A monoclonal antibody directed against a conformational epitope of the HIV-1 trans-activator (Tat) protein neutralizes cross-clade

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*Running Title: Tat broadly reactive neutralizing mAb

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Keywords: HIV-1; Tat; Oyi; broadly neutralizing monoclonal antibody; passive immunization.

Background: Tat, an essential HIV-1 replication factor and extracellular toxin acting on immune cells, is an interesting target to develop therapeutic antibodies.

Results: MAb 7G12 recognizes a conformational epitope of Tat, cross-neutralizes different Tat variants and blocks Tat uptake.

Conclusions: MAb 7G12 could be used in immunotherapy to restore patients immunity.

Significance: Development of alternatives to antiretroviral therapy is crucial.

SUMMARY

The identification of a neutralizing monoclonal antibody (mAb) against extracellular HIV-1 transactivator of transcription (Tat) is important for the development of an efficient HIV-1 treatment. Tat plays an essential role in HIV-1 pathogenesis, not only for HIV-1 replication, but also as an extracellular toxin able to disrupt the immune system. Previously, we showed that immunization of rabbits with Tat Oyi, a variant cloned from an African woman who did not develop AIDS following HIV-1 infection, raised antibodies able to recognize different Tat variants. We carried out mice immunization with Tat Oyi, and selected a mAb, named 7G12, which had the capacity to cross-recognize heterologous Tat variants by a common 3D epitope. These results highlighted that Tat variants were able to acquire a structure, in contrast to a number of studies showing that Tat is as an unfolded protein. MAb 7G12 also had the capacity to neutralize the biological activities of these Tat variants by blocking the cellular uptake of extracellular Tat. This is the first study using Tat Oyi to produce a mAb able to neutralize effectively activities of extracellular Tats from different
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HIV-1 subtypes. This mAb has an important potential in therapeutic passive immunization and could help HIV-1 infected patients to restore their immunity.

Developing an effective vaccine against HIV-1 remains an important task due to the high genetic variability of HIV-1. In addition, the virus capability to evade immune responses, particularly in immunologically weakened infected patients, highlights the challenge to find a target that induces broadly reactive antibodies. Besides, an efficient vaccine must elicit potent HIV-1 neutralizing antibodies. Conserved epitopes are generally considered to be the key approach to obtain such broadly neutralizing antibodies. Passive immunization studies with monoclonal antibodies (mAb) targeting the Env protein in simian-human immunodeficiency virus (SHIV) challenge showed promising results (1). However, other potential HIV-1 protein targets such as Tat (transactivator of transcription) should be considered due to its extracellular functions. Tat is one of the first proteins produced by infected cells (2). It is essential for initiation (3) and elongation (4) of HIV-1 genes expression. Moreover, Tat is secreted from HIV-1 infected cells and can cross membranes, inducing apoptosis in different immune cells and protecting HIV-1 infected cells and reservoirs cells against the cellular immune system (5). Extracellular Tat also plays a role in spreading the infection, by inducing the expression of chemokine receptors CCR5 and CXCR4, which are CD4 co-receptors for HIV-1 (6).

A clade B Tat variant, named Tat Oyi, has been cloned in the eighties from a seropositive patient who did not develop AIDS in a remote area of Gabon. HIV-1 Oyi genes were similar to genes of usual HIV-1 strains except the tat gene, which had mutations never found in other Tat variants (7). Previously, we showed that rabbit immunization with Tat Oyi variant rose antibodies able to recognize different Tat variants (8). A heterologous SHIV-BX08 challenge carried out on macaques vaccinated with Tat Oyi showed a reduced viremia in vaccinated monkeys. Furthermore, reservoir cells were no longer detectable (9). Thus, Tat Oyi has specific immunogenic features to generate neutralizing mAbs against Tat variants (8).

In this study, we immunized mice with Tat Oyi and screened mAbs for their cross-clade recognition. We selected one IgG1 mAb, named 7G12, showing an efficient cross-recognition against various HIV-1 subtypes. MAb 7G12 was able to neutralize the biological activities of Tat variants from the five main HIV-1 subtypes and to block Tat uptake. This is the first report of a broadly neutralizing mAb against Tat with a therapeutic potential.

EXPERIMENTAL PROCEDURES

Tat Variants and peptides Synthesis-Tat Oyi was assembled in solid phase synthesis as previously described (10). A Ser→Cys substitution at position 22 in Tat Oyi sequence (Fig. 1) allowed recovering biological activity of Tat Oyi and its use in neutralization assays with antibodies. Five peptides covering the full sequence with overlaps (1-22, 13-46, 38-72, 57-86 and 72-101) and respectively named peptide 1 to 5 were synthesized. Other synthesized Tats correspond to clade A (Ug11RP), clade D (Eli), circulating recombinant form AE (CM240), clade C (96Bw), and clade B, predominant in Europe and the Americas (HxB2) (Fig. 1). Purification and analysis were performed, as previously described (10). Purity and mass were controlled by mass spectrometry. After lyophilization, biological activity of Tat variants were checked by transactivation assays with HeLa P4 cells as previously described (11).

Immunization and monoclonal antibody production- Four BALB/c mice were immunized with 10 µg of synthetic Tat Oyi in 100 µl phosphate calcium gel adjuvant (Brenntag Biosector) by the subcutaneous route. Two weeks later, mice were boosted with the same preparation. Five weeks later, mice were boosted again with the same preparation by the intramuscular route. Three control mice were also immunized with the same adjuvant and protocol but without Tat protein. On day 45, mice were euthanized and terminally bled. The spleenocytes were immediately separated to hybridize with myeloma cells as described (12). Supernatants of
isolated hybridoma were screened by Enzyme Linked Immune-Sorbent Assay (ELISA) against Tat Oyi variant to identify producing clones. Then, they were sub-cloned by limiting dilutions (< 1.0 cell per well) twice and antibody positive clones were screened by ELISA against firstly Tat Oyi, then against Tats Ug11RP, ELI, CM240, 96BW and HxB2. Previously, a similar immunization with Tat Oyi of four rats has been performed and a mAb (IgG1), named 27A8, has been selected on its high recognition of Tat Oyi and was used as control. Selected hybridomas were cultured and mAbs were purified by protein G chromatography (Roche Diagnostics) according to the manufacturer’s instruction. After purification, antibodies were dialyzed against Hepes buffer 20mM, NaCl 120mM, pH 7.3 and concentrated at 1mg/ml.

**Detection of purified mAbs responses by ELISA**

- 96-well plates were coated with 100 ng of folded or denatured Tats or peptides 1 to 5 (1 µg/ml in Phosphate buffer 100mM, pH 4.5) or peptides pool (each peptide: 1 µg/ml in Phosphate buffer 100mM, pH 4.5). Tats (10µg/ml) were denatured by heating at 90°C during 20 min in presence of urea 3M, then diluted 10 fold with cold Phosphate buffer 100mM, pH 4.5. To control urea effect, folded Tats were coated in presence to 0.3M urea. Non-specific signal was controlled in coating 100ng of BSA by well.

- Following washing steps, the plates were blocked with 5% skim milk in PBS for 1 h. The antibody (1µg/ml) and the Tat variants (0 to 0.4µg/ml) solutions were prepared in Hepes buffer 20mM, NaCl 120mM, pH 7.3 at twice the final concentration. At time zero, similar volumes of antibody and antigen solutions were mixed and 100 µl were immediately (time zero of the kinetic analysis) transferred to three coated wells and one uncoated well. Each five min, the wells were filled with a sample of mAb/Tat mixture. Each sample was incubated in the wells for 4.5 min and removed. At the end of the kinetic analysis, plates were washed and incubated with HRP-conjugated anti-mouse IgG. After washing steps, 100 µl of ABTS substrate was added. The absorbance was measured at wavelength 405 nm one hour later. Association and dissociation rate constants (k_on and k_off) were then determined, allowing the calculation of equilibrium dissociation constant (K_D).

**Western Blot** - Tats (100 ng) were subjected to SDS-PAGE (15%) under reducing conditions (DTT 100mM and urea 6M in Laemmli sample buffer at 96°C for 10 min). Tats were then electro-transferred to nitrocellulose membrane (Schleicher & Schuell). After blocking with 5% skim milk, strips of the membrane were incubated for 1h with mAbs 7G12 or 27A8 at 1:2500. The secondary antibody was HRP-conjugated anti-mouse or anti-rat IgG (GE Healthcare) diluted 1/1000 and bands were revealed with H_2O_2 0.1%, diaminobenzidine tetra hydrochloride (Sigma) as substrate.

**Neutralization of Tat transactivation** - Neutralization of Tat transactivation was performed with HIV LTR transfected HeLa cells (HeLa-P4) and analyzed by monitoring the production of β-Galactosidase (β-Gal) as previously described (11). Briefly, Hela-P4 cells were incubated in DMEM with glutamine supplemented with 10% (v/v) heat-inactivated foetal calf serum and 50 units/ml neomycin. 2 × 10^5 cells/well were incubated in 400µl of DMEM supplemented with 0.01% (w/v) protamine (Sigma-Aldrich) and 0.1% (w/v) bovine serum albumin (BSA) (Sigma) in 24-well plates (Falcon). Lyophilized or denatured Tats were diluted at 5

**Measurement association rate constants of mAb 7G12 with Tats in solution** - An ELISA based method was used for measuring antigen/antibody association rate constants in solution (13). Briefly, 96-well plates were coated with 100 ng of Tat Oyi in Phosphate buffer 100mM, pH 4.5 for overnight at 4°C. One well on four was left uncoated. Following washing steps, the plates were blocked with 5% skim milk in PBS for 1 h. The antibody (1µg/ml) and the Tat variants (0 to 0.4µg/ml) solutions were prepared in Hepes buffer 20mM, NaCl 120mM, pH 7.3 at twice the final concentration. At time zero, similar volumes of antibody and antigen solutions were mixed and 100 µl were immediately (time zero of the kinetic analysis) transferred to three coated wells and one uncoated well. Each five min, the wells were filled with a sample of mAb/Tat mixture. Each sample was incubated in the wells for 4.5 min and removed. At the end of the kinetic analysis, plates were washed and incubated with HRP-conjugated anti-mouse IgG. After washing steps, 100 µl of ABTS substrate was added. The absorbance was measured at wavelength 405 nm one hour later. Association and dissociation rate constants (k_on and k_off) were then determined, allowing the calculation of equilibrium dissociation constant (K_D).
μM (or 10μM) in phosphate buffer 100mM pH 4.5. Tat (0.5 μM final) and described concentrations of antibodies were added to cells. Volumes were completed to 500μl with Hepes buffer 20mM, NaCl 120mM, pH7.3. When added together, denatured and folded Tat had a final concentration of 0.5 μM each. After 18h at 37°C, β-Gal production was measured with a β-Gal ELISA kit (Roche Diagnostics) according to manufacturer’s recommendations. Background against BSA was removed. Transactivation ratio was the amount of β-Gal in presence of Tat divided by the amount without Tat.

**Methyl Thiazolyl Tetrazolium (MTT) assay-** The Jurkat T-Cells quantity remaining after incubation with Tat, reflection of induced apoptosis, was measured with a MTT-based method as previously described (14). Jurkat T-cells were cultivated in RPMI 1640 medium with glutamine supplemented with 10% (v/v) heat-inactivated foetal calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin. 10⁵ cells in 100μl of RPMI with 0.1% BSA and 0.01% protamine A were transferred in wells of 96-wells plates. Lyophilized Tats were diluted at 65μM in phosphate buffer 100mM pH 4.5. Cells were incubated 48h at 37 °C with 10μl of Tats and 20μl of mAbs or Hepes buffer 20mM, NaCl 120mM, pH7.3. Control wells were adjusted with 10μl of phosphate buffer 100mM pH 4.5 and 20μl of Hepes buffer 20mM, NaCl 120mM, pH7.3 to verify cell viability. 0.5 mg of MTT (InterChim) was added and incubated 5 h. Formazan crystals were dissolved in 200μl dimethyl sulphoxide and absorbance was measured at 510 nm.

**Tat uptake-** Translocated Tat localization in nucleus and cytosol was evaluated by immunoblot in Jurkat and HeLa cells. Jurkat T-cells (or Hela cells) were incubated as described above. 10⁶ cells by well (24-well plate) were incubated in 400μl of RPMI (or DMEM) supplemented with 0.01% (w/v) protamine (Sigma) and 0.1% (w/v) BSA (Sigma). Lyophilized Tats were diluted at 50 μM in phosphate buffer 100mM pH 4.5. 50μl of Tat (5μM final) and 50μl of purified antibodies (50μg) or 50μl of Hepes buffer 20mM, NaCl 120mM, pH7.3 were added and cells were incubated for 2h at 37 °C. Cells were washed with 1ml PBS and poterized in lysis buffer (50mM Tris-HCl buffer pH 7.5, 150mM NaCl and protease inhibitors (Roche Diagnostics)). Complete lysis was controlled by microscopy. Pellet corresponding to the nuclear extract was obtained by centrifuging the lysate at 600 × g for 15 min at 4 °C. Supernatant was centrifuged at 100 000 × g for 1h at 4 °C and membrane pellet was retrieved. Cytoplasmic fraction (supernatant 2) was Trichloroacetic acid precipitated overnight at -20 °C. Final pellet was washed by 1ml of cold acetone. Nuclear, membrane and cytoplasmic pellets were subjected to SDS-PAGE (15%) under reducing conditions (DTT 100mM) and electro-transferred to nitrocellulose membrane (Schleicher & Schuell). Protein amounts were controlled by staining with Ponceau red (Sigma). After blocking with 5% skim milk, membrane was incubated overnight with an anti-Tat rabbit sera (1:1000) previously described (11). The secondary HRP-conjugated anti-rabbit antibody (GE Healthcare) was diluted to 1/5000 and bands were revealed with Immobilon Western chemiluminescent HRP substrat (Millipore). The intensity of bands was analyzed by densitometric imaging using the freely available Image J program (NIH). Densitometries in nucleus and cytosol were added to evaluate total translocated Tat without antibody (100%). Densitometries of each compartment in presence of antibodies were compared and expressed as percentage. Annexin 1, P-AC-Histone H3 and Fusin (Santa Cruz Biotechnology) antibodies were used as cytoplasmic, nuclear and membrane fractions control, respectively.

**Statistical analysis-** Statistical differences were analyzed by use of Mann-Whitney test. A P value < 0.05 was considered significant.

**RESULTS**

MAb 7G12 cross-recognizes Tat variants from the five main HIV-1 subtypes. Mice were immunized with Tat Oyi and one IgG1 mAb, named 7G12, was selected among 132 pre-screened clones for its broadly reactive immune response against a panel of Tat variants.
A broadly reactive neutralizing mAb

representative of main HIV-1 clades (Fig. 1). To characterize the cross-recognition, the affinities of mAb 7G12 for the different Tat variants were evaluated in ELISA (Fig. 2A). mAb 7G12 bound all Tats with a similar high affinity. Only one mAb, named 6E7, showed also a broadly reactive immune response but with ten times lower affinities (Fig. 2B). All other clones did not recognize all Tats, showed very low affinities or were IgM subtype. The control mAb named 27A8 bound only Tats Oyi and HXB2 with high affinities but not the others (Fig. 2C).

In order to quantify mAb 7G12 affinities for Tat variants, an ELISA-based method (13) was used to measure antigen/antibody association rate constants in solution. Antigen and antibody were mixed and aliquots were withdrawn at different time intervals to determine the amount of free antibodies. The disappearance of free antibodies reflected the time course of the association reaction. Affinity constant obtained for Tat Oyi was high ($K_D = 7 \pm 0.4$ nM). $K_D$ for Tats UG11RP (12 ± 0.5nM), Eli (5.7 ± 0.1nM), CM240 (3.2 ±0.1nM) and 96BW (7.4 ± 0.3 nM) were very similar. These results confirmed ELISA affinity curves (Fig. 2A) and suggested that mAb 7G12 recognized a common site on the surface of all Tat variants tested. Interestingly, sequences comparison shows only a small percentage of potential continuous epitopes (> 5 amino acids) between the different Tats (Fig. 1).

**MAb 7G12 recognizes a 3D epitope.** To map the epitopes recognized by mAbs 7G12, 6E7 and 27A8, ELISA was carried out as previously described (15), using 100 ng of different overlapping peptides spanning the entire sequence of Tat Oyi (Fig. 3A). mAb 7G12 did not recognize peptides while mAb 27A8 recognized peptide 5 covering the C-terminal sequence (72 to 100). Thus, mAb 27A8 recognized a linear epitope in the C-terminal domain, conserved only between Tats Oyi and HxB2 (identity 87.1 %) but not in others Tat variants. In contrast, mAb 6E7 recognized peptides 1 and 2, indicating that its epitope matched, at least partly, with the overlapping sequence (residue 13 to residue 22). Thus, mAb 6E7 showed a broad immune response against the panel of Tat variants because this linear epitope is highly conserved in the N-terminal domain of these proteins (Fig.1). These data suggested that mAb 7G12 did not recognize a linear epitope but a 3D epitope on the surface of Tat Oyi that corresponds to a particular folding highly conserved in Tat variants.

To test this hypothesis, ELISA with mAbs 7G12 and 27A8 were performed against folded and denatured Tats Oyi and HXB2 (Fig. 3B). Urea alone, used for complete denaturation, did not modify the ELISA responses for both mAbs, while high temperature drastically decreased mAb 7G12 binding to Tat without modifying 27A8 binding. mAb 7G12 did not recognize Tat variants following denaturation or a peptides pool, highlighting that this antibody recognized a 3D epitope and not a linear epitope. In contrast, mAb 27A8 recognized denatured Tat variants and peptides pool, confirming that this mAb was directed against a linear epitope. Moreover, mAb 7G12 was also unable to recognize Tat Oyi in Western blot analysis (Fig. 3C). In contrast, mAb 27A8 in the same dilution strongly recognized Tat Oyi.

**MAb 7G12 neutralizes Tat Oyi transactivation activity.** We studied the effects of mAb 7G12 (3D epitope) and of mAbs 6E7 or 27A8 (linear epitopes) on the Tat Oyi biological activities. The neutralizing effect of mAb 7G12 on transactivation activity of Tat Oyi was monitored with HeLa P4 cells (Fig. 4A). 50 µg of mAb 7G12 neutralized almost completely Tat Oyi (0.5µM) transactivation activity. The neutralization potency of mAb 7G12 correlated with the concentration of antibody. In our assays, the maximal neutralization was reached with 0.33 nmol (50µg) of mAb against 0.25 nmol of Tat, suggesting a molar-molar association. In contrast, mAb 27A8 only had a very low effect on transactivation activity of Tat Oyi (Fig. 6A).

To determine if the neutralizing effect of mAb 7G12 was due to recognition of a 3D epitope, transactivation activity was examined using 0.5µM of folded and denatured Tat Oyi (Fig. 4B). Denatured Tat Oyi had lost the transactivation activity but did not prevent the biological effect of folded Tat when both were added together. Interestingly, unfolded Tat did not
decrease the neutralizing effect of 50µg of mAb 7G12 when folded and denatured Tats were in competition. These demonstrated that the activity of Tat Oyi in solution was conformation dependent and blocked by the mAb 7G12, which recognized a 3D epitope.

\textit{MAb 7G12 inhibits Tat Oyi-induced apoptosis in lymphocytes.} Discordance between the transactivation and other Tat functions was previously observed (16). Thus, to confirm mAb 7G12 neutralizing effect, an inhibition assay was performed on Tat induced apoptosis (17). Viability of Jurkat lymphocytes cells was monitored in presence of Tat Oyi, using a tetrazolium salt-based colorimetric assay (Fig. 4C). 10 µM of Tat Oyi almost completely inhibited formazan production in Jurkat cells. This inhibition was correlated with the concentration of Tat Oyi up to 1µM. The neutralizing effect of mAb 7G12 was evaluated (Fig. 4D). 20 µg (1µM) of mAb 7G12 completely blocked the apoptosis of lymphocytes induced by Tat Oyi (5µM). Partial neutralization was observed up to 5µg (0.25µM) of mAb 7G12 but not with 1µM of mAb 27A8 (Fig. 6B).

\textit{MAb 7G12 blocks extracellular Tat uptake.} Exogenous Tat is able to enter cells, inducing apoptosis or activating transcription. We evaluated the ability of mAb 7G12 to neutralize Tat Oyi uptake using Jurkat cells (Fig 5). MAb 27A8, which recognized a linear epitope of Tat Oyi, was used as mAb control. As previously described (18, 19), most of internalized Tat was located in nucleus (78%) and only a small part was in cytosol (22%). MAb 27A8 only showed a non significant decrease in cytosolic fraction. In contrast, mAb 7G12 almost totally blocked Tat translocation as shown by the significant drop in the nuclear (-75.6%) and cytosolic (-98.5%) fractions. Very similar results were observed with HeLa cells (data not shown). Thus, mAb 7G12 was able to block Tat uptake, preventing apoptosis and transcription, more effectively than a mAb which recognized only a linear epitope.

\textit{MAb 7G12 cross-neutralizes biological activities of other heterologous Tat variants.} Cross-neutralizing activity of 50µg (0.66µM) of mAb 7G12 was evaluated using the transactivation assay with HeLa P4 cells and 0.5µM of Tat variants representative of the various HIV-1 subtypes including A, B, C, D, and the circulating recombinant form AE (Fig. 6A). MABs 27A8 and 6E7 were used as control at the same concentration. MAb 7G12 inhibited the transactivation activity of all Tat variants as well as with Tat Oyi. Precisely, mAb 7G12 inhibited 61.9%, 66.3%, 57.1%, 53.2% and 50.8% of transactivation activities of Tats UG11RP, HxB2, 96Bw Eli and CM240 respectively, compared to 63.7% observed with Tat Oyi. In contrast, mAb 27A8 showed a low inhibitory effect only for Tat Oyi and Tat HxB2 (12 % and 19.4% respectively) and no significant inhibition with others Tats. MAb 6E7 had no inhibitory effect on all Tat variants. We also examined the neutralizing effects of mAbs 7G12, 27A8 and 6E7 on inhibition of lymphocyte proliferation induced by 5µM of Tat variants (Fig. 6B). Unlike mAbs 27A8 and 6E7, mAb 7G12 completely reversed the proliferation inhibition induced by the Tat variants.

Thus, mAb 7G12 neutralized the biological activities of the different Tat variants tested with a similar efficiency, suggesting that the epitope recognized by this antibody was conserved on the variants surface and that their foldings play an important role for Tat activity.

\textbf{DISCUSSION}

Up to now, the main targets to obtain therapeutic antibodies to HIV-1 were the surface envelope glycoprotein gp120 and the transmembrane glycoprotein gp41 (20, 21, 22, 23, 24, 25, 26, 27, 28). These antibodies were designed to block virus uptake in cells but resistances emerged by selection of escape mutants. Most of these antibodies recognize linear epitopes, which can mutate easily or be modified after transcription (29, 30, 31). Recently, screening strategies in infected patients using new technologies have selected neutralizing antibodies efficient against a large number of variants of HIV-1 (32, 33, 34). These studies highlight that only a very limited number of antibodies in few patients are broadly neutralizing. Some mAbs against Tat were developed on mice (35, 36, 37) and tested on
humans (38, 39) but none of them had the capacity to cross-recognize Tat variants.

In this study, we used immunogenic property of Tat Oyi to obtain a mAb, named 7G12, selected for its high recognition of several genetically heterologous Tat variants. This specific feature of Tat Oyi has been described previously (8). Among these variants, Tat HxB2 sequence has the highest sequence identity (87.1%) with Tat Oyi, while Tat variants CM240, UG11RP, 96Bw and Eli have identities of 67.3%, 65.3%, 67.3% and 73.3% respectively. Moreover, identical amino acids among the 6 variants are gathered in sequence between residues 13 to 18 and residues 43 to 52 (Fig. 1). The sequence 13-18 corresponds to the core region of Tat and the sequence 43 to 52 appears to lack immunogenicity, although exposed to solvent (40). MAb 6E7, used as control, recognized a linear epitope located in the common sequence (residues 13 to 22) of the N-Terminus of Tat. MAb 6E7 also showed a broad immune response but with lower affinities than mAb 7G12. Moreover, mAb 6E7 was unable to block the biological activities of the different Tat variants, suggesting that this region was not reachable in active Tats.

MAb7G12 did not recognize any peptides covering the Tat Oyi sequence and the affinities were comparable for all these heterologous Tats. Thus, we concluded that this antibody recognized sites on a common surface in all Tat variants. This common surface required a conserved folding of Tat variants that plays an important role for Tat activity. This conclusion is in disagreement with studies showing that Tat is an unfolded protein (41). The absence of recognition of all Tat variants after urea/heat degradation in ELISA and on Western blot (data not shown) confirms the existence of a common 3D structure. The presence of a conserved folding in Tat variants is also supported by previous structural studies on three Tat variants with a biological activity (42). Accordingly, mAb 7G12 could be used to map the 3D epitope able to induce broadly cross-reactive antibodies. Preliminary recognition experiments with different Tat Oyi domains combinations have shown that the sequences 1-22, 38-53 and 93-101 were involved in proper folding conditions to be recognized by mAb 7G12 (data not shown).

Protection assay against acetylation on lysine residues could be an appropriate mean to map precisely the discontinuous epitope but needs further development.

We showed that mAb 7G12 neutralized two biological activities for all Tat variants tested. We used a higher Tat concentration in Jurkat cells apoptosis assay (5µM) compared to transactivation assay with HeLa cells (0.5µM) because these two cell lines are differently sensitive to the extracellular Tat. For instance, Jurkat T cells do not express caveolin and cannot internalize Tat by this pathway (43). Moreover, the full length form of Tat is less efficient for apoptosis than the short form, while no difference is observed for transactivation assay (44). In contrast, the quantity of antibodies used to obtain a high neutralization seemed less important in apoptosis assay (20µg) than in transactivation assay (50µg) but the final antibody concentrations were similar (0.2 µg/µl and 0.1µg/µl respectively). The lower Tat/antibody ratio needed to obtain complete neutralization in transactivation assay arises from the efficiency of the LTR promoter. Thus, a molar/molar ratio (0.25 nmoles of Tat and 0.33 nmoles of mAb) is crucial to block all Tat able to trigger β-Gal expression. In contrast, trigger apoptosis pathways is more difficult, especially in a stable cell line, and the neutralization is easier, needing a higher Tat/antibody ratio (0.65 nmoles of Tat and 0.14 nmoles of mAb). We observed a low neutralization of Tats Oyi and HxB2 transactivation activities with mAb 27A8 (Fig. 6A). This antibody recognized a common linear epitope in the C terminal domain of these two clade B variants. MAb 27A8 could interfere with their transactivation activities but with a very low efficiency compared to mAb 7G12. Moreover, mAb 27A8 was unable to prevent apoptosis activities of these Tats.

Intracellular pathways leading to Tat induced transactivation and apoptosis were probably distinct but both dependent on Tat uptake. We observed that mAb 7G12 was able to block Tat uptake in both cell lines. Kinetic conditions used resulted from data previously published (45) highlighting that internalization was almost completed after 2h. Experimental conditions were close to those of transactivation...
Tat broadly reactive neutralizing mAb assay but with 5µM (and not 0.5 µM) of Tat. A highest Tat concentration was used to obtain a good signal in nuclear and cytosolic fractions on immunoblot. However, due to experimental limitations, a molar/molar ratio between Tat and mAb was not reached in these conditions, explaining why 19% of Tat was still observed in nucleus (Fig 5). We assume that, with a molar/molar ratio, mAb 7G12 could completely block Tat uptake. MAb 27A8 had a low effect on Tat Oyi uptake, not significant in the assay. In molar/molar conditions, this effect could become significant as observed in figure 6A on transactivation activities of Oyi and HXB2 Tat variants. However, mAb 27A8 was inefficient compared to mAb 7G12 and did not inhibit the other variants without the specific C terminal linear epitope.

Up to now, only antiretroviral therapy (ART) is an efficient treatment against HIV-1 but does not eradicate virus (46). Stopping treatment triggers the viral production, due to the existence of reservoir cells (47). Passive immunization with a neutralizing antibody targeting other pathways would complement ART. Thus, mAb 7G12 properties are interesting for this purpose after humanization. We showed that Tat is a naturally folded protein in the blood of HIV infected patients and can generate antibodies against 3D epitopes (15). Thus, therapeutic antibodies recognizing 3D epitopes of Tat could be selected by competitive ELISA with mAb 7G12 on isolated B-cells from Long-Term Non-Progressor patients. Studies also suggested that mAbs combination might protect more effectively against HIV-1 (48). Thus, passive immunization, using broadly neutralizing antibodies against different targets, such as Tat and gp120, should be evaluated for therapeutic potential.
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FIGURES LEGENDS

FIGURE 1. Tat variants sequences. Sequences of Tat Oyi and Tat variants representative of the five main HIV-1 subtypes (in grey, identities).

FIGURE 2. MAb 7G12 cross-recognizes heterologous Tat variants. Affinity curves of mAbs (A) 7G12, (B) 6E7, and (C) 27A8 ranging from $10^4$ to 16 ng/ml were performed against different Tat variants: Oyi (white square), HxB2 (white diamond), UG11RP (white triangle), Eli (black square), CM240 (black diamond), 96Bw (black triangle). B/B0 corresponds to the OD measured for each Tat dilution divided by the maximal OD. The standard deviation measured is <0.02 (n=4).

FIGURE 3. MAb 7G12 recognizes a 3D epitope. Affinities (mean ± SD, n≥3) of mAbs 7G12, 27A8, and 6E7 (1:1000) with (A) peptides 1 to 5 (p1 to p5) overlapping Tat Oyi sequence (grey bars) and (B) folded or denatured Tat Oyi (black bars), Tat HxB2 (white bars) and a Tat peptides pool (grey bars) were measured in ELISA. In bar graphs, statistical significant differences (P<0.01) between folded Tats and either denatured proteins or peptides were indicated by (*). (C) Recognition of Tat Oyi on immunoblot by mAbs 7G12 and 27A8 (1:5000). Shown is a representative assay out of three experiments.

FIGURE 4. MAb 7G12 neutralizes Tat Oyi biological activities. Transactivation ratio (mean ± SD, n≥3) was measured on HeLa P4 cells with (A) 0.5µM of Tat Oyi in presence of different mAb 7G12 concentrations (black bars) and with (B) 0.5 µM of folded (f) or denatured (d) Tat Oyi or a mix of both (f+d) in presence of 50µg of mAb 7G12. Jurkat cells proliferation (mean ± SD, n≥3) without Tat (Ctrl, dark grey bars) was compared (C) in presence of different Tat Oyi concentrations or (D) in presence of 5µM Tat Oyi and different mAb 7G12 concentrations (black bars). In bar graphs, statistical significant differences (P<0.05) were indicated by (*).

FIGURE 5. MAb 7G12 blocks Tat uptake. Tat uptake into Jurkat cells was analyzed in absence or in presence of mAb 7G12 or 27A8 by anti-Tat immunoblotting on nuclear and cytosolic fractions. (A) Western blotting was performed with the indicated antibodies. Annexin I and P-AC-Histone H3 antibodies were used as control of cytosolic and nuclear fractions respectively. This blot is representative of three independent experiments. Fractionation efficiency was controlled in supplemental data with these antibodies and with a membrane specific antibody (Fusin). (B) Percentage of translocated Tat (mean ± SD, n=3) was analyzed by densitometry imaging. The sum of densitometries in nuclear and cytosolic fractions without antibodies (control) represents 100% of translocated Tat in each experiment. Total was the sum of percentages obtained in nuclear and cytosolic fractions. Statistical significant differences (P<0.05) between percentages total in absence versus in presence of antibodies were indicated by (*). Statistical significant differences (P<0.05) between percentages in absence versus in presence of antibodies in each compartment were indicated by (**).

FIGURE 6. MAb 7G12 cross-neutralizes the biological activities of other Tat variants. (A) Transactivation ratio (mean ± SD, n=3) of several Tat variants (0.5µM) were compared in absence (grey bars) or in presence of mAbs 7G12 (50µg, black bars), 27A8 (50µg, white bars) and 6E7 (50µg, dark grey bars) in HeLa P4 cells. (B) Jurkat cell proliferation (mean ± SD, n=3) without Tat (Ctrl, dark grey bars) was compared in assays with 5µM of several Tat variants in absence (grey bars) or in presence of mAbs 7G12 (20µg, black bars), 27A8 (20µg, white bars) and 6E7 (20µg, dark grey bars). In bar graphs, statistical significant differences (P<0.05) were indicated by (*).
Tat broadly reactive neutralizing mAb

1-------10-------20-------30-------40-------50-------
Cy1 C22 MEPVD IRLKP WKRPG SQPFKT TACTMN YCKRK CCLHHC QVGFPTK LG 1 YG RKR RQRDRAP
RxB2 MEPVD IRLKP WKRPG SQPFKT TACTMN YCKRK CCLHHC QVGFPTK LG 1 YG RKR RQRDRAP
Ug11LP NDMD VNLKPN SGSPPT TCHCNCY CKKCCFCQ LUFPITK AL1 YG RKR RQRDRAP
ELI NDMD VNLKPN SGSPPT TCHCNCY CKKCCFCQ LUFPITK AL1 YG RKR RQRDRAP
CM2 40 HELVD VNLKPN SGSPPT TCHCNCY CKKCCFCQ LUFPITK AL1 YG RKR RQRDRAP
96B W MEPVD IRLKP WKRPG SQPFKT TACTMN YCKRK CCLHHC QVGFPTK LG 1 YG RKR RQRDRAP
60------70------80------90------100------
Cy1 C22 QDSKTHQVSLSKQP ASQPRGDPT PGCYCKK ERET T PED 101
RxB2 QNSKTHQASLSKQP ASQPRGDPT PGCYCKK ERET T PED 100
Ug11LP QSMKOKOMPQIPQPTGQIGKETGPE SKKEDKT T PED 101
ELI QGGQAKQVPI QKOPSSQPRGDPT PGCYCKK ERENT 99
CM2 40 QSSKDHQNPQIPQPLPRRNPQDPKQ SKKETASKA T QCD 101
96B W PSSESHONLISEQQPLPRQGMNTIGSE CKKVESKTEADFPF 101

Fig 1
Fig 2
Tat broadly reactive neutralizing mAb

Fig 3
Tat broadly reactive neutralizing mAb

Fig 4
Fig 5
A broadly reactive neutralizing mAb

B

FIG 6
A monoclonal antibody directed against a conformational epitope of the HIV-1 trans-activator (Tat) protein neutralizes cross-clade
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J. Biol. Chem. published online February 23, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M111.319863

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