Intercellular traffic of human immunodeficiency virus type 1 transactivator protein defined by monoclonal antibodies

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Abstract Monoclonal antibodies (mAbs) directed against the amino-terminal region (N-terminal sequence 2–19) of transactivator protein (tat) of HIV-1 have been shown to inhibit intercellular transactivation mediated by the extracellular tat protein. The intracellular transactivation was not significantly affected by anti-tat mAbs. The specificity of anti-tat mAbs in abolishing the transactivating potential of extracellular tat is documented by studies with mAbs to HIV-1 reverse transcriptase, or to a human mammary cancer protein. None of these antibodies showed any inhibitory effect on intercellular transactivation. Specific interaction of anti-tat IgG with tat protein expressed in Jurkat cells is further supported by experiments on immunoblotting. Extracellular tat is responsible for signals which induce a variety of biological responses in HIV-infected cells, as well as in uninfected cells. The fact that anti-tat mAbs can abolish the intercellular traffic of tat protein offers a unique strategy in the development of vaccines against AIDS.

Key words: Anti-tat immunoglobulin G; Transactivation; Passive vaccination; Extracellular tat signal; Kaposi's sarcoma

1. Introduction

The tat protein encoded by the human immunodeficiency virus type 1 (HIV-1) is a potent *trans*-activator of gene expression from the viral long terminal repeat (LTR). The domains that are essential for *trans*-activation, a Pro-xaa$_3$-Pro triad, a cysteine-rich metal binding sequence motif, and a cluster of basic residues, are present within the N-terminal 57 residues of tat protein. In recent years, additional functions for tat have been detected which are attributed to its extracellular release from infected cells. Tat uptake not only enhances HIV-1 transcription in infected cells, it also affects a range of host cell genes in both infected and uninfected cells. These include activation of tumor necrosis factor (TNF), interleukin-6 (IL-6), CD-4, IL-2, IL-2Rα, IL-10, transforming growth factor-β1 (TGF-β1), and CD95 ligand [1–5].

In this communication we have compared the intracellular and intercellular transactivation mediated by the tat protein. Intracellular transactivation designates the phenomenon where a single cell population was cotransfected with plasmids containing tat-expressing construct and the HIV-1 LTR construct hybridized to the chloramphenicol acetyltransferase (CAT) gene. Intercellular transactivation was studied by transfecting two cell populations separately by either of these plasmids, and then co-cultivating the two cell populations together. We found that addition of tat IgG to the culture medium abolishes intercellular transactivation, but has little effect on intracellular transactivation. This is attributed to the interdiction of extracellular tat by the monoclonal antibodies (mAbs). This is further supported by the fact that antibodies directed against HIV-1 reverse transcriptase or specific to a human mammary cancer protein failed to influence the intercellular transactivation potential of tat protein. Specific interaction of anti-tat IgG to tat protein expressed in Jurkat cells is also documented in experiments with immunoblotting. The studies offer tat protein as a unique target for active and passive vaccination against AIDS.

2. Materials and methods

2.1. Chemicals

The culture medium (RPMI 1640) and fetal calf serum were supplied by Gibco (Eggenstein). [^{14}C]Acetyl-CoA (4 mCi/mmol) was obtained from NEN/Dupont (Dreieich). All other reagents were analytical grade of high purity obtained from Merck (Darmstadt) or Serva (Heidelberg).

2.2. Plasmids

The *tat* gene (clone 1) was cloned by way of cDNA cloning, using poly(A)-selected RNA from HIV-1 (BH 10)-infected cells [6,7]. The HIV-1 LTR-CAT construct was obtained by inserting clone 15 DNA into pSV0-CAT at the *Hin*dIII site [6,8], and the resulting plasmid was termed pC15CAT. The plasmid pCV1 was obtained by inserting viral cDNA containing the *tat* gene (clone 1) into the mammalian expression vector pCV which contains duplicated SV40 origins of replication (*ori*), adenovirus major late promoter, splice sites from adenovirus and mouse immunoglobulin genes, mouse dihydrofolate reductase cDNA, and SV40 polyadenylation signal. We are grateful to Dr. S.K. Arya for the preparation of these plasmids.

2.3. Transfection and CAT assay

Jurkat cells (1×10^7) were washed with serum-free medium and incubated at 37°C for 1 h in 1 ml serum-free medium containing 50 mM Tris-HCl (pH 7.3), DEAE dextran (M_r 500 000, 250 μg/ml) and 15 μg of each plasmid DNA, pCV 1 and pC15CAT. The cells were then washed with RPMI medium (without serum) and incubated in 10 ml serum-containing medium at 37°C. At this time, the test compounds were added at the desired concentrations in parallel batches. 44 h after the incubation, cells were washed with phosphate buffered saline and suspended in 80 μl of 0.25 M Tris-HCl (pH 7.8), and cellular extracts were prepared by three cycles of freezing (in liquid nitrogen) and thawing (37°C). All cellular extracts were adjusted to equal protein concentration and heated for 15 min at 65°C to inactivate deacetylases in the extract. For CAT assays, 30 μl of the cell extract was mixed with 20 μl of 100 mM Tris-HCl (pH 7.8) in a 6 ml glass scintillation vial. The vial was transferred to a water bath set at 37°C and 200 μl of a freshly prepared CAT reaction mixture (100 μl of 0.25 M Tris-HCl, pH 7.8, 50 μl of 5 mM chloramphenicol, 10 μl [^{14}C]acetyl-CoA, 0.1 μCi, and 40 μl water) was added. To this mixture, 5 ml of a water-immiscible scintillation fluid (Econofluor, NEN/Dupont) was carefully overlaid and incubated at 37°C for 4 h. All transfections were performed in triplicate for each set of experiments.

2.4. Monoclonal antibodies

BALB/c mice were immunized subcutaneously with 100 μg of a

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recombinant tat protein expressed in *Escherichia coli*, emulsified in Freund's complete adjuvant, followed by three inoculations of 50 μg tat protein in incomplete Freund's adjuvant at 3 week intervals. Eighty-four hours after the last booster, splenic lymphocytes (approximately 10^8 cells) were mixed with P3-X63-Ag8.653 mouse myeloma cells (4×10^7 cells). The fusion procedure, cell culturing and cloning of hybridomas were similar to the procedures described by Galfre et al. [9]. Hybridoma cultures were screened for antibodies to tat protein by enzyme-linked immunosorbent assay (ELISA), using a direct antigen-coated plate method. One of the clones, designated C3.2D7, was found to secrete antibodies reacting with the N-terminal sequence (2–19) of tat protein. The sequential tat peptides were synthesized by the solid-phase method on a fully automated peptide synthesizer (Beckman, 990) with PAM resin. Compositional analysis was performed on the peptides to confirm primary structure. For preparing ascites fluid containing anti-tat mAb cells from clone C3.2D7 (1×10^7 cells) were injected into pristane-primed BALB/c mice, and ascites was collected (2–6 ml per mouse) 2 weeks later. Further purification was carried out using protein G-Sepharose columns.

Hybridoma clones secreting mAb to HIV-1 reverse transcriptase were generated by the same procedure, and the details have been described elsewhere [10,11]. Hybridoma clones secreting antibodies to a 80 kDa protein from human mammary carcinoma cells (T47D) were generated by a similar procedure. The purification of this protein has been described elsewhere (A. Chandra, G. Bosma and P. Chandra, in preparation).

2.5. Immunoblotting of tat protein

Jurkat cells were transfected with a tat-expressing vector pCV1; non-transfected cells served as control. The transfection procedure and the preparation of cell lysates is described in Section 2.3. Lysates from pCV1-transfected and non-transfected cells were fractionated by SDS-polyacrylamide slab gel (PAGE) electrophoresis [10]. The proteins were transferred to nitrocellulose sheets using an electroblotting device (LKB Transphor, unit 2005) overnight at 35 V. The nitrocellulose sheets were incubated in phosphate-buffered saline (PBS) containing 5% milk powder and 0.08% antifoam for 3 h at 37°C. The sheets were washed thrice with PBS containing 0.05% Tween 20 and incubated in PBS containing 2.5% bovine serum albumin (BSA), 2.5% normal horse serum, 0.05% Tween 20 and affinity-purified (see Section 2.4) monoclonal anti-tat antibodies (clone C3.2D7). After an overnight incubation at 4°C the sheets were washed thrice with PBS containing 0.05% Tween 20. The sheets were incubated in PBS containing 2.5% BSA, 2.5% normal horse serum, 0.05% Tween 20 and biotinylated horse anti-mouse IgG (Vector, BA-2000, 1:1000) for 1 h at 37°C. After washing thrice with PBS containing 0.05% Tween 20 the sheet were incubated in PBS containing 2.5% BSA, 2.5% normal horse serum, 0.05% Tween 20 and horseradish peroxidase-avidin D conjugate (Vector, A-2004, 1:1000) for 1 h at 37°C. After the final washing the sheets were developed at room temperature in phosphate buffer (pH 7.4) containing 4-chloro-α-naphthol (0.75 mg/ml), methanol (25%) and 0.12% solution of hydrogen peroxide (30%). The sheets were washed twice with bidistilled water and air-dried.

2.6. Indian ink staining

The transfer membrane was washed twice with PBS containing 0.3% Tween 20. It was then immersed in PBS containing 0.3% Tween 20, 0.4% (v/v) Indian ink and placed on a shaking platform until appearance of bands. The sheets were washed twice with bidistilled water and air-dried.

3. Results

Our interest was to compare the effects of Tat-IgG on the intracellular and intercellular transactivation mediated by the transactivator protein of HIV-1. For studying intracellular transactivation Jurkat cells were co-transfected with a plasmid expressing tat protein (pCV1) and another plasmid that contained the construct HIV-1 LTR hybridized to the reporter gene CAT (pC15CAT). For studying intercellular transactivation we transfected Jurkat cells separately with pCV1, or pC15CAT, and cocultivated the two cell populations to analyze HIV-1 LTR transactivation.

The effect of tat-IgG on the intracellular (Fig. 1A) and intercellular (Fig. 1B) tat-mediated transactivation is depicted in Fig. 1. Quantitatively the magnitude of transactivation mediated by the intercellular traffic of tat protein is only 22% of that of intracellular transactivation. These quantitative differences are probably due to the nature of the experimental conditions in the two systems. Intercellular system is dependent on the release and uptake of functionally active tat protein, whereas in the intracellular transactivation the tat protein functions within the same cell. This also explains the differences in the inhibitory effects of tat IgG on intracellular and intercellular transactivation systems. Since the immunoglobulins cannot enter the cells, the intracellular transactivation is not significantly affected by antibodies to tat protein (Fig. 1A). However, the intercellular traffic of tat protein can be blocked by the capture of extracellular tat protein by tat-specific antibodies. This would result in the inhibition of intercellular transactivation which is dependent on the availability of functionally active extracellular tat protein. The immune complexes formed between the extracellular tat protein and tat IgG cannot be taken up by the cells. Fig. 1B depicts the effect of tat IgG on intercellular transactivation. At a concentration of 7 μg/ml of tat IgG, a 70% inhibition of intercellular transactivation was achieved.

The second question we asked was whether the inhibition of intercellular transactivation involves a specific interaction between tat protein and tat IgG. To examine this aspect we studied the effect of different antibodies on the intercellular transactivation mediated by the extracellular tat protein (Table 1). Monoclonal antibodies to HIV-1 reverse transcriptase at 7 μg/ml had no effect on the intercellular transactivation potential of tat protein. Also the mAb to a human mammary carcinoma protein did not affect the transactivation potential

Table 1
Effect of different monoclonal antibodies on the intercellular transactivation mediated by tat protein

Antibody used	Antibody concentration (μg/ml)	pg CAT/10 μg protein (% CAT activity)
–	0	4519 (100)
HIV-1 RT IgG (4F8)[a]	7	4221 (93.4)
–	0	2956 (100)
Ma-Ca IgG (C7C)[b]	7	2733 (92.4)
–	0	4925 (100)
TAT IgG (C3.2.D7)	7	1445 (29.3)

[a]Designates hybridoma clone 4F8 secreting antibodies to HIV-1 reverse transcriptase generated in our laboratory.
[b]Designates hybridoma clone C7C secreting antibodies to a 80 kDa protein present only in human mammary tumor cells. This hybridoma clone was generated in our laboratory.
Each value is the arithmetic mean of three individual experiments. The studies with individual antibodies were carried out at different times. For this reason, the controls (without antibody) for each set of experiment are mentioned separately.

of tat in this system. These results convey the message that inhibition of intercellular transactivation by tat IgG involves a specific interaction between tat protein and the antibody. The fact that anti-tat IgG reacts only with tat protein is further supported by experiments on the immunoblotting of tat protein expressed in Jurkat cells (Fig. 2). Immunoreactivity of anti-tat IgG with proteins separated by SDS-PAGE from Jurkat cells transfected with the tat-expressing plasmid pCV1 (lane 2) or non-transfected Jurkat cells (lane 1) is depicted in Fig. 2B; lane 3 depicts the immunoreactive profile of a partially purified tat protein expressed in *E. coli*. The immunoreactive band at 14.5 kDa was observed only in lanes 2 and 3. There was no immunoreactive band detectable in proteins separated from extracts of non-transfected Jurkat cells (lane 1).

Cultivation of Jurkat cells transfected with pC15CAT in conditioned medium from cultured cells transfected with pCV1 showed no transactivation potential (data not shown). This would indicate that the tat protein released from cultured cells is degraded during centrifugation and other experimental manipulations. The fact that synthetic tat [12], recombinant tat [13–15] and synthetic peptides corresponding to different regions of the tat protein [16] are able to catalyze transactivation in cells transfected with HIV-1 LTR-CAT indicates that the transactivating potential of tat protein released from the cell is independent of any tat binding protein, or any cell-cell contact [17]. This conclusion is also supported by studies on exogenous tat functions other than the transactivating potential [18,19].

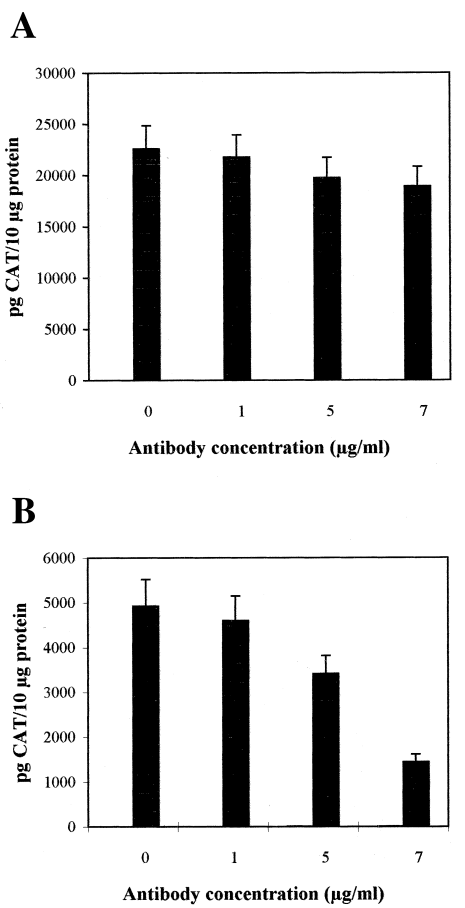

Fig. 1. Effect of anti-tat mAbs directed to the N-terminal region (2–19) of tat protein on intracellular (A) and intercellular (B) transactivation mediated by tat protein. All transfections were done in triplicate for each set of experiments. The values depict an arithmetic mean (±8–14%) of three individual experiments.

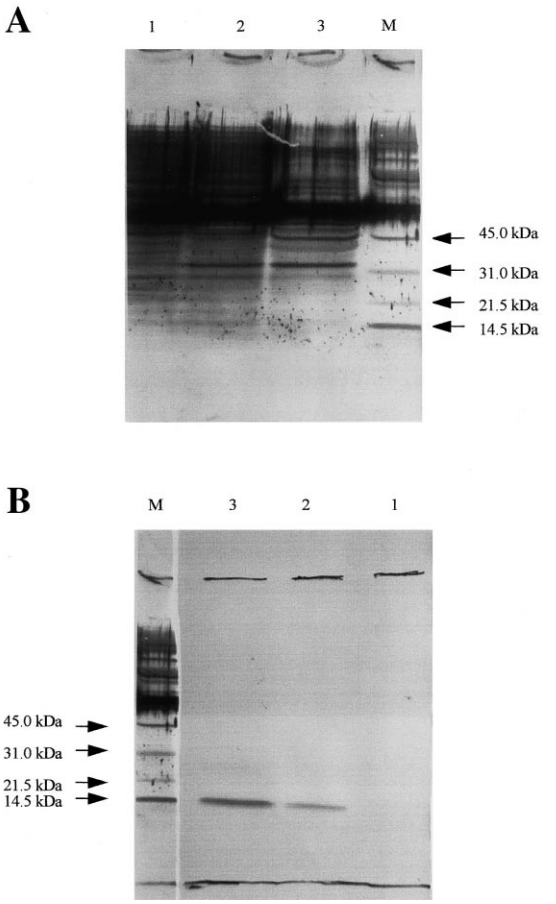

Fig. 2. Immunoreactivity of anti-tat IgG with proteins separated by SDS-PAGE: A: Indian ink staining. B: Immunoblot. Lane 1: non-transfected Jurkat cells; lane 2: Jurkat cells transfected with the tat-expressing plasmid pCV1; lane 3: partially purified tat protein expressed in *E. coli* cells. Experimental details are described in Section 2.

4. Discussion

Tat protein is responsible for signals which induce a variety of biological responses in HIV-infected cells, as well as in uninfected cells. For this reason, tat protein is an attractive target for therapeutic manipulation of HIV-1 infection. For many years, attempts have been made in our laboratory to develop compounds which block the transactivation potential of tat protein [7,20–22]. This strategy is useful in combating the dysregulation of infected cells in promoting large amounts of virus replication. Extracellular tat protein, secreted by the HIV-1-infected cells, is responsible for signalling dysregulation of the metabolic machinery of uninfected cells leading to immune suppression and T-cell apoptosis, a condition commonly found in patients with HIV-1 infection. In addition to its immunologic dysregulation and apoptosis, tat protein induces proliferation of AIDS-Kaposi's sarcoma lesions. In a recent study [23] we have shown a correlation of the antibody status to tat protein in sera of HIV-infected asymptomatic

patients ($n = 30$) and HIV-infected patients with Kaposi's sarcoma lesions. HIV-infected patients with Kaposi's sarcoma ($n = 36$) did not possess IgG to tat protein. The fact that monoclonal antibodies to tat protein can abolish the intercellular traffic of transactivator, as evident from the data presented here, can be applied to the concept of using tat protein for AIDS vaccination. This approach is different from the known vaccination strategies, since it is directed to a regulatory product of the virus that is essential to pathogenesis rather than to the virion itself. Using an inactivated but immunogenic tat protein (tat toxoid) a phase 1 clinical trial on 14 asymptomatic HIV-infected patients has recently been reported by Gringeri et al. [24]. Although virologic parameters were not assessed, they observed the production of anti-tat antibodies and cell-mediated immunity in treated patients. We have recently evaluated the anti-tat antibody status in 30 HIV-1-infected asymptomatic individuals [20] and found that all the asymptomatic patients had anti-tat IgG in their sera. Epitope analysis has revealed that anti-tat antibodies in these sera were directed towards the functional domains of tat protein. In our opinion the application of anti-tat directed vaccines will be more useful in pathological situations where the anti-tat antibody titer is very low or absent. This is indeed the situation in the later stage of HIV-1 infection, as well as in AIDS patients with Kaposi's sarcoma [23]. These are highly immune-suppressed persons with very limited ability to synthesize antibodies. For this reason, we suggest that passive immunization with anti-tat antibodies is a useful approach to abolish the signals mediated by the extracellular tat protein.

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