Efficient mucosal delivery of the HIV-1 Tat protein using the synthetic lipopeptide MALP-2 as adjuvant

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A major requirement for HIV/AIDS research is the development of a mucosal vaccine that stimulates humoral and cell-mediated immune responses at systemic and mucosal levels, thereby blocking virus replication at the entry port. Thus, a vaccine prototype based on biologically active HIV-1 Tat protein as antigen and the synthetic lipopeptide, macrophage-activating lipopeptide-2 (MALP-2), as a mucosal adjuvant was developed. Intranasal administration to mice stimulated systemic and mucosal anti-Tat antibody responses, and Tat-specific T cell responses, that were more efficient than those observed after i.p. immunization with Tat plus incomplete Freund’s adjuvant. Major linear B cell epitopes mapped within aa 1–20 and 46–60, whereas T cell epitopes were identified within aa 36–50 and 56–70. These epitopes have also been described in vaccinated primates and in HIV-1-infected individuals with better prognosis. Analysis of the anti-Tat IgG isotypes in serum, and the cytokine profile of spleen cells indicated that a dominant Th1 helper response was stimulated by Tat plus MALP-2, as opposed to the Th2 response observed with Tat plus incomplete Freund’s adjuvant. Tat-specific IFN-γ-producing cells were significantly increased only in response to Tat plus MALP-2. These data suggest that Malp-2 may represent an optimal mucosal adjuvant for candidate HIV vaccines based on Tat alone or in combination with other HIV antigens.

Key words: AIDS / Intranasal vaccination / Mucosal adjuvant / Tat

1 Introduction

The development of a vaccine against HIV/AIDS represents the only realistic hope to contain the spread of HIV infection, particularly in developing countries [1]. So far, most of the vaccination approaches have been based on the envelope antigens, in an attempt to induce neutralizing antibodies able to prevent infection (sterilizing immunity) [2, 3]. Unfortunately, no envelope-based vaccine prototype has been able to promote sterilizing immunity against homologous challenge [3]. The high variability of envelopes among different HIV clades prevents their use for the development of a universal vaccine. Therefore, the control of disease onset or progression appears as the most realistic end-point for an HIV/AIDS vaccine candidate. Towards this goal, innovative vaccine strategies are being presently developed to induce immune responses capable of controlling viral infection and preventing disease development or progression (non-sterilizing immunity).

The implementation of “reverse vaccinology” approaches has facilitated the development of vaccine candidates based on the regulatory proteins of HIV-1 [4]. In particular, recent studies have demonstrated that the HIV-1 Tat protein is an attractive vaccine candidate since: (i) Tat is expressed very early upon virus entry and even prior to virus integration [5]; (ii) it plays a key role in the viral life cycle [6, 7]; (iii) its sequence is highly conserved among different HIV subtypes, particularly in the functional and immunodominant regions of the product of the I exon [8]; (iv) cross-reactive anti-Tat immune
responses are elicited by natural infection with viruses belonging to different clades [8]; (v) the anti-Tat immune response correlates with non-progression in infected individuals [9–12]; and (vi) biologically active Tat targets DC and is efficiently taken up, induces DC maturation and antigen-presenting function, and drives Th-1 type immune responses [13]. Pre-clinical studies performed in macaques have demonstrated that parenteral immunization with biologically active Tat protein or tat DNA is safe, immunogenic and protects animals from challenge with the highly pathogenic virus SHIV89.6P [14–16]. Finally, Tat has been shown to be safe and immunogenic also in humans [17].

Most HIV infections occur either during sexual intercourse or as a result of mother-to-child transmission by breastfeeding. The efficient stimulation of both systemic and local mucosal immune responses may contribute to contain infection at the level of the portal of virus entry, reducing also the risk of viral transmission to uninfected individuals [18, 19]. Thus, the development of a vaccine candidate that is amenable for administration by the mucosal route constitutes a priority. However, antigens delivered mucosally are usually poorly immunogenic (because of e.g. degradation, rapid clearance and/or poor penetration). One of the potential strategies to overcome this problem is the use of mucosal adjuvants [20–23]. Unfortunately, only few mucosal adjuvants have been identified so far. Furthermore, their intrinsic toxicity and side-effects render difficult their implementation for the formulation of human vaccines. Although non-toxic derivatives have been generated to solve this problem [21, 24, 25], recent studies suggested that even non-toxic derivatives may lead to potentially severe side-effects [26]. Thus, mucosal adjuvants with adequate safety and efficacy profiles are urgently needed.

We have recently found that a synthetic derivative (S-[2,3-bispalmitoyloxypropyl] cysteinyl-GNDESNIFFKEK) of the Mycoplasma-derived macrophage-activating lipopeptide-2 (MALP-2) is a potent adjuvant when co-administered with antigens by the mucosal route [27]. MALP-2 is a powerful inducer of chemokines and cytokines as a result of signaling via Toll-like receptors 2 and 6 [28–30], which enhances humoral and cellular antigen-specific immune responses. In the present study we have investigated whether the HIV-1 Tat protein can be efficiently delivered by the intranasal (i.n.) route using MALP-2 as a mucosal adjuvant. Our results demonstrate that co-administration of Tat and MALP-2 stimulates cellular and humoral immune responses at both the mucosal and systemic levels. In addition, we were able to promote not only the production of Tat-specific antibodies at distant mucosal sites (i.e. the genitourinary tract), but also an efficient stimulation of IFN-γ-producing cells.

This suggests that MALP-2 may be a useful tool for the development of Tat-based mucosal vaccine formulations against HIV/AIDS.

2 Results

2.1 Intranasal vaccination with HIV-1 Tat protein co-administered with MALP-2 elicits systemic and mucosal humoral responses

To evaluate whether biologically active Tat protein can be efficiently delivered by the mucosal route using MALP-2 as adjuvant, mice were immunized via the i.n. route with Tat protein alone (10 μg/dose) or Tat admixed with MALP-2 (0.5 μg/dose) on days 0, 14 and 21. As a positive control, a group of animals was vaccinated by the i.p. route with Tat emulsified with incomplete Freund’s adjuvant (IFA).

As shown in Fig. 1A, i.n. immunization with Tat protein alone resulted in the induction of very low antibody titers only after the second boost (end-point titer = 1:123). In contrast, when MALP-2 was used as mucosal adjuvant, a rise in the antibody titers was observed after the first boost (end-point titer = 1:1,400), and by the end of the immunization protocol the titers were >1:135,000 (Fig. 1A). Interestingly, the overall efficiency of the serum antibody responses obtained using MALP-2 as a mucosal adjuvant was similar to that observed in the positive control group, in which animals were vaccinated by the i.p. route using IFA, which is an extremely potent adjuvant (Fig. 1A).

To investigate the capacity of MALP-2 to facilitate the stimulation of mucosal responses against Tat, the production of anti-Tat IgA was examined in lung and vaginal lavages from immunized animals (Fig. 1B). Whereas i.n. immunization with Tat alone induced very low levels of Tat-specific IgA in lung lavages (≈0.8% of the total IgA), a significant increase in the levels of antigen-specific IgA was detected in the animals immunized with Tat+MALP-2 (≈7.6%). Interestingly, the co-administration of MALP-2 also resulted in the stimulation of efficient IgA production in the genital tract, as demonstrated by the presence of high concentrations of Tat-specific IgA in vaginal lavages (≈6.9%). In contrast, mucosal responses against Tat were absent in animals vaccinated with Tat+IFA (data not shown).

2.2 Epitope mapping of anti-Tat systemic and mucosal antibody responses

In an attempt to identify the regions encompassing the linear B cell epitopes recognized after i.n. vaccination with the Tat protein co-administered with MALP-2,
epitope-mapping studies were performed. The most frequently recognized epitope was within residues 1–20 of the Tat sequence (Fig. 1C), as previously found in humans and monkeys [31–33]. This peptide was recognized by 80% of the vaginal lavages, and by all the lung lavages and sera from mice immunized with MALP-2+Tat. The serum antibodies from animals immunized with Tat+IFA also recognized this linear epitope. An additional B cell epitope was identified between residues 46–60, which was recognized by vaginal lavages (20%), lung lavages (50%) and sera (50%) from Tat+MALP-vaccinated mice, and by 100% of the sera from Tat+IFA-immunized animals. Finally, an epitope within the 73–86 sequence was recognized by 50% of the sera from animals immunized with Tat+IFA by the i.p. route.

2.3 Intranasal vaccination with biologically active Tat plus MALP-2 induces cellular immune responses, which map to specific epitopes of Tat, in both spleen and lymph nodes

The cellular immune responses induced by vaccination were evaluated by assessing the proliferative capacity of cells from either spleens or sub-mandibular lymph nodes; cells were isolated at day 31 after primary immunization and re-stimulated in vitro with Tat. Intranasal vaccination with Tat alone induced either no (draining lymph node) or a barely detectable (spleen) cellular proliferation, as already found in monkeys vaccinated with Tat alone [14]. In contrast, co-administration of Tat and MALP-2 triggered the induction of lymphoproliferative
Additional studies were performed to identify the regions encompassing the T cell epitopes recognized after vaccination with Tat+MALP-2. The strongest proliferation of splenocytes was observed after re-stimulation with peptides encompassing residues 36–50 and 56–70 (Fig. 2C). A proliferative response was also observed with peptides 1–20, 11–25 and 46–60, whereas no responses were observed against peptides 21–40, 66–80 and 76–90. No proliferation against Tat peptides was seen in mice immunized with Tat alone or Tat+IFA. These results indicate the presence of a strong T helper activity in the mice vaccinated with Tat+MALP-2.

### 2.4 Analysis of T helper responses after i.n. vaccination with Tat co-administered with MALP-2

To identify the dominant Th subpopulation stimulated by the different vaccination protocols, the subclass distribution of the Tat-specific IgG was evaluated in sera from vaccinated mice. The IgG1:IgG2a ratio was first determined, since these isotypes are considered to be the main ones stimulated by Th2 and Th1 cells, respectively [34]. Vaccination with Tat+IFA by the i.p. route stimulated a Th2-dominant response pattern (Fig. 3A). In contrast, the ratio was reduced when Tat was co-administered with MALP-2 by the i.n. route, suggesting a shift towards a Th1 response (Fig. 3A), which is consistent with the adjuvanticity of Tat toward Th1-type responses [13]. However, when all the IgG isotypes were considered, a different pattern emerged. As expected, in animals vaccinated with Tat+IFA the dominant isotype was indeed IgG1. In contrast, the IgG2b subclass was the prevalent isotype in animals vaccinated with Tat+MALP-2 (68% of total Tat-specific IgG, Fig. 3B). A similar pattern of IgG2a, IgG2b and IgG3 was observed in mice vaccinated with Tat alone, by the i.n. route (Fig. 3B); however the concentration of serum IgG was low.

To further characterize the main responding Th subpopulation, the content of IFN-γ, IL-2, IL-4, IL-5, IL-6 and IL-10 was measured in supernatants from spleen cells re-stimulated in vitro (Fig. 3C). In animals vaccinated with Tat+MALP-2, IFN-γ and IL-2 were the most prominent cytokines in comparison to the negative control group, suggesting that a dominant Th1 response pattern was stimulated. Some IL-6 was also detected in the supernatants of stimulated cells from Tat+MALP-2-vaccinated mice. However, the values were not significantly different from those in the controls (p>0.05). In contrast, in mice vaccinated with Tat+IFA, IL-6 was the dominant cytokine (Fig. 3C). Secreted IL-4, IL-5 and IL-10 were below the detection limit (data not shown). The observed cytokine responses were consistent with the IgG isotype response patterns observed in all groups (Fig. 3B).

### 2.5 Intranasal vaccination with biologically active Tat protein co-administered with MALP-2 induces Tat-specific IFN-γ-producing cells

We next evaluated by ELISPOT whether i.n. vaccination with biologically active Tat protein and MALP-2 was also able to induce IFN-γ-producing cells, which constitute a correlate for protection. For this reason, splenocytes from vaccinated animals were incubated for 16 h in the absence or presence of a pool of Tat peptides that were known to be targets for CTL in the murine system (ACTNCYCKKCCFHCQVCFIT [aa 21–40], WKHPGSQ-
PKTACTNC [aa 11–25], SQPKTACTNCYCCKKC [aa 16–30], KPQTSQSRGDPTGPK [aa 71–85] and QPKTACTNC [aa 17–25] [35] and unpublished data), and the number of IFN-γ-secreting cells was determined by ELISPOT. The results demonstrated that the frequency of IFN-γ-secreting cells was significantly higher in mice vaccinated with Tat protein + MALP-2 than in the other groups (Fig. 3D). This suggests that i.n. vaccination with Tat+MALP-2 leads to a modulation of the immune response towards the Th1 type, which has been associated with protection in non-human primate infection models.

3 Discussion

Recent studies suggest that HIV-1 regulatory proteins are promising vaccine targets for the induction of non-sterilizing immunity (i.e. control of viral infection and disease progression). In particular, it was shown that vaccination with biologically active Tat protein or tat DNA blocks virus replication and disease onset in vaccinated monkeys upon pathogenic virus challenge [14–16]. However, novel mucosal vaccination approaches aimed at inducing not only systemic but also mucosal immunity are urgently needed to either prevent or contain HIV infection.

We have recently demonstrated that i.n. co-administration of MALP-2 and soluble antigens results in a potent enhancement of humoral and cellular immune responses at both systemic and mucosal levels [27]. Thus, we decided to evaluate whether biologically active Tat protein can be efficiently delivered by the i.n. route using MALP-2 as mucosal adjuvant. The well-established synthesis, defined structure and stability of MALP-2 render it an attractive adjuvant for vaccine formulations. Furthermore, in contrast to traditional protein adjuvants, the poor immunogenicity of MALP-2 facilitates its incorporation in different vaccine formulations (i.e. it has a reduced risk of impaired responses resulting from pre-existing immunity).

The obtained results indicate that all arms of the immune system are efficiently stimulated when biologically active Tat is co-delivered with as little as 0.5 μg of MALP-2. High anti-Tat antibody titers were detected in sera from immunized animals, even after a single boost. The titers were as high as those elicited by i.p. immunization with Tat+IFA. In particular, secretory antibodies were only stimulated in animals vaccinated using MALP-2 as adjuvant. Moreover, Tat-specific IgA was detected not only at the local site of immunization (i.e. the respiratory tract), but also at the genital tract (=6.9% of the total IgA), which represents the portal of entry for most cases of heterosexual transmission.

Epitope-mapping studies revealed the presence of a major linear B cell epitope within residues 1–20, both in sera and in vaginal or lung lavages. An additional B cell epitope was identified between residues 46–60. These results are in agreement with previous reports in which B cell linear epitopes were identified in the murine BALB/c system [36, 37]. This is an important finding, since in vitro experiments have indicated that mAb against the N-terminal activation domain of Tat protein (Tat<sub>32,31</sub> and Tat<sub>3,12</sub>) and the basic region (Tat<sub>40,42</sub>) inhibit HIV-1 Tat-mediated LTR transactivation, and HIV-1 infection and replication in acutely and persistently infected human CD4<sup>+</sup> cells [36, 38, 39]. Of further importance is the fact that specificity against the same B cell epitopes was also found in sera of Tat-vaccinated monkeys, as well as in HIV-1-infected individuals [32, 33] and unpublished data), and that vaccination of monkeys with peptides encompassing these epitopes was able to control chronic viremia after challenge [40].

The in vivo relevance of these epitopes is also supported by epidemiological data, since it has been recently reported that individuals with anti-Tat antibodies do not appear to progress to AIDS [9, 11, 12, 41, 42] and that the presence of high antibody levels to Tat<sub>40,50</sub> and Tat<sub>56,60</sub> is associated with undetectable viral load [12]. Thus, mucosal vaccination with biologically active Tat protein + MALP-2 induces antibodies against epitopes that are associated with a better prognosis.

In animals vaccinated by the i.p. route with Tat emulsified in IFA, IgG1 was the dominant subclass (a classical Th2 type response pattern), whereas in those vaccinated with Tat+MALP-2, IgG2b was the major isotype. This bias does not seem to be a direct effect of MALP-2 alone, since IgG1-dominant responses were observed using other antigens in combination with MALP-2 [27]. Thus, the particular response pattern may depend on the combination of MALP-2 with Tat. The recent demonstration that biologically active Tat also exhibits immunomodulatory properties supports this hypothesis [13]. Interestingly, an increase in TGF-β has been observed in mononuclear cells from AIDS patients and HIV-infected monocytes, as well as in Tat-treated human bone marrow macrophages, and it seems that TGF-β stimulates IgG2b production [43–47]. This is consistent with the absence of IL-4 in the supernatants of spleen cells from mice vaccinated with Tat+MALP-2, since IgG2b is down-regulated by IL-4 [48]. Very little is known about the potential effector functions of IgG2b; however, IgG2b is the isotype with the highest affinity for Fc-RII and Fc-III Y receptors [49]. Whether the significant induction of mucosal IgA is also due to TGF-β stimulation remains to be elucidated.
In addition to humoral responses, strong Tat-specific proliferative T cell responses were also observed after immunization with Tat+MALP-2. Mapping studies showed that the major epitopes are encompassed within the residues 36–50 and 56–70. Of note, the 36–50 region was found to be a T-helper epitope also in vaccinated mice and macaques, as well as in infected humans [33, 50, 51]. The determination of the dominant cytokines produced by spleen cells from vaccinated animals suggests that Th1 cells are predominantly stimulated when Tat is delivered by the mucosal route using MALP-2 as adjuvant. These data are in agreement with the observed serum IgG response pattern. On the other hand, ELISpot studies demonstrated that, in contrast to what observed after i.p. vaccination with Tat+IFA, the mucosal immunization protocol with MALP-2 resulted in an efficient stimulation of Tat-specific IFN-γ-producing cells. This may be of relevance, since Tat-specific CTL, besides Tat-specific induction of TNF-α, was the only correlate of protection in monkeys vaccinated with HIV-1 Tat protein or DNA [14, 16].

Mucosal vaccination has already proven advantageous with respect to other routes, particularly in the context of a mass immunization campaigns to be carried out in developing countries, since it ensures high acceptance, compliance, minimization of cross-contamination and easy administration logistics. Our results suggest that MALP-2 is a very promising adjuvant for a mucosal immunization protocol with Tat as antigen. This protocol evokes humoral and cellular responses at both systemic and mucosal levels which mimic those observed in successfully immunized monkeys, and, more importantly, in HIV-positive individuals with good prognosis. Thus, MALP-2 plus Tat alone, or Tat combined with other regulatory or structural antigens, may constitute a promising vaccine candidate for the development of preventive and therapeutic mucosal vaccine strategies against HIV/AIDS.

4 Materials and methods

4.1 Animals and cell cultures

Female BALB/c (H-2^d) mice (6 weeks old) were purchased from Harlan-Winkelmann (Germany). Cells were grown in RPMI 1640 supplemented with 10% FCS, 100 U/ml of penicillin, 50 μg/ml of streptomycin and 1 mM L-glutamine (GIBCO BRL, Germany).

4.2 Antigen production

The Tat protein (aa 1–86) from the HTLV-IIIB isolate, BH10 clone (clade B), was expressed in Escherichia coli, purified by heparin-affinity chromatography and high-performance liquid chromatography and stored at −80°C. The purified Tat protein was fully monomeric [13, 14, 52], as determined by PAGE, Western blotting and high-performance liquid chromatography analysis, and had full biological activity as assessed by a virus transactivation assay and by uptake studies with dendritic cells [9, 10, 13, 14, 52]. MALP-2 was synthesized according to established protocols [53].

4.3 Immunization protocols

Groups of five mice were immunized on days 0, 14 and 21 by the i.n. route (10 μl per nostril) with Tat (10 μg) with or without MALP-2 (0.5 μg). The negative control group received only PBS, whereas the positive controls were vaccinated by the i.p. route (200 μl) with 10 μg of Tat protein emulsified in IFA (Sigma Chemie, Germany).

4.4 Sample collection

Serum samples were collected on days 0, 13, 20 and 31. Mice were killed on day 31 and vaginal and lung lavages were obtained by flushing the organs, with a final volume of 0.5 and 1 ml of PBS supplemented with 5% FCS and 40 μM PMSF, respectively. Lavages were then centrifuged (10 min at 13000×g) to remove debris and supernatant fluids were stored at −20°C. Sub-mandibular lymph nodes and spleens were removed and pooled for the analysis of cellular immune responses.

4.5 Detection of anti-Tat IgG in serum

Antibody titers were determined by ELISA using microtiter plates coated with 100 μl/well of Tat (1 μg/ml in 0.05 M carbonate buffer, pH 9.6) as previously described [27]. Endpoint titers were expressed as the reciprocal log of the last dilution that gave an optical density at 405 nm of 0.1 units above the values of the negative controls after 15 min of incubation.

4.6 Measurement of anti-Tat IgG isotypes

The amount of Tat-specific IgG subclasses present in serum samples was determined using an isotype-specific ELISA, as previously described [27].

4.7 Determination of total IgA and anti-Tat IgA

The amount of total and Tat-specific IgA present in lung and vaginal lavages was determined by ELISA, as previously described [27]. To compensate for variations in the efficiency of recovery of secretory antibodies among animals, the results were normalized and expressed as percentage of Tat-specific IgA with respect to the total amount of IgA present in the sample.
4.8 Epitope mapping

Epitope mapping was performed by ELISA. Due to the limited amount recovered, vaginal and lung lavages were tested simultaneously for epitope-specific IgG and IgA. Preliminary tests performed to detect Tat-specific IgG, IgA and IgG+IgA in these samples confirmed the absence of interference in this experimental setting. Purified synthetic peptides spanning the HTLV-IIIb, BH-10-clone-derived linear sequence of the Tat protein (aa 1–86) were designed to partially overlap the Tat sequence (aa 1–20, 21–40, 36–50, 46–60, 56–70, 65–80 and 73–86). All peptides were plated in 96-well plates at 250 ng/well (diluted in 200 μl of PBS with Ca2+ and Mg2+) and incubated overnight at 4°C. Wells were then blocked with PBS containing 1% BSA and 0.05% Tween-20 for 90 min at 37°C, and washed five times with PBS containing Tween-20 (0.05%). Sera (1:100), vaginal lavages (1:9) or lung lavages (1:8) diluted in blocking buffer were added to the wells (100 μl) and incubated at 37°C for 90 min. To correct for nonspecific binding, each sample was tested against each peptide and blocking buffer. Plates were washed and 100 μl of horseradish-peroxidase-conjugated goat anti-mouse-IgG (Sigma, MO, USA) or a mixture of goat anti-mouse-IgG and -IgA (lavages) was added to each well and incubated for 90 min at 37°C. After five washes, peptide-bound antibodies were detected by the addition of ABTS solution (Roche Diagnostics, Germany) for 50 min at 37°C.

4.9 T cell proliferation assays

Proliferation assays were performed in triplicates, as previously described [27]. The epitope mapping was carried out by re-stimulation of spleen cells for four days with partially overlapping synthetic peptides (aa 1–20, 11–25, 21–40, 36–50, 46–60, 56–70, 66–80 and 73–90) at a concentration of 50 μg/ml each. These peptides span the entire Tat sequence (aa 1–86) derived from the HTLV-IIIb, BH-10 clone. Peptide aa 76–90 comprises aa 76–86 derived from BH-10 plus aa 87–90 derived from the Tat-sequence of the HIV-1 clade B strain 89.6. The results are expressed as the ratio of the mean [3H]thymidine uptake of the stimulated:non-stimulated samples. A stimulation index >3 was considered positive.

4.10 Cytokine determination

Culture supernatants from proliferating cells were collected on days 2 and 4, and stored at −70°C. Determinations of IFN-γ, IL-2, IL-4, IL-5, IL-6 and IL-10 were performed by ELISA using commercial antibodies (Pharmingen), according to the manufacturer's instructions.

4.11 ELISPOT assay

To determine the concentration of IFN-γ-secreting cells, the murine IFN-γ (Euroclone, UK) ELISPOT kit was used. Cells (5×10^5/well) were incubated for 16 h in the absence or presence of a mixture of Tat peptides that were known to be targets for CTL in the murine system (see Sect. 2.5). After 16 h of culture, cells were removed and the colored spots were counted under a light microscope.

4.12 Statistical analysis

Comparisons between experimental groups were made by using Student's t-test. p<0.05 was considered as significant.

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References

10. van Baalen, C. A., Pontesilli, O., Huisman, R. C., Geretti, A. M., Klein, M. R., de Wolf, F., Miedema, F., Gruters, R. A. and Oster-
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