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Increasing the humoral immunogenic properties of the HIV-1 Tat protein using a ligand-stabilizing strategy

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Dedicated to the memory of André Ménez.

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Summary Tat is regarded as an attractive target for the development of an AIDS vaccine. However, works suggest that Tat is a poorly immunogenic protein and therefore we attempted to increase its immunogenic potency. As we observed that Tat is highly sensitive to enzymatic degradation *in vitro* we tried to make it less susceptible to proteolysis using ligands. We complexed Tat101 with various sulfated sugars and observed that some of these ligands made the protein more resistant to proteolysis and more immunogenic. In a more thorough study, we observed that a low-molecular-weight heparin fragment, called Hep6000, altered both the cell-binding capacity and transactivating activity of Tat101, suggesting that this sulfated polysaccharide can make the protein less toxic. Sera raised against Tat101 and Tat101/Hep6000 similarly bound mainly to the N-terminal region of the protein, indicating that formation of the complex does not alter the B-cell immunodominant region. Anti-Tat101/Hep6000 antisera neutralized the transactivating activity of Tat101 more efficiently than anti-Tat101 antisera. Altogether, these results indicate that stabilization of Tat101 using sulfated sugars increases its immunogenicity and might be of value in increasing its vaccine efficacy.

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Introduction

Several proteins from human immunodeficiency virus (HIV) are considered as attractive candidates for the formation of a therapeutic or a prophylactic vaccine against AIDS. Amongst these proteins, the transcriptional transactivator (Tat) could be particularly relevant since non-progression in infected individuals is correlated with the presence of high anti-Tat antibody titers [1—4] and of cytotoxic T-cells [5,6]. Furthermore, Tat is released by infected cells [7,8] and has numerous biological activities that might contribute either to the impairment of the immune response [9—11] or to viral dissemination [12—14] and pathogenesis [15—17], suggesting that the anti-Tat antibody response could contribute to protection through neutralization of these extracellular activities. Last, macaques immunized with biologically active Tat [18,19], Tat DNA [20] or recombinant vaccinia vectors expressing Tat and Rev proteins [21] are protected against a viral challenge.

Several additional observations also suggest that Tat may be an immunogen particularly suitable for vaccination. Thus, it was proposed to target monocyte-derived dendritic cells, enhance their maturation, function, and antigen (Ag)-specific T-cell responses [22]. Furthermore, it can trigger a humoral immune response in mice in the absence of adjuvant [23]. In contrast, Tat also has a number of less positive aspects. In macaques, the wild-type Tat protein had to be injected nine times in the presence of an adjuvant to raise an immune response sufficient to protect against a viral challenge [18]. Furthermore, extensive vaccination regimens with a Tat toxoid only attenuates disease in SHIV89.6PD intrarectally challenged macaques [24], while fewer immunizations failed to protect macaques against an intravenous challenge with SHIV89.6PD [25], suggesting that the immune response raised by the toxoid is inefficient. From these observations, we speculated that Tat has the unusual capacity to trigger the complex events required for the initiation of a humoral immune response, but needs to be modified to induce a strong and lasting neutralizing immune response in humans.

We previously showed that protein stability influences susceptibility to proteolysis by pronase (unpublished results), cathepsins and Ag-presenting cells (APCs) and thus controls the ability to stimulate T helper cells *in vitro* [26]. These observations, which are in agreement with those previously published by So et al. [27,28], suggest that protein stability can influence immunogenicity. The validity of this assumption was confirmed by a recent report showing that protein immunogenicity is increased when its susceptibility to enzymatic degradation is decreased [29]. Therefore, we anticipated that the immunogenicity of Tat could be enhanced if one could limit its susceptibility to proteolysis. This was all the more important as Tat is a highly flexible unfolded polypeptide [30] and it is well known that unfolded proteins are degraded very efficiently by proteases [31]. To make Tat more resistant to proteolysis, we decided to use an approach that could protect numerous putative cleavage sites of the protein. This approach consists in using Tat ligands, and was considered since previous reports showed that formation of complexes makes it possible to limit susceptibility of proteins to proteolysis [32—34].

In the present study, we first showed that Tat101 is highly susceptible to enzymatic degradation. Second, we showed that this susceptibility decreases when Tat is incubated with appropriate ligands, such as a heparin fragment of molecular mass of 6000 Da (Hep6000). Third, we showed that, in the presence of Hep6000, Tat loses its transactivating activity and its ability to bind cells. Fourth, we showed that, in mice, the Tat/Hep6000 complexes give an anti-Tat antibody response which is 6—100 times higher than that given by free Tat101 or a Tat101toxoid, and that the anti-Tat101/Hep6000 sera neutralize the transactivating activity of Tat101 more efficiently than the anti-Tat101 sera. From these observations, we conclude that stabilization of Tat101 by Hep6000 is an efficient way to increase the ability of the protein to raise a neutralizing humoral immune response, in mice. This stabilizing strategy led to the definition of a new Tat candidate vaccine whose prophylactic or therapeutic efficacy should be examined in non-human primates. Furthermore, we suggest that the ligand-stabilizing strategy might be an efficient way to increase the intrinsic immunogenicity of any candidate Tat vaccine able to bind sulfated polysaccharides, because Hep6000 also increases the humoral immune response raised against a Tat86 protein and a Tat101 derivative deprived of transactivating activity, and because the effect is also provided by pentosan polysulfate.

Materials and methods

Ags and anti-Tat monoclonal antibodies

Tat101, Tat86, Tat86C(22—37)Scam, Tat101R(52,53)Q and the 18 overlapping Tat peptides of 15 amino-acids long were chemically synthesized as previously described [23]. Wild-type heparin, heparin 6000 (Hep6000), heparin 3000 (Hep3000), dextran sulfate (DS) were purchased to Sigma—Aldrich. Pentosan polysulfate was kindly provided by doctor J.-F. Dhelys. For the preparation of the anti-Tat mAbs, Tat101/Hep6000 (5 μg) was repeatedly injected subcutaneously (s.c.) in BALB/c mice, in the absence of adjuvant. Three days before the fusion, Tat101 was injected intravenously. Splenocytes from immunized mice were then fused with the P3X63Ag8.653 mouse myeloma cell line (ATCC# CRL-1580) [35] according to standard protocols [36]. The specific hybridomas were identified using an enzyme immunoassay. Briefly, wells of microplates (Maxisorp® Immunoplate, Nunc, Denmark) were coated overnight with Tat or Tat peptides diluted to 10 μg/ml in a carbonate buffer (100 mM, pH 9). Wells were subsequently saturated with PBS containing 0.5% bovine serum albumin. After washings, the supernatants of the hybridomas (100 μl) were incubated overnight on the coated plates and binding was revealed using a goat anti-mouse IgG-peroxidase conjugate (Sigma—Aldrich) and 3,3′,5,5′-tetramethyl-benzidine (Sigma—Aldrich, France). ELISA experiments showed that the anti-Tat mAbs were not capable to bind Hep6000 (data not shown). The antibody-producing hybridomas were then subcloned and frozen. Monoclonal antibodies were produced in ascitic fluid by intraperitoneal injection of hybridoma cell lines into pristane-treated BALB/c mice. Experiments involving animals were conducted according to the institutional guidelines for animal care.

Proteolysis experiments

Susceptibility to trypsinolysis and chymotrypsinolysis of Tat101 and of three other unrelated proteins (hen-egg lysozyme, toxin alpha and a double domain derived from protein A, called ZZ) were investigated. The four proteins (5 μg/19 μl) were each incubated for 10 min at 37 °C with or without trypsin in a 50 mM phosphate buffer pH 7 (enzyme/substrate = 1/200). The samples were then subjected to reverse phase chromatography using an HPLC column to determine the amount of residual full-length antigen (ratio between the area of the peak corresponding to the antigen after 10 min with the enzyme and the area of the peak corresponding to the antigen in the absence of enzyme).

Kinetics of proteolysis of Tat101: Tat101 (10 μg/100 μl) was incubated at 37 °C with trypsin or chymotrypsin, in a 50 mM phosphate buffer pH 7 (enzyme/substrate = 1/200). The enzymatic reaction was stopped at various times (30 s, 1 min, 2 min, 5 min and 6 min). The samples were submitted to reverse phase chromatography coupled to mass spectrometry (LC—MS) to determine the Tat fragments generated at various times of digestion.

Susceptibility to proteolysis in the absence or presence of ligand:

- Analysis using high-performance liquid chromatography: Tat101 (10 μg/100 μl) was preincubated for 5 min at 37 °C or 1 h RT in a 50 mM phosphate buffer pH 7 in the absence or presence of either Hep6000 or Hep3000 (Sigma—Aldrich, France). Trypsin (enzyme/substrate = 1/200 or 1/600) was then added and the mixtures were incubated for 15 min at 37 °C. The reaction was stopped by the addition of a mixture containing TFA, NaCl and urea (2%, 1.6 M and 4.7 M final, respectively). The samples were injected into a C18 reverse phase HPLC column and the degradation profile was examined at 214 nm. The same procedure was used for the control peptide Tatp3.
- Analysis using an anti-Tat monoclonal antibody: Tat101 or Tat86 or Tat101 derivative (2.5 μg) was incubated with chymotrypsin (enzyme/substrate = 1/50). After 2 h at 37 °C, the reaction was stopped by addition of phenylmethylsulfonyl fluoride (PMSF) (5 mM final). Phosphate buffer pH 7 (300 μl) was added and the mixtures were serially diluted in an ELISA plate and left overnight at 4 °C. The plates were saturated with phosphate buffer, pH 7.2, containing 0.3% bovine serum albumin (BSA) and incubated for 1 h at 37 °C. The plates were then washed and the anti-Tat17S monoclonal antibody was added to the wells. After 2 h of incubation at room temperature, the plates were washed and antibody binding was revealed using a goat anti-mouse IgG-peroxidase conjugate (Jackson-ImmunoResearch, PA, USA) and 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) as substrate. The proportion of Tat that had resisted chymotrypsin was taken as the ratio between Tat in the presence of chymotrypsin and Tat in the absence of chymotrypsin, for an optical density of 1. The same ratio was calculated for the Tat derivatives. The stability of one Tat derivative, as compared with wild-type Tat, was determined by dividing the two ratios (ratio Tat derivative/ratio wild-type Tat).

Immunization of mice

For the immunizations in the absence of adjuvant, groups of four BALB/c mice (IFFA CREDO, France) were injected twice subcutaneously at 14-day intervals at the tail base with 100 μl of a PBS solution containing 5 μg of Tat101 or Tat101 derivative previously incubated with or without Hep6000. For the immunizations in the presence of Alum, groups of four BALB/c mice were injected once intraperitoneally with 100 μl of a PBS solution containing 5 μg of Tat101 or Tat101 derivative previously incubated with or without the different sulfated polysaccharides. Blood samples were collected 14 and 28 days after the last injection. For the assessment of the T-cell response, BALB/c mice were injected twice s.c. with 20 μg of Tat101 previously incubated in the presence or absence of Hep6000.

T cell stimulating assay

Thirty days after immunization of the mice, spleens were harvested and suspended in a proliferation medium containing 1% fetal bovine serum (FBS). Cells (5×10^5 cells/well) were cultured at 37 °C with serial dilutions of the different Ags. The presence of IL-2 in culture supernatants was determined after a 24-h period by measuring the proliferation of an IL-2-dependent cytotoxic T cell line (CTLL), using methyl^{3}H thymidine ([^{3}H] TdR; 5 Ci/mmol, Amersham, England).

Ab titration by enzyme immunoassay

ELISA plates were coated overnight with either Tat101 or Tat101 derivatives (0.1 μg/well) in 0.05 M phosphate buffer pH 7.4 at 4 °C. Plates were then saturated with 0.1 M phosphate buffer pH 7.4 containing 0.3% BSA. Individual antisera were serially diluted in the same buffer containing 0.1% BSA and incubated in the wells overnight at 4 °C. Binding of Abs was assessed using a goat anti-mouse IgG peroxidase conjugate and ABTS. The titers were defined as the highest serum dilution giving an absorbance value of 0.6 above the negative control. For this control we used pooled sera collected before immunization of mice. For subclass IgG analysis binding of the anti-Tat101 antibodies was assessed according to the same procedure using a goat anti-mouse IgG1 peroxidase, and a goat anti-mouse IgG2a peroxidase (Bethyl laboratories). For IgM analysis, binding was assessed using a goat anti-IgM biotinylated and streptavidin-peroxidase (Amersham, UK).

Mapping of the B-cell epitopes

ELISA plates were coated overnight with the 18 overlapping peptides (1 μg/well) as described above. Pooled antisera collected 28 days after the immunizations were serially diluted in 0.1 M phosphate buffer pH 7.4 containing 0.1% BSA and incubated in the wells overnight at 4 °C. Binding of Abs was assessed using a goat anti-mouse IgG peroxidase conjugate and ABTS as substrate.

Cell-binding assay

Tat101 was preincubated in a PBS buffer pH 7.4 containing 0.5% BSA for 15 min at 4 °C with or without serial dilutions of Hep6000. The mixtures were then added to murine splenic cells (2×10^5 cells/well). After 30 min of incubation at 4 °C, cells were washed and an anti-Tat mAb conjugated to fluorescein (anti-Tat12S, specific to the N-terminal region of Tat) was added. After 30 min at 4 °C, cells were washed and binding of the fluorescent antibody was assessed by FACS analysis. Percentage of binding was expressed as a ratio (binding of antiTat12S in the presence of Tat101 and Hep6000 least binding in the absence of Tat101 and Hep6000 divided by binding of antiTat12S in the presence of Tat101 least binding in the absence of Tat101 and Hep6000).

Transactivation assay

Transactivation assay was performed as previously described [23]. Tat101 was preincubated with or without Hep6000. The mixtures were added to a HeLa cell line over-expressing green fluorescent protein (EGFP), in the presence of 100 μM chloroquine. After 3 h at 37 °C, 10% FBS containing Dulbecco's modified Eagle's medium (DMEM) was added. 45 h later, cells were harvested and fluorescence intensity was determined by FACS analysis.

Neutralization assay

The neutralizing capacity of the antisera was evaluated by measuring their ability to inhibit the transactivating activity of Tat101 using a HeLa cell line previously transfected with a plasmid coding for the LTR of HIV-1 and for β-galactosidase, called P4 [37] and kindly provided by Dr. Olivier Schwartz. In these experiments, the pooled antisera result from bleeding made at day 28 (immunizations in the absence of adjuvant) or day 42 (immunizations with Alum) after the last injection. They were serially diluted in a DMEM medium supplemented with 100 μM chloroquine in the presence of a fixed amount of Tat101 (15 nM). The mixtures were then transferred to 96-well plates containing P4 cells (10^4 per well). After 2 h of incubation at 37 °C, a DMEM medium containing sodium pyruvate (10 mM), antibiotics and 10% FBS was added. 60 h later the medium was removed and cells were lysed using PBS containing $MgCl_2$ (5×10^{-3} M), NP40 (0.1%) and CPRG (6×10^{-6} M) (Roche) as substrate. The β-galactosidase activity was then measured at 570 nm using an ELISA reader. For each antiserum, inhibition was defined as the ratio between the β-galactosidase activity in the presence of the pooled antisera and its activity in the presence of a non-immune serum.

Results

Tat101 is highly susceptible to proteolysis

As Tat is a highly flexible unfolded polypeptide [30] and as denatured proteins are highly susceptible to enzymatic proteolysis [31], we examined the degradation of Tat101 in mild conditions of proteolysis (E/S = 1/200). We compared Tat101 to three unrelated natively folded proteins, i.e. hen egg-white lysozyme (HEL) [38], toxin alpha from *Naja nigricollis* [39], and ZZ, a double domain derived from protein A of *Staphylococcus aureus* [40]. We incubated the various proteins for 10 min at 37 °C, with or without either trypsin or chymotrypsin. Following HPLC chromatography of the samples, we measured the area of the peak corresponding to the full-length protein. 103.3 ± 4.6% of toxin alpha, 105.9 ± 5.6% of lysozyme, 94.9 ± 2.4% of ZZ, and 4.5 ± 5.9% of Tat101 were found after trypsinolysis. 106.3 ± 5.6% of toxin alpha, 105.3 ± 4.5% of lysozyme, 104 ± 8.3% of ZZ, and 6.7 ± 1.1% of Tat101 were found after chymotrypsinolysis. These results indicated that, in mild conditions of proteolysis, Tat101 is strongly degraded by the two enzymes, whereas the three other proteins remain mainly intact, which strongly suggests that Tat101 is highly susceptible to proteolysis. To study the kinetics of proteolysis of Tat101, we incubated it with trypsin and chymotrypsin for various times and then submitted the samples to HPLC chromatography coupled with mass spectrometry (LC—MS), to identify the fragments generated by each enzyme. Thus, we observed that, after only 30 s of incubation with trypsin (Fig. 1 top), one cleavage (residues K19-T20) occurred in the vicinity of the cysteine-rich region and another (residues K29-C30) in the cysteine-rich region between residues 22 and 37. Several other cleavages appeared later in other areas of Tat101, particularly in the basic-rich region between residues 49 and 57. In the presence of chymotrypsin (Fig. 1 bottom), a first cleavage (residues 38—39) also occurred after only 30 s of incubation, but the cut was generated in the core area that contains most of the hydrophobic residues of Tat101. Several other cleavages appeared later in other areas of the protein. These results indicated that Tat101 is susceptible to tryptic and chymotryptic proteolysis.

Heparin fragments decrease susceptibility of Tat101 to proteolysis

We postulated that the high susceptibility of Tat101 to proteolysis might reduce its immunogenicity. This assumption prompted us to try to decrease the proteolytic susceptibility of Tat101. We decided to complex Tat101 with two heparin fragments of respective molecular masses 6000 Da (Hep6000) and 3000 Da (Hep3000), since Tat is a heparin-binding protein [41] which also binds with various affinities to low-molecular-weight heparin fragments [42,43]. To examine whether the two ligands can make Tat101 less susceptible to degradation, we preincubated Tat101 with or without either Hep3000 or Hep6000, and then added trypsin. After 15 min of incubation, we stopped the enzymatic reaction and injected the samples into an HPLC column. As shown in Fig. 2, two peaks were observed in the absence of enzyme. The peak of retention time 9.4 min was found in all the chromatograms and corresponds to the urea used to improve dissociation of the ligands. The heterogeneous peak with a retention time ranging from 21 to 23 min corresponds to full-length Tat101. This fraction contains the fully reduced protein and a mixture of Tat101 proteins with at least one intramolecular disulfide bond appearing during the incubation in PBS buffer (see Fig. 1B and [23]). In the presence of trypsin, the peak corresponding to the full-length protein

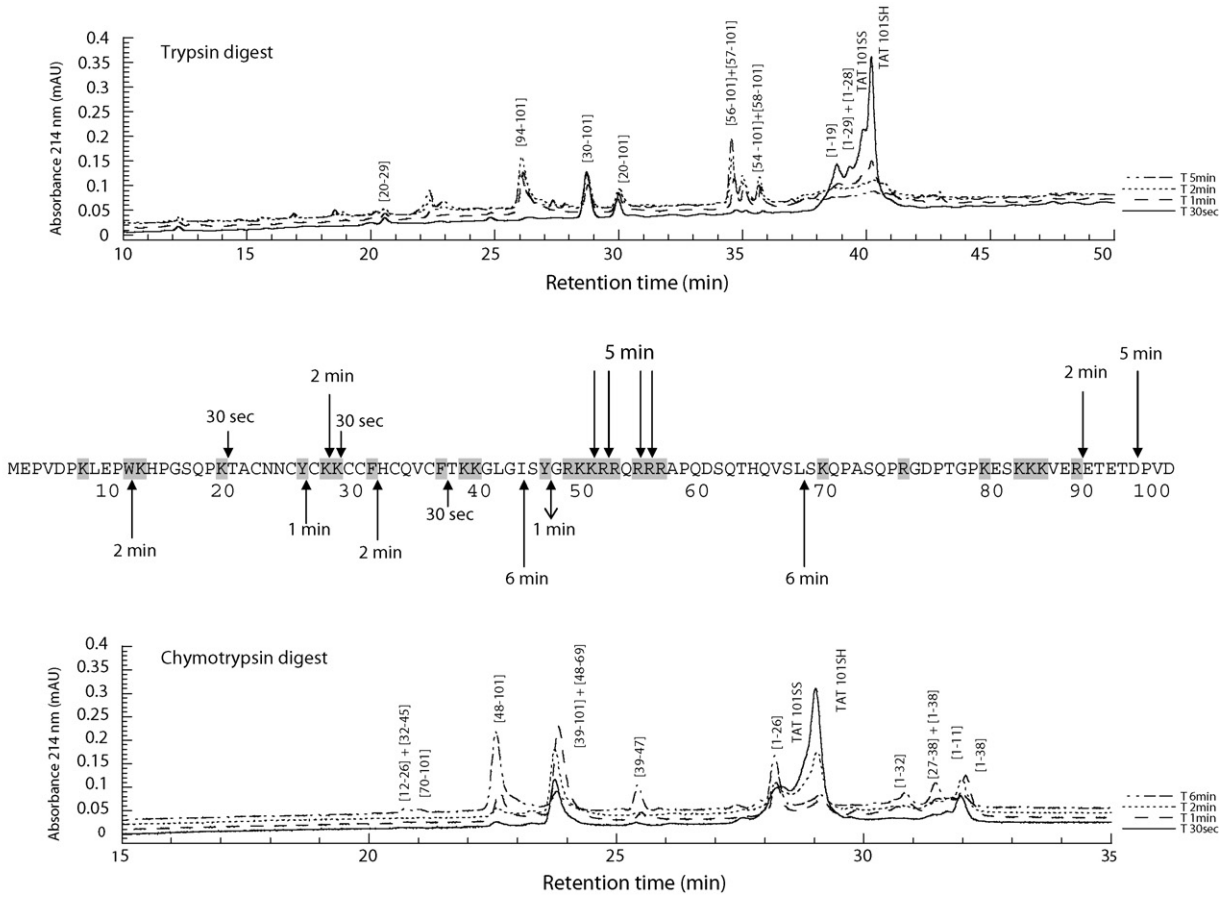

Figure 1 Tat101 is highly susceptible to proteolysis. Tat101 was incubated for various times at 37 °C with trypsin or chymotrypsin. The samples were then submitted to a reverse phase chromatography coupled to mass spectrometry (LC—MS) to determine the Tat fragments generated at various times of digestion. The arrows and times shown along the sequence indicate respectively the location of the cleavage sites and their time of appearance.

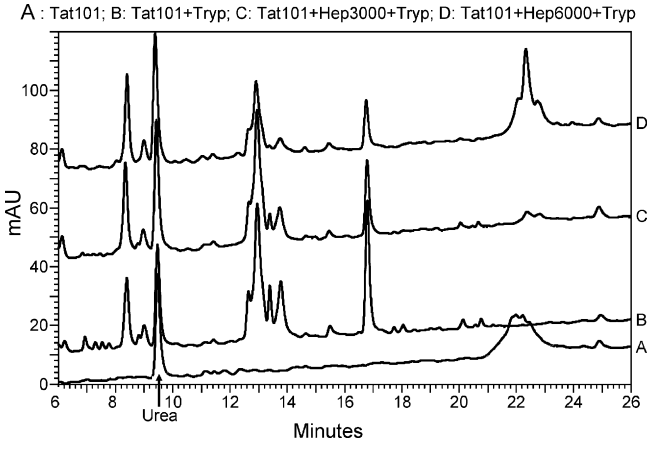

Figure 2 Hep6000 makes Tat101 less susceptible to proteolysis. Tat101 was preincubated in the absence or presence of either Hep3000 or Hep6000. Trypsin was added for 15 min at 37 °C. The samples were then injected into an HPLC column in order to estimate the residual amount of full-length Tat101. The data are representative of two separate experiments.

vanished while numerous peaks corresponding to Tat fragments were found at retention times ranging between 6 and 21 min. In the presence of heparin3000, a similar profile of degradation products was observed. However, the sum of their areas represented 61.2% of that found in the absence of sulfated polysaccharide. Furthermore, a tiny peak corresponding to full-length Tat101 was found at 22.3 min. In the presence of Hep6000, degradation products were also observed but the sum of their areas represented 40% of that found in the absence of sulfated polysaccharide. Furthermore, a peak corresponding to full-length Tat101 was found at 22.5 min. These results indicated that the two heparin fragments decreased the susceptibility of Tat101 to proteolysis and that Hep6000, which has a higher affinity for Tat ([42,43] and our unpublished results), is more efficient than Hep3000. To evaluate whether this occurs through direct inhibition of the enzyme, we did control experiments using a Tat peptide called Tatp3 (sequence WKHPGSQPKTACNNC), which does not bind heparin and which is efficiently cleaved by trypsin (not shown). We incubated Tatp3 for 15 min with or without trypsin and either Hep6000 or Hep3000, stopped the enzymatic reaction, and injected the samples into an HPLC column to estimate the residual amount of full-length peptide (data not shown). After trypsin treatment, 16% of Tatp3 remained intact in the absence of the sulfated

polysaccharides and, in their presence, this value was not increased. We concluded that Hep6000 and Hep3000 do not alter the enzymatic activity and that it is the binding of these fragments to Tat101 that makes the protein antigen less susceptible to trypsin action.

We then used an immunoenzymatic assay to examine whether the two previous ligands can protect a B-cell antigenic site from proteolysis. We preincubated Tat101 with or without each of these two ligands and added trypsin or chymotrypsin. After 2 h of incubation, we stopped the enzymatic reaction using PMSF and coated the mixtures on ELISA plates. Finally, we added a monoclonal anti-Tat antibody, called anti-Tat17S. Anti-Tat17S is specific to the region 41—50 (not shown) which is cleaved early by chymotrypsin (see Fig. 1 bottom). After chymotrypsinolysis, binding of the antibody to the plates was 5.1 and 8.3 times higher when Tat was previously incubated with Hep3000 and Hep6000, respectively, indicating that both ligands preserve the antigenic site. After trypsinolysis, antibody binding was not increased when Tat was previously incubated with Hep3000. In contrast, it was 24.9 times higher when Tat was previously incubated with Hep6000, indicating that Hep6000 also protects the B-cell epitope against trypsinolysis. As Hep6000 was more protective than Hep3000, we examined the biological and immunological properties of a Tat101/Hep6000 complex.

Hep6000 alters the ability of Tat101 to bind spleen cells and abrogates its transactivating activity

Tat has a considerable number of biological activities [44] some of which might contribute to its toxicity [9—11,15—17]. Therefore, it is difficult to evaluate if all these activities are abrogated when Tat is complexed with Hep6000. However, numerous Tat biological activities depend on binding to cells [13,45—50]. Therefore, to have a first indirect estimate of the abrogation of such activities we studied whether Hep6000 inhibits binding of Tat101 to murine splenic cells. As shown in Fig. 3A, binding of Tat101 to splenocytes decreased as a function of the amount of Hep6000. Similar results were obtained with various cell lines (not shown), indicating that the ligand alters Tat101 binding to numerous cell types and suggesting that formation of Tat101/Hep6000 complexes might alter some of the biological activities that depend on binding to cellular targets. We then evaluated if Hep6000 influences the transactivating activity of Tat101. We preincubated Tat101 with or without the sulfated polysaccharide and added the mixtures to a HeLa cell line transfected with a plasmid coding for the LTR sequence of HIV-1 and green fluorescent protein [23]. As shown in Fig. 3B, in its free form, Tat101 transactivated efficiently as it increased EGFP expression by the HeLa cell line. In contrast, no EGFP overexpression was observed in the presence of Hep6000, indicating that the sulfated polysaccharide alters the transactivating activity of Tat101. Therefore, abrogation of both cell-binding capacity and transactivating activity suggests that the sulfated polysaccharide might inactivate several biological activities of Tat101 and thus might make it less toxic.

Heparin6000 increases the immunogenicity of Tat101

To evaluate if Hep6000 increases the immunogenicity of Tat101, we immunized groups of BALB/c mice with either Tat101 or Tat101/Hep6000. After two immunizations in the absence of adjuvant, the free protein raised a humoral immune response (Fig. 4A), but the resulting anti-Tat101

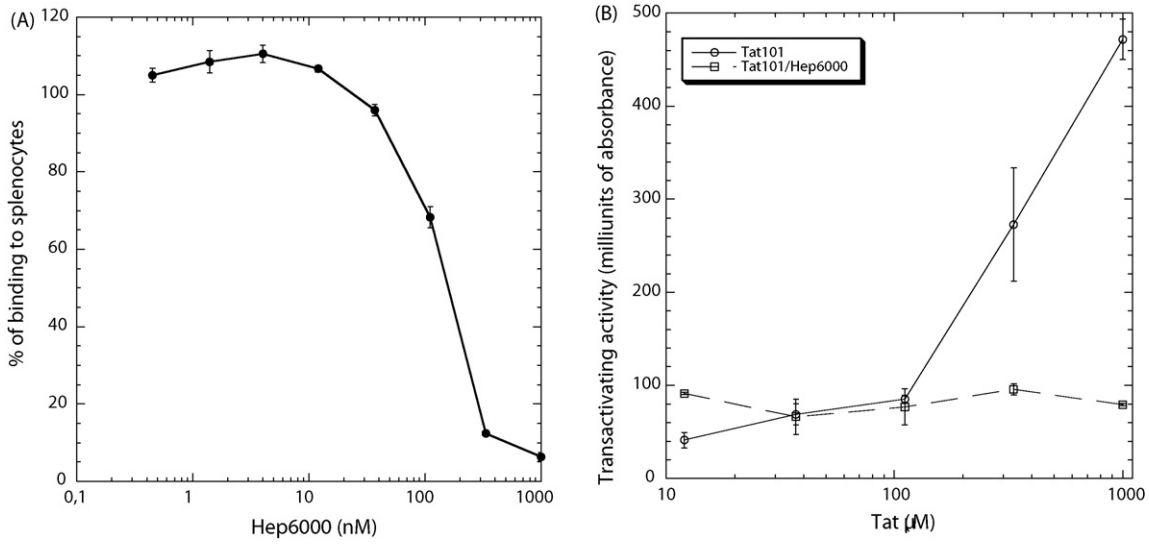

Figure 3 Hep6000 alters the ability of Tat101 to bind cells and to transactivate. (A) A fixed amount of Tat101 was preincubated with serial dilutions of Hep6000. The mixtures were incubated with splenocytes and after 30 min the N-terminal specific anti-Tat12S monoclonal antibody conjugated to fluorescein was added. Antibody binding was assessed by FACS analysis. The data shown are representative of three separate experiments. (B) A HeLa cell line stably transfected with a plasmid coding for the LTR sequence of HIV-1 and for the sequence of EGFP was incubated with Tat101 with or without Hep6000. After a 45-h incubation period, EGFP expression was examined using FACS analysis. The data shown are representative of two separate experiments.

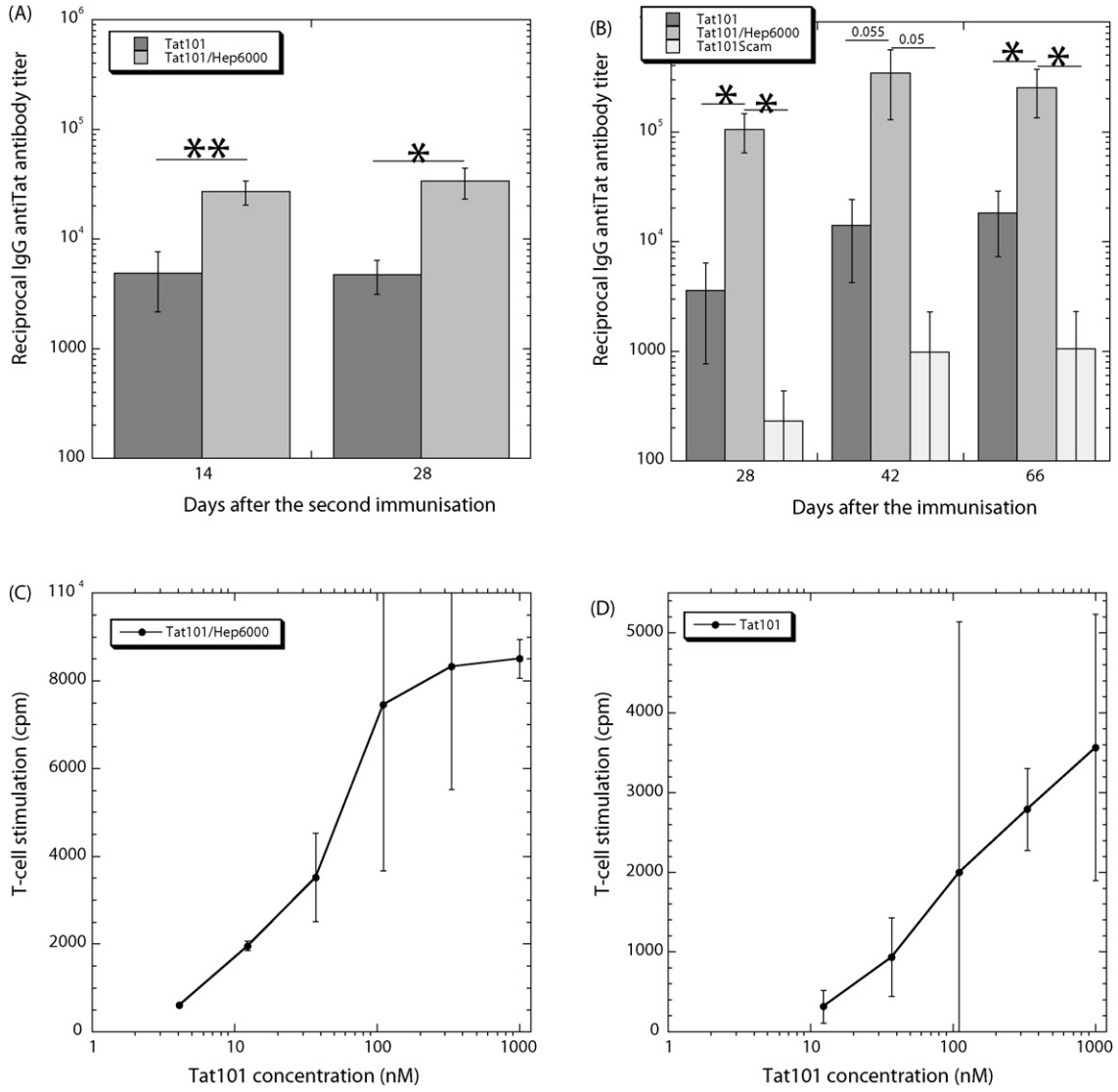

Figure 4 Immune response of BALB/c mice immunized with Tat101/Hep6000. (A) Groups of four BALB/c mice were immunized twice s.c. at a 14-day interval in the absence of adjuvant with 5 μg of Tat101 previously incubated with or without Hep6000. Mice were bled on different days after the second immunization and the presence of anti-Tat Abs was assessed using an enzyme immunoassay. (B) Tat101 was previously incubated with or without Hep6000. Tat101/Hep6000, Tat101 and Tat101Scam were then mixed with Alum and injected once i.p. into groups of four BALB/c mice. Mice were bled on different days after the immunization and the presence of anti-Tat Abs was assessed using an enzyme immunoassay. $^{*}p$-value < 0.05; $^{**}p$-value < 0.005. (C) Tat101 (20 μg) was preincubated with Hep6000. The mixture was injected twice s.c at a 14-day interval into BALB/c mice. 30 days later, spleens were collected and splenocytes were challenged with serial dilutions of Tat101. After a culture period of 24 h, each supernatant was assayed for its capacity to stimulate incorporation of (^{3}H) thymidine in an IL2-dependent CTLL. (D) Tat101 was injected in the absence of Hep6000 and its ability to raise a T-cell response was assessed using a protocol similar to that described in C.

antibody titer was roughly six times lower than that given by the Tat101/Hep6000 complex, also injected with no adjuvant, indicating that Hep6000 increases the immunogenicity of Tat101. To evaluate whether the oligosaccharide provides a similar enhancement in the presence of an adjuvant, we mixed Tat101 and Tat101/Hep6000 with Alum and immunized groups of BALB/c mice with these samples. We also immunized mice with a Tat101 toxoid, called Tat101Scam. The Tat101 toxoid was previously prepared by alkylation with iodoacetamide, since it was previously shown that this reagent inactivates Tat [51]. Thus, we observed that the complex raises an anti-Tat antibody response approximately 10- and 100-fold higher ($p < 0.05$) than that provided by the free Tat101 molecule and Tat101Scam, respectively (Fig. 4B). Therefore, in the presence of adjuvant, Hep6000 increases the humoral immune response directed against Tat101 and makes the protein more immunogenic than a chemically inactivated Tat101 protein.

We previously observed that Tat101 is capable to raise a T-cell response in mice, in the absence of adjuvant [23]. We, next, examined whether Tat101 remains capable to raise a T-cell response when it is injected in the presence of

Hep6000. We injected BALB/c mice twice at a 14-day interval with the mixture or with Tat101 alone, in the absence of Alum. Thirteen days after the second immunization, we collected spleens and incubated the splenocytes with serial dilutions of Tat101. After 24 h at 37 °C, IL-2 was found in the cell supernatants from Tat101/Hep6000 immunized mice (Fig. 4C) and Tat101 (Fig. 4D), indicating that Tat101 remains capable to raise a specific T-cell response when it is injected in the presence of Hep6000.

The predominant B-cell epitopes of Tat101 are preserved in the Tat101/Hep6000 complex

To assess whether immunization with Tat101/Hep6000 could provide an antigenic profile different from that generated during the immunization with free Tat101, we mapped the Tat101 B-cell epitopes using pooled sera resulting from the immunizations performed with Tat101 or Tat101/Hep6000 previously mixed with Alum. We assessed the binding of the sera to ELISA plates previously coated with eighteen 15mer peptides overlapping the polypeptide chain. The two sera had similar abilities to bind to peptides 1, 2 corresponding to the region 1—20 of Tat and to peptides 15, 16 corresponding to the area 71—85 of Tat101 (Fig. 5A and B). Therefore, the injection with Hep6000 did not modify the pattern of antibody response raised against the immunodominant B-cell epitope located in the N-terminal region of the molecule and against the subdominant B-cell epitope located in the region 71—85. To determine whether Hep6000 changes the polarization of the immune response, we determined the proportions of anti-Tat IgM, IgG_1 and IgG_{2a}. We found that the anti-Tat antibodies resulting from the immunizations with Tat101 and Tat101/Hep6000 were predominantly of the IgG_1 isotype (not shown), indicating that the isotypic profile is preserved and suggesting that the immune response remains mainly Th2 polarized in the presence of Hep6000.

Neutralizing potency of the anti-Tat antisera

We investigated whether the transactivating activity of Tat101 is neutralized *in vitro* by pooled antisera resulting from the immunizations described above (see Fig. 4A and B). We mixed a fixed amount of Tat101 with or without the different pooled antisera and added these mixtures to a HeLa cell-line previously transfected with a plasmid coding for the LTR of HIV-1 and β-galactosidase [37]. After 2 h of incubation at 37 °C, we added a DMEM medium containing 10% FBS. Sixty hours later, we lysed the cells and revealed the β-galactosidase activity using CPRG as substrate. For the immunization in the presence of Alum, the ability to inhibit the transactivating activity followed the order anti-Tat101Scam serum < anti-Tat101 serum < anti-Tat101/Hep6000 serum (Table 1) and therefore varies according to the sera and to the anti-Tat101 antibody titer (see Fig. 4). For the immunization in the absence of adjuvant, the anti-Tat101 serum inhibited the activity less efficiently than the anti-Tat101/Hep6000 serum. These observations indicated that the Tat101/Hep6000 complex raises the highest neutralizing antibody response and that the neutralizing capacity is correlated with the level of anti-Tat antibody response.

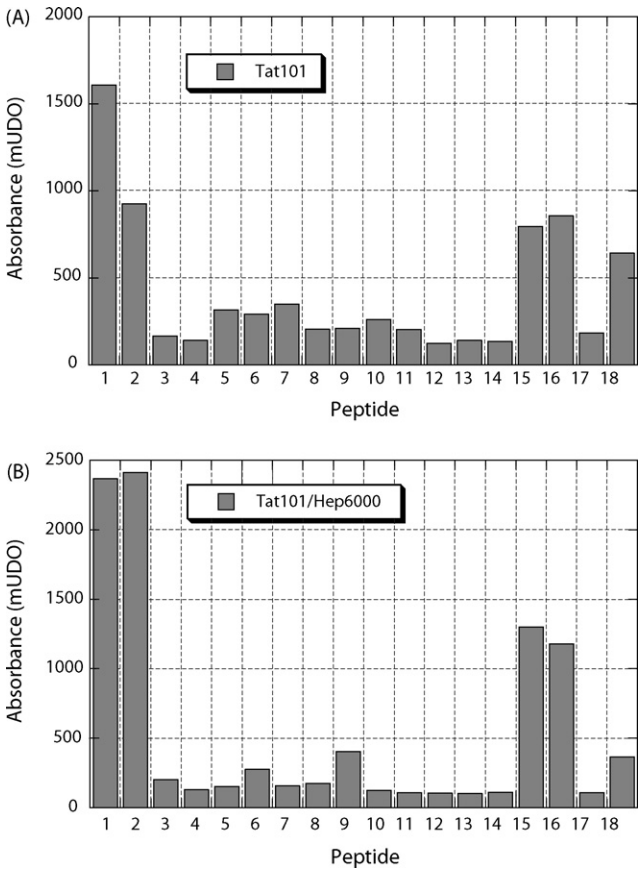

Figure 5 Mapping of the Tat101 B-cell epitopes resulting from the immunizations with or without heparin6000. Pooled sera collected 28 days after the immunizations with Tat101 (A) or Tat101/Hep6000 (B) were tested for their ability to bind 18 overlapping 15-mer peptides representing the sequence of Tat101. Binding of the anti-Tat Abs to the peptides was assessed using an enzyme immunoassay.

Table 1 Neutralizing potency of the anti-Tat antisera

Antiserum raised against	% of inhibition of βGal activity	
	1/45 dilution	1/135 dilution
Tat101 adjuvant-free	67	11
Tat101/Hep6000 adjuvant-free	93.5	59
Tat101 + Alum	54	23
Tat101/Hep6000 + Alum	87.8	43.4
Tat101Scam + Alum	24	0

Tat101 was mixed with different pooled antisera or non-immune serum. The mixtures were then added to P4 cells and β-galactosidase expression, which reflects the transactivating activity of Tat101, was examined using CRPG as substrate. Percentage of inhibition was defined as the ratio between the β-galactosidase activity in the presence of the antiserum and its activity in the presence of non-immune serum. The data shown are representative of two separate experiments.

Proteolytic stability and immunogenicity of various Tat molecules in the presence of different glycosaminoglycans

To assess whether or not the increase in proteolytic stability and in immunogenicity is a property restricted to the combination between Hep6000 and Tat101, we examined whether the phenomenon is also observed with pentosan-polysulfate (PPS) and dextran sulphate (DS) [52—54] and Tat86. We also used Tat101R(52,53)Q, since this disubstituted Tat101 derivative is devoid of transactivating activity [23], and as it binds heparin with an affinity similar to Tat101 (not shown). We first investigated susceptibility to proteolysis. PPS and DS made Tat101 and Tat86 less susceptible to proteolysis and Hep6000 made Tat101R(52,53)Q more resistant to enzymatic activity (Table 2). We injected five groups of BALB/c mice, without adjuvant, with Tat86, Tat86/Hep6000, Tat86/PPS Tat86/DS, and a Tat86 protein previously inactivated by alkylation of its seven cysteines (Tat86Scam). The anti-Tat86 antibody response followed the order: anti-Tat86Scam < anti-Tat86 < anti-Tat86/DS < anti-Tat86/PPS < anti-Tat86/Hep6000 (Fig. 6A). When Tat86 was associated with PPS or Hep6000, the antibody titers were significantly higher ($p < 0.05$) than when it was injected in a free form. However, the difference was not significant when DS was used as ligand. When we immunized BALB/c mice with Tat101R(52,53)Q in its free form, or previously complexed to Hep6000 (Fig. 6B), the presence of Hep6000 increased the anti-Tat101R(52,53)Q antibody response approximately 3-fold. The difference was not significant at day 14 ($p = 0.072$) and became significant at day 28 ($p < 0.03$). Altogether these results indicate that the increase in proteolytic stability and in immunogenicity is a property shared by complexes associating different Tat molecules and different sulfated polysaccharides.

Table 2 Proteolytic stability of various Tat molecules in the presence of different sulfated polysaccharides

Antigen	Relative amount of Ag bound by mAb antiTat17S after chymotrypsinolysis
Tat86	1
Tat86/Hep6000	4.3
Tat86/Dextran	2.9
Tat86/PPS	5.6
Tat101	1
Tat101/PPS	11.1
Tat101/Dextran	3.3
Tat101R(52,53)Q	1
Tat101R(52,53)Q/Hep6000	3.9

Tat101 was preincubated in the absence or presence of either DS or PPS. Tat86 and Tat101R(52,53)Q were preincubated with or without heparin6000. Chymotrypsin was added and the mixtures were incubated for 2 h at 37 °C. The reaction was stopped with PMSF and susceptibility to proteolysis was assessed using the enzyme immunoassay described in the second paragraph of section ''Heparin fragments decrease susceptibility of Tat101 to proteolysis''.

Discussion

The goal of this work was to increase the immunogenicity of Tat in order to improve its efficacy as a potential

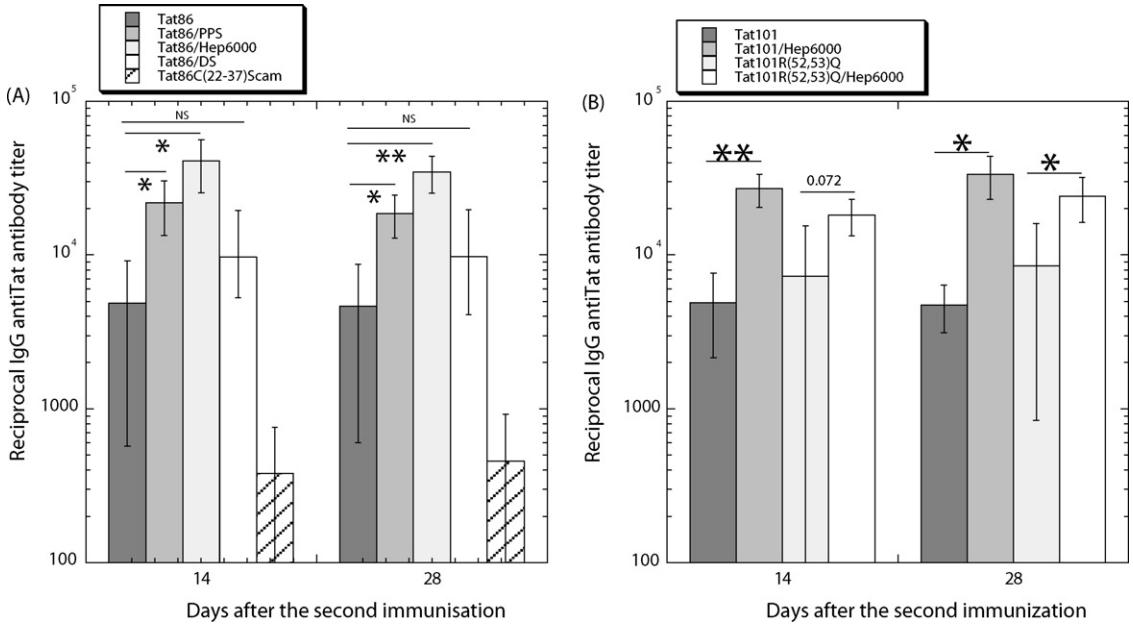

Figure 6 Immunogenicity of various Tat molecules in the presence of different sulfated polysaccharides. (A) Tat86 was preincubated in the absence or presence of either PPS, or DS or Hep6000. BALB/c mice were injected twice at a 14-day interval in the absence of adjuvant with the different mixtures or Tat86C(22—37)Scam (5 μg per dose). Mice were bled 14 and 28 days after the immunization and the presence of anti-Tat Abs was assessed by an enzyme immunoassay. (B) Tat101 and Tat101R(52,53)Q were preincubated with or without Hep6000. BALB/c mice were injected as described in (A).

vaccine. To increase the immunogenic potency of Tat we made it more resistant to proteolysis. We chose this strategy because three types of observations suggested that this stabilizing approach could particularly suit with our objective. Firstly, our laboratory [26] and others [27,28] had previously shown that protein stability influences proteolysis by pronase, cathepsins and APCs and thus controls T-cell presentation, suggesting that immunogenicity depends on Ag stability. This assumption was recently validated by studies carried out in the mouse model [29]. Secondly, NMR studies showed that a Tat protein of 86 residues is extremely flexible and deprived of α-helix and β-sheet [30], suggesting that the Tat86 proteins, which are mainly encoded by laboratory isolates, are natively unfolded polypeptides [55] and might therefore be degraded very efficiently by proteases [31]. Furthermore, the Tat proteins of 101 residues, which are mainly encoded by primary isolates, might have similar structural characteristics since we observed that the Tat101 protein used in this work gives a circular dichroism spectrum similar in shape to that of Tat86 protein (not shown) with the same negative signal at 200 nm, which is characteristic in both cases of a lack of organized secondary structure. These data suggest that Tat101 is also a natively unfolded polypeptide. Thirdly, we observed that Tat101 is more susceptible to proteolysis by trypsin than three other unrelated proteins, and that it is cleaved in hardly 30 s of incubation with trypsin or chymotrypsin for E/S ratios 10—30 times lower than those classically used in this type of experiment, indicating that it is particularly sensitive to enzymatic action.

As we had no information concerning the cleavage sites generated during the *in vivo* degradation of Tat, we decided to stabilize it using ligands. This approach proved to be effective in making other proteins less susceptible to proteolysis [32—34]. Furthermore, it did not require any modification in the protein sequence and thus allowed the preservation of the T-cell epitopes. As Tat is a heparin-binding protein [41] and binds to heparin fragments [42,43], we evaluated whether its proteolytic susceptibility is decreased in the presence of two heparin fragments, Hep6000 and Hep3000, which differ in their affinity for Tat [42,43]. We found that Hep3000, which has the lowest affinity for Tat, is poorly protective, while Hep6000 makes the protein significantly more resistant to trypsinolysis and chymotrypsinolysis. The protective effect is not due to a direct inhibition of the enzymes since in control experiments we observed that a peptide that does not bind to Hep6000 and to Hep3000 is similarly cleaved in the presence or absence of these sulfated polysaccharides (unpublished results). Therefore, we concluded that Hep6000 makes Tat less susceptible to proteolysis, presumably by masking some of its cleavage sites.

As Hep6000 was more protective, we evaluated its effect on the immunogenicity of Tat101. We first monitored the anti-Tat immune response of BALB/c mice injected, with either Tat101 or Tat101/Hep6000, with or without adjuvant. In the presence of Hep6000, the anti-Tat antibody titers were 6—10 times higher than those given by the free protein. Furthermore, the anti-Tat101/Hep6000 sera neutralized the transactivating activity of Tat101 more efficiently than the anti-Tat101 sera. Lastly, splenocytes from mice immunized with Tat101/Hep6000 secreted IL-2 when incubated *in vitro* with Tat101. These results therefore indicated that Hep6000 increases the neutralizing antibody response raised against Tat101 and that the protein Ag remains capable to raise a specific T-cell response. Further examination showed that the anti-Tat antibodies present in the sera resulting from the immunizations with Tat101 and Tat101/Hep6000 are both of the IgG1 subclass (not shown), and that the sera both recognize the area 1—20 of Tat, which is immunodominant in mice [56,57], monkeys [25], and humans [57], and the sub-dominant regions 71—85. We concluded that Hep6000 increases the neutralizing antibody response raised against Tat101 while preserving the Th2 polarization, the T-helper cell response, and most of the predominant B-cell epitopes.

The large variety of Tat biological activities [44], some of which might contribute to make it toxic [58], prompted us to evaluate whether Hep6000 can affect these activities. We first found that Hep6000 inhibits binding to splenocytes and to various cell lines (not shown), suggesting that it could alter the numerous biological activities of Tat that require cell binding [13,45—50]. We then examined the transactivating activity of Tat101 and observed that Hep6000 inhibits this activity and thus behaves like other sulfated sugars [42,43,52,54] with respect to this activity. We hypothesize that the sulfated polysaccharide can inhibit several activities of Tat101 and might thus contribute to its inactivation. This assumption must now be validated by experiments designed to determine if the Tat101/Hep6000 complex is safe in vivo.

Interestingly, resistance to proteolysis and increase in immunogenicity are not properties restricted to the wild-type Tat101 and Hep6000 pair. Thus, Tat101 is made less susceptible to chymotrypsinolysis in the presence of other polysaccharide ligands, such as pentosan polysulfate and dextran sulphate [52—54]. Moreover, Hep6000 and PPS increase the immunogenicity of a Tat protein of 86 residues. Lastly, the $Tat101_{R(52,53)Q}$ derivative, which is deprived of transactivating activity [23], raises a higher humoral immune response when it is injected with Hep6000. Therefore, we postulate that most of the Tat proteins can be made more resistant to proteolysis and more immunogenic if they are previously associated with appropriate sulfated sugars. It is interesting to note that Tat86/PPS raised antibody titers roughly 40 times higher than those raised by Tat86Scam, because Tat86Scam is a Tat86 protein previously inactivated by alkylation of its seven cysteines according to a protocol similar to that used to prepare a toxoid of Tat86 [51] that could only attenuate the clinical signs of the disease in macaques challenged with SHIV89.6PD [24]. We therefore postulate that such a toxoid might raise a stronger immune response following its stabilization using appropriate sulfated polysaccharides. We postulate that this stabilizing approach might be used to increase the immunogenicity of other Tat candidates vaccines and, in particular, that of a disulfide-mediated Tat dimer that we previously showed as being capable to raise an immune response in the absence of adjuvant [23].

The stabilizing approach described in this report offers several advantages. Firstly, it increases the immunogenicity of Tat and, in particular, the neutralizing antibody response. Secondly, it does not modify the Tat sequence and thus preserves its T-cell epitopes. Thirdly, it preserves the predominant B-cell epitopes of Tat and its T-stimulating capacity. Fourthly, it makes possible to inhibit the transac-

tivating activity of Tat101 and its capacity to bind cells and might thus contribute to its inactivation. Fifthly, it can be applied easily to other Tat candidate vaccines [23,24,59]. Our data suggest that the stabilizing approach offers the opportunity to produce new, highly immunogenic, candidate vaccines whose toxicity and therapeutic or prophylactic effectiveness needs now to be evaluated in non-human primates.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2008.02.057.

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