID_{50}	−	−	− (+)	−	+ (−)	−	+	+	+
54222	Tat (6 μg, ID)	10 MID_{50}	−	−	− (+)	−	+ (−)	−	+	+	+
(B) 54920	*tat* DNA (0.5 or 1 mg, IM)	10 MID_{50}	−	−	− (+)	−	+ (−)	−	+	+	+
55122		10 MID_{50}	−	−	− (+)	−	+ (−)	−	+	+	+
55361		10 MID_{50}	−	−	− (+)	−	+ (−)	−	+	ND	+
PR2		10 MID_{50}	−	−	− (+)	−	+ (−)	−	+	ND	+
37	*tat* DNA (0.2 mg, ID)	10 MID_{50}	+	+	+	+	+	+	−	ND	− (E)
54219	Vector-CpG (1 mg, IM)	10 MID_{50}	−	−	+	+	−	− (+)	−	−	+
55123	Control RIBI	10 MID_{50}	+	+	+	+	+	+	−	−	− (E)
55129	Control Alum	10 MID_{50}	+	+	+	+	+	+	−	−	+
2	Naïve	28 MID_{50}	+	+	+	+	+	+	ND	ND	−
12	Naïve	2.8 MID_{50}	+	+	+	+	+	+	ND	ND	−

[a] p27-Gag antigenemia determined by antigen-capture ELISA: +, >20 pg/ml.

[b] Measured by a branched DNA signal amplification assay and by a quantitative–competitive RT-PCR.

[c] Proviral DNA copies per μg of DNA were determined by a semi-quantitative DNA-PCR.

[d] Virus isolation and cytoviraemia (quantitation of productively infected PBMCs) were determined by co-culturing for up to 30 days PBMCs with CEM × 174 cells at 1:1 ratio or at decreasing ratios, respectively.

[e] *Anti*-SIV Abs determined by a commercial ELISA.

[f] Staining performed on whole blood; total CD4 single positive cells determined within the lymphocyte gate.

[g] Specific killing measured after 2 week in vitro expansion of *anti*-Tat CTLs: +, >5%.

[h] TNFα production in PBMCs, $CD8^+$ and $CD8^-$ T cells upon Tat stimulation: +, >15.6 pg/ml (ELISA test).

[i] inhibition of viral replication by *anti*-CD3 stimulated $CD8^+$ lymphocytes (co-culture with autologous *anti*-CD3 stimulated $CD4^+$ T cells superinfected with SHIV89.6P). +, >50%; −, 0–50%; E, enhancement.

As shown in Table 2, panel B, vaccination with *tat* DNA was immunogenic. *Anti*-Tat antibodies were transient and at low titres in the four animals vaccinated IM with pCV-*tat*. In contrast, the monkey inoculated ID with 0.2 mg of pCV-*tat*, developed specific antibodies that reached titres up to 1:800, remained high throughout the follow-up period, and weakly neutralised Tat activity as detected by the rescue assay. As expected no *anti*-Tat antibodies were observed at any time in the animal inoculated with pCV-0, or in the control animals.

T helper responses to Tat or tetanus toxoid (TT), for which all animals had been vaccinated, were detected in three out of four pCV-*tat*-inoculated monkeys, whereas the DTH response to Tat resulted always negative in all vaccinated macaques, except for the monkey inoculated ID (Table 2, panel B).

Anti-Tat CTLs were detected in all the macaques vaccinated IM with *tat* DNA whereas no CTLs were detected in the monkey vaccinated ID, in the animal injected with pCV-0 and in the control animals (Table 2, panel B).

All animals from the DNA arms were challenged IV with the same SHIV89.6P 14–18 weeks after the last boost and on the same day of the macaques from the Tat protein arms. Upon virus challenge and during all the follow-up period, all the macaques vaccinated IM with pCV-*tat* were negative for p27 antigenaemia, plasma viraemia, and virus isolation (Table 3, panel B). Accordingly, in these animals the SIV proviral DNA was undetectable or was only sporadically detected at very low copy number (range 1–8 copies/μg DNA). In contrast, all the controls and the monkey vaccinated ID with 0.2 mg of pCV-*tat* had detectable levels of both p27 and viral RNA in plasma, infectious virus was repeatedly isolated from the peripheral blood, and a high proviral copy number was detected early after the challenge and remained detectable for the entire follow-up period (Table 3, panel B and C). Of note, the monkey vaccinated with pCV-0 resulted negative for both antigenaemia and plasma viraemia. However, virus was isolated 18 weeks after challenge and proviral DNA was detected at week 14 after challenge (55 proviral copies) indicating that some level of control was exerted by the natural immunity boosted with the CpG sequences, but it was more potent and complete only in the presence of *anti*-Tat CTLs (Table 3, panel B).

In all the macaques vaccinated IM with pCV-*tat* and in the animal injected with pCV-0 the *anti*-SIV antibodies titres were undetectable or low (range 1:2–1:100) and transient (Table 3, panel B). In contrast, they increased and remained high in the monkey vaccinated ID with pCV-*tat*, and in the controls (Table 3, panel B and C). *Anti*-SIV antibodies were not detected in the naive control monkey infected with 28.5 MID_{50}, a relatively uncommon feature that correlates with fast progression (Dykhuizen et al., 1998).

Consistent with the data of the virological assays, the number of $CD4^+$ T cells remained in the normal range after the viral challenge and during all the follow-up period in all monkeys injected IM with pCV-*tat* or pCV-0 (Table 3, panel B). In contrast, in the infected animals the $CD4^+$ T cells decreased and remained persistently below the baseline values during the following weeks (Table 3, panel B and C). The naive control monkey infected with 28.5 MID_{50} had a progressive and severe $CD4^+$ T cells decline and had to be euthanised.

Thus, similarly to what observed with the Tat protein immunisation with *tat* DNA was safe, induced a broad immune response capable of blocking virus replication to undetectable levels, preventing the CD4 T cell decline and disease onset. Protection correlates (100%) with a $CD8^-$ T cell-mediated activity that includes both antigen specific CTLs and a Th-1 type cytokine production and non-antigen specific immune responses such as CD8-NCA activity, suggesting that Tat vaccine can also potentiates the innate immunity (Table 3, panel B).

Thus although Tat strategy does not block virus entry, it can efficiently control virus replication and render 'abortive' the natural infection. This is likely to occur against different virus strains as suggested by studies in HIV-infected Ugandan patients that, although infected by different strains have antibodies recognising Tat from clade B (Buttò et al., in preparation).

Of importance, this blockade correlates in 100% of the animals with the presence of a cellular immunity, in particular with *anti*-Tat CTLs and, as a correlate of CTL activity, TNFα production upon Tat stimulation of $CD8^+$ T cells (Cafaro et al., 1999, 2001). In addition, all protected monkeys had a potent and stable CD8-NCA activity that was not mediated by beta-chemokines (Goletti et al., submitted). The protective role of *anti*-Tat CTLs and CD8-NCA activity was even more clear-cut in the DNA vaccination study since no humoral *anti*-Tat response was detected in these monkeys upon immunisation (Cafaro et al., 2001).

Similar results were reported with an *anti*-SIV Tat vaccine. Mks immunised with viral vectors (Semliki Forest virus and modified vaccinia Ankara) expressing the SIV-Tat and -Rev were protected upon challenge with a pathogenic SIV (Osterhaus et al., 1999), stressing the importance of targeting early regulatory genes. Again, no toxic effects were observed. These results have been recently confirmed (Osterhaus, personal communication).

6. Concluding remarks

Taken together, this evidence indicates that the immunisation with Tat may block HIV replication both in individuals exposed to the virus after vaccination and in seropositive patients, reducing HIV infection and favouring its control by the mounting immune response, especially CTLs. It is conceivable that soon after initiation of virus replication the infected cells will express on their membrane epitopes from the first viral products, that is the regulatory proteins Tat, Rev and Nef. In the Tat vaccinated individual these cells will be immediately recognised and either killed by CTLs or the viral replication suppressed by CD8-NCA activity. A very limited, apparently harmless, and undetectable (as indicated by very sensitive PCR assays in our monkeys studies) viral replication may occur. We hypothesise that this limited viral replication, rather than being dangerous, may actually provide additional viral antigens to drive an immune response against other viral components. As a consequence, the *anti*-HIV immune response will be boosted (against Tat), broadened and strengthened (against the other viral proteins), making the individual more resistant to further challenges and possibly capable of eventually clearing the virus. In essence, the vaccine will modify the virus–host dynamic at the very beginning of the infection and this will profoundly impact on the evolution of the infection.

Two new protocols have confirmed in a larger number of animals the data of safety and immunogenicity and the animals are scheduled for challenge shortly to evaluate the efficacy. Together, 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 organised and expected to be started in Italy within the year 2001/2002.

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