Effects of different routes of administration on the immunogenicity of the Tat protein and a Tat-derived peptide

Running title: Tat protein administered by different routes

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Abstract

The use of the Tat protein of HIV in vaccines against AIDS showed promising results in primate and human studies. To characterize the impact of the administration route on the induction of humoral responses at systemic and mucosal levels, we compared intradermal, intramuscular and mucosal immunizations with Tat and a Tat-derived peptide. Mice were immunized with the Tat protein by different routes and the titer and isotype of anti-Tat antibodies were assessed in serum and mucosal lavages. Intramuscular and intradermal administrations showed comparable immunogenicity, while the mucosal administration was unable to induce IgM in serum and IgG at mucosal sites but showed superior immunogenicity in terms of IgA induction. Anti-Tat antibodies were also obtained upon vaccination with the immunodominant Tat 1-20 peptide which was, however, less immunogenic than the whole Tat protein.

The Tat protein of HIV plays a key role in the viral life cycle and progression to AIDS [1-4] and anti-Tat humoral and cellular responses correlate with disease control in HIV-infected individuals. [5-11] Thus, the inclusion of Tat in preventive and therapeutic vaccines has been pursued by several groups showing promising results in nonhuman primates [12-16] and in phase I and II clinical trials.[16-19] However, the development of vaccines able to induce protective responses has to face some major challenges such as: i) the compliance requested for mass immunizations; ii) the induction of immune responses in mucosal tissues, which are the predominant sites of HIV acquisition; [20] and iii) the isotypes of antibodies elicited, which may influence the level of vaccine efficacy. [21] These factors may be modulated by the administration route. Intramuscular (IM) administration is widely used for vaccinations, although intradermal (ID) administration has been shown to be safe, well tolerated, less painful [22-24] and to require lower doses [22,25,26] due to the higher number of professional antigen presenting cells found in the skin than in the muscle. [27] Therefore, the ID route may be a relevant strategy for mass immunization, and clade B Tat protein has been administered by this route in two phase II clinical trials [17 and unpublished data] after having shown superior immunogenicity when administered ID rather than subcutaneously in phase I clinical trials. [18,19] At the same time, the oral administration is gaining interest for its high compliance and the capability of inducing mucosal responses. [28] However, to our knowledge, neither IM nor oral administration of the Tat protein have ever been compared to the ID route.

Thus, to assess the effects of the administration routes on the immunogenicity of the Tat protein, groups of mice were immunized with 30 μg of Tat [17] by ID, IM route or through the oral mucosa (OM) in the absence of adjuvants. ID and IM injections were performed in two sites on the back while, for OM immunization, mice were deprived of water 6 h before immunization and the immunogen was delivered in the mouth through a pipett tip. The dose of 30 μg was chosen because preliminary experiments showed that ID immunization with 30 μg of Tat protein induced higher anti-Tat IgG and IgM titers, as compared to immunization with 1 and 7.5 μg of protein (Fig. 1), and

its injection was safe and well tolerated by mice as demonstrated by periodical observation of spontaneous activity (Irwin Test) and histological studies of organs (not shown). [29]

Thus, animals were immunized at days 1, 14 and 28 by ID, IM or OM route. Serum and mucosal samples were collected at day 42 and tested for the presence of anti-Tat antibodies. [30,31]

Anti-Tat IgG were present in sera of all animals (Fig. 2A) with titers comparable among groups (Fig. 2B). In contrast, anti-Tat IgM in serum were present at similar levels in all mice injected IM or ID, but not in mice immunized OM (Fig. 2A and C). Interestingly, the OM route was the only one capable of inducing serum anti-Tat IgA antibodies in 71% of vaccinated mice (Fig. 2A and D).

To determine whether Tat immunization elicited antibodies in mucosal secretions, we next assessed anti-Tat humoral responses in vaginal and intestinal lavages. As shown in Fig. 3A, OM immunization did not induce anti-Tat IgG in vaginal secretions, that were barely detected in some mice injected ID or IM (Fig. 3B), while the different routes of administration did not show significant differences in terms of anti-Tat IgA responses (Fig. 3C). Analysis of intestinal lavages showed that the ID and IM routes induced higher titers of anti-Tat IgG than the OM administration (Fig. 3D-E), whereas only the OM route of immunization induced anti-Tat IgA in intestinal secretions (Fig. 3D and F, $p<0.05$). Thus, while IM and ID administration of Tat were comparable for location and titers of antibodies elicited, the OM route showed a different pattern of responses inducing preferentially IgA both in serum and in mucosal lavages. Further studies aimed at assessing dose-dependence of immune responses and biodistribution of the Tat protein may be important to define the most effective vaccination protocol. This is particularly relevant for OM immunization since the antigen may have several fates (mainly adsorbed through the oral mucosa, but also part of it may be ingested).

It has been recently shown that antibodies directed against the N-terminus region of Tat, which is the most immunogenic in terms of humoral responses, [32,33] protect monkeys from infection acquisition, [15] suggesting that the Tat 1-20 peptide constitutes an interesting candidate for a preventive vaccine. To assess how different routes of administration may affect humoral

immunogenicity of peptide vaccines, mice were vaccinated 3 times (at days 1, 14 and 28), by ID or OM route, with Tat 1-20 peptide (EPVDPRLEPWKHPGSQPKT, synthesized by solid phase method and purified by HPLC to >98% purity by UF Peptides, University of Ferrara, Italy), encompassing an immunodominant Tat epitope, [32,33] at the dose of 7 μg. This dose contains the same number of molecules of 30 μg of Tat protein. Serum samples were collected at day 42 to assess systemic humoral responses against the Tat 1-20 peptide. As shown in Fig. 4A-B, IgG antibodies were almost absent in mice vaccinated with the peptide, irrespective to the administration route, while they were present in all mice vaccinated with the whole protein (Fig. 2A), suggesting that the peptide is, at least in the absence of a proper adjuvant or delivery system, much less immunogenic than the whole Tat protein. Anti-Tat 1-20 IgA were absent in all groups while, consistent with what previously observed, anti-Tat 1-20 IgM were present at higher frequencies in mice immunized ID rather than OM, although differences did not reach statistical significance (Fig. 4A and C). All mice possessing antibodies against the peptide were also able to recognize the whole protein (not shown).

Interestingly, our results show that the Tat protein is immunogenic when administered orally. OM administration of the Tat protein failed in eliciting IgM but induced serum IgG responses and both serum and mucosal IgA. Although we did not evaluate immune responses induced by OM immunization with other proteins, which may differ from Tat for water solubility, stability and cell penetration capacity, these results show that this strategy may be further explored as a way to induce IgA mucosal responses. This may be of particular relevance for the development of vaccines against HIV/AIDS or other infectious diseases, which are transmitted mostly by mucosal routes. [34]

The immunogenicity of oral administration, which may be further boosted by proper adjuvants or different vaccination schedules, [28,35] could be due to the binding properties of Tat. Indeed, some studies have shown that the oral administration of antigens capable of binding to mucosal surface induces strong immune responses. [36] In fact, Tat has been reported to bind to heparan sulphate

proteoglycans present in the extracellular matrix and on the cell membranes [37,38] as well as to integrins present on the cellular surface of professional antigen presenting cells. [39]

However, some issues still need to be solved in the field of mucosal vaccines. Intriguingly, although mucosal responses were not investigated, data from the RV144 phase 3 trial suggests that serum IgA may actually abrogate vaccine efficacy conferred by serum anti-Env IgG. [40] Moreover, it is still unclear whether optimal mucosal immune responses are elicited by vaccines delivered mucosally or by systemic immunizations inducing strong immune responses crossing-over into mucosal sites. Although, ID, IM and OM routes induced comparable amounts of IgG in serum, the OM route almost failed in inducing IgG in intestinal and vaginal lavages, which were present at higher frequency and titers in ID and IM immunized animals. These differences may depend on the site of antigen presentation and thus to a different location of actively secreting plasmacells.

In conclusion, this study demonstrates that the IM administration of the Tat protein induced humoral responses comparable to those elicited by the ID route. Moreover, we explored the oral vaccination, a promising option which needs more formulation efforts to improve its efficiency further to the clarification of serum IgA role in protection from acquisition and progression to AIDS.

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Legends to figures

Figure 1. Characterization of anti-Tat humoral responses in sera. Serum samples of mice immunized three times ID with Tat protein at the dose of 1, 7.5 or 30 µg were collected at day 42 from retro-orbital plexus and the presence of anti-Tat IgG and IgM was evaluated by Elisa test. Briefly, 96-well plates were coated with Tat (100 ng/200 µl/well) in 0.05 M carbonate buffer (pH 9.6) for 18 hours at 4°C. Plates were washed with PBS containing 0.05% Tween 20 (Sigma-Aldrich, Milan, Italy), and incubated for 90 minutes at 37°C with blocking buffer. The following blocking buffers were used: PBS containing 0.05% Tween 20 and 1% BSA (for IgG) and PBS containing 0.05% Tween 20 and 3% BSA (for IgM). After extensive washes, serial dilutions of each serum were dispensed in duplicate wells (100 µl/well) and incubated for 90 minutes at 37°C. Plates were washed again before the addition of 100 µl/well of HRP-conjugated goat anti-mouse IgG or IgM (Sigma), and incubated at 37°C for 90 minutes. After incubation, plates were washed five times and subsequently a solution of 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (Roche) was added. The absorbance values were measured at 405 nm with an automatic plate reader (SUNRISE TECAN Salzburg-Austria). The cut-off value was estimated as the mean OD of 3 negative control sera plus 0.05. Each OD value was subtracted of the blank and cut-off values to obtain a net OD value and IgG titers calculated by intercept function. (**A**) Titers of serum anti-Tat IgG. (B) Titers of serum anti-Tat IgM. *$p<0.05$, **$p<0.01$ according to two-tailed Mann Whitney test. Results of 2 independent experiments are shown. Dots represent single mice and lines represent the means +/- SEM.

Figure 2. Characterization of anti-Tat humoral responses in sera. Serum samples of mice immunized three times ID, IM or OM with Tat protein (30µg) were collected at day 42 and the presence of anti-Tat IgG, IgM and IgA was evaluated by Elisa test. Elisa test for IgA evaluation was performed using PBS containing 0.1% Tween 20 and 1% BSA as block buffer. (**A**) Proportion of

295 mice that developed serum anti-Tat IgG, IgM and IgA. Frequencies of anti-Tat positive mice were
296 compared among different groups using two-tailed Fisher's exact test. Anti-Tat positivity was
297 determined by titers >100, 50 and 25 for IgG, IgM and IgA respectively. (**B**) Titers of serum anti-
298 Tat IgG. (**C**) Titers of serum anti-Tat IgM. (**D**) Titers of serum anti-Tat IgA. * $p<0.05$, **$p<0.01$
299 according to two-tailed Mann Whitney test. Results of 2 independent experiments are shown. Dots
300 represent single mice and lines represent the means +/- SEM.
301
302 **Figure 3**. Characterization of anti-Tat humoral responses in mucosal lavages. Mucosal samples of
303 mice immunized three times ID, IM or OM with Tat protein (30µg) were obtained at day 42 by
304 repeated flushing and aspiration with 0.5 ml of PBS containing a protease inhibitor cocktail (Roche,
305 Mannheim, Germany). The presence of anti-Tat IgG and IgA was evaluated by Elisa test.
306 Proportion of mice that developed anti-Tat IgG and IgA in (**A**) vaginal and (**D**) intestinal lavages.
307 Frequencies of anti-Tat positive mice were compared among different groups using two-tailed
308 Fisher's exact test. Anti-Tat positivity was determined by titers >50 and 25 for IgG and IgA
309 respectively. Titers of anti-Tat IgG in (**B**) vaginal and (**E**) intestinal lavages. Titers of anti-Tat IgA
310 in (**C**) vaginal and (**F**) intestinal lavages. * $p<0.05$ according to two-tailed Mann Whitney test.
311 Results of 2 independent experiments are shown. Dots represent single mice and lines represent the
312 means +/- SEM.
313
314 **Figure 4**. Characterization of anti-Tat 1-20 peptide humoral responses in sera. Serum samples of
315 mice immunized three times ID or OM with the Tat 1-20 peptide (7µg) were collected at day 42 and
316 the presence of anti-Tat 1-20 peptide IgG, IgM and IgA was evaluated by Elisa test (plates were
317 coated with Tat 1-20 peptide at the dose of 100ng/200µl/well). (**A**) Proportion of mice that
318 developed serum anti-Tat 1-20 peptide IgG, IgM and IgA. Frequencies of anti-Tat 1-20 peptide
319 positive mice were compared among different groups using two-tailed Fisher's exact test. Anti-Tat
320 1-20 peptide positivity was determined by titers >100, 50 and 25 for IgG, IgM and IgA respectively.

(**B**) Titers of serum anti-Tat 1-20 peptide IgG. (**C**) Titers of serum anti-Tat 1-20 peptide IgM. Results of 2 independent experiments are shown. Dots represent single mice and lines represent the means +/- SEM.