EFFECTS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 TAT PROTEIN ON EXPRESSION OF INFLAMMATORY CYTOKINES BY HUMAN ASTROCYTIC AND MONOCYTIC CELLS

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Peiqin Chen

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

To my wife, daughter, and parents.

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LIST OF ABBREVIATION

AIDS	Acquired immunodeficiency syndrome
ADC	AIDS dementia complex
AP-1	Activator protein-1
CAT	Chloramphenicol acetyltransferase
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
BSA	Bovine serum albumin
CSF-1	Colony stimulating factor-1
DAG	Diacylglycerol
DAB	3,3' Diaminobenzidine
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetraacetic-acid
EGF	Epithelial growth factor
ELISA	Enzyme-linked immunosorbent assay

FBS	Fetal bovine serum
HA	Herbimycin A
HIV-1	Human immunodeficiency virus type 1
HRP	Horse radish peroxidase
IFN	Interferon
IL	Interleukin
IP3	Inositol (1,4,5)-trisphosphate
LBP-1	Leader binding protein-1
LPS	Lipopolysaccharides
LTR	Long terminal repeat sequence
MK-801	1-amino-3,5-dimethyladamantine (memantine) and dizocylpine
NBQX	6-Nitro-7-sulphamoylbenzo(f)quinoxaline-2-3-dione
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
NK	Natural killer
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
PAF	Platelet activating factor

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PKA	Protein kinase A
PKC	Protein kinase C
PIP2	Phosphatidylinositol 4,5-bisphophate
PLC	Phospholipase C
PTK	Protein tyrosine kinase
RNA	Ribonucleic acid
mRNA	Messenger RNA
RRE	Rev response element
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
SSC	Saline-sodium citrate
SSPE	Saline-sodium phosphate-EDTA
TAR	Transactivating response element
TGF	Transforming growth factor
TLCK	Nα-p-Tosyl-l-lysine chloromethyl ketone

TNF	**Tumor necrosis factor**
USF-1	**Upstream stimulatory factor-1**

I. ABSTRACT

Effects of recombinant Tat_{1-72} on the expression of inflammatory cytokines were investigated using a human monocytic cell line (THP-1) and a human astrocytoma cell line (U373 MG). Tat induced a differential expression of mRNA for cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in THP-1 and U373 cells. The increases in gene transcriptions lead to corresponding increases in the production of cytokine proteins in THP-1 cells. However, in U373 cells, Tat only induced production of IL-6 protein while the inductions of IL-1β and TNF-α proteins by Tat were not observed. To determine the mechanism(s) by which Tat induced the expression of cytokines, we first examined the role of nuclear factor kappa B (NF- κB). Tat-induced expression of IL-1β and IL-6 in U373 cells, and IL-1β and TNF-α in THP-1 cells were completely blocked by an inhibitor of NF-κB activation (TLCK), indicating that NF-κB mediates Tat induced-cytokine production. Further the inhibition of phospholipase C (PLC), protein tyrosine kinase (PTK) or protein kinase A (PKA) blocked the expression of IL-1β and TNF-α. Whereas inhibition of protein kinase C (PKC) had no effect on Tat induction of cytokine expression, suggesting that PLC, PKA and PTK are involved in Tat induced expression of the cytokines probably through activating NF-κB.

II. INTRODUCTION

Since the identification of the first case of the acquired immunodeficiency syndrome (AIDS) in 1981 (Gottlieb *et al.* 1981), the epidemic has spread world wide, currently infecting 20 million people and carries a nearly 100 percent mortality rate. Despite identification of HIV-1 as the etiological agent of AIDS in 1983 (Barre-Sinoussi *et al.* 1983), the pathogenesis of AIDS is still not well understood and effective approaches for prevention of human immunodeficiency virus type 1 (HIV-1) infection and treatment of the disease are still to be developed.

HIV-1 is a member of lentivirus subfamily of retroviruses. The main target cells of HIV-1 are helper T lymphocytes which exhibit the surface marker CD4 (also called T4) and cells of the monocyte/macrophage lineage. It can also infect other types of cells in the immune and central nervous systems. The major consequences of HIV-1 infection are slow, progressive, degenerative changes in the immune and central nervous systems.

The impairment of the immune system is characterized by progressive depletion of T4-cells. The most important effector systems of the immune defense (production of antibodies, cytotoxic T lymphocyte (CTL), natural killer (NK) cells, monocytes/ macrophages) require assistances from T4-cells. This assistance consists of cytokines, mainly including Interleukin-2 (IL-2) and Interferon-γ (IFN-γ), produced and secreted only by activated T4-cells. The depletion of T4-cells results in the second failure of all T-helper cell-dependent effector systems. A direct consequence of the failure is repeatedly opportunistic infections which are the main cause of death in AIDS patients.

Approximately one third of individuals with AIDS eventually have neurologic complications resulting in cognitive, motor, and behavioral dysfunction, which are directly attributable to infection of the brain by HIV-1 (Price and Brew, 1988; Lipton, 1994). A severe degree of neuronal injury often occurs in the brain tissue of these patients. (Ketzler *et al.* 1990; Everall *et al.* 1991; Oster *et al.* 1995). The neuronal loss may account for the clinically apparent neurological deficits. However, there is little evidence that neurons themselves are infected by HIV-1 . Thus, indirect mechanisms for HIV-mediated neuron damage are postulated. Microglia and macrophages are the major cell type productively infected with HIV-1 in the brain (Vazeux *et al.* 1987; Kure *et al.* 1990). However, the mere presence of a small number of virus-infected brain macrophages and microglia is not enough to explain the severe clinical and pathological brain abnormalities. This suggests that brain injury in AIDS requires a cellular amplification and/or activation, which is necessary for the generation of cellular and viral toxins that cause brain tissue injury and sustained viral infection. HIV- infected brain macrophages produce and release some cellular factors such as cytokines (Tyor *et al.* 1992; Wesselingh *et al.* 1993), nitric oxide (NO) (Bukrinsky *et al.* 1995), and other soluble factors (Griffin *et al.* 1994; Giulian *et al.* 1990; Pulliam *et al.* 1991). Some viral proteins such as gp120 and Tat can be released from the infected cells. These cellular factors and viral proteins could act as neurotoxins or as cellular activators on neighboring uninfected neural cells, resulting in damage to the cells or release of harmful substances by uninfected cells.

Tat, one of HIV-1 regulatory proteins, has been identified as a transactivator of viral gene expression (Arya *et al.* 1985; Sodroski *et al.* 1986). Tat controls the synthesis of all viral proteins including the production of Tat itself. In combination with cellular factors, it greatly

enhances viral gene expression and replication by interacting with transactivating response (TAR) element in viral long terminal repeat sequence (LTR) (Gaynor, 1995). Tat can also be released from infected cells (Ensoli *et al.* 1990) and produce influences on uninfected cells. It has been shown that Tat induces apoptosis in uninfected lymphocytes (Li *et al.* 1995), suggesting that Tat may play a role in depletion of T lymphocytes in patients with AIDS. Extracellular Tat has been shown to be associated with toxicity in neuronal and glial cells (Sabatier *et al.* 1991; Magnuson *et al.* 1995) (Weeks *et al.* 1995; Nath *et al.* 1996; New *et al.* 1997). Tat also upregulates the production of several inflammatory cytokines in the immune system (Rautonen *et al.* 1994; Gibellini *et al.* 1994; Buonaguro *et al.* 1992; Brady *et al.* 1995) and central nervous system (CNS) (Philippon *et al.* 1994; Cupp *et al.* 1993).

Cytokines are a group of polypeptides with a broad range of activities on a variety of target cells. The roles of cytokines in HIV-induced immunodeficiency have been an intensive field of research. (Tomar, 1994; Hu *et al.* 1994; Fuller and Haynes, 1994; Meyaard and Miedema, 1994; Yoshioka *et al.* 1995). Cytokines, which exert a wide variety of effects to accelerate inflammation, are called "inflammatory cytokines" or "proinflammatory cytokines", and are mainly composed of IL-1β, TNF-α, IL-6, TGF-β1 (Transforming growth factor) and IFN-γ. In addition, these cytokines have many other functions and play active roles in physiological and pathological states. There is increasing evidence that inflammatory cytokines are associated with some central nervous system disorders (Benveniste, 1992; Campbell, 1995; Rothwell and Hopkins, 1995) including HIV-1 infection in brain (Gallo *et al.* 1989; Wesselingh *et al.* 1993). Some inflammatory cytokines may induce neurotoxicity (Chao *et al.* 1995b) and thus play an important role in the pathogenesis of HIV-1-induced

neurological damage and dysfunction.

Traditionally, brain function and dysfunction were interpreted in terms of neuronal activity whereas roles of non-neuronal cells were neglected. Astrocytes, a predominant cell type within the brain (comprising up to 30% of brain volume), were viewed as the type of cells that function exclusively as passive physical support for neurons. It is now well known that a reciprocal relationship exists between neurons and astrocytes, and that this association is vital for mutual differentiation, development, and functioning of both cell types (Vernadakis, 1996; Hertz *et al.* 1996). It is now clear that astrocytes play a prominant role in normal brain development and physiological processes. Astrocytes are importantly involved in the pathogenesis of certain nervous system disorders including AIDS dementia (Rogers *et al.* 1988; McGeer *et al.* 1988; Dickson *et al.* 1991; Peudenier *et al.* 1991; Hofman *et al.* 1989). The roles of astrocytes in chemical-induced CNS injury have been reviewed (Aschner and LoPachin, Jr. 1993).

A diffuse chronic inflammation often exists in the CNS of patients with AIDS dementia complex (ADC). The chronic inflammation undoubtedly plays a promotive role in the development of HIV-induced neurodegeneration. In addition to effects of HIV-1 itself, its products, and induced chemicals, the roles of inflammatory cytokines in maintenance and promotion of chronic inflammation in CNS can be predicted. In the brain tissue infected by HIV-1, astrocytes may be activated by HIV-1 and its gene products to produce inflammatory cytokines.

In view of Tat's effects as a pleiotropic factor on HIV-1 and cellular processes, we postulate that Tat may play an important role in the development of AIDS dementia through

its own toxic effect on neural cells and/or its activation of certain important cells in CNS such as macrophages and astrocytes, resulting in release of some cellular factors and toxins and subsequent CNS injury. In the present research project, Tat protein was assessed for its effects on expression of inflammatory cytokines (IL-1β, IL-6 and TNF-α) by a monocytic cell line (THP-1) and an astrocytoma cell line (U373). Further to determine the mechanism(s) of Tat induction of the cytokines, signal pathway(s) involved in activation of the cytokines was explored. The long term goal of the project is to better understand the underlying pathophysiological mechanisms of HIV dementia which would ultimately help develop novel and better therapies to prevent and treat HIV-1 dementia.

III. LITERATURE REVIEW

1. General structure of HIV-1 virion

HIV-1 virions are particles with a relatively simple structure measuring 80-130 nm in diameter (Fig. 1). They consist of an inner electron-dense core (nucleoid) and an outer envelope. The core is composed of ribonucleoprotein complex (combination of two identical molecules of genomic viral RNA and the nucleocapsid protein) surrounded by a capsid protein. Three functional proteins are also present in the viral core: protease, reverse transcriptase (RT), and integrase. The envelope consists of a host cell-derived bilayer lipid membrane and two viral proteins, the outer surface glycoprotein and the inner transmembrane glycoprotein which spans the viral lipid membrane. An inner coat core protein is intimately associated with the inner surface of the viral membrane.

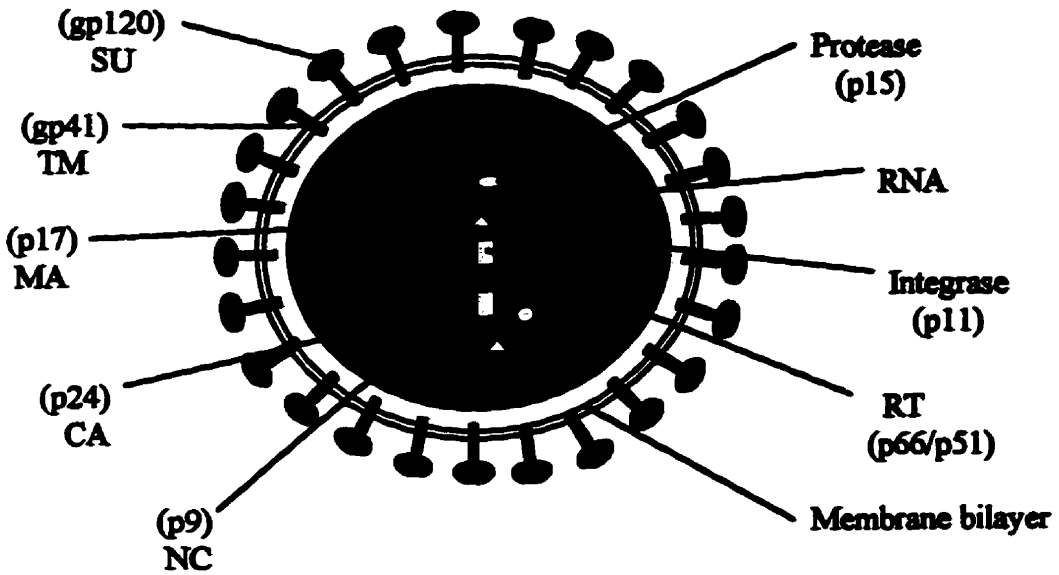


Fig. 1 General structure of HIV-1 virion

2. HIV-1 genomic organization and expression of viral proteins

2.1. Structural genes and their products

The genome of HIV-1 is ~9.8 kilobases (kb) in length (Muesing *et al.* 1985; Ratner *et al.* 1985) (Fig. 2). In common with other retroviruses, HIV-1 contains two LTRs and three major genes: *gag*, *pol*, and *env*. The *gag* gene codes for structural proteins of the core. The Gag precursor p55 gives rise, by proteolytic cleavage, to the smaller proteins p24--the capsid (CA) protein, p17 -- he matrix (MA) protein, p9 -- the nucleocapsid (NC) protein, and p6 (NC). The *pol* gene encodes at least three functional enzymes: protease, RT, and integrase. These proteins originate from $Pr160^{gag\text{-}pol}$, a fusion protein between the Gag and the Pol

proteins (Jacks *et al.* 1988), and are required for virus replication. RT is composed of two subunits (p66 and p51) (di Marzo Veronese *et al.* 1986) (Lightfoote *et al.* 1986). This enzyme has multiple catalytic activities, including a DNA polymerase activity that copies either RNA or DNA templates and ribonuclease H (RNase H) activity that specifically degrades the RNA strand of an RNA-DNA hybrid duplex (Hansen *et al.* 1987; Hansen *et al.* 1988). The protease, a protein of 11 kDa (p11), is responsible for specific cleavage events leading to the release from polyprotein of the mature protease, RT, and integrase. The viral protease is also responsible for processing the Gag polyprotein precursors (Cann and Karn, 1989) (Hansen *et al.* 1988). HIV-1 integrase is a 31 kDa protein, which is essential for integration of retroviral DNA into the host cell chromosome (Brown, 1990). The envelope proteins are synthesized initially as a nonglycosylated 88 kDa precursor (p88). The size of this protein after glycosylation is 160 kDa (gp160). The gp160 precursor is cleaved in the Golgi by a cellular protease to produce a 120 kDa outer surface glycoprotein (SU) and a 41 kDa transmembrane (TM) protein, both of them are involved in virus entry, cell fusion, and cell cytolysis (Lasky *et al.* 1987; Hwang *et al.* 1991; Gallaher, 1987; Kowalski *et al.* 1987).

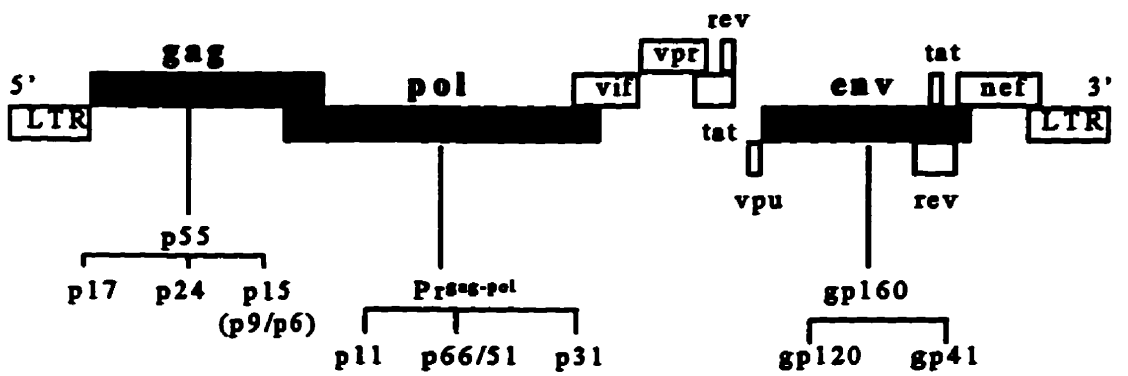


Fig. 2. Genome organization of HIV-1

2.2. Regulatory genes and their products

Tat The *tat* gene consists of two exons which are located at the 3' end of the viral genome. The first exon, preceeding the *env* gene, encodes 1- 72-amino acids (a 14 kDa protein) that contains the major functional domains and exerts major functions of full-length Tat protein (86 amino acids, 16 kDa) (Seigel *et al.* 1986; Conant *et al.* 1996; Garza, Jr. *et al.* 1996). The second exon, within the *env* gene, codes for an additional 14 amino acids that may be involved in Tat binding to cell surfaces via integrin-mediated cell adhesion (Brake *et al.* 1990). More information about Tat will be given in the next chapter.

Rev The Rev protein, another regulator of HIV-1 viron expression, is also encoded by two exons which partly overlap the *tat* exons. This 19 kDa protein is also required for viral replication (Feinberg *et al.* 1986). Unlike Tat, Rev interacts with a cis-acting RNA loop structure called the Rev responsive element (RRE), located in the *env* gene (Felber *et al.* 1989; Malim *et al.* 1989). It has been shown that Rev increases the half-life of RRE-containing HIV-1 mRNA (Felber *et al.* 1989), promoting their transport from the nucleus to the cytoplasm (Guyader *et al.* 1987; Ellison *et al.* 1990; Felber and Pavlakis, 1993) and their efficient translation (Arrigo and Chen, 1991) (Hadzopoulou Cladaras *et al.* 1989).

Nef The *nef* gene is contained in a single open reading frame at the 3' end of the genome overlapping the *env* gene and the 3' LTR. Nef is a 25- to 27-kDa myristylated phosphoprotein that is associated with the cytoplasmic membrane (Allan *et al.* 1985; Franchini *et al.* 1986). The inhibition of Nef as a negative factor on HIV-1 LTR-specific gene expression and viral replication is still in controversy (Niederman *et al.* 1989; Hammes *et al.* 1989; Kim *et al.* 1989; Admad-N 1988). It has been shown that Nef downregulates

expression of CD4 on the surfaces of infected cells (Rhee and Marsh, 1994). Nef is probably important for pathogenesis in vivo since it has been demonstrated that the presence of an intact *nef* gene is required to prolong SIV infection and induce pathogenesis in infected macaques (Kestler, 3d *et al.* 1991). In addition, HIV-1 genome encodes several accessory proteins including Vpu, Vif, and Vpr.

2.3. Accessory genes and their products

Vpu These proteins are involved in viral assembly, maturation, infectivity, and release. Vpu is a 15 to 20 kDa protein and appears to be associated with cytoplasmic membranes of infected cells (Strebel *et al.* 1989). It has been shown that the presence of the Vpu protein significantly increases the number of virus particles released into the supernatants of infected $CD4^+$ T cell lines (Klimkait *et al.* 1990; Strebel *et al.* 1988).

Vif Vif, a 23 kDa cytoplasmic protein is classified as a late gene product because its expression is dependent on Rev (Garrett *et al.* 1991). Vif-defective mutant viruses do not affect expression of viral proteins (Kan *et al.* 1986; Strebel *et al.* 1989). However, the Vif-defective viruses have been reported to be ~1,000 fold less infectious than wild-type virus (Fisher *et al.* 1987; Kan, 1991).

Vpr The Vpr protein of HIV-1 is a 15 kDa protein that is dispensable for HIV-1 replication in culture (Ogawa *et al.* 1989). This protein is present in mature virus particles (Cohen *et al.* 1990). The Vpr protein accelerates the replication and cytopathic effect of HIV-1 in CD4+ T-cells, with the most pronounced effect exerted early in infection (Cohen *et al.* 1990; Ogawa *et al.* 1989).

2.4. LTR

At the 5' and 3' ends of HIV proviral genome, there are identical viral sequences of DNA termed the long terminal repeat (LTR). The LTR contains three subregions, U3, R, and U5 (Fig. 3), which comprises 513 bp and play a role in viral integration and transcription (Varmus, 1988). The 5' LTR can be divided into three functional regions designated the modulatory, core, and TAR elements (Demarchi *et al.* 1993). The modulatory region contains a number of defined motifs which bind cellular transcription factors to either upregulate or downregulate viral RNA transcription. These motifs include three activator

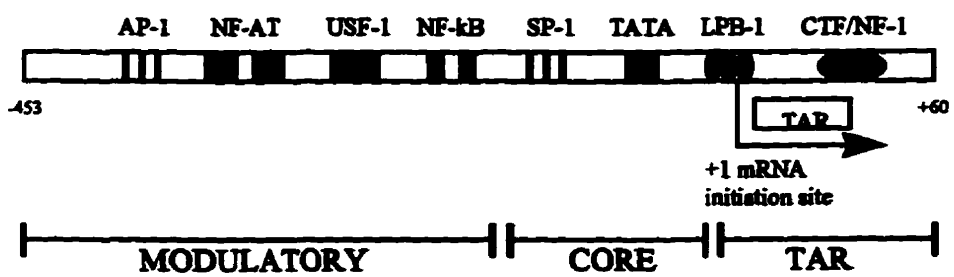


Fig. 3. Structure of HIV-1 LTR

protein-1 (AP-1) binding sites, nuclear factor of activated T cells (NF-AT) binding sites, upstream stimulatory factor-1 (USF-1) binding sites, and two nuclear factor kappa B (NF-κB) binding sites. The core region consists of three SP-1 binding sites, a TATA binding site, and three leader binding protein-1 (LPB-1) binding sites. TAR, a unique region in the LTR, is the binding site of HIV-1 protein Tat, which is a potent transactivator of transcription and is absolutely required for viral infectivity (Cullen and Garrett, 1992; Felber *et al.* 1989).

3. HIV-1 Tat protein

3.1. Sequence and functional regions of Tat

The sequence of the 86 amino acid Tat protein may be divided into four functional regions (Fig. 4), the acidic region, an acidic group of amino acid residues at the N-terminus (from 1 to 21), the cysteine-rich region (from 22 to 37), comprising seven cysteine residues, the core region (from 38 to 47), and the basic region (from 38 to 57). The acidic region has been suggested to form an amphipathic α-helical structure which may constitute an activating domain of Tat (Rappaport *et al.* 1989). The cysteine-rich domain is also critical for Tat transactivation function (Garcia *et al.* 1988; Kuppuswamy *et al.* 1989; Rice and Carlotti, 1990) and metal binding and dimerization have been proposed for this region (Garcia *et al.* 1988; Frankel *et al.* 1988a). Some studies showed that the first 47 amino acid residues of Tat exerts the transactivation function of Tat in cell-free transcription systems (Jeyapaul *et al.* 1991; Jeyapaul *et al.* 1990). The core and basic regions are highly conserved among all known Tat proteins. The core domain is required for sequence- specific RNA binding (Churcher *et al.* 1993; Kuppuswamy *et al.* 1989; Rice and Carlotti, 1990). A recent study has demonstrated that soluble peptide analogs of the Tat core domain are able to effectively block LTR transactivation (Kashanchi *et al.* 1997), suggesting that the core domain is important for Tat transactivation. The basic domain of Tat, composed of nine basic amino acids, has been found to have several different functions. It is required for the nuclear and nucleolar localization of Tat (Hauber *et al.* 1987; Kuppuswamy *et al.* 1989) (Ruben *et al.* 1989; Siomi *et al.* 1990) and essential in mediating Tat binding to TAR RNA of HIV-1

(Dingwall *et al.* 1989; Weeks *et al.* 1990; Cordingley *et al.* 1990). This domain is also involved in Tat transactivation (Valvatne *et al.* 1996). The core and basic regions have been shown to be neurotoxic (Nath *et al.* 1996; Philippon *et al.* 1994). The second exon of *tat*, which codes for 14 additional amino acids, is dispensable to the transcription and replication of HIV-1. However, this portion of Tat contains an RGD sequence that may be important in Tat binding to cell surfaces via integrin- mediated cell adhesion (Brake *et al.* 1990). The relationship of Tat structures and its functions is summarized in Table 1.

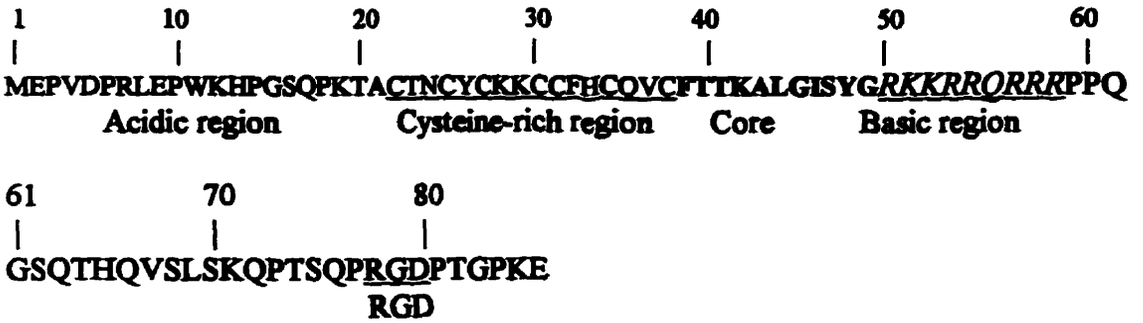


Fig. 4 Tat amino acid sequence and its functional regions of HIV-1_{BRU}

Table 1. Major functional regions of Tat

Region	Position	Functions
Cysteine-rich region	22-37	Metal binding, dimerization, transactivation
Core region	38-48	TAR RNA binding, transactivation, neurotoxicity
Basic region	49-57	TAR RNA binding, nuclear and nucleopar localization, transactivation, neurotoxicity

3.2. Biological effects of Tat protein

Tat transactivation of HIV-1 gene expression has been well studied. Further studies suggest that the primary effect of Tat appears to be at the level of transcription elongation. Some experiments indicate that in the presence of *tat*, the level of short transcripts generated from the HIV-1 promoter decreased, while the level of elongated transcripts was found to increase (Kao *et al.* 1987; Laspia *et al.* 1990; Feinberg *et al.* 1991). In the past few years, it has become quite clear that the function of Tat is not limited to regulation of viral gene expression. Many studies suggest that Tat is involved in regulation of certain cellular processes. Tat can enhance the growth of Kaposi's sarcoma (KS) cells (Vogel *et al.* 1988; Ensoli *et al.* 1990; Ensoli *et al.* 1991; Ensoli *et al.* 1993) and Tat-expressing transgenic mice develop liver cancer (Vogel *et al.* 1991). These observations suggest that Tat may influence growth properties of cells as a growth factor or as a tumor promoter. Tat increases production of inflammatory cytokines such as TNF-β in monocytic and T-lymphocytic cell lines (Buonaguro *et al.* 1992), TGF-β1 in an astrocytic-derived cell line (Cupp *et al.* 1993), and IL-6 in endothelial cells (Hofman *et al.* 1994). Tat induces apoptosis in lymphocytes (Li *et al.* 1995) and neurotoxicity in neuroblastoma cell lines (Sabatier *et al.* 1991) and human fetal neurons (Magnuson *et al.* 1995; Nath *et al.* 1996) *in vitro*, suggesting that Tat may play an important role in the pathogenesis of AIDS dementia.

4. Cellular signal transduction pathways and Tat

4.1. Protein kinases

Many cellular processes require some form of transmembrane signalling. Protein phosphorylation is an important event in transmembrane signal transduction in eukaryotic

cells (Cohen, 1992). A number of protein kinases have been identified. In general, they are divided into serine/threonine kinases and tyrosine kinases on the basis of the phosphate accepting residues in the substrate proteins. Several signal transduction pathways, which are involved in PKC, PKA, and PTK, have been well studied (Sweet and Hume, 1996; Sugden and Bogoyevitch, 1995; Foster, 1993; Duff and Berk, 1995). PKC belongs to the serine/ threonine kinase family that consists of at least 12 isotypes in mammalian cells, indicated by Greek symbols, α, βI, βII, γ, δ, ε, η, ι, ζ, λ, θ and μ. Binding of various ligands to their cell membrane receptors leads to activation of G-proteins. The active G-proteins are then able to activate different effector systems. One of the signalling mechanisms is activation of membrane-bound PLCβ to hydrolyze the lipid precursor PIP2 at the third position of the glycerol backbone liberating two second messengers, inositol (1,4,5)-trisphosphate (IP3) (Alexander *et al.* 1985) and diacylglycerol (DAG) (Griendling *et al.* 1986). IP3 stimulates the release of Ca^{2+} from intracellular stores , and the elevated concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and DAG stimulate the activity of PKC, resulting in phosphorylation of a large number of proteins (Nishizuka, 1986) and changes of cellular processes (De Graan *et al.* 1991; Linden and Routtenberg, 1989; Costa *et al.* 1992). Extracellular Tat protein has been found to be associated with an increase in cytoplasmic PKC activity (Conant *et al.* 1996). However, the consequences of increased PKC activity is unknown.

Another signalling mechanism regulated by G-protein is activation of adenylate cyclase leading to an increase in intracellular cAMP, which then acts as a second messenger by activating PKA. PKA phosphorylates substrate proteins, leading to a variety of physiological responses (Scott, 1991; Sugden and Bogoyevitch, 1995).

PTK also plays an important role in cellular signal transduction pathways. PTKs can be divided into two groups, so called receptor and non-receptor kinases. Receptor kinases are represented by TGF-1 receptor such as receptors for platelet-derived growth factor (PDGF), epithelial growth factor (EGF) and colony stimulating factor-1 (CSF-1). These kinases possess a transmembrane region which divides the ligand binding domain from the intracellular kinase domain. Non-receptor kinases lack the transmembrane or extracellular regions but attach to the membrane and may interact with receptors of the membrane whereas some of non-receptor PTKs associate with intracellular parts of membrane receptors (Rudd *et al.* 1993; Weiss and Littman, 1994). Binding of some ligands to their receptors activates PTKs, resulting in phosphorylation of target proteins including membrane-associated PLCγ (Weiss *et al.* 1991; Secrist *et al.* 1991). Activated PLCγ exerts the same function as does PLCβ. It has been reported that PTK mediates LPS induction of cytokines (Geng *et al.* 1993; Shapira *et al.* 1994).

4.2. NF-κB

NF-κB is a member of the Rel protein family which is divided into two groups based on differences in their structures, functions, and modes of synthesis (Baeuerle and Henkel, 1994; Siebenlist *et al.* 1994). The first group comprises p50 (NF-κB1) and p52 (NF-κB2), which are synthesized as precursor proteins of p105 and p100 kDa, respectively. The second group consists of p65 (RelA), Rel (c-Rel), RelB, and Drosophila Rel proteins dorsal and Dif. The proteins of both groups share a common region called Rel homology domain that includes DNA binding and dimerizations domains and a nuclear localization signal. The proteins of the second group possess one or more transcriptional activation domains. Members of both

groups of Rel proteins can form homo- and heterodimers. NF-κB is a p50/p65 heterodimer.

NF-κB is a pleiotropic transcription factor that participates in regulation of a wide variety of genes whose products are involved in diverse biological processes including immune response, inflammation, and cell differentiaion and growth control (Grilli *et al.* 1993; Shapira *et al.* 1994; Grimm and Baeuerle, 1993). Typically, NF-κB inactively exists in the cytoplasm complexed to its inhibitor IκBα (Baeuerle and Henkel, 1994). A variety of stimulation of cells, including cytokines, LPS, bacteria, and viruses (Grilli *et al.* 1993), results in phosphorylation of IκBα by unidentified kinases and dissociation of IκBα from NF-κB. The liberated NF-κB translocates to the nucleus and activates a variety of cellular and viral promoters including that of HIV-1 (Grilli *et al.* 1993; Siebenlist *et al.* 1994). Thus, NF-κB acts as a mesenger to transmit the gene induction signal from the cytoplasm or cell surface to the nucleus. Activation of NF-κB requires the dissociation of NF-κB/IκBα complexes. It has been shown that various treatments of cells result in phosphorylation of IκBα followed by degradation of the modified IκBα by some cellular proteases (Finco *et al.* 1994; Lin *et al.* 1995).

Extracellular Tat protein has been found to increase NF-κB binding activity in primary human astrocytes (Conant *et al.* 1996) and in the HL3T1 cell line that contains LTR-CAT construct (Demarchi *et al.* 1996). These data suggest that some effects of Tat on cellular processes, such as induction of cytokines, may be mediated by NF-κB. Some studies have indicated that NF-κB is involved in effects of Tat on certain cellular processes. Tat has also been shown to increase TNF-α and TNF-β gene expression in *tat*-transfected T-lymphocytic and monocytic cell lines (Buonaguro *et al.* 1992). Further studies have shown that the NF-κB

binding sites of the TNF-β promotor are required for Tat-mediated activation and a predicted stem-loop structure in the TNF-β mRNA leader region, which resemblies the TAR of the HIV-1 LTR, is essential for TNF-β activation by Tat (Buonaguro *et al.* 1994). Activation of NF-κB is associated with the activation of some kinases (Koong *et al.* 1994; Siebenlist *et al.* 1994). LPS-induced activations of PTK and PKA result in increases in NF-κB activity (Geng *et al.* 1993). LPS and Tat protein have been found to be associated with increases in PKC activity (Geng *et al.* 1993; Conant *et al.* 1996). However, the LPS- or Tat-associated increases in PKC activity were demonstrated not to be responsible for the increase in NF-κB binding activity (Geng *et al.* 1993; Conant *et al.* 1996).

5. HIV-1 infection in CNS and the AIDS dementia complex

Infection by HIV-1 is often complicated by an array of CNS disorders (Levy *et al.* 1985; Navia *et al.* 1986). In addition to opportunistic infections or neoplasms, some disorders are directly caused by HIV-1 infection of the CNS. The most important of these is a progressive dementing illness that is seen in both adults and children and has been described by a variety of terms, including subacute encephalitis, AIDS dementia, or encephalopathy, and the AIDS dementia complex (ADC) (Snider *et al.* 1983; Epstein *et al.* 198; 5Navia *et al.* 1986; Price *et al.* 1988). ADC's clinical picture is characterized by a constellation of cognitive, motor, and behavioral dysfunction (Navia *et al.* 1986).

5.1. Cell types infected by HIV-1

Detectable infection appears to preferentially involve diencephalic structures, particularly the globus pallidus, but also other basal ganglia nuclei, the substantia nigra, and deeper white

matter, with less frequent infection of the cerebral cortex (Rosenblum *et al.* 1989; Kure *et al.* 1990). Numerous studies have shown that the cells chiefly infected by HIV-1 in the brain are monophagocytes including brain macrophages, microglia, and multinucleated giant cells , which are actually derived from infected macrophages and likely microglia (Spencer and Price, 1992; Atwood *et al.* 1993). In addition, nonproductively HIV-1-infected astrocytes were also detected in the brain specimens from pediatric AIDS cases with well-documented HIV-1- associated dementia (Tornatore *et al.* 1994; Epstein and Gendelman, 1993). However, there is little evidence for the infection of oligodendrocytes and neurons by HIV-1.

5.2. Pathology of HIV-associated dementia

All of the pathological changes are mainly observed in subcortical regions rather than the cortex. (Navia *et al.* 1986; Budka, 1989; Budka, 1991; Budka, 1990). The diffuse white-matter pallor accompanied by astrocytic reaction (i.e. astrocytosis) is the most common of these pathological findings. This change involves particularly the central white matter and diencephalic nuclei. Multinucleated cells are found in a subgroup of patients who usually have severe clinical disease. Reactive macrophage infiltrates accompany the multinucleated cells and are present in the perivascular region and parenchyma. Quantitative studies of cellular pathology have shown that there is a loss of neurons from the frontal cortex of AIDS patients ranging from 18-38% (Everall *et al.* 1991; Ketzler *et al.* 1990) and a loss of up to 50% of large neurons in the neocortex of patients with HIV-1 encephalitis (Bito *et al.* 1992). A loss of synaptic density and vacuolation of dendritic processes have also been observed (Gnann, Jr. *et al.* 1987).

5.3. Mechanisms of HIV-1 damage to the CNS

Mechanisms of HIV-1-induced brain injury have not been well understood. Generally, HIV-1 infection in brain is not abundant and does not seem to correlate with clinical severity of disease or histopathology. This suggests that indirect mechanisms may play a major role in brain injury. Virus-coded products or cell-coded products released by infected or reactive cells might exert toxic effects on neurons or other neural cells.

5.3.1. Effects of HIV-1 proteins

gp120 Picomolar concentrations of the envelope protein gp120 increase levels of intracellular calcium ($[Ca^{2+}]_i$), and lead to death of rodent hippocampal neurons or retinal ganglion cells (Dreyer *et al.* 1990; Lo *et al.* 1992; Brenneman *et al.* 1988). The toxic effect of gp120 is blocked by calcium channel antagonists such as nimodipine and nifedipine (Dreyer *et al.* 1990). This type of neuronal injury is similar to effect of glutamate on NMDA receptors, a subtype of glutamate receptors that mediate excitatory neurotransmission in the brain. A test of the association between gp120 toxicity and N-methyl-D-aspartic acid (NMDA) receptors showed that NMDA antagonists, 1-amino-3,5-dimethyladamantine (memantine) and dizocipine (MK-801), prevent the gp120-induced neuronal injury (Dahl *et al.* 1990; Lipton *et al.* 1991). However, there is no evidence that the toxicity is caused by direct binding of gp120 to the NMDA receptor. Thus, this gp120-induced NMDA receptor activation may be indirect. When endogenous glutamate is degraded by using guanosine triphosphate (GPT), the neurons are protected from gp-120-induced injury (Lipton *et al.* 1991), indicating that endogenous glutamate is required for gp120 toxicity. These data suggest that activation of NMDA receptors and existence of glutamate are necessary for gp120-induced neuronal injury.

Lipton reported that depletion of monocytoid cells from cultures of mixed glia and neurons eliminates toxicity of gp120 (Lipton, 1992), indicating that macrophages/ microglia may be necessary to mediate the neurotoxic effects of gp120. Macrophages stimulated by gp120 release arachidonic acid and its metabolites which increase the release of glutamate and/or inhibit its reuptake, and thus enhance its actions at the NMDA receptor, resulting in excitatory neurotoxicity (Bito *et al.* 1992; Miller *et al.* 1992; Giulian *et al.* 1993; Genis *et al.* 1992). The gp120-stimulated macrophages secrete some inflammatory cytokines, whose effects are discussed below.

Tat There is increasing evidence that Tat is also implicated in HIV-1-associated CNS injury. Tat protein induces cytopathic effects in cultured rodent glioma and neuroblastoma cells and affects rat brain synaptosomal membrane (Sabatier *et al.* 1991; Dalgleish, 1992). Some experiments have shown that Tat causes an inflammatory reaction and apoptosis of neurons in mouse brain (Philippon *et al.* 1994). Tat also causes cytotoxicity to human neuronal cell lines and fetal neurons (Weeks *et al.* 1995; Nath *et al.* 1996). Tat-induced neurotoxicity can be prevented by 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2-3-dione (NBQX); an antagonist of non-NMDA receptors, suggesting that, unlike that of gp120, the Tat -induced neurotoxic effects may be mediated by non-NMDA excitatory amino acid receptors (Magnuson *et al.* 1995; Nath *et al.* 1996).

Peptides derived from the basic domain of Tat increase expression of TNF-α, IL-1, and IL-6 in mouse brain, which may in turn be responsible for some of the pathological changes in brain (Philippon *et al.* 1994). Besides, Tat protein has also been shown to stimulate the production of TGF-β1 in human astrocytic glial cells (Cupp *et al.* 1993). Because certain

cytokines have been suggested to be associated with CNS disorders (Rothwell and Hopkins, 1995; Chao *et al.* 1995a), the neurotoxic effects of Tat may be at least in part mediated by cytokines in brain.

5.3.2. Effects of inflammatory cytokines

Certain inflammatory cytokines have been suggested to be involved in neuropathogenesis of HIV-1 infection. IL-1, IL-6, and TNF-α can upregulate HIV-1 expression/replication in vitro (Poli and Fauci, 1992b; Edfjall *et al.* 1996), suggesting that these cytokines may play a role in maintaining productive HIV-1 infection and activating latent HIV-1 infection in the brain. IL-1 may also disrupt the blood brain barrier (Quagliarello *et al.* 1991), stimulate macrophages/ microglia to release toxins such as β amyloid precusor protein and NO (Buxbaum *et al.* 1992; Lee *et al.* 1993). NO production in the brain correlates with the presence of AIDS dementia (Adamson *et al.* 1996). Increased expression of IL-6 in transgenic animals caused neuronal degeneration and astrocytosis (Campbell *et al.* 1993). A low concentration of IL-6 (at 1 ng/ml) produced neuronal vacuolization (Yeung *et al.* 1995). TNF-α has been shown to induce cytotoxicity and apoptosis in human neuronal cell lines (Tauber *et al.* 1992; Talley *et al.* 1995) and in oligodendrocytes (Wilt *et al.* 1995). Oligodendrocytes are myelin-producing cells. Thus, increased production of TNF-α may contribute to the demyelinization, noted in patients with ADC. Astrocytosis is a very frequent and wide-spread pathologic change occuring in adult and developing brains of HIV-1-infected individuals (Funata *et al.* 1991; Budka, 1990). IL-1 and TNF-α can induce astrocytosis (Giulian *et al.* 1988; Selmaj *et al.* 1991).

5.3.3. Roles of macrophages/microglia in CNS injury with AIDS

Macrophages/microglia play an important role in HIV-1-associated neurological disorders. These cells are the major cell types that support productive HIV-1 infection in the CNS of patients with AIDS. Accumulating evidence has indicated that soluble factors released by HIV-infected macrophages/microglia are neurotoxic (Pulliam *et al.* 1991). These soluble factors include viral products, such as gp120, Tat, and neurotoxins such as eicosanoids, nitric oxide, cysteine, superoxide anions, platelet activating factor (PAF), and inflammatory cytokines (Gendelman *et al.* 1994; Lipton *et al.* 1995). Macophages stimulated by the HIV-1 envelope protein gp120 have also been shown to release some of these factors (Wahl *et al.* 1989; Bukrinsky *et al.* 1995). Eicosanoids and PAF cause neuronal damage by increasing calcium ions and the release of glutamate (Genis *et al.* 1992; Lipton, 1994). The released glutamate contributes to further injury to neighboring neurons. NMDA antagonists can largely inhibit neuronal injury produced by the culture fluid of HIV-infected or gp120-stimulated macrophages (Dreyer *et al.* 1990; Lipton *et al.* 1991; Savio and Levi, 1993).

5.3.4. Roles of astrocytes in CNS injury with AIDS

Astrocytes are the most numerous of neural cells, outnumbering neurons 10:1. Astrocytes extensively exist in the grey and white matters of CNS and intimately contact neurons. Astrocytes have a wide range of functions within the CNS and play very important roles in maintaining normal brain physiology. Astrocytes regulate certain ions, metabolites, and neurotransmitters to maintain the microenvironment of neurons. They are important in the metabolism of neurotransmitters such as glutamate, an excitatory transmitter. Extracellular glutamate is maintained at a low level mainly by a high-affinity uptake system in astrocytes.

Once taken up by astrocytes, glutamate is converted to glutamine via glutamine synthetase that almost exclusively exists in astrocytes. Glutamine is then transported to glutaminergic neurons, where it is reconverted to glutamate via another enzyme, glutaminase (Hertz *et al.* 1979). Thus, astrocytes maintain the normal excitability of the neurons by uptake of glutamate from the synaptic clefts and transport of glutamine to the neurons. Additional functions of astrocytes include contribution to the structural integrity of the blood-brain barrier through its end feet's surrounding of the CNS capillaries and induction of the formation of tight junction between the endothelial cells, proliferation in response to CNS injury, and participation in immune and inflammatory processes in brain through presenting antigens and secreting cytokines. Thus, any abnormality of astrocyte functons potentially impair neurons.

In brains of patients with AIDS, functions of astrocytes are perturbed due to effects of multiple factors. Certain neurotoxins such as arachidonic acid and metabolites produced by gp120-stimulated and HIV-1-infected marophages inhibit the reuptake of glutamate by astrocytes (Barbour *et al.* 1989). Accumulated glutamate and other neurotoxins may cause overstimation of NMDA receptors on the neurons, resulting in increases of intracellular calcium in neurons and excitatory neurotoxicity.

IV. Materials and methods

1. Cell culture U373 cells, a human astrocytoma cell line, was maintained in MEM medium with 10% (v/v) fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U penicillin/ml, 100 μg streptomycin/ml and 0.25 μg amphotericin/ml at 37°C. THP-1 cells, a human macrophage-derived cell line, was cultured in RPMI 1640 medium with 10% (v/v) FBS, 100 U penicillin/ml, 100 μg streptomycin/ml, 0.25 μg amphotericin/ml and 5.5 μM 2-mercaptoethanol at 37°C. Both cell lines were obtained from American type culture collection.

2. HIV-1 Tat protein

Recombinant Tat (1 to 72 amino acids) was prepared in our laboratory as previously reported (Ma and Nath, 1997). The recombinant protein was identified by Western blot analysis and its purity was examined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). An LTR-chloramphenicol acetyltransferase (CAT) assay was used to test the transactivation properties of Tat (Ma and Nath, 1997).

3. Immunoabsorption of Tat protein Antisera to Tat was prepared by immunizing rabbits with recombinant Tat protein in our laboratory. Immunoabsorption of Tat was performed as described previously (Magnuson *et al.* 1995). Briefly, 500 μl of Tat antiserum (1:40) was incubated with 50 μl of protein A-sepharose beads at 4°C for 4 hours. The mixture was centrifuged at 2000 rpm for 3 minutes, washed with phosphate buffered saline (PBS) two times and then incubated with 20 μg of Tat protein at room temperature for 90 minutes. The mixture was centrifuged at 2000 rpm for 3 minutes and the supernatant was collected for later experiments.

4. Treatment of cultures with Tat or pharmacological agents

1) Determination of Tat effects U373 and THP-1 cells (1.5 x 10^6 cells/ml) were treated with PBS, Tat (0.1 μM), lipopolysachoride (LPS) (1 μg/μl) (Sigma), or Tat-immunoabsorbed solution for various times (see legends) at 37°C and then cells and supernatants were collected for analysis of cytokine expression by RT-PCR or enzyme-linked immunosorbent assay (ELISA).

2) Time course U373 and THP1 cells (1 x 10^6 cells/ ml) were incubated with Tat protein at a final concentration of 0.1 μM for 0, 1, 3, 6, 12, 24, and 48 hours, and then the cells and supernatants were harvested for analysis by RT-PCR or ELISA.

3) Dose response U373 and THP1 cells (1.5 x 10^6 cells/ml) were incubated in the presence of different concentrations of Tat (0.001 μM, 0.01 μM, 0.1 μM, 1 μM) for 4, 6 or 16 hours following which the cells and culture supernatants were collected for determining the production of cytokines by RT-PCR or ELISA.

4) Other treatments of cells Cells were pretreated with 100 μM TLCK (Sigma), an inhibitor of chymotrypsin-like proteases for 30 minutes, or with 20 μM H7 (Calbiochem), a blocker of PKC, 10 μM H89, a blocker of PKA, 2.5 μM herbimycin A (HA) (Sigma), a selective inhibitor of PTK, or 10 μM U73122 (Calbiochem), a blocker of PLC, for 3 hours, respectively, prior to the addition of Tat. Cells and culture supernatants were collected for analysis after another 4, 6 or 16 hours of incubation with Tat (0.1 μM) or LPS (1 μg/μl). These inhibitors alone were not found to affect the viability of the cells using trypon blue staining technique (data not shown).

5. Preparation of cell lysates Cell lysates were prepared for ELISA by freezing and thawing

7.5×10^5 cells in 100 µl of fresh medium in dry ice with ethanol for three cycles followed by centrifugation at 5,000 g for 10 minutes and collecting the supernatants (Arend *et al.* 1991).

6. Extraction of total RNA Extraction of total cellular RNA was performed as previously published (Jovelin *et al.* 1995), with minor modifications as follows. Cells were washed with cold PBS and resuspended in 1 ml of 0.5% SDS-Tris- saturated phenol (pH 7.8) and then vortexed for 30 seconds. 150 µl of 2 M sodium acetate (pH 4.0) and 350 µl of diethyl pyrocarbonate (DEPC)-treated distilled water were added to each sample. The samples were centrifuged at 10,000 g for 10 minutes and then the upper phases containing RNA were transferred to fresh Ependorf tubes. One volume of phenol:chloroform (50:1) was added to each sample and mixed to further remove proteins and lipids in the samples. The samples were centrifuged at 10,000 g for 10 minutes, and the upper phases were transferred to fresh Ependorf tubes. After adding 2 volumes of absolute ethanol to each sample, the samples were left at -80 °C for 30 minutes. The samples were centrifuged at 10,000 g for 15 minutes and washed in 500 µl of 80% and then absolute ethanol, in succession. The RNAs were dried in air and then resuspended in 8 µl of DEPC-treated water for cDNA synthesis. The purity of RNA samples was examined on 1 % agarose gels as previously described (Davis *et al.* 1986). No DNA signal was observed (data not shown).

7. First strand cDNA synthesis The first strand cDNA synthesis kits (Pharmacia) were used to produce single complemental DNA chains from the total RNA. According to the manufacturer's protocol, 8 µl of RNA was incubated at 65°C for 10 minutes and then mixed with 5 µl of Bulx mixture (including reverse trancriptase), 1 µl of dithiothreitol (DTT) and 1 µl of primers. The reactions were incubated at 37°C for 1 hour.

8. Oligonucleotide primers and probes Oligonucleotides of sense and antisense primers (for IL-1β, IL-6, TNF-α, and β-actin) were synthesized by GIBCO according to previous designations (Yamamura *et al.* 1991). Internal oligonucleotide probes were designed based on the sequences of these cytokine genes. The sequences of the primers and probes are listed in Table 2.

Table 2. Oligonucleotides sequences for primers and probes

	Primers (5'→3')	Fragment size (bp)	Probes
IL-1β	5' GACACATGGGATAACGAGGC 3' ACGCAGGACAGGTACAGATT	248	CTGCACGCTCCGGGACTCACACCA
IL-6	5' ATGTAGCCGCCCCACACAGA 3' CATCCATCTTTTTCAGCCAT	190	AATTCGGTACATCCTCGACGGCATCT
TNF-α	5' TCTCGAACCCCGAGTGACAA 3' TATCTCTCAGCTCCACACCA	125	CAAGCTGAGGGGCAGCTCCAGTGG
β-actin	5' GTGGGGCGCCCCAGGCACCA 3' CTCCTTAATGTCACGCACGATTTC	540	GAGACCTTCAACACCCCAGCCATGT

9. Polymerase chain reaction PCR was conducted using a programmable thermal controllor PTC100™ (MJ Research, Inc) in a 50 μl reaction volume including 2.5 U Taq DNA polymerase (Roche), 30 nM of each primer, 150 μM of dNTP, 5 μl of 10 x PCR buffer, and 1.5 μl of cDNA solution. Samples were amplified according to a published protocol (Yamamura *et al.* 1991), with minor modifications. Briefly, samples were first heated at 95°C for 5 minutes and then amplified by 32 or 35 cycles of denaturation at 94°C for 1 minute and

annealing-extension at 65°C for 2 minutes. β-actin primers, included in each reaction, served as an internal control for comparison of applied cDNA amounts of different samples.

10. Radioactive labeling of oligonucleotide probes Radioactive probes were prepared according to the following protocol (Davis *et al* 1986): 10 pmol of probe was mixed with 1 μl of T4 kinase (GIBCO), 4 μl of 5 x buffer, 5 μl of ^{32}P-ATP solution (Du Pont), and 9 μl of sterile water. The mixtures were incubated at 37°C for 1 hour followed by 10 min of incubation at 68°C. The free ^{32}P was removed and the ^{32}P-labeled probes were collected using Bio-spin 6 columns (Bio-Rad) according to the manufucturer's instruction. The column was inverted several times to resuspend settled gel. The top cap was removed and then the snap-off tip was snapped off to allow excess buffer to drain by gravity. The column was placed in a collection tube and centrifuged for 2 min at 1,100 x g. The sample was mixed with (80 μl Tris 10 mM)-EDTA (1 mM) buffer and very carefully applied to the center of the column, and then centrifuged for 4 min at 1,100 x g. The labeled probe was collected in a tube.

11. Southern blot and hybridization PCR products were separated on a 1.5% agarose gel and then transfered to GeneScreen *Plus* nylon membranes (Du Pont) according to the manufacturer's protocol as following: the gel was soaked in 0.25 N HCl for 10 minutes and then denatured in 0.4 N NaOH for 30 minutes with gently shaking. The nylon membrane was cut to exactly the same size as the gel. The membrane was pre-wetted in distilled water for a few seconds and then equilibrated in 0.4 N NaOH for 10 minutes. A capillary blot was set up using 0.4 N NaOH as transfer solution and the buffer was allowed to flow through the membrane by capillary action overnight. The membrane was prehybridized in prehybridization

solution [10 x Denhardt's solution, 1% SDS, 5 x SSPE (saline-sodium phosphate-EDTA)] for 1 hour at 65°C. The radioactive probes were mixed with 0.9 ml of distilled water and 30 μg of salmon sperm DNA solution (30 μg/ml) and added into the prehybridization solution at a final concentration of 5×10^5 cpm/ml for each probe. The membrane was incubated at 65°C overnight, and then washed with wash solution [2 x SSC (saline-sodium citrate), 1% SDS] for 5 minutes at room temprature twice and then washed for 15 minutes at 65°C twice. Following washing in another wash solution (0.1 x SSC, 0.1% SDS), the membrane was exposed to Kodak X-AR films at -80°C using an intensifying screen.

12. ELISA

1). TNF-α. Determination of TNF-α release from THP-1 and U373 cells was completed using a modification of the recommended protocol provided by the manufacturer (Pharmingen). The 96 well-plates were coated with 2 μg/ml of purified mouse anti-human recombinant TNF-α (Pharmingen) in coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6) overnight at 4^0C and washed twice with wash buffer (0.5 ml of Tween-20 in 1L PBS, pH 7.4). The plate was incubated with blocking buffer [1% BSA (bovine serium albumin)/PBS, pH 7.4] for 1 hour at room temperature followed by removal of blocking buffer. 50 μl of sample or standard solution was added into each well, incubating overnight at 4^0C. The plate was washed twice in wash buffer and incubated with 50 μl of biotin-labeled mouse anti-human TNF-α antibody (0.5 μg/ml) (Pharmingen) in dilution buffer (0.05% Tween-20/PBS, pH 7.4) for 45 minutes at room temperature. Following 2 washes, each sample was incubated with 50 μl of 1:4000 goat anti-mouse IgG linked to alkaline phophatase-conjugated streptavidin (Jackson Laboratories) for 30 minutes at room

temperature. The plate was washed twice and incubated with phophatase substrate (1 μg/μl) (Sigma) disolved in substrate buffer (disolve 101 mg $MgCl_2$ in 900 ml ddH_2O plus 97 ml of diethanolamine, pH 9.8) for 30 minutes at room temperature. The concentrations of TNF-α were measured at 405 nm wavelength using a microtiter plate reader.

2). IL-1β and IL-6. ELISA kits were provided by R&D System. ELISA for IL-1β or IL-6 was performed according to the manufacturer'instructions. Briefly, 200 μl of standard or sample (1:5 dilution for IL-6) was added to each well which had been precoated with a murine monoclonal antibody (2 μg/ml) against IL-1β or IL-6, incubating for 2 hours at room temperature followed by 3 time wash in wash buffer. 200 μl of IL-1β or IL-6 conjugate (1:1000) (polyclonal antibody against IL-1β or IL-6 conjugated to horse radish peroxidase (HRP) was added to each well, incubated for 1 hour for IL-1β or 2 hours for IL-6 at room temperature. Following 3 time wash, 200 μl of substrate solution (hydrogen peroxide and chromogen) was added to each well. After the plates were incubated for 20 minutes at room temperature, 50 μl of stop solution (2 N sulfuric acid) was added to each well. The optical density of each well was determined within 30 minutes using a microtiter plate reader set to 450 nm with a 570 nm correction wavelength.

13. SDS-PAGE: A discontinuous buffer system was used (Laemmli *et al.* 1970). The protein was dissolved in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) ß-mercaptoethanol, and 0.01% (w/v) bromophenol blue and heated in boiling water for about 4 minutes. After loaded, the gels were run in running buffer (0.025 M Tris-HCl, pH 6.6, 0.192 M glycine and 0.1% SDS) at 150 volts until the tracking dye reached the bottom of the gel. The molecular weight markers (GIBCO) were used to

estimate molecular weight of the protein. The gels were used for Coomassie brilliant blue staining or Western blot.

14. Coomassie blue staining: The SDS-PAGE gel was placed in the staining buffer [20% (v/v) methanol, 7.5% (v/v) acetic acid, 0.04% (w/v) Coomassie brilliant blue] for 1-2 hours with gentle shaking. The gel was then destained in the destaining buffer [20% (v/v) methanol and 7.5% (v/v) acetic acid] at room temperature until protein band(s) could be clearly seen.

15. Western Blot: Western blot was performed according to a standard protocol (Davis *et al.* 1986) with some modifications. Briefly, the gel was immersed in transfer buffer containing 25 mM Tris-HCl, pH 6.6, 192 mM glycine and 20% (v/v) methanol, pH 8.3 for 10 minutes. A piece of nitrocellulose membrane (Bio-Rad, 0.45 μm) was cut to the same size as the gel and presoaked in the transfer buffer for 30 minutes and then the membrane and gel were placed between two 3 MM chromatography papers prewet in transfer buffer. Electroblotting was performed in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 15 volts for 30 minutes. The membrane with bound proteins was blocked with 5% (w/v) skimmed milk in 1 x PBS, pH 7.4 (PBS-5% (w/v) skim milk) for 30 minutes and incubated with Tat antisera (NIH, AIDS Repository) [1:1000 in PBS-5% (v/v) skim milk] at room temperature for two hours and then washed three times over 15 minutes in washing buffer (PBS with 0.05% (v/v) Tween 20). The second antibody (goat anti- rabbit monoclonal or polyclonal antibody conjugated with HRP (Chemicon) was diluted to 1:1500 in PBS- 0.05% Tween 20 and applied to the membrane, incubated at room temperature for 90 minutes followed by three washes in washing buffer and then develop the blot in a developing solution [1x PBS, pH 7.4, 0.05% (w/v) 3,3' diaminobenzidine (DAB) (Sigma), 0.06% (v/v) H_2O_2

(Sigma)] for 5 minutes. The reaction was quenched by rinsing the membrane in dH_2O.

16. Tat transactivation assay HL3T1 cells containing HIV-1 LTR-CAT construct was obtained from Drs. B. K. Felber and Dr. G.N. Pavlakis through AIDS Repository, NIH. The cells were propagated in DMEM with 10% (v/v) FBS as described above. Tat transactivation assay was performed as previously described (Frankel *et al.* 1988b; Felber and Pavlakis, 1988) with minor modifications and CAT activity was measured by a simple phase extraction assay (Seed and Sheen, 1988).

1) Preparation of ^{3}H-chloramphenicol for CAT assay ^{3}H-chloramphenicol (1 μCi/μl) was purchased from Du Pont. To prepare a 0.2 μCi/μl ^{3}H-chloramphenicol stock, 960 μl of 100% ethanol and 40 μl of 100 mg/ml of unlabelled chloramphenicol were added into 250 μl of 1 μCi/μl ^{3}H-chloramphenicol. This stock solution was stored at -20°C. To create a working solution of 0.01 μCi ^{3}H-chloramphenicol, the 0.2 μCi/μl ^{3}H-chloramphenicol stock was diluted 20-fold in ddH_2O. The mixture was then extracted with an equal volume of xylenes by vigorous shaking. The phases were separated by centrifugation at 10,000 x g for 1 minute, and the top xylenes phase was discarded. The extraction was repeated one more time. This working solution of 0.01 μCi ^{3}H-chloramphenicol was used for the CAT assay.

2) CAT assay $1x10^6$ HL3T1 cells were plated into 60 mm^2 culture dishes for 24 hours to 70% confluence. The cells were washed twice with 3 ml serum-free DMEM. 1 ml of DMEM with 10% (v/v) FCS (with scrape-loading) or 0.5% (v/v) FCS (without scrape-loading), Tat (0.1 to 0.5 μM), and chloroquine (100 μM) in the presence or absence of Tat was added. The cells were scraped off the dishes with a rubber policeman and resuspended carefully and evenly. Cells were cultured at 37°C for 24 hours and then washed twice with 5 ml PBS. The

cells were then incubated with 1 ml TEN solution containing 40 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0 and 150 mM NaCl for 5 minutes on ice, scraped off dishes by a rubber policeman, and transferred into a microcentrifuge tube, again, on ice. The cells were then centrifuged at 400 g for 1 minute and the cell pellets were resuspended in 100 µl of ice-cold 0.25 M Tris buffer (pH 7.5) and lysed by freezing in dry ice/ethanol for 5 minutes and thawing at 37°C for 5 another minutes. This freeze-thaw cycle was repeated twice. The cell lysates were then centrifuged at 4°C for 5 minutes. Supernatants were saved for the CAT assay. The protein concentration was determined by a Biorad assay. 50 µl of cell extract was mixed with a 50 µl of the solution containing 0.004 µCi ^{3}H-chloram-phenicol, 25 µg of butyryl-CoA (Sigma) , 0.2 M Tris buffer, pH 8.0 and incubated for 60 minutes at 37°C. The acylated ^{3}H-chloramphenicol was extracted by vigorously mixing the reaction solution with 200 µl of tetramethyl-pentadecane/ xylenes (2/1, v/v). The top organic phase was transferred into a scintillation vial containing 4 ml of scintillation fluid and quantitated by a Beckman LS5000CE counter.

V. RESULTS

1. Analysis of purity of recombinant Tat $_{1-72}$

Purified recombinant Tat_{1-72} was analyzed by 15% SDS-PAGE under reducing condition followed by coomassie blue staining (Fig. 5A). The major band at 14 kDa represents monomeric form of Tat_{1-72}. The purity of recombinant Tat was estimated to be > 90%. Western blot analysis with anti-Tat sera further confirmed that the 14 kDa protein was indeed recombinant Tat_{1-72}. (Fig. 5B).

2. Analysis of Tat_{1-72} transactivation activity

The transactivation activity of Tat_{1-72} for the LTR region of the HIV genome was analyzed using HL3T1 cells with the LTR-CAT construct. Fig. 6 shows that Tat_{1-72} significantly increased LTR-CAT activity (Tat alone) compared to cells without Tat_{1-72} treatment ($p < 0.005$). To determine if the effect was specific for Tat_{1-72}, Tat_{1-72} was preincubated with monoclonal antibody to Tat. The result shows that the transactivation activity of Tat_{1-72} was completely blocked by anti-Tat antibody (Fig. 6). These data demonstrate that recombinant Tat_{1-72} was biologically active.

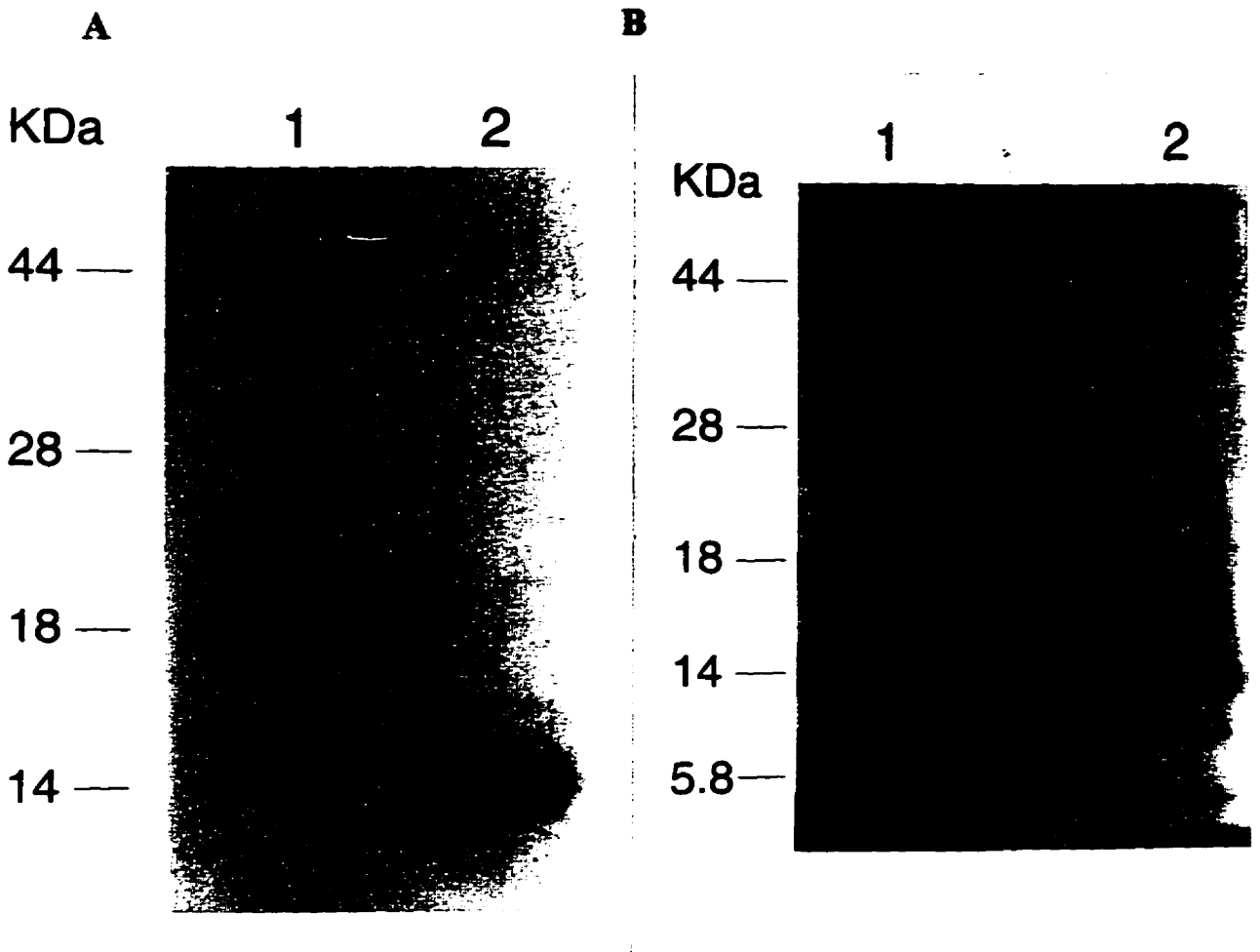


Fig. 5. Analysis of purity of recombinant Tat_{1-72}. (A): Purified recombinant Tat_{1-72} was analyzed by 15% SDS-PAGE followed by coomassie blue staining. Lane 1 shows molecular weight markers. Lane 2 shows a single band at 14 kDa representing recombinant Tat_{1-72} protein. (B): Recombinant Tat_{1-72} was further analyzed by Western blot analysis. Lane 1 shows pre-stained molecular weight markers. Lane 2 shows that Tat antisera recognise a single band at 14 kDa.

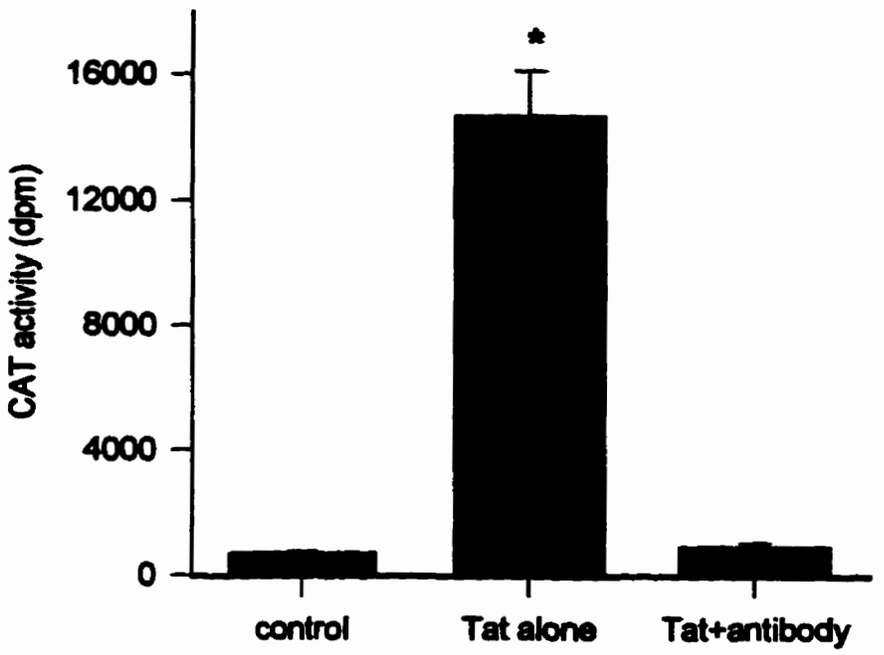


Fig. 6. Transactivation of HIV-1 LTR Tat_{1-72}. As described in the Materials and Methods section, HL3T1 cells were analyzed either untreated (control), following treatment with 150 nM Tat_{1-72} or with 150 nM Tat_{1-72} preincubated with a monoclonal antibody to Tat. A significant transactivation was noted with Tat_{1-72} alone compared to the control group (* $p < 0.005$, Student's *t* test). The values represent the mean $\pm$ SEM of two experiments performed in triplicate.

3. Specific induction of cytokine mRNAs by Tat in human astrocyte-derived cells

To study whether HIV-1 Tat protein affects expression of inflammatory cytokines in human astrocyte-derived cells, we first examined the levels of IL-1β, IL-6 and TNF-α mRNAs expressed by U373 cells exposed to recombinant Tat protein. Cells treated with LPS were utilized as a positive control for activation of cytokine gene expression. RT-PCR was conducted to evaluate the gene transcription of the cytokines. To semi-quantify amplified transcripts of interest, β-actin, a housekeeping gene, was also amplified in each reaction. Because the gene of β-actin is expressed stably, it allows the comparison of the expression of cytokine mRNA between different samples. The results show that mRNAs for IL-1β and IL-6 were induced by Tat or LPS by 6 hours poststimulation (Fig. 7 A and B). However, TNF-α mRNA was only marginally induced by Tat (Fig. 8).

To confirm that Tat activation of the gene expression was specific, Tat was immuno-absorbed to Tat antiserum conjugated to protein A beads and the remaining solution (Tat-im) was applied to the cells. The PCR results indicate that expression of IL-1β and IL-6 mRNAs was not induced by the solution from which Tat had been immunoabsorbed (Fig. 7 A and B), demonstrating that recombinant Tat is responsible for the activation of the gene transcriptions.

To further confirm the effect of Tat, we incubated cells with different concentrations of Tat ranging from 1 nM to 1000 nM for 6 hours. The results indicate that Tat increased the expression of IL-1β and IL-6 mRNAs in a dose-dependent manner (Fig. 9A and B). Even a very low concentration of Tat (1 ~10 nM) was capable of inducing detectable IL-1β and IL-6 mRNAs in U373 cells.

To determine the timing of the mRNA expression of IL-1β and IL-6 by U373 cells exposed to Tat, we studied the relative abundances of the cytokine mRNAs at different time points after application of Tat using semi-quantitative RT-PCR. Fig.10A shows the mRNA expression of IL-1β at different time points (0, 1, 3, 6, 12, 24, and 48 h) after 100 nM Tat treatment. The expression of IL-1β mRNA increased by 1 hour, peaked by 6 hours, and returned to the baseline by 48 hours after treatment. The kinetics of Tat- induced IL-6 mRNA expression shows a similar pattern to that of IL-1β (Fig. 10B).

4. Induction of cytokine proteins in human astrocyte-derived cells

To determine whether the increases in the gene transcription lead to increases in synthesis and secretion of corresponding cytokine proteins, we examined levels of IL-1β protein in the supernatants of U373 cultures by ELISA; sensitivity of detection was 3.9 pg/ml. IL-1β protein could not be detected in samples treated with Tat or LPS. To determine the presence of membrane associated IL-1β protein, we examined the level of IL-1β protein in cell lysates. Even at the highest dose of Tat (1 μM) used we were unable to detect an increase in the production of IL-1β. In response to LPS (1 μg/ml), however, a low level of IL-1β (17.3 $\pm$ 0.8 pg/ml, n=2) was induced. Similarly we were unable to detect the presence of TNF-α protein in the culture supernatants of U373 cells treated with different concentrations of Tat (1 nM-1000 nM) for 16 hours or incubated with 100 nM Tat for different time points (1, 3, 6, 12, 24, and 48 hours) (data not shown).

In contrast, Tat-treated U373 cells produced high leves of IL-6 protein (Fig. 11). Untreated U373 cells produced 0.34 ng/ml of IL-6 in the culture supernatants. Following Tat

treatment (100 nM) for 16 hours, there was 7 fold increase in IL-6 production (2.47 ng/ml). A further increase in Tat concentration to 1000 nM resulted in 3.62 ng/ml of IL-6 production. Time course experiments showed that IL-6 production significantly increased by 6 hours ($p < 0.005$). IL-6 protein level further increased beyond 24 hours (Fig. 12), even though the expression of IL-6 mRNA graduately decreased after 12 hours. This suggests continued protein production and accumulation without degradation or uptake.

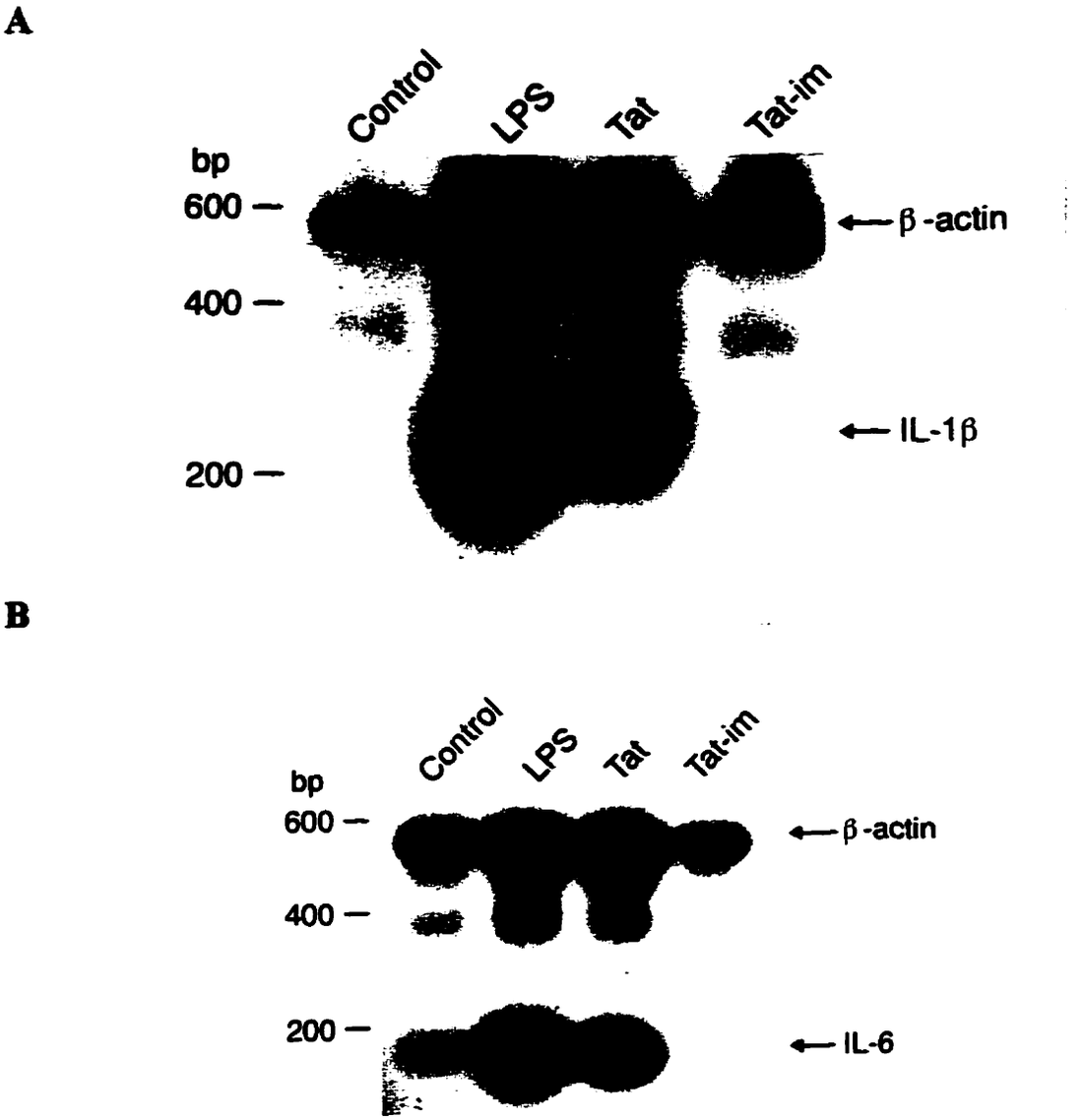


Fig. 7. Effect of Tat on expression of IL-1β and IL-6 mRNA by human astrocyte-derived cells. U373 cells were treated with PBS, LPS (1 μg/ml), Tat (100 nM), or Tat-immunoabsorbed solution (Tat-im) respectively for 6 hours and then the expression of IL-1β and IL-6 mRNAs was analyzed by RT-PCR and Southern hybridization. The sizes of the amplified IL-1β (A) and IL-6 (B) bands are 248 bp and 190 bp, respectively. Both cytokines were induced by LPS and Tat but not by Tat-immunoabsorbed solution.

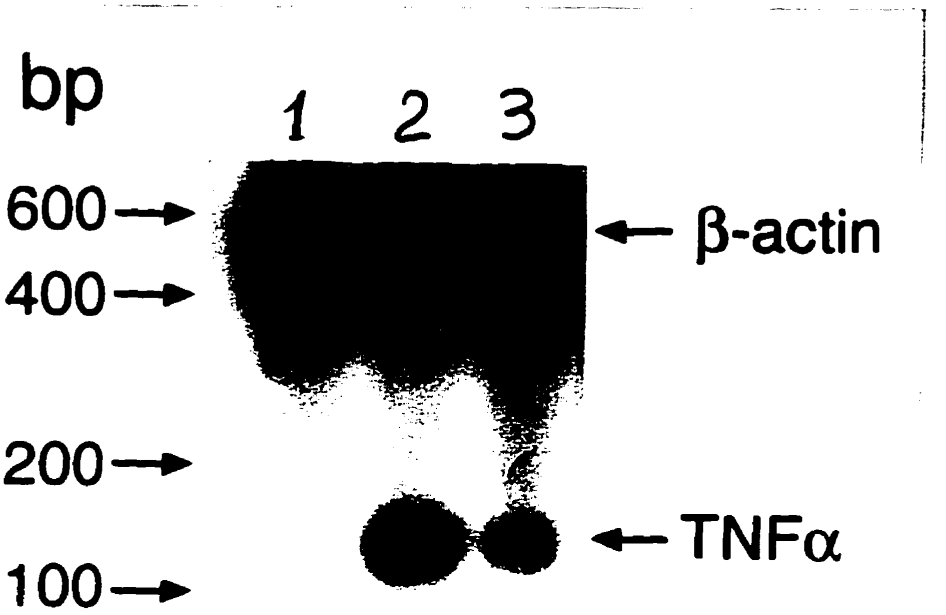
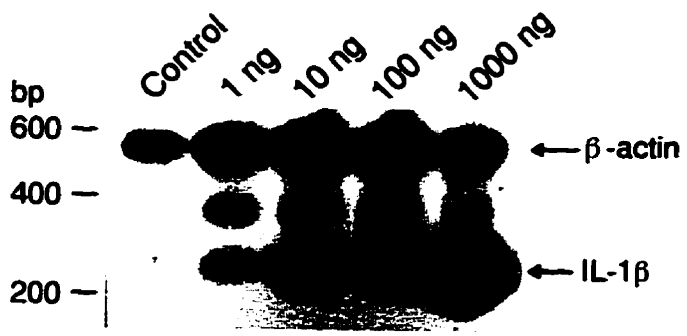


Fig. 8. Effect of Tat on expression of TNF-α mRNA by human astrocyte-derived cells. U373 cells were treated with PBS (Lane 1), LPS (1 μg/ml) (Lane 2), or Tat (100 nM) (Lane 3) for 6 hours. Expression of TNFα mRNA was analyzed by RT-PCR and Southern hybridization. The amplified TNF-α band is located at 125 bp. TNF-α mRNA could be induced by Tat and LPS.

A



B

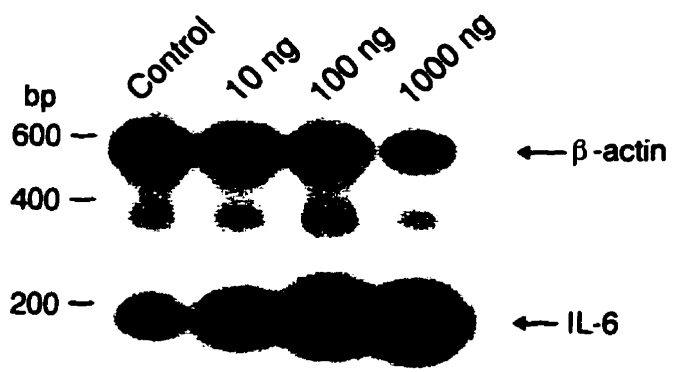
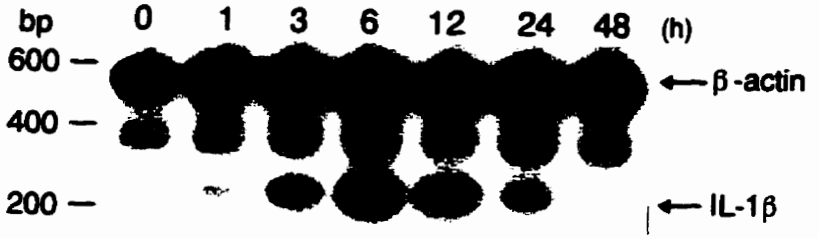


Fig. 9. Response of Tat on IL-1β and IL-6 mRNA expression in U373 cells. 7.5 x 10^5 U373 cells were incubated with 1, 10, 100, or 1000 nM Tat for 6 hours and analyzed by RT-PCR followed by Southern hybridization. The dose-response pattern for IL-1β mRNA (A) and IL-6 mRNA (B) was similar.

A



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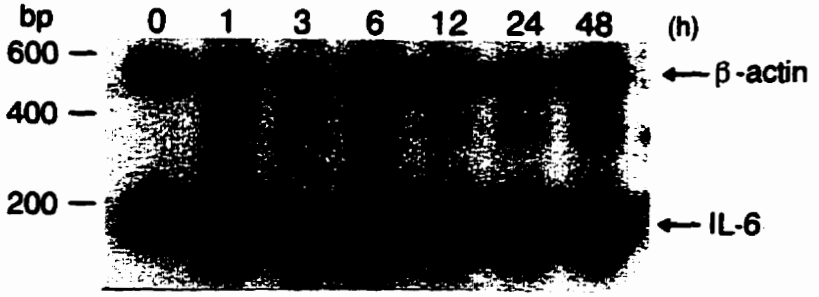


Fig. 10. Kinetics of Tat-induced IL-1β and IL-6 mRNA expression by U373. U373 cells were incubated with 100 nM Tat for the indicated time points. Total cellular RNA was extracted followed by analysis of IL-1β (A) and IL-6 (B) mRNA expression with RT-PCR and Southern hybridization.

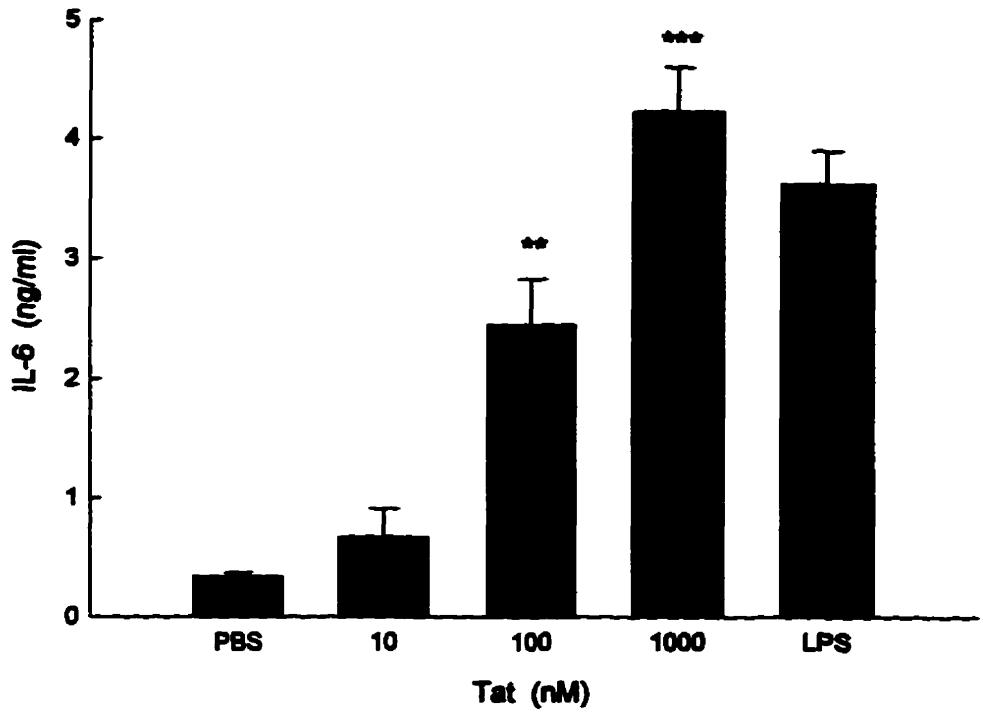


Fig. 11. Effect of different concentrations of Tat on IL-6 protein in U373 cells. U373 cells (1.5×10^6 cells/ml) were treated with PBS, 10, 100, 1000 nM Tat or LPS (1 μg/ml) for 16 hours and examined by ELISA for the production of IL-6. The values represent the means ± SEM of three experiments done in duplicate. ** $p < 0.01$, *** $p < 0.005$ versus the control group (PBS).

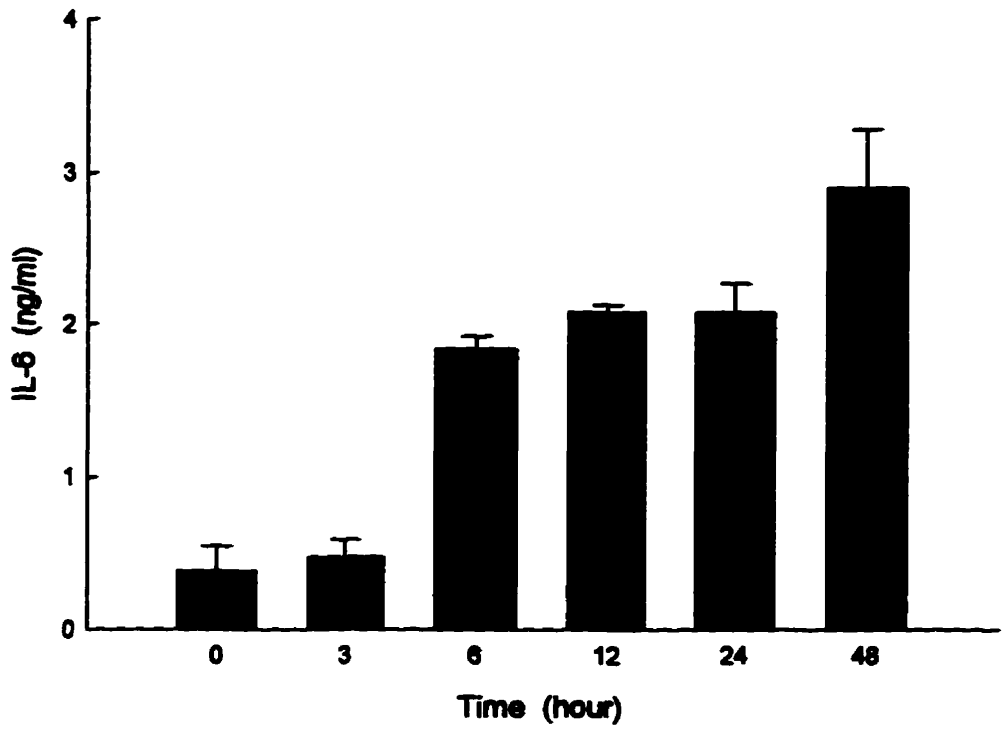


Fig. 12. Time course of IL-6 protein released by U373 cells stimulated with Tat. U373 cells (1×10^6 cells/ml) were incubated with Tat (100 nM) for the indicated time points and culture supernatants were analyzed by ELISA for the expression of IL-6 protein. Values represent means $\pm$ SEM of three experiments performed in duplicate. A significant increase in IL-6 release was noted by 6 hours ($p < 0.005$ versus the control group) with further increase thereafter.

5. Tat induction of cytokine mRNAs in human macrophage-derived cells

We examined the expression of IL-1β, IL-6 and TNF-α mRNAs by Tat-treated THP-1 cells. Tat induced expression of IL-1β and TNF-α mRNAs in dose-dependent fashion (Fig. 13A and B). However, IL-6 mRNA was only marginally induced by 100 nM Tat (data not shown). Kinetic analysis of TNF-α induction revealed that TNF-α mRNA production reached a peak by 4 hours and returned to baseline levels by 12 hours (Fig. 14).

6. Induction of cytokine proteins in macrophage-derived cells

Consistent with the results of RT-PCR, exogenous Tat stimulated the production and release of IL-1β and TNF-α protein by THP-1 cells in a dose-dependent manner (Fig. 15 and 16). The production of IL-1β was significantly induced by 100 nM Tat (34.3 pg/ml versus 8.67 pg/ml for the control group, $p < 0.01$) and a further induction by 1000 nM Tat was noted (178 pg/ml, $p < 0.005$) (Fig. 15). The specificity of the recombinant Tat was confirmed using Tat-immunoabsorbed solution. Solutions from which Tat had been immunoadsorbed, did not show any significant cytokine induction (Fig. 15). 100 nM Tat significantly increased the production of TNF-α (2.03 ng/ml versus 0.3 ng/ml for the control group, $p = 0.00\ 45$) and 1000 nM Tat further increased the levels of the cytokine (5.12 ng/ml, $p = 0.0011$) (Fig. 16). Kinetic analysis of TNF-α protein production dramatically increased by 4 hours, peaked by 6 hours, and returned to baseline by 48 hours poststimulation (Fig.17). Compared to U373 cells, THP-1 cells produced a much lower level of IL-6 protein. The cells treated with 1 μM Tat produced 252 pg/ml (Fig. 18), which is approximately one sixteenth of the amount of IL-6 produced by U373 cells stimulated with the same dose of Tat (4230 pg/ml). The effects

of Tat on expression of cytokines were outlined in Table 3.

Table 3. Tat induction of cytokine expression in THP-1 and U373 cells

	IL-1β		IL-6		TNF-α	
	mRNA	protein	mRNA	protein	mRNA	protein
THP-1	++	+	+	+	++	+++
U373	+++	-	+++	+++	+	-

" - ": no induction; " + ": marginal induction (100 nM Tat can induce mRNA expression; 100 nM Tat induces increases in cytokine concentration by < 100 pg/ml); " ++ ": moderate induction (10 nM Tat can induce mRNA expression; 100 nM Tat increases cytokine concentrations by > 100 pg/ml but < 1000 pg/ml); " +++ ": strong induction (1 nM Tat can induce mRNA expression; 100 nM Tat stimulates increases in cytokine protein concentrations by more than 1000 pg/ml versus the control group).

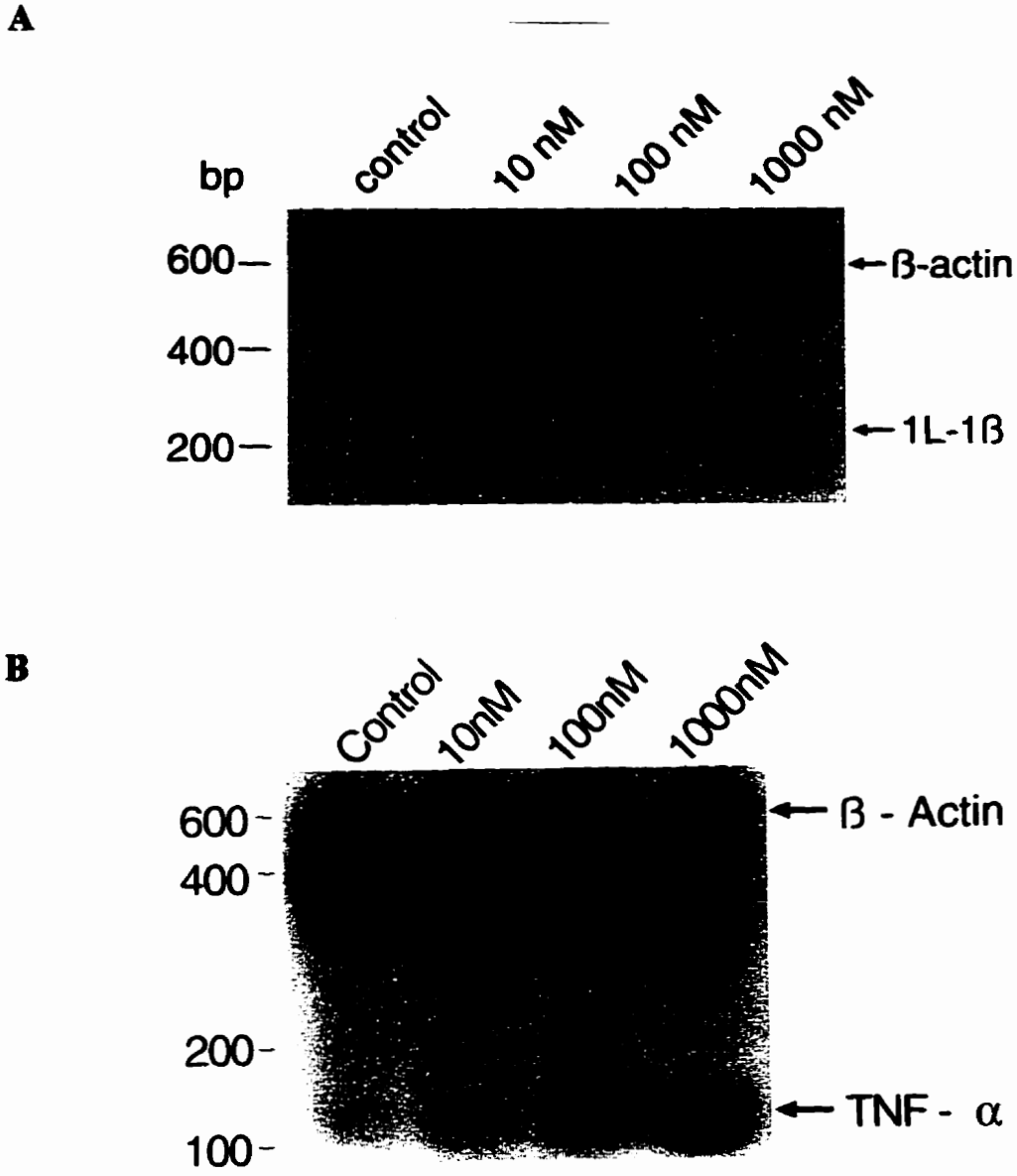


Fig. 13. Induction of IL-1β and TNF-α mRNAs by different doses of Tat in human monocytoid cells. THP-1 cells (7.5×10^5 cells) were incubated with 10, 100, 1000 nM Tat for 4 hours and total RNA was extracted for analyses of IL-1β (A) or TNF-α (B) mRNA expression by RT-PCR and Southern hybridization. Both cytokines were induced dose-dependently by Tat.

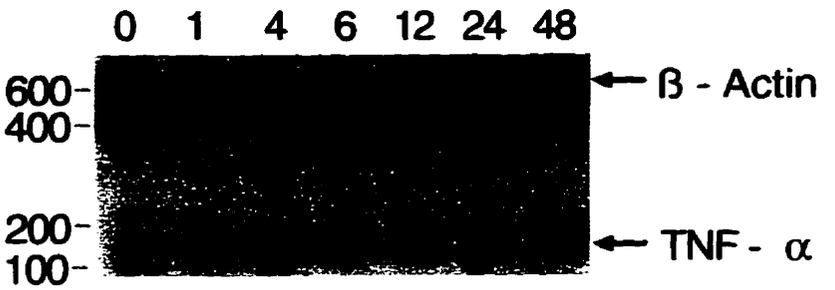


Fig. 14. Kinetics of expression of TNF-α mRNA in THP-1 cells stimulated with Tat. THP-1 cells (7.5×10^5 cells) were treated with 100 nM Tat for the indicated time points and TNF-α mRNA levels were analyzed by RT-PCR followed by Southern hybridization. Data show time-dependent changes in THP-1 mRNA levels.

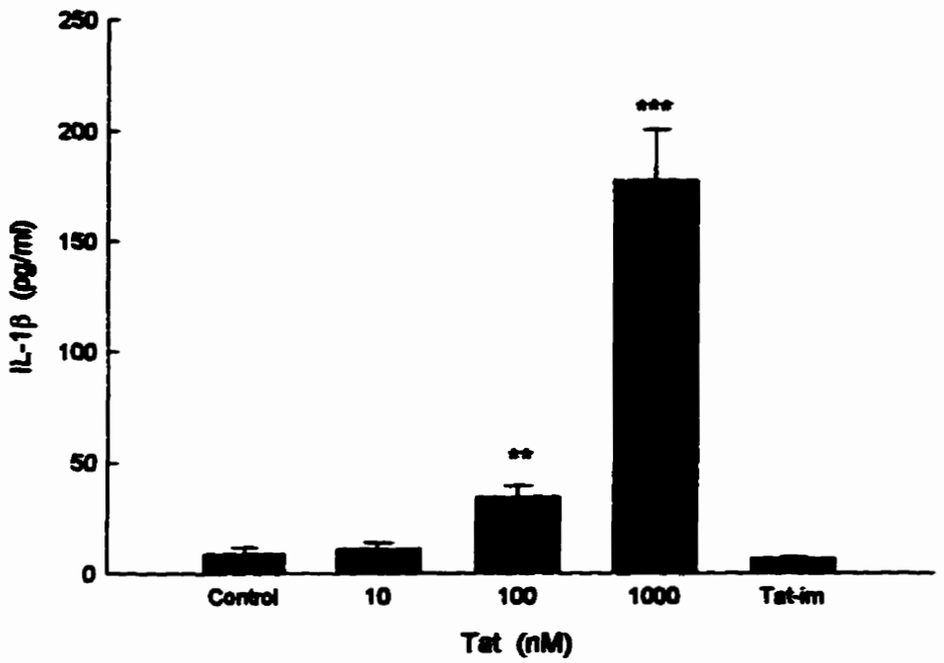


Fig. 15. Monocytoid cells produce IL-1β protein in response to different doses of Tat. THP-1 cells (1.5×10^6 cells/ml) were treated with PBS, Tat-im, or the indicated doses of Tat for 16 hours followed by quantitation of IL-1β production in culture supernatants by ELISA. Values represent means $\pm$ SEM of four experiments. A significant increase in IL-1β production was seen with 100 nM Tat, with a further increase with 1000 nM Tat (** $p < 0.01$, *** $p < 0.005$). However, no induction of IL-1β was observed with Tat-im.

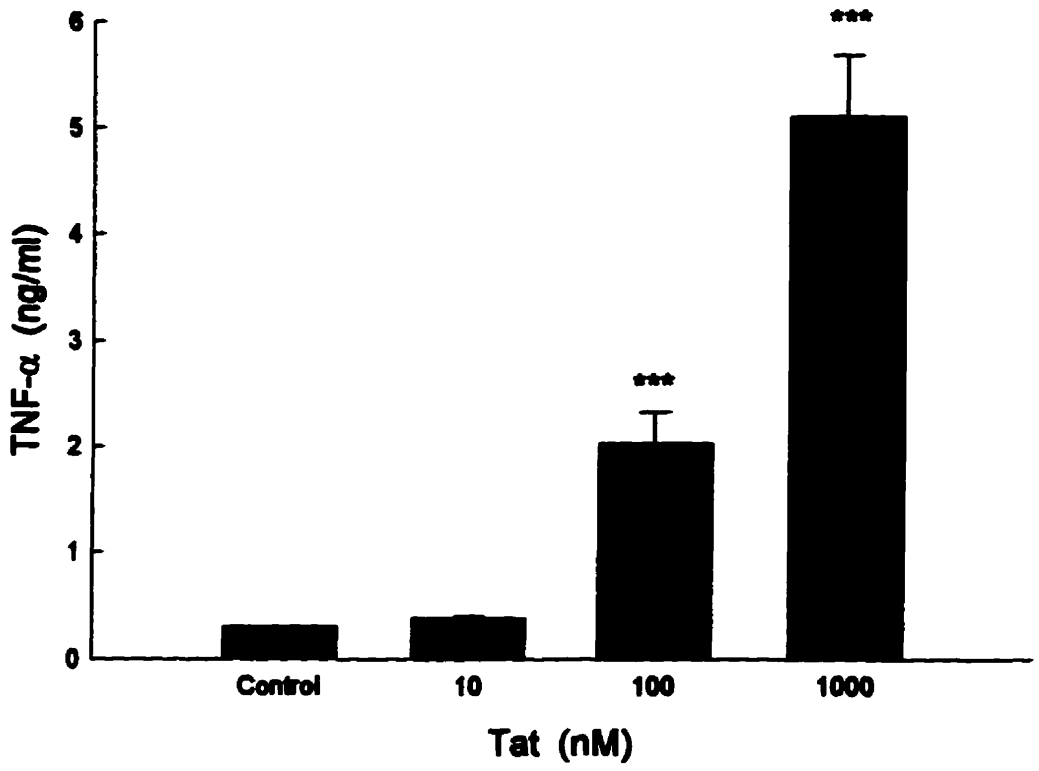


Fig. 16. Tat induction of TNF-α production by monocytoid cells. THP-1 cells (1.5 x10^6 cell/ml) were incubated with PBS or the indicated doses of Tat for 4 hours and culture supernatants were analyzed by ELISA. Data represent mean ± SEM of three experiments done in duplicate. TNF-α production with Tat was induced in a dose-dependent manner (*** $p < 0.005$, Student' *t* test).

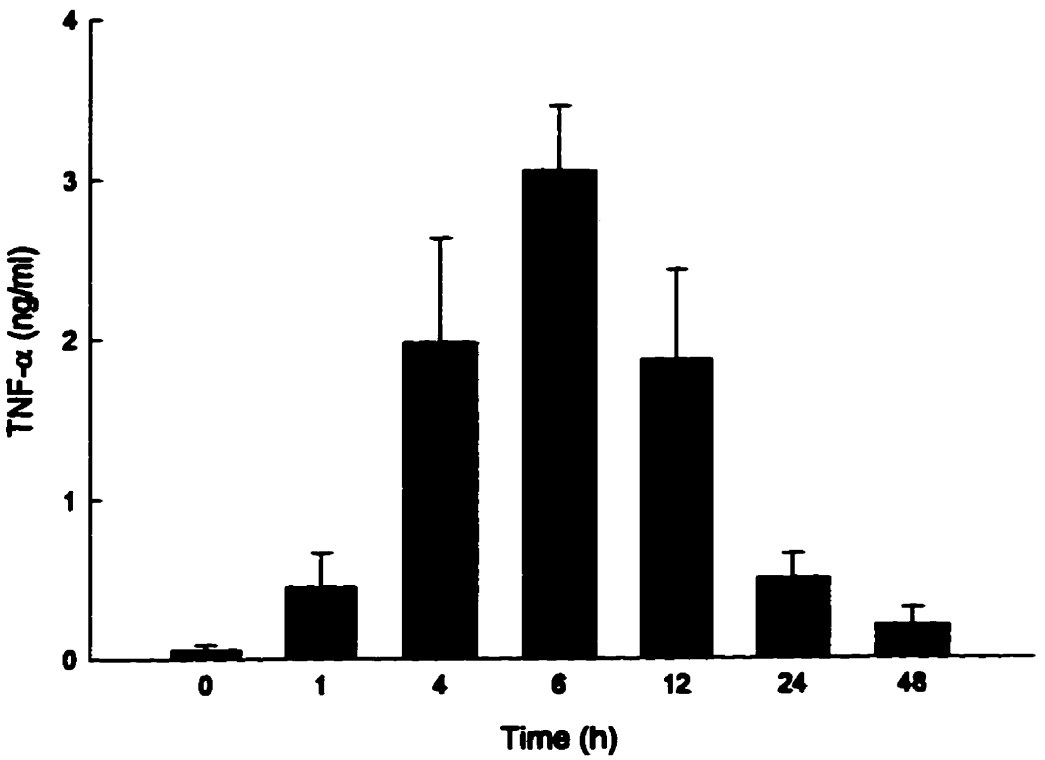


Fig. 17. Time course of TNF-α production by THP-1 cells stimulated with Tat. THP-1 cells (1 x 10^6 cells/ml) were incubated with 100 nM Tat for the indicated time points and TNF-α levels were determined in culture supernatants by ELISA. The data were presented as mean ± SEM of three experiments done in triplicate. Maximal induction of TNF-α was noted at 6 hours.

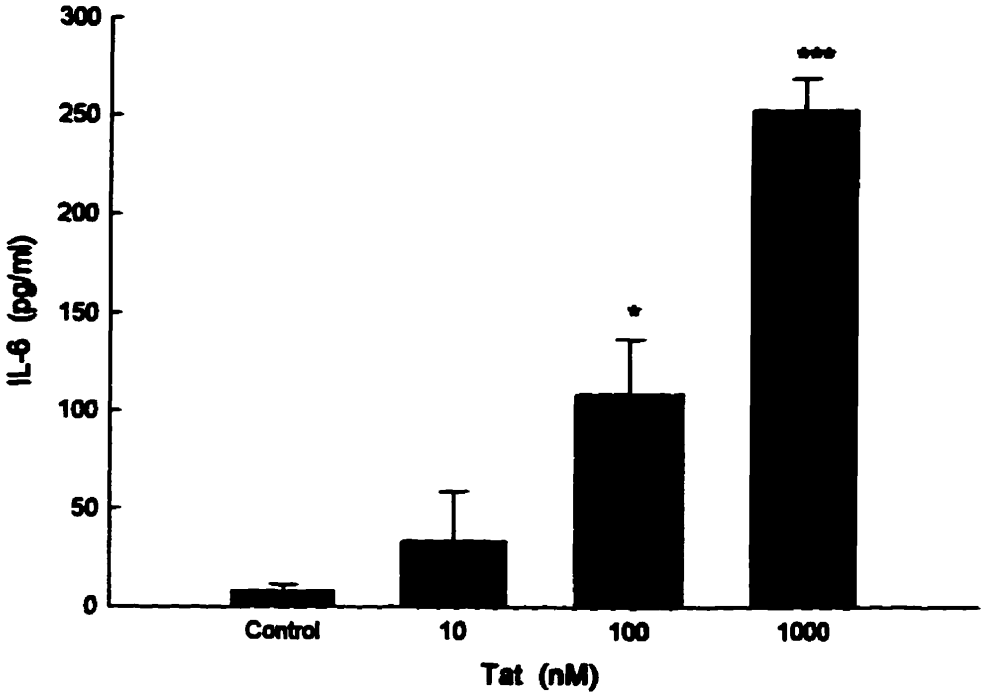


Fig. 18. Tat induction of IL-6 production by THP-1 cells. Cells (1.5×10^6 cells/ml) were treated with PBS or the indicated doses of Tat for 16 hours and culture supernatants were analyzed by ELISA. The data were expressed as mean $\pm$ SEM of three experiments. Tat-induced IL-6 production was noted in a dose-dependent manner (* $p < 0.05$, *** $p < 0.005$ versus the control group).

7. Tat-induced activation of cytokine gene transcription is mediated by NF-κB

TLCK, an inhibitor of cytoplasmic trypsin-like proteases which degrade phosphorylated IκBα, was utilized to determine the role of NF-κB in Tat-induced activation of the cytokine genes. The results indicate that pretreatment of U373 cells with TLCK prevented subsequent Tat induction of IL-1β and IL-6 mRNAs (Fig. 19A and B). TLCK also blocked Tat-induced expression of IL-1β and TNF-α mRNAs (Fig. 20A and B) and protein (Fig. 21 and 22) in THP-1 cells, suggesting that IκBα degradation and subsequent NF-κB activation may be required for Tat's effect.

8. Roles of protein kinases and PLC in Tat induction of expression of the cytokines

To determine if cellular protein kinases and PLC are associated with Tat induction of the cytokine expression, several inhibitors of protein kinases and PLC were used to evaluate possible roles of these enzymes in Tat induction of gene expression. THP-1 cell line was chosen to evaluate effects of the kinases on the expression of IL-1β and TNF-α mRNAs and proteins which were stably expressed in this cell line after Tat treatment. H7, an inhibitor of PKC, did not affect Tat induced expression of IL-1β and TNF-α mRNAs (Fig. 23A and B, lane 3), and no significant inhibition of productions of IL-1β and TNF-α proteins proteins was observed (Fig. 24 and 25). However, HA, a blocker of protein tyrosine kinase, reduced Tat-induced increases in IL-1β mRNA (Fig. 23A, lane 4) and inhibited TNF-α mRNA expression induced by Tat (Fig. 23B, lane 4). The productions of IL-1β and TNF-α proteins were inhibited (Fig. 24 and 25). A selective inhibitor of protein kinase A (H89) inhibited Tat induction of IL-1β and TNF-α mRNAs (Fig. 23A and B, lane 5) and abolished the

productions of IL-1β and TNF-α proteins (Fig. 24 and 25). The doses of the inhibitors we used in the experiments neither significantly affected the viability of treated cells nor expression of the cytokines (data not shown). These data suggest that PKA and PTK may be directly or indirectly implicated in Tat-induced phosphorylation of IκBα.

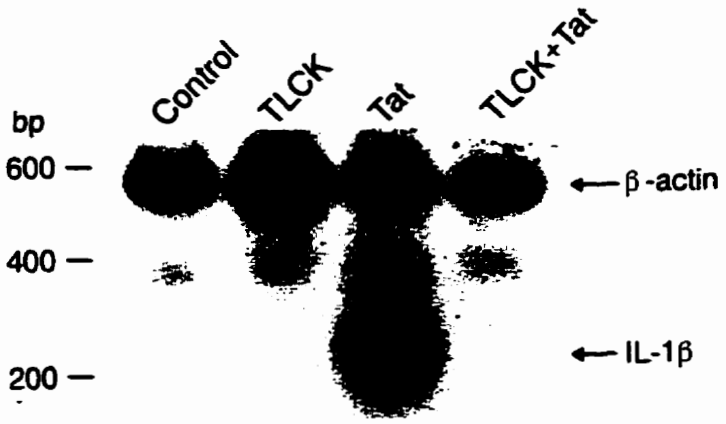
When THP-1 cells were preincubated with a blocker of PLC (U73122) followed by treatment of Tat, Tat inductions of both IL-1β and TNF-α mRNAs (Fig. 23A and B, lane 2) and proteins (Fig. 24 and 25) were completely inhibited, indicating that PLC is also importantly involved in Tat activation of the cytokine expression. Role of these cellular enzymes in Tat-induced expression of cytokines was summarized in Table 4.

Table 4. Effects of inhibitors of some cellular enzymes on Tat induction of cytokine expression in monocytic cells

Inhibitors	Targets	IL-1β	TNF-α	Dosage (μM)	Source
TLCK	NF-κB	+	+	100	Sigma
U73122	PLC	+	+	10	Calbiochem
H7	PKC	-	-	20	Calbiochem
H89	PKA	+	+	10	Calbiochem
HA	PTK	+	+	2.5	Sigma

+ : inhibition; - : no inhibition.

A



B

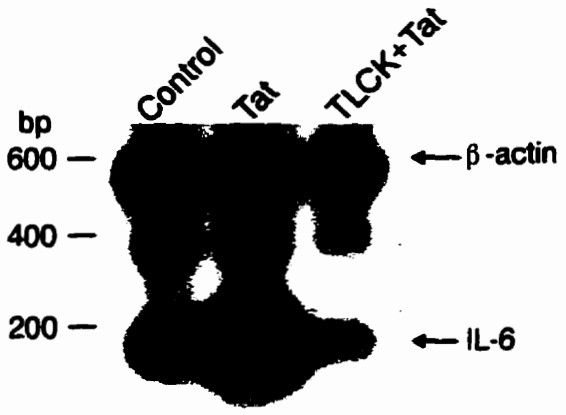
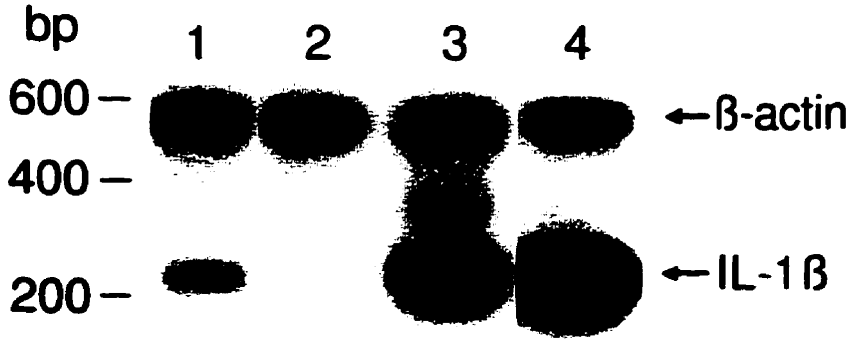


Fig. 19. TLCK blocked Tat-induced expression of IL-1β and IL-6 mRNAs. 7.5 x 10^5 U373 cells were treated with PBS, 100 nM Tat alone, or preincubated with 100 μM TLCK for 30 minutes followed by treatment with 100 nM Tat for 6 hours and IL-1β (A) or IL-6 (B) mRNA expression was analyzed by RT-PCR and southern hybridization. Tat-induced expression of IL-1β and IL-6 mRNAs was completely blocked by TLCK.

A



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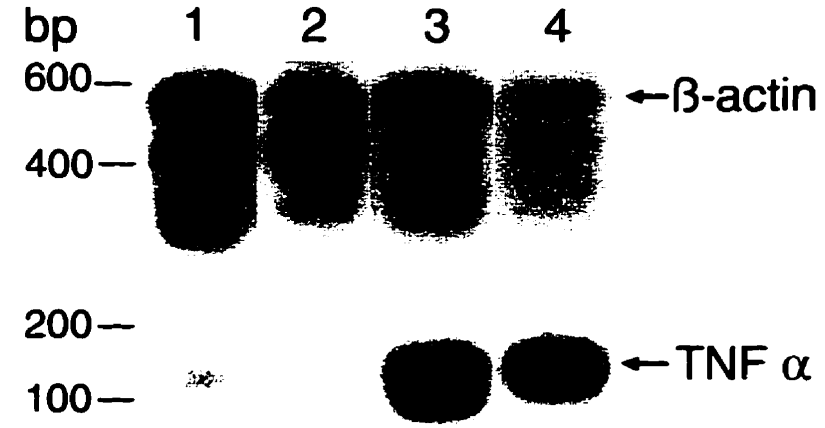


Fig. 20. Role of NF-κB in Tat-induced expression of IL-1β and TNF-α mRNAs in THP-1 cells. Cells were treated with PBS (lane 1), 100 nM Tat alone (lane 3) or 1 μg/ml LPS (lane 4) for 4 hours, or preincubated with 100 μM TLCK for 30 minutes followed by treatment with 100 nM Tat for 4 hours (lane 2). Expression of IL-1β (A) and TNF-α (B) mRNA was analyzed by RT-PCR and southern hybridization. A complete block in the production of both cytokines was observed with TLCK.

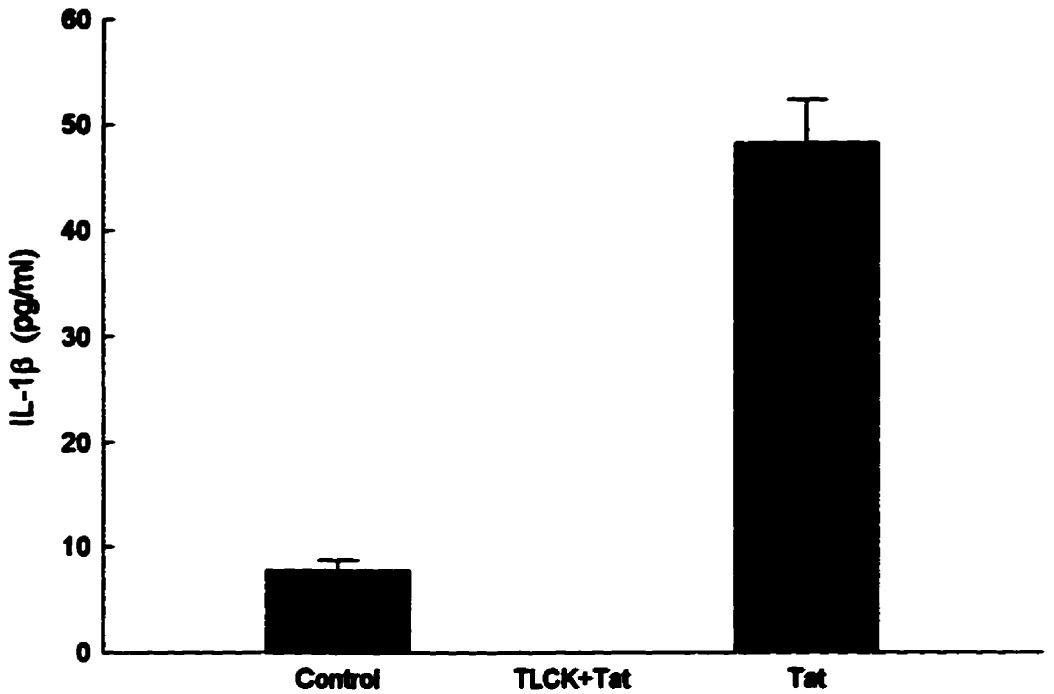


Fig. 21. Role of NF-κB in Tat induction of IL-1β protein in THP-1 cells. Cells (1.5 x 10^6 cells/ml) were stimulated with PBS or 100 nM Tat alone, or preincubated with 100 μM TLCK for 3 hours followed by treatment with 100 nM Tat for 16 hours. The supernatants were analyzed by ELISA. Data represent mean ± SEM of three experiments. A complete inhibition of IL-1β production was noted with TLCK.

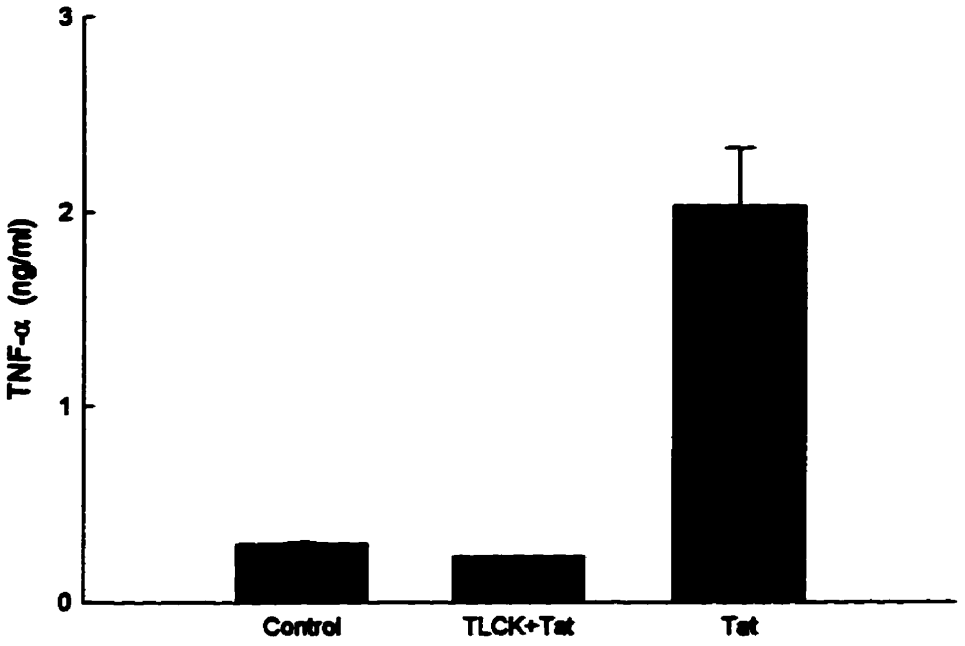
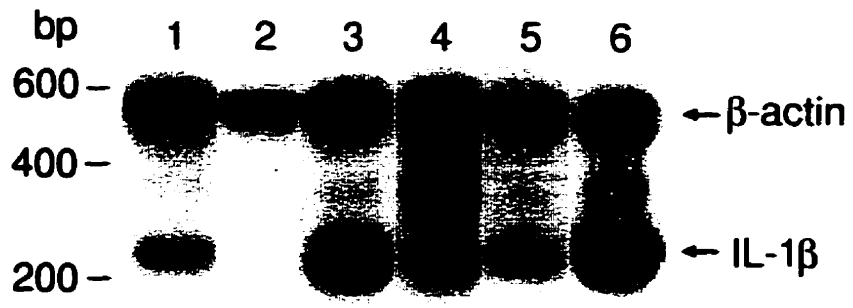


Fig. 22. Role of NF-κB in Tat-induced expression of TNF-α protein in THP-1 cells. Cells were treated with PBS or 100 nM Tat alone for 4 hours, or pretreated with 100 μM TLCK for 3 hours followed by treatment with 100 nM Tat for 4 hours. Culture supernatants were analyzed using RT-PCR and Southern hybridization. The results represent the mean ± SEM of three experiments done in duplicate. A complete block of TNF-α production was seen in the presence of TLCK.

A



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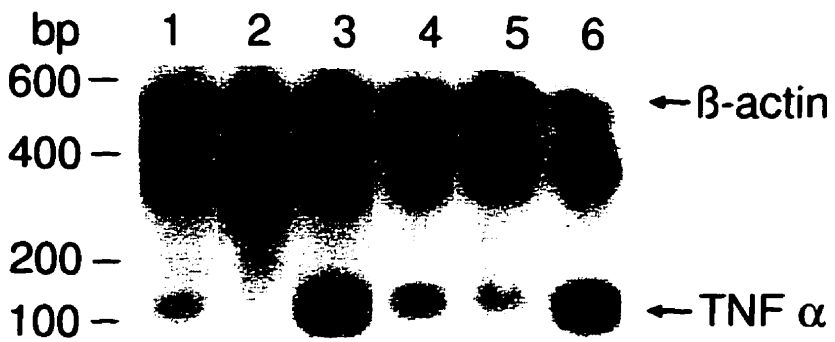

Fig. 23. Role of protein kinases and PLC in Tat-induced IL-1β and TNF-α mRNA expression in THP-1 cells. Cells were treated with PBS (lane 1) or 100 nM Tat alone (lane 6) for 4 hours, or pretreated with inhibitors of protein kinases and PLC for 3 hours followed by treatment with 100 nM Tat for 4 hours. The induction of IL-1β (A) and TNF-α (B) mRNAs was determined by RT-PCR and Southern hybridization. A and B: lane 1, cells treated with PBS; lane 2, U73122 (10 μM) + Tat; lane 3, H7 (10 μM) + Tat; lane 4, HA (2.5 μM) + Tat; lane 5, H89 (10 μM) + Tat. Both cytokine mRNA induction by Tat was inhibited by inhibitors of PLC (lane 2), protein tyrosine kinase (lane 4), and protein kinase A (lane 5).

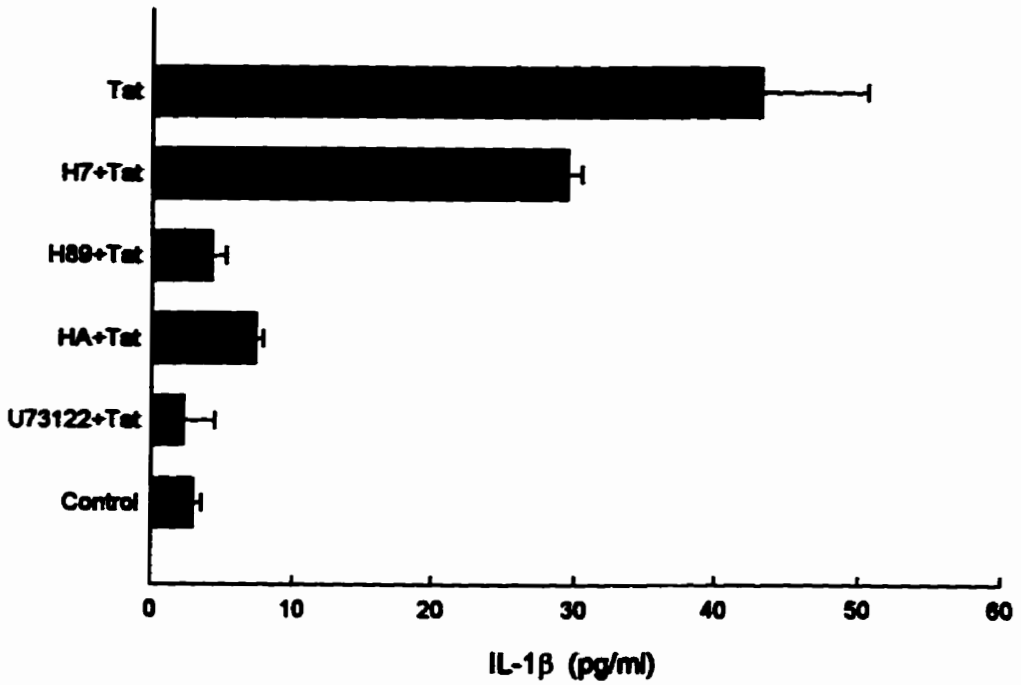

Fig. 24. Role of protein kinases and PLC in Tat-induced IL-1β expression. THP-1 cells were treated with PBS or 100 nM Tat alone for 16 hours, or preincubated with blockers of PLC (U73122), PTK (HA), PKA (H89) , or PKC (H7) for 3 hours each, followed by treatment with 100 nM Tat for 16 hours. Culture supernatants were analyzed for IL-1β by ELISA. Values represent the mean ± SEM of three experiments. Significant inhibitions of IL-1β induction by Tat were noted with all compounds except H7.

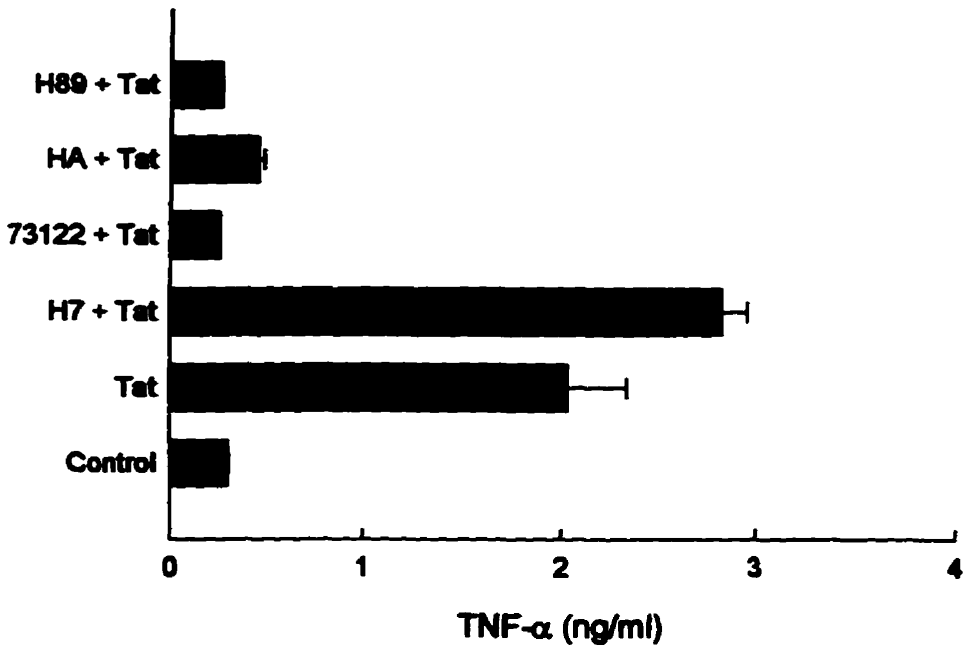

Fig. 25. Role of protein kinases and PLC in Tat induction of TNF-α in human macrophage-derived cells. THP-1 cells were treated with PBS or 100 nM Tat for 16 hours, or preincubated with U73122 (10 μM), H7 (20 μM), HA (2.5 μM), or H89 (10 μM) for 3 hours followed by treatment with 100 nM Tat for 16 hours. Concentrations of IL-1β in culture supernatants were determined by ELISA. Each value represents the mean ± SEM of three experiments conducted in triplicate.

VI. DISCUSSION

The recombinant Tat protein used in this study is the product of the first exon of HIV-1 *tat* gene which comprises the first 72 amino acids of the 86-amino acid full length Tat protein. Although the full length Tat is the most abundantly expressed form in infected cells, a stop codon following the splice donor permits the expression of a 72 amino acid truncated protein that exerts most of functions of Tat protein. Several important functional regions, such as the basic domain and core region, are included in the protein. These regions are responsible for causing neural damage through activation of excitatory amino acid recepors (Nath *et al.* 1996) and induction of cytokines in mouse brain (Philippon *et al.* 1994), binding to the integrin α_v β_5 (Vogel *et al.* 1993) and transactivation of the HIV genome (Feinberg *et al.* 1991) as well. Recombinant Tat_{1-72} induces NF-κB activity in glial cells when presented extracellularly (Conant *et al.* 1996). The *tat* gene when expressed in cells induces expression of cytokines such as TNF-β in monocytic and T lymphocytic cell lines (Buonaguro *et al.* 1992), TGF-β1 in an astrocytic cell line (Cupp *et al.* 1993), and IL-6 in brain endothelial cells (Hofman *et al.* 1994). In the present research, we demonstrate that exogenous recombinant Tat_{1-72} differentially activates expression of several inflammatory cytokines in monocytic and an astrocytoma cell lines.

1. Sources of inflammatory cytokines in CNS

Within the CNS, IL-1β is primarily produced by activated microglia (brain macrophages) (Lee *et al.* 1993; Sebire *et al.* 1993). HIV-infected macrophages also release high levels of

IL-1β (Genis *et al.* 1992; D'Addario *et al.* 1990). Our study shows that extracellular Tat increased the expression of IL-1β mRNA in macrophage-derived cells. This elevation of expression at the transcriptional level led to a significant increase in production of IL-1β protein that was released from the macrophages. In contrast, even though the astrocytoid cells showed an increased expression of IL-1β mRNA in response to Tat, a corresponding increase in extracellular release of IL-1β protein was not observed with either Tat- or LPS-treatment. Since stimuli such as IL-1α or IL-2 result in production of cell-associated IL-1β (Dinarello, 1987; Numerof *et al.* 1990), we also examined the cell lysates of the astrocytoid cells for the presence of IL-1β. No significant induction of cell-associated IL-1β by Tat was observed while LPS induced only a small amount of IL-1β expression. The results are consistent with previous findings that LPS could not induce IL-1β protein in human fetal astrocytes (Lee *et al.* 1993). It is known that transcription and translation of IL-1 are distinct and dissociated processes. Some stimuli like recombinant C5a, β-glucan polymers, or calcium ionophore can induce IL-1β transcription but not translation (Schindler *et al.* 1990b; Schindler *et al.* 1990a; Schindler *et al.* 1990c). Thus, it is likely that Tat may induce IL-1β transcription only in astrocytes. Our data also show that monocytic cells are the major source of IL-1β in the CNS and Tat may at least in part contribute to the elevation of IL-1β levels in the CNS of patients with AIDS dementia. The increase in IL-1β not only induces astrocytosis (Giulian and Lachman, 1985; da Cunha *et al.* 1993) and promotes HIV-1 replication (Poli and Fauci, 1992a), but also induces other inflammatory cytokines such as TNF-α in astrocytes (Lee *et al.* 1993), resulting in further CNS injury.

In human brain, the major cellular sources of TNF-α are activated microglia and macrophages. The production of TNF-α by astrocytes depends on the types of stimuli used. For example, while IL-1β can induce TNF-α production by astrocytes, no response to LPS has been observed (Lee *et al.* 1993). Some studies have shown that some stimuli like LPS can not induce human astrocytes to produce TNF-α (Lee *et al.* 1993). In this study, we could not detect any production of TNF-α protein in Tat- or LPS- treated U373 cells although TNF-α mRNA was marginally induced. However, under certain circumstances, astrocytes also release TNF-α. It has been shown that IL-1β induces TNF-α expression in human astrocytes and astrocytoma cells (Lee *et al.* 1993; Bethea *et al.* 1992). As we have indicated early, Tat can not induce production of TNF-α protein in astrocyte-derived cells (U373), but it can activate macrophage-derived cells (THP-1) to produce IL-1β. Thus, the Tat-induced IL-1β may be able to stimulate astrocytes to express TNF-α in the brain. This may be very important for the pathogenesis of AIDS dementia since astrocytes are the most numerous cell type in the brain and very important cells for neuron functions. Astrocytes are capable of promoting HIV-1 replication in macrophages by producing TNF-α (Vitkovic *et al.* 1990) and many studies have shown that TNF-α is neurotoxic (Tauber *et al.* 1992; Wilt *et al.* 1995).

Although astrocytoid cells were not capable of producing IL-1β and TNF-α proteins after Tat treatment, Tat-treated astrocytoid cells expressed a high level of IL-6 protein and the high level of IL-6 protein persisted for a longer time in the culture supernatants. Many studies have shown that astrocytes are an important source of IL-6 in CNS (Benveniste *et al.* 1990; Lieberman *et al.* 1989; Yasukawa *et al.* 1987). IL-6 has prominent effects on CNS: these central effects include activation of the hypothalamic-pituitary-adrenal axis, reduction

of food intake, induction of fever, and neuronal growth (Akira *et al.* 1990). Overexpression of IL-6 in CNS may cause harmful influences on brain function. Increased levels of IL-6 produced neuronal degeneration in transgenic animals (Campbell *et al.* 1993). IL-6 expression is elevated in the brain of some patients with Alzheimer's disease or acute infection of the CNS (Huell *et al.* 1995; Houssiau *et al.* 1988), suggesting that IL-6 may play a role in HIV-associated dementia.

We postulate that in the brain of patients with AIDS, macrophages and microglia may initially produce IL-1β and TNF-α due to stimulation by Tat and other viral products, but the amounts of these inflammatory cytokines are limited because of the small number of macrophages/microglia. When astrocytes are stimulated by IL-1β released by macrophages/ microglia, much greater amounts of TNF-α and IL-6 may be produced. The increased cytokines in the brain may play a crucial role in promotion of HIV-1 replication, induction of astrocytosis, disturbance of the blood brain barrier, release of toxins, and finally CNS damage.

2. Mechanisms of Tat induction of cytokines

The mechanism of transactivation of Tat in HIV-1 gene transcription has been well studied. Binding of Tat protein to the TAR element in the LTR of HIV-1 genome may be required for activation of viral gene transcription (Weeks and Crothers, 1991; Calnan *et al.* 1991; Cordingley *et al.* 1990). Some recent studies have showed that Tat stimulation of RNA polymerase II is associated with Tat-induced phosphorylation of this enzyme (Parada and Roeder, 1996). However, mechanism(s) of Tat activation of cellular genes is unclear. In a

recent study, Ma and Nath *et al.* have shown that full length Tat (1-86 amino acids) is mainly present within the nucleus when presented extracellularly to astrocytes whereas Tat_{1-72} mainly binds to the cell membrane (Ma and Nath, 1997). We postulate that there may be two possible mechanisms for the Tat effect. One is that extracellular Tat translocates into the nucleus and directly or indirectly interacts with the promoters of the cytokine genes, resulting in activation of the gene transcription. Another is that Tat binds to specific receptors on the surface of cells and triggers certain signal transduction pathway(s), leading to expression of cytokine genes.

Stimulation of various receptors on cell membranes result in the degradation of IκBα and activation of NF-κB. NF-κB is an important transcriptional factor which can be activated by a variety of stimuli in a membrane receptor-mediated manner (Brown *et al.* 1993; Henkel *et al.* 1993; Thanos and Maniatis, 1995; Roulston *et al.* 1995). This process is rapid and transient, and independent of new protein synthesis (Conant *et al.* 1996). The kinetic analysis presented here shows that increases in expression of IL-1β, IL-6 and TNF-α mRNAs can be observed at 1 ~ 3 hours and reaches a peak at 4 ~ 6 hours after Tat treatment. This process is basically consistent with the time course of Tat-induced NF-κB activation (Demarchi *et al.* 1996). This time match between NF-κB activation and the expression of the cytokine genes suggests that NF-κB may play a direct and an important role in Tat-induced activation of the cytokine gene transcriptions although other cellular factors such as Sp1 may be also essential for gene transactivation of Tat (Buonaguro *et al.* 1994). To further determine role of NF-κB in Tat induction of the cytokine expression, TLCK, an inhibitor of NF-κB activation that prevents IκBα from being degraded by yet unidentified chymotrypsin-like proteases, was utilized. We

observed that Tat induction of IL-1β, IL-6, and TNF-α mRNA and IL-1β and TNF-α protein expressions was completely inhibited by TLCK. Similarly, LPS induction of IL-1β mRNA was also blocked by TLCK. It has been shown that LPS binds to CD14 molecules on the surface of monocytes and triggers signal transduction pathway(s) (Gegner *et al.* 1995), resulting in activation of NF-κB (Delude *et al.* 1994). Tat may act through a similar way as LPS although the specific membrane receptor for Tat has not yet been identified. These data indicate that NF-κB may mediate Tat activation of IL-1β, IL-6 and TNFα by translocating into the nucleus and initiating expresson of the cytokine genes.

Binding of many ligands to their receptors on the surface of cells activates PLC. PLC is a membrane-bound enzyme which triggers signal transductions through hydrolyzing the lipid precursor phosphatidyl-inositol 4,5-bisphophate liberating two secondary messengers, IP3 (Alexander *et al.* 1985) and DAG (Wilt *et al.* 1995). Our experimental results show that an inhibitor of PLC blocked Tat induction of IL-1β and TNFα mRNAs and proteins in THP-1 cells, suggesting that the effects of Tat may be PLC-dependent. The above blocking experiments indicate that, like LPS, Tat_{1-72} activation of the cytokine expression is a membrane receptor-mediated event.

Activation of NF-κB depends on phosphorylation and subsequent degradation of IκBα. However, the kinases which phosphorylate IκBα have not been identified. It has been known that PKC, PKA, and PTK are involved in phosphorylation of a number of proteins and many signal transduction pathways. Thus, we examined the possible roles of these kinases in Tat induction of cytokine expression. Activation of PLC produces IP3 and DAG. IP3 stimulates the release of Ca^{2+} from intracellular stores, and the elevated $[Ca^{2+}]_i$ and DAG stimulate the

activity of PKC. Activation of PKC in turn results in phosphorylation of many proteins (Nishizuka, 1986) and expression of several genes (De Graan *et al.* 1991; Linden and Routtenberg, 1989; Costa *et al.* 1992). Tat has been shown to increase activity of PKC (Conant *et al.* 1996). However, the activation of PKC by Tat didn't induce an increase in NF-κB activity (Conant *et al.* 1996). Thus, it is not surprising that Tat induction of IL-1β and TNFα mRNAs was not blocked by a blocker of PKC, indicating that PKC may not be involved in phosphorylation of IκBα.

Ligand-receptor interactions stimulate adenylyl cyclases on the inner membrane surface, resulting in an increase of cAMP (Krupinski, 1991). PKA is a cAMP-dependent protein kinase. Some hormones, such as prostaglandin E2, act through PKA (Baylink *et al.* 1996). To determine whether PKA was implicated in the Tat effect, H89, a selective PKA inhibitor, was used in the present study. A previous study has shown that H89 reduces LPS induction of TNF-α and IL-1β mRNAs (Geng *et al.* 1993). We demonstrated that Tat activation of the expression of TNF-α and IL-1β mRNAs and proteins was blocked by H89, indicating that PKA may mediate the Tat effect.

It has been shown that LPS and some polypeptide growth factors activate PTK activity and may initiate several signal pathways (Mustelin and Burn, 1993). In the present study, we observed that Tat induction of expression of IL-1β and TNF-α mRNAs was dramatically reduced by a PTK inhibitor and the protein expressions were also inhibited, suggesting that PTK is also associated with Tat activation of the cytokine expression. Because both PTK and PKA have been shown to be implicated in activation of NF-κB (Geng *et al.* 1993), IκBα may be directly or indirectly phosphorylated by these kinases. It has been reported that PTK may

mediate TCR-induced tyrosine phosphorylation and activation of PLCγ (Graber *et al.* 1992; June *et al.* 1990). Because activation of PLCγ increases PKC activity by mediation of DAG (Griendling *et al.* 1986), PTK may mediate activation of PKC. However, the PLC-mediated down stream events involved in Tat-induced expression of the cytokines are unclear. In addition, because either PKA or PTK inhibitor blocked the expressions of the cytokines, both kinases may act on the same pathway. However, the relationship between PTK and PKA is to be dertermined.

VII. Summary

In this study, we demonstrate that recombinant Tat_{1-72} induced differential expression of inflammatory cytokines IL-1β, IL-6 and TNF-α mRNA transcripts in a human macrophage-derived cell line (THP-1) and a human astrocyte-derived cell line (U373). The increases in the transcription led to increased productions of all of three cytokines in THP-1 cells. However, U373 cells could only be induced to produce IL-6 protein while the translations of IL-1β and TNF-α were not observed despite differential increases in transcripts. Tat induction of IL-1β and TNF-α was totally blocked by an inhibitor of NF-κB, indicating that the Tat effects are mediated by NF-κB. In addition, Tat induction of the cytokines was downregulated by inhibitors of PLC, PKA and PTK but not PKC, suggesting that PLC, PKA and PTK may be associated with Tat-induced activation of NF-κB by directly or indirectly phosphorylating IκBα, an inhibitor of NF-κB activity. Because activation of PLC, PKA or PTK is a result of cellular receptor stimulation, Tat induction of cytokine expression may be a cellular receptor-mediated event. The above data indicate that Tat may play a role in pathogenesis of AIDS dementia by inducing inflammatory cytokines in the CNS of patients with AIDS.

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