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Vaccine 21 (2003) 2082–2090

Vaccine

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Red blood cell-mediated delivery of recombinant HIV-1 Tat protein in mice induces anti-Tat neutralizing antibodies and CTL

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Received 26 July 2002; received in revised form 5 November 2002; accepted 25 November 2002

Abstract

The immunotherapeutic potential of biologically active HIV-1 Tat protein coupled to autologous red blood cells (RBCs) was evaluated in a mouse model. HIV-1 Tat expressed in *Escherichia coli* and purified to homogeneity was found to be active in viral *trans* activation and efficiently internalised by monocyte-derived dendritic cells (MDDCs). The product of HIV-Tat biotinylation and coupling to RBCs by means of a biotin–avidin–biotin bridge, (RBC-Tat), showed no *trans* activation activity and was still efficiently internalized by MDDCs as compared to uncoupled Tat.

Balb/c mice were then immunized with 10 μg of soluble Tat in complete Freund's adjuvant or with 40 ng of Tat coupled on RBCs surface and boosted at week 3, 6 and 25 with 5 μg soluble Tat in incomplete Freund's adjuvant or with 20 ng of RBC-coupled Tat, respectively. Anti-Tat antibody response was similar in both groups; however, 2/6 animals immunized with soluble Tat and 6/6 animals immunized with RBC-Tat developed anti-Tat neutralizing antibodies. In addition, at week 28 cytolytic anti-Tat CTLs were detected in all animals although they were slightly higher in mice immunized with RBC-Tat. These results indicate that RBC-mediated delivery of HIV-1 Tat, in amounts 250 times lower than soluble Tat, is safe and induces specific CTL responses and neutralizing antibodies.

Keywords: HIV-1 Tat; Antigen-delivery system; Red blood cells

1. Introduction

Most HIV-1 vaccine strategies based on structural gene products have failed to protect from infection due to the homologous or heterologous viruses with production of escape virus variants [1,2]. Vaccines with live, attenuated viruses appear to protect against most heterologous virus challenge, at least in non-human primates [3–6], but the appearance of revertant viruses and the limited accessibility of relevant cross-neutralizing epitopes [7–10], prevents their use in humans. This has led to the concept that containment of infection and block of disease onset is at present a more achievable target of HIV/AIDS vaccine development [11].

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 leading to the production of escape virus variants [12,13]. In particular, Tat is produced early after infection and is essential for virus replication and infectivity [14–16]. In acute infection of T cells by HIV, Tat is released extracellularly by infected cells [17,18] and is taken up by neighbour cells [19,21]. Tat is also immunogenic and antibodies (Ab) against Tat have been found to correlate with delayed disease progression [22–24] and may exert protective effects by inhibiting both HIV replication and the effects of extracellular Tat [19,25]. Moreover, Tat is efficiently taken up by monocyte-derived dendritic cells (MDDCs), promotes their maturation and antigen (Ag)-presenting functions [26] directing Th1 and CTL responses against itself and other Ags since it enters the major histocompatibility complex (MHC) class I pathway [27]. Finally, vaccination of monkeys with a biologically active Tat protein or DNA has been shown

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0264-410X/02/$ – see front matter
PII: S0264-410X(02)00746-6

to be safe, immunogenic and to contain infection with the highly pathogenic SHIV89.6P [28,11].

In HIV-1, as well as in many other infections, the cellular immune response appears to be crucial in mediating protection. Many currently available vaccines generate humoral immune responses but are relatively inefficient in generating cellular immune responses (TH1 or $CD8^+$ T-cell responses) limiting their applications [29]. Various approaches have been developed to route exogenous Ags to the MHC class I processing pathway and thus to induce $CD8^+$ T-cell responses [30,31]. Particulate Ags coupled to latex beads or encapsulated in liposomes, recombinant bacteria or apoptotic cells, have been demonstrated to elicit both $CD4^+$and $CD8^+$ T-cell responses [30–33].

RBCs have been proposed as a potential Ag delivery system because they can be conjugated to protein Ags via biotin–avidin–biotin bridges [34]. Furthermore, RBCs coated with viral Ags induce protective immune responses higher than those obtained by using classical adjuvants both in mice and cats [35,36]. We have previously shown that human MDDCs efficiently and rapidly internalize RBC-Tat inducing specific $CD4^+$ and $CD8^+$ T-cell responses ([37] and data not shown). Here we report that inoculation of RBC-Tat in mice is safe, immunogenic and elicits anti-Tat neutralizing Ab and CTL at doses 250-times lower than those needed to obtain a similar response with soluble Tat in Freund's adjuvant. Thus, the administration of very low amounts of a particulate form of Tat without any adjuvant appears to be more immunogenic than biologically active soluble Tat.

2. Materials and methods

2.1. Tat protein expression and purification

The Tat protein was expressed in *Escherichia coli* and purified by heparin sepharose affinity chromatography through a modification of published methods [19,26,38]. Further details are being published elsewhere (Magnani et al., in preparation).

The purified Tat protein is >95% pure and fully monomeric, as tested by sodium dodecyl sulphate polyacrylamide gel elettroforesis (SDS-PAGE) (Fig1A), and possesses full biological activity as tested by the rescue assay [19,39] and by MDDCs uptake ([37] and data not shown). The Tat protein was stored lyophilized at −80 °C and resuspended in degassed phosphate buffered saline (PBS) before use, as previously reported [19,26,38].

2.2. Biotinylation of the Tat protein

The Tat protein was biotinylated by incubation with *N*-hydroxysuccinimidobiotin (NHS–biotin PIERCE, Rockford, IL) in *N*,*N*-dimethylformamide (DMF) as previously described [34]. Excess biotin was removed by Alkilamine Beads (Pierce Chemical) at 4 °C for 18 h and the protein concentration was determined by the Bradford method (Bio-Rad, Richmond, CA). The biotinylation of Tat was tested by Western blotting with streptavidin-HRP conjugated Ab (Fig. 1B).

2.3. Coupling the biotinylated Tat protein to mouse erythrocytes

Mouse blood was collected in heparinized tubes. RBCs were obtained by centrifugation of fresh blood at 1800 × *g* for 10 min at 4 °C. Plasma and buffy coat were removed by aspiration and the packed cells resuspended in PBS and washed twice. Washed RBCs at 10% hematocrit (Ht) were coupled to biotinylated Tat by means of avidin–biotin bridges as previously described [40]. The percentage of RBC-Tat conjugates was evaluated by flow cytometry (FACScan, Becton-Dickinson, Mountain View, CA) using a rabbit fluorescein isothiocyanate (FITC)-conjugated anti-Tat Ab. (Fig. 2).

The amount of Tat bound to RBCs was estimated considering that the relative number of biotin molecules bound to one mouse RBC is 1000 [35]. Assuming that each biotin can bind a molecule of avidin and that avidin has from

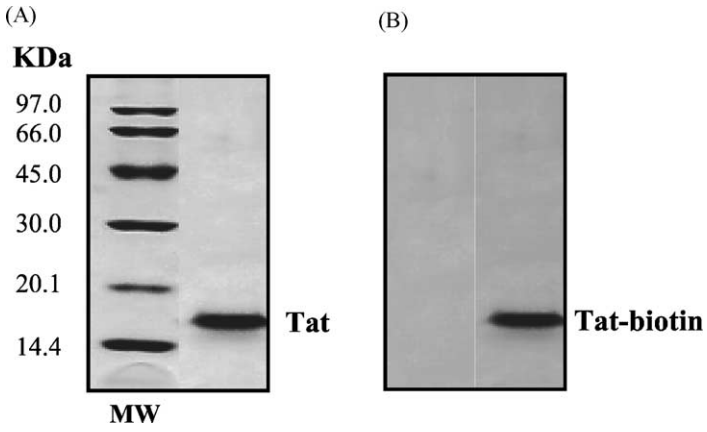

Fig. 1. SDS-PAGE of Tat protein expressed in *E. coli* and purified by heparin sepharose affinity chromatography. The monomeric form of Tat protein is >95% pure (A). Western blotting of biotinylated Tat stained with streptavidin-HRP conjugated Ab (B).

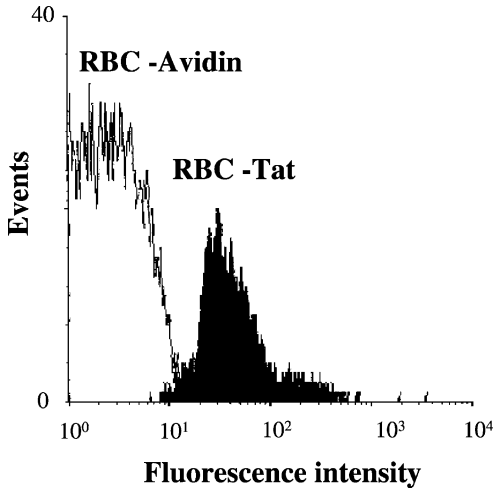

Fig. 2. Flow cytometry analysis of RBC-Tat stained with a rabbit anti-Tat antibody (solid curve). As control, RBC-conjugated avidin were stained with the same antibody (open curve).

one to three available sites for further binding of biotinylated proteins, we have estimated that every RBC binds approximately 1000–3000 molecules of biotinylated Tat. Based on this estimate, 200 μl of RBCs (10% Ht) should have bound at maximum 40 ng of biotinylated Tat protein [41].

2.4. Immunization with the Tat protein

Two groups of 10 female BALB/C mice from Nossan (Milan, Italy) were immunized intra-peritoneally (IP) with the Tat protein, 10 μg in 200 μl of an emulsion prepared with Freund's adjuvant and PBS (group 1), or 40 ng of Tat protein coupled to an autologous RBC-suspension (200 μl) at 10% Ht as described above (group 2).

Eight control mice (group 3) were injected with 200 μl of biotinylated ubiquitin coupled to an autologous RBC-suspension at 10% Ht. (40 ng). Boosts were given at 3, 6, and 25 weeks after the first immunization with 5 μg of soluble Tat (group 1) or 20 ng of RBC-coupled Tat (group 2). Blood samples were drawn by retro-orbital puncture from anesthetized mice at 0, 3, 6, 8, 12, 18, 26, 28 weeks after the first immunization (week 0). Sera were prepared by centrifugation of clotted blood at $1800 \times g$ for 5 min, stored at −80 °C and utilized for the anti-Tat serology and neutralization assay.

At the time of the last bleeding, mice were sacrificed and spleens were collected for analysis of cell-mediated immune responses (CTL assay).

2.5. Anti-Tat serology

The presence of serum anti-Tat specific immunoglobulins (IgG) was determined by an ELISA assay. Briefly, 96-well microplates (IMMULON I, Dinex) were coated overnight for 18 h at 4 °C with 50 ng per well of purified Tat protein in 0.05 M sodium carbonate–bicarbonate buffer pH 9.6. Plates were washed 5 times with PBS pH 7 containing 0.05% Tween 20 (PBS/Tween 20).

Sera were diluted in blocking solution (PBS/Tween 20 containing 1% BSA, 200 μl per well), plates were washed 5 times with PBS/Tween 20 prior to the addition of 100 μl per well of horseradish peroxidase-labeled goat anti-mouse IgG (Bio-Rad, Richmond, CA) diluted 1:1000 in blocking solution and incubated for 60 min at 37 °C. Plates were then washed 5 times with PBS/Tween 20, and 100 μl of ABTS substrate (Boerhinger Mannheim, Mannheim Germany) were added for 30 min at room temperature. The absorbance was measured at 405 nm with a microplate reader (BioRad).

2.6. Anti-Tat Ab isotyping

IgG isotyping was determined in mouse sera as described earlier, using goat anti-mouse IgG1, IgG2, IgG2a, IgG2b and IgG3 (Sigma). Immune complexes were detected with a horseradish peroxidase-labeled rabbit anti-goat Ab (Sigma) and ABTS substrate.

2.7. Purification of spleen cells

Mononuclear cells were purified from the spleens of sacrificed animals by Ficoll-Histopaque 1083 (Sigma) density gradients, washed twice with PBS, and resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT). Macrophages were removed by plastic adherence for 1 h at 37 °C. Cells were counted by trypan blue exclusion dye.

2.8. Target cells for CTL assay

Monolayer cultures of murine BALB/C 3T3 fibroblasts were grown in DMEM (Sigma) supplemented with 10% FBS and transfected with the pRPneo-c/Tat vector or pRPneo-c control plasmid previously described [42], using the calcium coprecipitation technique. Stable clones of BALB/C 3T3-Tat expressing cells and BALB/C 3T3 expressing pRPneo-c/Tat and pRPneo-c (referred to as BALB/C-control cells) were selected with G418 (350 μg/ml, Sigma). Tat-expression was monitored following transfection of cells (5×15^5) with pU3RCAT (1 μg) where transcription of the reporter *CAT* gene is driven by the HIV-1 LTR promoter [43]. Cat activity was measured 48 h later, using amounts of cell extracts normalized to total protein content, as described [43].

2.9. Tat-specific CTL response

Mononuclear cells were purified from mouse spleen as described above and seeded (2×10^5 per well) in

96-U-bottomed-well plates. Tat-specific CTL response was assayed on fresh, unstimulated lymphocytes and on lymphocytes stimulated for 5 days by co-cultivation at a ratio of 20:1 with BALB/c 3T3-Tat expressing cells and BALB/c 3T3-control cells. For the killing assay BALB/c 3T3-Tat expressing cells and BALB/c 3T3-control cells were labelled with $Na_2{}^{51}CrO_4$ (NEN, Brussels, Belgium) 0.1 μCi/10^6 cells for 1 h at 37 °C, washed 3 times with RPMI 1640 containing 10% FBS and seeded in triplicate (5×10^3 per well) in 96-U-bottomed-well plates in the presence of the effector cells (E/T ratio: 100:1, 33:1 for CTL fresh and 75:1, 25:1 for CTL cocultured). Percent specific lysis was calculated as 100 × (cpm sample − cpm medium)/(cpm Triton-X-100 − cpm medium).

Spontaneous release (i.e. ^{51}Cr spontaneously released from target cells in the absence of effectors) was always below 20%, while the maximum release corresponded to the ^{51}Cr released, in the absence of effectors, from target cells after lysis with 1% Triton-X-100 (Sigma, Milan, Italy).

2.10. Neutralization assays

HLM1 cells (1.5×10^5), a HeLa-CD4$^+$ cell line containing an integrated copy of HIV-1 Tat-defective provirus the replication of which is rescued by the addition of exogenous Tat [19], were seeded in 24-well plates in 500 μl of Dulbecco's modified essential medium containing 10% of FBS (FBS, Hyclone, Logan, UT). After 24 h of culture, the medium was replaced with 300 μl of fresh medium containing 2.5 μg/ml of recombinant Tat protein pre-incubated alone or with diluted murine plasma overnight at 4 °C under rotation. Each plasma was tested in duplicate. Negative controls were represented by sera obtained from preimmune mice. After 48 h, rescue of HIV-1 replication was monitored in the culture supernatants by an enzyme immunoassay (EIA) for the detection and quantification of HIV p24 core Ag (Innogenetics N.V.). Neutralization values were expressed as the percentage of inhibition of virus rescue assuming as 100% the rescue obtained with Tat alone or pre-incubated with control sera, as previously described [44].

3. Results

3.1. Biotinylation and coupling of Tat to RBCs and uptake by antigen-presenting cells

Recombinant HIV-1 Tat was produced in *E. coli* and purified to homogeneity by affinity chromatography [19,26,38] through a modification of described methods (Magnani et al., in preparation). The final preparation (Fig. 1A) was biotinylated by a *N*-hydroxysuccinimido derivative of biotin as described in the Section 2 and further analysed by SDS-PAGE and Western blotting. A single protein band with an electrophoretic mobility identical to that of the native Tat was always observed (in more than eight different preparations). The biotinylated protein was identified by staining with streptavidin-peroxidase in Western blotting experiments (Fig. 1B). Murine RBCs were separately biotinylated and coupled to biotinylated Tat by avidin. The procedure for coupling biotinylated Ag to RBCs is described in detail elsewhere [41,45] and provides RBC populations that carry surface bound Ag in amounts greater than 80%. In fact, as shown in Fig. 2, almost all RBCs were recognized by a FITC-labelled anti-Tat Ab. Conversely, omission of avidin or addition of native Tat resulted in the absence of FITC-labelled RBCs (Fig. 2). Thus, biotinylated Tat can be coupled to biotinylated RBCs by avidin.

The trans-activating activity of biotinylated Tat was then tested and compared with native Tat. Native Tat was biologically active in a transactivation assay, (1562 ± 71 pg/ml of p24 for 10 ng of Tat protein) [19,39], in contrast, biotinylated Tat or RBC-Tat were not (36.3±pg/ml of p24 for 10 ng of biotinylated Tat protein) (Fig. 3). However, both native and biotinylated Tat, like RBC-Tat were efficiently internalized by MDDCs ([37] and data not shown]). Thus, coupling of Tat to RBCs results in the loss of its transactivating activity but not in its ability to target and be internalized by MDDCs.

3.2. Safety of RBC-Tat upon vaccination in mice

Four-week-old Balb/c female mice were immunized IP with either 10 μg per soluble Tat in complete Freund's adjuvant (10 mice group 1) or 200 μl of RBCs at 10% hematocrit coupled with 40 ng of Tat (10 mice group 2). As controls, 8 mice (group 3) were injected IP with 40 ng each of bovine ubiquitin coupled to RBCs. Based on previous experiments [45] all animals were boosted at week 3, 6 and 25. Group 1 received 5 μg of soluble Tat (half the amount used for the initial immunization), this time in incomplete Freund's adjuvant at week 3 and 6 with 5 μg of soluble Tat in PBS at week 25. Group 2 was boosted with 20 ng of RBC-Tat (half

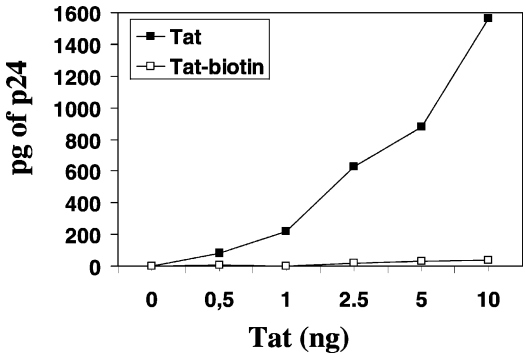

Fig. 3. Rescue assays. The HLM-1 cell line containing an integrated non-reversible Tat-defective provirus was used for rescue assays in order to determine the biological activity of recombinant Tat protein (closed square) or biotinylated Tat (open square). The proteins were added to the cells and the supernatants were collected 72 h later for p24 antigen capture assays.

the amount used for the initial immunization), at week 3, 6 and 25. Controls received 20 ng of RBC–ubiquitin (half the amount used at week 0) at week 3, 6 and 25. Mice were followed for 28 weeks and blood samples were taken at the time of the first immunization (week 0) and subsequently at week 3, 6, 8, 12, 15, 18, 26, and 28 when they were sacrificed (see Section 2) (Fig. 3). We have monitored mouse survival, weight loss, spleen and lymphnodes weight, histological sections at the site if injection and of liver and spleen. No toxicity was ever detected in the vaccinated animals throughout the immunization period. In addition, vaccination with Tat induced both humoral and cellular anti-Tat specific responses.

3.3. Anti-Tat humoral responses

Anti-Tat Ab responses were detected in all immunized mice and reached titers of 1:7181 and 1:7646 in mice immunized, respectively with Tat and Freund's adjuvant or RBC-Tat (Table 1), remaining stable throughout the immunization schedule (Fig. 4).

Immunization of mice with RBC-coupled Ags through a biotin–avidin–biotin bridge also elicited the production of anti-avidin Ab [35]. Thus, we evaluated whether the administration of Tat coupled to RBCs affected the production of anti-avidin Ab. As the controls, the induction of anti-avidin Ab in mice receiving ubiquitin coupled to RBCs was evaluated. Both control animals receiving RBC–ubiquitin and mice receiving RBC-Tat seroconverted by week 6 and developed an anti-avidin Ab titer of 11,255 and 13,051, respectively (Table 1). Thus, the administration of Tat coupled to RBCs was not immunosuppressive [46].

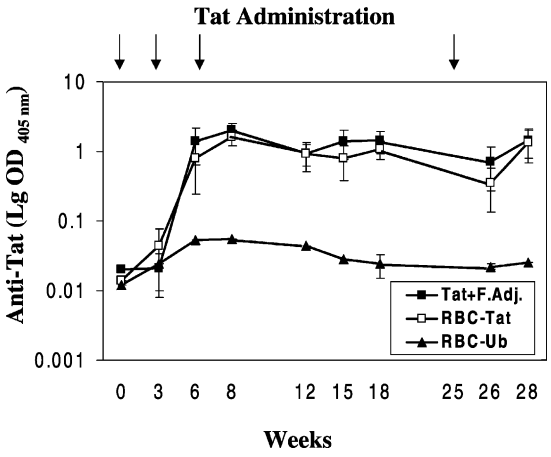

Fig. 4. Schematic representation of the immunization schedule. Boosts were given at 3, 6, and 25 weeks after the first administration (week 0). Bleedings were performed at week 0, 3, 6, 8, 12, 15, 18 and 26, at 28th week mice were sacrificed. Measurement of anti-Tat antibody ($OD_{405\,nm}$) in sera from mice immunized with Tat and Freund's adjuvant (closed square), RBC-Tat (open square), RBC–ubiquitin (closed triangle) were performed at the same time of the bleeding. The presence of serum anti-Tat specific immunoglobulins (IgG) was determined by an ELISA assay. The antibody response was assayed on mouse sera 1:500 diluted using Tat protein as the antigen. Immune complexes were detected with a horseradish peroxidase-labeled goat anti-mouse IgG antibody diluted 1:1000 and ABTS substrate. The values are the mean ± S.D. of eight mice for each group The antibody response product in mice immunized with Tat and adjuvant is not significantly different from that of mice immunized with RBC-Tat ($P > 0.05$) in the several time of bleedings.

3.4. RBC-Tat immunization induces neutralizing Ab

The induction of Ab neutralizing Tat activities may be of importance. In fact, during acute HIV infection of T cells, Tat is released into the extracellular milieu and is taken up both by infected cells, where it trans-activates virus gene expression and replication [17,20,21,39], and by uninfected cells, where it favours virus transmission by inducing the expression of CCR5 and CXCR4 chemokine receptors [47,48]. Using HLM1 cells containing an integrated copy of an HIV-1 tat-defective provirus, the ability of sera from immunized mice to inhibit the rescue of HIV-1 upon Tat addition was evaluated. As shown in Table 1, two out of six mice in the group receiving soluble Tat developed Tat neutralizing activity as compared to 6/6 mice receiving RBC-Tat. Thus, RBC-Tat induces antibodies which neutralize Tat activity (Table 1).

3.5. IgG isotypes and anti-Tat CTL activity

In the mouse model, IgG1 and IgG3 isotypes are suggestive of a Th2-like immune response whereas the prevalence of IgG2a isotypes suggests of a Th1 response [49–51]. The identification of the distinct IgG subclasses by ELISA showed that up to 30 and 20–25% of the total anti-Tat IgG were IgG1 and IgG3, respectively, whereas IgG 2a isotypes constituted up to 40% of total anti-Tat IgG. The results were similar in mice vaccinated with Tat protein in Freund's adjuvant and in mice inoculated with RBC-Tat. (Fig. 5) Thus, both Th1 and Th2 types of immune responses were induced by RBC-Tat immunization.

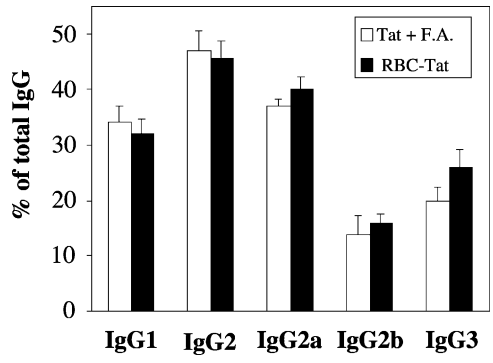

Fig. 5. Isotype analysis of anti-Tat IgG in mice immunized with Tat in Freund's adjuvant or with RBC-Tat. Results are expressed for each IgG subclass as the cumulative percentage of the total IgG. The difference in the percentage of IgG subclass in the two groups is not significant ($P > 0.05$). The values are the mean ± S.D. of eight mice for each group.

Table 1
Immune response in mice immunized with Tat or RBC-Tat

Immunogen	No. of mice	IgG anti-Tat titers[a]	IgG anti-avidin titers[b]	Neutralization activity[c] (%)
Tat + Freund's adjuvant	1A	ND	0	ND
	2A	9,154	0	ND
	3A	5,304	0	0
	4A	7,563	0	0
	5A	8,177	0	45.94
	6A	9,491	0	ND
	7A	ND	0	ND
	8A	6,211	0	4.89
	9A	5,073	0	0
	10A	6,482	0	0
Mean values		7,181 ± 1,680	0	9
RBC-Tat	1R	7,983	11,666	ND
	2R	5,087	12,572	ND
	3R	3,704	11,900	30
	4R	8,347	14,322	12.41
	5R	5,110	ND	22.88
	6R	8,802	14,796	46.29
	7R	10,314	14,265	ND
	8R	7,701	ND	ND
	9R	10,606	ND	38.84
	10R	7,532	1,1840	30.14
Means values		7,646 ± 1,721	13,051 ± 1,358	30.09 ± 11
RBC–ubiquitin	1C	0	11,603	0
	2C	0	ND	0
	3C	0	14,252	0
	4C	0	7,128	0
	5C	0	12,272	0
	6C	0	13,576	0
	7C	0	ND	0
	8C	0	8,704	0
Mean values		0	11,255 ± 2,795	0

[a] Analysis by ELISA of antibody responses to mice immunized with Tat and Freund's adjuvant (group 1) or to mice immunized with RBC-Tat (group 2). Titers were determined by linear regression analysis of serial 1:100, 1:1000, 1:10,000 dilutions. Results are the mean ± S.D. of eight mice for group 1 and then for group 2. The antibody response product in mice immunized with Tat and Freund's adjuvant is not significantly different from that of mice immunized with RBC-Tat ($P > 0.05$).

[b] Titers of antibody anti-avidin in mice immunized with RBC-Tat and RBC–ubiquitin, indicating that the Tat protein administered conjugated on RBC is not immunosuppressive. Results are the mean ± S.D. of eight mice for group 1 and six mice for group 3. The anti-avidin response in mice immunized with RBC–ubiquitin is not significantly different from that of mice immunized with RBC-Tat ($P > 0.05$).

[c] Percentage of neutralization of Tat-mediated transactivation by anti-Tat antibodies developed in each group of mice (groups 1–3) at the time of killing. Neutralization values were expressed as the percentage of inhibition of virus rescue assuming as 100% the rescue obtained with Tat alone or pre-incubated with preimmune sera (control sera). Results are the mean ± S.D. of six mice for each group. ND: not determined.

The induction of specific CTL was also investigated. Mononuclear cells purified from the spleen of immunized mice served as effectors. Control 3T3 fibroblasts, transfected with a pRPneo-c/Tat or pRPneo-c control plasmid were used as targets. Tat expression was monitored following transfection of cells with pU3RCAT where transcription of the reporter *CAT* gene is driven by an HIV-1 LTR promoter. Cytotoxic activity of the culture, assayed in standard 5 h ^{51}Cr-release assays, occurred both in mice treated with Tat and Freund's adjuvant and in mice treated with RBC-Tat but not in control mice. The anti-Tat CTL activities were comparable in the two groups of immunized animals (Fig. 6A and B). Thus, vaccination with RBC-Tat induced both cellular and humoral specific responses in mice.

4. Discussion

The immunogenicity of HIV-1 Tat has already been well documented in a number of studies [44,52–54]. Ab and CTL responses to Tat have been detected in humans and shown to correlate with non progression to AIDS [27,55,56]. Results from vaccination of non-human primates with biologically active Tat protein or Tat DNA showed containment of the infection in 9 out of 12 vaccinated cynomolgus monkeys for up to 2 years in follow-up studies [44,52,57]. Of importance, the use of a biologically active Tat protein for vaccination appears to be critical for the induction of a specific CD8$^+$ CTL response [19,27]. In this regard, it has been recently reported that biologically active Tat is

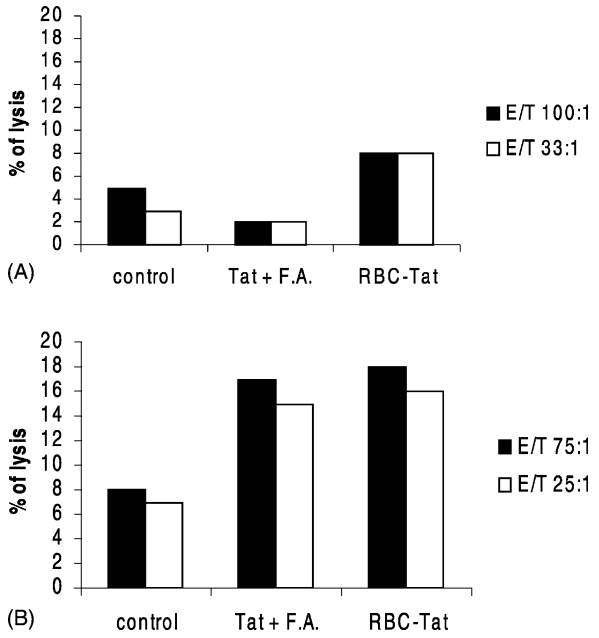

Fig. 6. CTL assays. Cytotoxic activity of mononuclear cells from mouse immunized with Tat in Freund's adjuvant or Tat bound to RBC was assayed in standard 5 h ^{51}Cr-release, as described in Section 2. All assays were performed in (A) with non-stimulated (ns) lymphocytes or in (B) with lymphocytes stimulated for 5 days by co-culture with 3T3-Tat expressing cells. The anti-Tat CTLs activities were not significantly different in the two groups of immunized animals ($P > 0.05$).

selectively and very efficiently taken up by MDDCs, promoting their maturation and Ag-presenting functions, driving Th1-type immune responses [26]. Notably these effects are abolished or greatly hampered by oxidation/inactivation of the protein [26]. Thus, biologically active Tat can act as both Ag and adjuvant, capable of driving T-cell mediated immune responses. However, the pleiotropic biological effects exerted by Tat on both viral and cellular gene expression [55,56,58–61], modulating the growth of several cell types [48,62–64] and acting on uninfected cells in a paracrine fashion [18,19,39,65–68], have been perceived as potentially harmful [69] and the use of oxidized Tat or of transdominant mutants lacking viral *trans* activation activity have been proposed [46–49]. Although vaccination with biologically active Tat protein or Tat DNA has been proven safe in mice, monkeys and humans [70], the use of Tat coupled to autologous RBCs through a biotin–avidin–biotin bridge represents an attractive alternative. In fact, biotinylation of native Tat abolishes the transactivating activity but does not affect recognition and uptake of Tat by MDDCs, a property considered essential to elicit cellular immunity ([26–37] and data not shown). Further, the bridged RBC-Ag delivery system has several potential advantages over conventional and new Ag delivery systems. The immunization strategies based on autologous RBCs, do not require adjuvants. In fact, the results reviewed in this paper and previous data [34–36,40,41] prove that this delivery system is capable of eliciting a significant humoral and cellular-mediated response, comparable or superior to than that obtained with Freund's adjuvant. Erythrocytes are naturally removed from circulation by macrophages, that are able to process and present antigens associated to MHC molecules to the immune system. [71]. In this regard we have recently shown that, in vitro administration to MDDCs of Tat in a particulate form, such as bound to RBCs, strongly enhances its imuunogenicity by measuring Tat-specific CD4$^+$ and CD8$^+$ T-cell responses ([37] and data not shown). Moreover should this antigen-delivery system also offers different advantages for vaccination in terms of prolonged intervals between immunizations and in the number of immunizations needed. In fact in mice RBCs are removed from circulation over a period of 8–10 days or longer ensuring prolonged uptake processing and presentation of the RBCs bound antigen by professional APCs. Finally, the amount of antigen is very low as compared to that as conventional administration and may represent a further advantage in terms of cost reduction when peptides or recombinant proteins have to be used. Here we show that in mice, vaccination with as little as 40 ng of Tat bound on the RBC surface, using an amount 250 times lower than that utilized to immunize with soluble Tat, is safe and induces both cellular and humoral immune responses, including specific CTLs and Ab capable of neutralizing the biological effects of Tat. Of note, both responses have been shown to be important. Anti-Tat CTLs have been found to inversely correlate with progression to AIDS in HIV-1 infected individuals [72], to exert significant immune pressure on the virus early after infection of macaques with SIV leading to the appearance of slowly replicating and apparently less pathogenic escape mutants [73], and to correlate with protection against a pathogenic SHIV challenge in monkeys vaccinated with Tat [28,44,57]. Similarly, Tat neutralizing Abs have been shown to significantly limit virus spreading in vitro [25,74] and may prevent, in vivo, HIV-1 transactivation in infected resting cells and the many biological effects of Tat on uninfected cells. However, HIV-1 Tat protein has been reported to suppress immune responses when administered in mice together with another Ag [46]. We have previously reported that immunization of mice with Ags coupled to RBCs through a biotin–avidin–biotin bridge elicits the production of anti-avidin Ab [45]. Therefore, we took advantage of our system and asked the whether injection in mice of Tat coupled to RBCs would suppress the induction of anti-avidin Ab. Indeed, both control animals receiving RBC–ubiquitin and mice receiving RBC-Tat seroconverted by week 6 and developed comparable anti-avidin Ab titers (Table 1), indicating that the administration of Tat coupled to RBCs is not immunosuppressive.

In conclusion, the delivery of very small amounts of Tat by autologous RBCs is safe, immunogenic and represents a further advancement in our knowledge and a further step in the design of a new vaccine which should certainly include Tat as one of the key antigens necessary in a prophylactic or therapeutic anti-HIV-1 vaccine.

Acknowledgements

This work was supported by grants from Project AIDS for vaccine development N.45 C/1.1, from the Istituto Superiore di Sanita (ISS) Rome ITALY, by grants from the AIDS National Program, The Italian Concerted Action on HIV-AIDS vaccine development (ICAV), ISS, Rome, and from the Associazione Nazionale per la lotta contro l'AIDS (ANLAIDS), Italy.

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