Important B-cell epitopes for neutralization of human immunodeficiency virus type 1 Tat in serum samples of humans and different animal species immunized with Tat protein or peptides

E. Moreau, G. Belliard, C. D. Partidos, F. Pradezinsky, H. Le Buanec, S. Muller and C. Desgranges

Correspondence
C. Desgranges
claude.desgranges@chu-stlouis.fr

1E03-34 INSERM, Institut de Génétique Moléculaire, Hôpital St Louis, 27 rue Juliette Dodu, 75010 Paris, France
2UPR9021 CNRS, Institut de Biologie Moléculaire et Cellulaire, 67000 Strasbourg, France
3Centre de Recherches des Cordeliers, Université Pierre et Marie Curie, 75005 Paris, France

The Tat regulatory protein of human immunodeficiency virus type 1 (HIV-1) is secreted by infected cells and plays a key role in viral pathogenesis and replication. Tat protein has been proposed as a target antigen for vaccine design since anti-Tat antibodies may interfere with virus spread and disease progression. The aim of this study was to analyse the serum antibody response of mice, rabbits, macaques and humans immunized with recombinant Tat, synthetic Tat, Tat toxoid or Tat peptides and to examine the biological properties of these antibodies in terms of Tat-induced transactivation and HIV-1 replication. Only sera with antibody specificity to both N-terminal and basic functional domains were able to inhibit extracellular Tat-dependent transactivation significantly in vitro. Antibodies from a human subject immunized with Tat also reduced HIV-1 replication in acutely infected T cells and blocked reactivation of virus replicating low levels in chronically infected cells by exogenous Tat. These results demonstrate that immunization with Tat protein or a combination of synthetic Tat peptides elicits the production of Tat-neutralizing serum antibodies and suggest that Tat vaccination could be used to block in vivo extracellular Tat autocrine/paracrine transactivation of HIV-1 replication.

INTRODUCTION

The Tat protein of human immunodeficiency virus type 1 (HIV-1) is critical in AIDS pathogenesis, particularly in the progression of HIV infection and the development of Kaposi’s sarcoma and neurological disorders (Gallo, 1999; Goldstein, 1996; Rubartelli et al., 1998). Tat is an 86–101 aa transcriptional activator encoded by two exons and is highly conserved in both inter- and intra-patient variants (Goldstein et al., 2001). Tat is one of the first genes expressed during HIV-1 infection and is essential for viral gene expression and virus production (Dayton et al., 1986). Despite its nuclear localization and function, Tat is also released extracellularly by infected cells (Frankel & Pabo, 1988) and endocytosed by neighbouring cells where it translocates to the nucleus in an active form (Ensoli et al., 1993). Extracellular Tat contributes towards disabling the cellular immune response by inducing apoptotic cell death (Bartz & Emerman, 1999; Westendorp et al., 1995) and inhibiting T-cell proliferation (Chirmule et al., 1995; Viscidi et al., 1989). It also interacts with a variety of surface receptors, including integrin receptors and members of the vascular endothelial growth factor family (Albini et al., 1998; Brake et al., 1990a), and activates various intracellular signal transduction pathways (Borgatti et al., 1997; Ganju et al., 1998; Gibellini et al., 1998). Finally, extracellular Tat favours viral infection by increasing expression of HIV coreceptors in susceptible cells (Huang et al., 1998; Secchiero et al., 1999).

Anti-Tat antibodies are present in the sera of HIV-1-infected patients (Reiss et al., 1991; Rodman et al., 1993). Epidemiological studies have shown that high titres of anti-Tat antibodies correlate with the maintenance of long-term non-progression status in the course of HIV-1 infection (Re et al., 2001; Richardson et al., 2003; Zagury et al., 1998). Natural IgM antibodies reacting with Tat may influence the course of AIDS progression and provide an early initial defence against the pathological effects of the Tat protein after HIV infection (Rodman et al., 1993, 2001). In vitro, anti-Tat antibodies abrogate Tat-dependent TNF-α secretion by monocytes (Bennasser et al., 2002), inhibit Tat-induced Kaposi’s sarcoma cell proliferation (Ensoli et al.,...
1990) and block Tat transactivation of the HIV-1 LTR (Brake et al., 1990b). Furthermore, monoclonal antibodies interfering with extracellular Tat modulate HIV-1 replication in infected cell cultures (Moreau et al., 2004; Re et al., 1995; Steinaa et al., 1994), suggesting that Tat may be a possible target for specific immunotherapy in HIV-1-infected patients. Immunization with a chemically inactivated Tat toxoid elicits high titres of anti-Tat antibodies in the serum of human volunteers (Gringeri et al., 1999).

Two immunodominant human B-cell epitopes with very limited antigenic polymorphism have been identified in the N-terminal (aa 1–20) and basic (aa 44–61) regions of the Tat protein (Noonan et al., 2003). Vaccination with short synthetic peptides covering these epitopes (aa 4–16 and 53–66) induced only moderate protection after virus challenge in macaques (Goldstein et al., 2000). However, encouraging results have been obtained in animals vaccinated with the active Tat protein (Cafaro et al., 1999) or its toxoid form (Pauza et al., 2000), although no protection was observed against challenge with a highly pathogenic strain of simian/HIV (Silvera et al., 2002).

In this study, we analysed the antibody response against Tat in the sera of rabbits, monkeys, mice and humans immunized with the full-length recombinant Tat protein, synthetic Tat, Tat toxoid or Tat synthetic peptides. The capacity of these serum antibodies to block extracellular Tat transactivation and to reduce virus replication in infected cell cultures was analysed with regard to their fine specificity.

**METHODS**

**Proteins and peptides.** Recombinant Tat (rTat) was a gift from Aventis Pasteur. Briefly, a GST–Tat plasmid that encoded the 86 aa Tat protein (Herrmann & Rice, 1995) was used for expression in *Escherichia coli*. GST–Tat was purified from bacterial lysates using a single-step procedure under non-denaturing conditions and Tat was cleaved from the fusion protein by digestion with thrombin (Rhim et al., 1994). In addition, buffer was supplemented with 5 mM diethiothreitol to prevent Tat oligomerization during storage in aliquots at –80°C. The synthetic 86 aa Tat protein (sTat) was synthesized using optimized 9-fluorenylmethoxycarbonyl (Fmoc) chemistry protocols with a multichain peptide synthesizer (Neirmark & Briand, 1993; O. Chaloin & J. P. Briand, unpublished data). The sTat protein was lyophilized and stored at –20°C to prevent oxidation. Just before use, it was dissolved in PBS, pH 7.4. Both rTat and sTat were manipulated on ice to prevent denaturation and kept away from light. Tat toxoid was generated from rTat by carboxymethylation of cysteine residues with iodoacetamide. This chemically inactivated Tat protein retains full antigenicity but lacks the biological properties, rTat protein was diluted in DMEM/10% FCS overnight. To assay the transactivation ability of Tat preparations, rTat and sTat were dissolved in DMEM/10% FCS (final volume 500 μl) supplemented with 100 μM chloroquine (Sigma) and added to the cells. After 24 h, cells were washed twice with PBS and incubated for an additional 24 h in fresh DMEM/10% FCS. Cells were lysed and CAT production was assayed with a CAT ELISA kit (Roche Diagnostics) following the manufacturer’s instructions. To test the anti-Tat antibody neutralizing properties, rTat protein was diluted in DMEM/10% FCS (final volume
500 µl) supplemented with 100 µM chloroquine to a final concentration of 25 ng ml⁻¹ and incubated with various dilutions of immune serum at 37 °C for 1 h. The mixture was then added to the cells and the procedure carried out as described above.

Inhibition of virus replication. H9 cells (1 × 10⁵) were infected with the HIV-1 IIIB laboratory strain (p24 concentration of 200 ng ml⁻¹) at 37 °C in 5% CO₂ overnight. Cells were washed twice in culture medium and seeded (4 × 10⁶ cells per well) in 96-well plates with or without different dilutions of antisera. The cultures were incubated at 37 °C in 5% CO₂ for 5 days. Aliquots of culture supernatants were sampled daily and replaced by fresh medium containing the respective concentrations of antibodies. Virus replication was monitored by quantification of HIV-1 p24 core antigen using a capture ELISA as described previously (Cartier et al., 1999).

Anti-Tat antibodies were added to long-term chronically HIV-1 IIIB-infected H9 cells with or without rTat protein (100 ng ml⁻¹) for 4 or 6 days and the p24 concentration measured as described above.

RESULTS

Reactivity of serum with Tat protein and peptides

The reactivity of serum samples with different synthetic Tat peptides and rTat and sTat proteins was measured by ELISA and titres are presented in Table 1. The mapping of different B-cell epitopes recognized by the immune sera was performed using overlapping 15-mer peptides spanning the entire Tat sequence (1–102) and the results are shown in Table 2.

Antisera from mice Mur1 and Mur2 immunized by the intranasal route with sTat protein displayed similar antibody titres to sTat and rTat proteins and to peptides covering the N-terminal region. However, only the Mur1 antiserum exhibited specific reactivity with peptides 44–61 and 45–59 from the basic region of Tat. In Western blots, both antisera stained a 14 kDa band that corresponded to Tat protein (Fig. 1a).

Serum from rabbit Rab1 immunized with peptide 8–53 displayed a high IgG antibody response to the N-terminal Tat domain (peptides 1–15, 1–20, 5–19 and 9–23) and was able to recognize rTat and sTat in ELISA. Rab1 antibodies also reacted with rTat in Western blotting (Fig. 1b). As expected IgG antibodies from rabbit Rab2 immunized with peptide 19–53 exhibited a weak or no reactivity against peptides covering the N-terminal region of the protein. However, they reacted strongly with peptides 37–51, 41–55 and 44–61 overlapping the core and the basic domains of Tat. Rab2 antiserum weakly recognized rTat and sTat in ELISA but bound strongly in Western blotting to the denatured rTat (Fig. 1b). IgG antibodies from rabbit Rab3 immunized with peptide 44–61 were highly specific to the immunizing peptide (titre = 102 400). They did not cross-react with any other overlapping peptides of this region including peptides 41–55 and 45–59. While Rab3 antibodies exhibited a high IgG titre against sTat (titre = 12 800) they recognized rTat only weakly in ELISA (titre = 800) and not at all in Western blotting (Fig. 1b). Rabbit Rab4 immunized with rTat produced a strong antibody response to the N-terminal domain (peptides 1–15 and 1–20) as well as a moderate response to peptide 44–61 encompassing the basic region (titre of 3200). Rab4 antibodies also recognized rTat both in ELISA (titre = 102 400) and in Western blotting (Fig. 1b). Strikingly, Rab4 antiserum displayed only weak reactivity to sTat in ELISA (titre = 800).

Antisera from macaques Mac1 and Mac2 immunized with a mixture of Tat peptides (1–20, 44–61 and 1–61) displayed high antibody specificity to the N-terminal region (titres = 51 200 and 102 400, respectively). Mac1 also mounted a high antibody response to the basic peptide 44–61, whereas Mac2 produced an equivalent response to peptides from

<table>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>sTat 1–86</td>
<td>12 800</td>
</tr>
<tr>
<td>Mur2</td>
<td>sTat 1–86</td>
<td>12 800</td>
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<tr>
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<td>44–61</td>
<td>–</td>
</tr>
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<td>51 200</td>
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<td>1–20+44–61+1–61</td>
<td>102 400</td>
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<tr>
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<td>Hum2</td>
<td>Tat toxoid 1–86</td>
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ELISA reactivity pattern of the anti-Tat sera

Sera were diluted to give an absorbance value of 1.6–2.0 with rTat or immunizing peptides. Results are presented as the arithmetic means of absorbance values from two independent experiments. NT, Not tested.

<table>
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<th>rTat 1–43</th>
<th>rTat 25–39</th>
<th>rTat 33–47</th>
<th>rTat 43–59</th>
<th>rTat 53–67</th>
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Interestingly, this antiserum was the only one that reacted to the N-terminal and basic regions of Tat (Tables 1 and 2, Fig. 1d). Rab1 serum had no neutralizing effect on Tat transactivation and Rab2 and Rab3 antibodies exhibited only weak inhibition of transactivation (<30% at a dilution 1:100). In contrast, Rab4 antiserum displayed a robust neutralizing effect on Tat activity (>60% at a 1:200 dilution; Fig. 2b). Interestingly, this antiserum was the only one that reacted with both active and denatured rTat protein and also with the N-terminal and basic regions of Tat (Tables 1 and 2).

Among the three monkey antisera tested, Mac1 antibodies displayed the strongest inhibition of Tat transactivation (>80% at a 1:100 dilution), which was retained at a 1:200 serum dilution (Fig. 2c). Antibodies from Mac2 and Mac3 also inhibited Tat activity but this effect was diminished when the serum was further diluted. It is important to note that all these antisera reacted with rTat protein and with the N-terminal and basic regions of Tat. In this group of

The two Hum1 and Hum2 sera collected from individuals immunized with Tat toxoid displayed a broad range of antibody specificities. In particular, they recognized peptides 1–20, 8–53 and 44–61, covering the N-terminal and the basic regions of Tat, and also the C-terminal RGD motif present in residues 77–79 not seen with the other antisera. Antibodies from both Hum1 and Hum2 sera bound rTat and sTat proteins in ELISA, and in Western blotting stained a band that corresponded to rTat protein (Fig. 1d).

### Inhibition of extracellular Tat LTR transactivation

Exogenous Tat can enter HL60 cells harbouring an LTR-CAT plasmid and transactivate CAT production. Both rTat and sTat were active in this assay and displayed significant transactivation properties in the same range of concentration (data not shown). However, due to limited availability of material, the ability of Tat antibodies to interfere with transactivation was tested with rTat only. Dilutions of Tat antisera were pre-incubated with rTat before addition to the reporter cell line. Pre-immune sera used as controls did not exhibit significant inhibition of CAT production and were considered as a negative control for each experiment. In contrast, antibodies from mouse Mur1 diluted 1:100 were able to block more than 40% of Tat transactivation and this inhibition was retained at a 1:200 serum dilution. Mur2 antibodies did not neutralize Tat activity (Fig. 2a). It was noticeable that the only difference between these two antisera was their reactivity with the basic region of Tat (Tables 1 and 2).
antisera, Mac1 antibodies bound peptide 44–61 with the highest titre (204 800).

The two Hum1 and Hum2 sera from Tat toxoid-vaccinated subjects contained antibodies with a high potential to inhibit Tat transactivation (Fig. 2d). At a 1 : 200 dilution, both immune sera inhibited more than 80% of the control transactivation and 40 and 50% inhibition was still detectable at a 1 : 1000 for Hum1 and Hum2, respectively (Fig. 2d). These two sera were also positive with the rTat protein and with the N-terminal and basic regions.

Inhibition of virus replication

After 4 days, newly infected H9 cells produced 625 ng HIV-1 p24 core antigen ml$^{-1}$ in culture supernatants. As shown in Fig. 3, mouse Mur1 and Mur2 antibodies did not display any visible effect against virus replication. Also, antibodies from rabbits Rab1, Rab2 and Rab3 failed to inhibit HIV-1 replication, whereas antibodies from rabbit Rab4 exerted a 25% inhibition of p24 production. Antibodies from Mac1 and Mac2 monkeys blocked 40 and 25% of virus replication, respectively, whereas Mac3 antibodies did not show any significant inhibition of p24 production. In contrast, a 1 : 20 dilution of Hum1 serum inhibited up to 75% of virus replication. No inhibition was observed with a 1 : 100 dilution of any of the animal and human sera (data not shown). Hum2 antibodies were not tested in this assay as our serum sample was exhausted in previous studies.

Fig. 3. Inhibition of HIV-1 IIIB replication by anti-Tat antibodies. Production of p24 HIV antigen by newly infected H9 cells in the presence of Tat antibodies (dilution 1 : 20) was measured after 3 days of culture. Non-immune sera did not interfere with virus replication. Results are expressed as a percentage of p24 production, taking the value obtained with the respective control antibodies in the culture medium as 100%. Each value is the arithmetic mean of triplicate experiments.
antigen ml\(^{-1}\) in the supernatant and the addition of Hum1 antiserum did not significantly inhibit virus replication (48 ± 2 ng ml\(^{-1}\)). The same effect was observed at day 6 (148 ± 5 versus 152 ± 7 ng ml\(^{-1}\)). These findings support the view that anti-Tat antibodies do not directly affect virus replication.

To examine whether Tat antibodies could play a role in virus replication by interfering with circulating Tat, we tested the effect of Tat alone or Tat incubated in the presence of anti-Tat antibodies. As expected, in the presence of 100 ng rTat protein ml\(^{-1}\) and a negative anti-Tat serum (used as a control at a 1:100 dilution), HIV replication in chronically infected H9 cells was enhanced twofold as measured by the production of p24 in culture supernatants (104 ± 17 and 270 ± 17 ng ml\(^{-1}\) after 4 and 6 days, respectively; Fig. 4). Most interestingly, we observed that the addition of Hum1 antibodies (serum dilution 1:100) neutralized the Tat effect by significantly reducing p24 production (70 ± 8 and 175 ± 14 ng ml\(^{-1}\) after 4 and 6 days, respectively; Fig. 4).

**DISCUSSION**

In the complex pathogenesis of AIDS, extracellular Tat protein induces immune suppression, enhances virus replication and promotes disease progression. In humans, humoral immune responses against Tat seem to be associated with the maintenance of a non-progression status in long-term stable, HIV-1-infected subjects (Re et al., 2001; Richardson et al., 2003; Zagury et al., 1998). In these slow or non-progressing individuals with a broad antibody response against a variety of HIV-1 antigens, the role of anti-Tat antibodies has not been clearly demonstrated. To understand better the possible protective effect of anti-Tat antibodies, one approach is to determine precisely the specificity of antibodies with Tat-neutralizing properties. Therefore, in this study we used short and long peptides, as well as rTat and sTat proteins (86 aa), to analyse the specific IgG antibodies elicited in different animals and humans immunized with Tat protein or fragments. The specificity of sera was analysed with respect to their capacity to inhibit the 

![Fig. 4. Virus replication of HIV-1 IIIB in chronically infected H9 cells. Production of HIV p24 antigen by chronically infected H9 cells in the presence of Hum1 antibodies diluted 1:100 and rTat protein (100 ng ml\(^{-1}\)) was measured after 4 and 6 days of culture. Each value is the arithmetic mean of triplicate experiments. Non-immune human serum was used as a control.](image)

Previous studies have reported that the Tat N terminus and basic regions contain immunodominant epitopes in mice (Boykins et al., 2000), rabbits (Goldstein et al., 2001), macaques (Tikhonov et al., 2003) and humans (Re et al., 2001; Tahtinen et al., 1997). In the present study, we first confirmed that these two regions contained dominant B-cell epitopes recognized by IgG antibodies present in sera from Mur1, Rab4, Mac3, Hum1 and Hum2 raised against the full-length 86 aa protein, administered either as rTat, sTat or Tat toxoid. Antibodies from two monkeys immunized with a cocktail of synthetic peptides (1–20, 1–61 and 44–61) reacted equally well with peptides 1–20 and 44–61, though the antibody titre to peptide 44–61 was eightfold higher in Mac1 than in Mac2. With the exception of the antisera Rab3 raised against peptide 44–61, all sera examined in our study reacted with rTat and sTat in ELISA and/or with rTat in its denatured form in Western blotting. However, Rab4 antibody titre to sTat was significantly lower than that measured for the rTat protein. In addition, Rab4 serum reacted with all long Tat fragments but only with the 1–15 short peptide. Epitope recognition by Rab4 antibodies could be highly dependent on the folding of the Tat protein preparation. Rab2 antibodies bound weakly to rTat and sTat in ELISA but reacted with denatured rTat, suggesting that the epitopes recognized by Rab2 antibodies in the core region are probably buried within the folded Tat structure and exposed when the protein is tested in denaturing conditions. Rab3 serum raised against the basic region peptide 44–61 reacted with immunizing peptide but not with the other large Tat fragments (8–53, 19–53) or overlapping short peptides in any assay. Rab3 antibody reactivity was low with rTat in ELISA but dramatically increased when sTat was used as an antigen. It has been shown previously that the binding of antibodies to the basic region of Tat is highly dependent on protein...
folding (Moreau et al., 2004; Tosi et al., 2000). Moreover, amino acid residues flanking the basic region can hinder the binding of specific antibodies to the minimal antigenic sequence (Goldstein et al., 2001). It is likely that the epitope structure as presented by sTat is similar to the folding of the peptide 44–61 used for immunization. This particular structure could be absent in overlapping peptides or in rTat in its active form or under the denaturing conditions used in Western blotting.

The ability of antisera to neutralize exogenous Tat transactivation was assessed by an LTR–CAT assay. It is well established that monoclonal or polyclonal antibodies directed against the various epitopes of the Tat protein can abolish LTR transactivation induced by exogenous Tat (Tikhonov et al., 2003; Tosi et al., 2000). Our results extend these findings by demonstrating that only immune sera with antibody responses to both N-terminal and basic regions (namely Mur1, Rab4, Mac1, Hum1 and Hum2) were able to block extracellular transactivation significantly. Moreover, it seems that the extent of their neutralizing activity correlated with responses toward the basic domain of Tat. Thus, in the case of Mac2 and Mac3, sera that had low IgG titres to peptide 44–61 displayed a weak neutralizing potential against Tat compared with Mac1 serum. However, it was noticeable that Hum1 and Hum2 sera displayed the highest neutralizing ability, although they had moderate antibody titres against both N-terminal and basic regions. It is well known that the neutralizing potential of serum antibodies to a given antigen does not systematically correlate with antibody titres measured in ELISA (Alape-Giron et al., 1997; Simonsen et al., 1987) but to the antibody affinity for the target antigen (Olszewska et al., 2000). Hum1 and Hum2 antibodies are likely to display a high affinity for rTat protein.

Neutralization of extracellularly secreted Tat by antibodies leads to partial inhibition of virus replication in cultures of T-cell lines newly infected with HIV-1, but requires high concentrations (µg ml⁻¹) of monoclonal antibodies in culture medium (Moreau et al., 2004; Re et al., 1995; Steinaa et al., 1994). The Hum1 serum that exhibited this activity may have been the only one that contained enough specific antibodies to abrogate Tat autocrine/paracrine activity significantly.

In chronically infected cells producing stable low levels of p24 antigen, the presence of Hum1 antibodies did not reduce virus production. This may be due to lower expression of extracellular Tat in these cells compared with Tat production in acutely infected cells (Ensoli et al., 1993). However, addition of soluble active Tat protein to chronically infected cells enhanced virus replication, as previously demonstrated by Boykins et al. (2000), and this activation was totally inhibited by Hum1 antibodies. These results suggest that anti-Tat antibodies in vivo could block the Tat protein secreted by HIV-productive cells that could reactivate virus replication in neighbouring chronically infected cells.

In conclusion, the data presented in this study demonstrate that immunization with the whole 86 aa Tat protein or a multi-epitope peptide cocktail leads to the production of Tat-specific antibodies able to neutralize exogenous Tat-driven transactivation and disrupt the autocrine/paracrine effects of the protein involved in virus replication in vitro. Therefore, with regard to the pleiotropic effects of extracellular Tat in the course of HIV-1 infection, our findings lend weight to the conclusion that active or passive Tat immunization could be used advantageously as a support therapy in association with other antiretroviral drugs.

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