Transient Exposure to HIV-1 Tat Protein Results in Cytokine Production in Macrophages and Astrocytes

A HIT AND RUN PHENOMENON*

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The pathological correlates of dementia due to human immunodeficiency virus (HIV) infection are glial cell activation and cytokine dysregulation. These findings occur in the setting of small numbers of productively infected cells within the brain. We determined whether exposure of susceptible cells to Tat protein of HIV could result in the production of select proinflammatory cytokines. In a dose-responsive manner, Tat induced interleukin (IL)-1β production in monocytic cells, while astrocytic cells showed an increase in mRNA for IL-1β, but had a translation block for IL-1β protein production. Conversely, IL-6 protein and mRNA productions were strongly induced in astrocytic cells and minimally in monocytic cells. IL-1β and IL-6 production were independent of tumor necrosis factor-α production. An exposure to Tat for a few minutes was sufficient for sustained releases of cytokines for several hours. This prolonged cytokine production is likely maintained by a positive feedback loop of Tat-induced nuclear factor κB activation and cytokine production that is independent of extracellular calcium. Thus a transient exposure may be sufficient to initiate a cascade of events resulting in cerebral dysfunction and a "hit and run" approach may be in effect. Hence cross-sectional measurement of viral load in the brain may not be a useful indicator of the role of viral products in the neuropathogenesis of HIV dementia.

The pathogenesis of dementia associated with HIV1 infection involves the complex interactions of viral products and cytokines, which eventually result in neuronal dysfunction and cell loss. Several studies have shown that viral proteins such as Tat and gp120 can induce cytokine dysregulation in macrophages and glial cells as well as cause neurotoxicity (1). However, it remains uncertain as to why infection of the brain is limited in comparison with the severity of the clinical presentation. In fact, macrophage infiltration, glial cell activation and not viral load in the brain seem to correlate best with the severity of dementia (2, 3). In keeping with these observations cytokine levels in brain and cerebrospinal fluid are elevated in patients with HIV dementia (2, 4).

The Tat protein of HIV is of particular interest, since it is released extracellularly from unruptured, HIV-infected lymphoid and microglial cells (5, 6). Tat exits from cells via a leaderless secretory pathway in the absence of permeability changes (7). This protein thus has the opportunity to interact with other uninfected cells. Further Tat can be detected in mononuclear cells within the brain of patients with HIV encephalitis (8) and in the sera of HIV-infected individuals (9). Tat-mRNA levels are also elevated in the brains of patients with HIV dementia (10, 11).

It has been shown previously that the Tat protein of HIV can induce macrophage infiltration in the brain (12) likely via production of monocyte chemoattractant protein-1 by astrocytes (13). Tat also causes glial cell activation for several days post intracerebroventricular inoculation (12). Furthermore, a Tat-derived peptide when injected into the brain causes cytokine dysregulation (14) similar to that observed in patients with HIV infection (10, 15). In this study, we examine the possibility that a transient exposure of cells to Tat may be sufficient to induce a sustained production of cytokines and we determine the role of extracellular calcium and autoregulation in cytokine production.

EXPERIMENTAL PROCEDURES

Cell Culture—Peripheral blood monocytes were isolated by a Percoll gradient technique, and human fetal astrocyte cultures were prepared by differential adhesion as described previously (16). The human astrocytoma cell line U373 and monocytic cell line THP-1 were obtained from American Type Culture Collection (Rockville, MD). Human astrocytes and U373 cells were maintained in minimal essential medium with heat-inactivated 10% (v/v) fetal bovine serum and 1 mM sodium pyruvate. Monocytes were cultured in RPMI with 10% fetal bovine serum. All cell types were supplemented with 100 μg streptomycin/ml and 0.25 μg amphotericin/ml. THP-1 cells were cultured in RPMI medium with 10% fetal bovine serum and 5.5 μM β-mercaptoethanol. The cells were cultured to approximately 95% confluence in 24-well plates (~1 × 10⁵ cells/well).

HIV-1 Tat Treatment—Highly purified (>95%) recombinant Tat protein was prepared from the tat gene encoding the first 72 amino acids as outlined previously (17). The functional properties of this protein were confirmed using a transactivation assay in HL6T1 cells containing the HIV-1 long terminal repeat, chloramphenicol acetyltransferase construct (17). For experiments designed to determine the effect of a transient exposure to Tat, cells were treated with 100 ng/ml Tat for either 5, 30, or 60 min, following which the cells were washed five times and then reincubated in culture media without Tat protein. In each case, culture supernatants were collected at 30, 90, and 180 min following reincubation without Tat and analyzed for the presence of IL-1β, IL-6, and TNF-α. For experiments designed to determine the role of
TNF-α in IL-1 and IL-6 production, the monocytes and astrocytes were preincubated with antisera to TNF-α (0.5 μg/ml; R&D Systems) for 30 min followed by incubation with 100 ng/ml Tat for either 5, 30, or 60 min. The cells were then washed and reincubated with culture media containing antisera to TNF-α. Culture supernatants were then analyzed by ELISA. Cells from two different donors were analyzed in triplicates. Data from a representative experiment are shown.

To establish dose profiles for each cell type, Tat was used at 0, 10, 100, and 1000 ng/ml concentrations for 4 h with THP-1 cells for IL-1β and IL-6 mRNA detection in THP-1 cells and for 6 h with U373 cells. IL-1β and IL-6 proteins were analyzed in culture supernatants, 16 h post-Tat treatment. Since IL-1β could not be detected in cell culture supernatants of U373 cells, cell extracts of U373 cells were also measured at 1, 3, 6, 12, 24, and 48 h following treatment with 1 μg/ml Tat for IL-1β by ELISA. Cells were stimulated with lipopolysaccharide from Escherichia coli type 055:B5 (Sigma) 1.0 μg/ml as positive controls. Negative controls included mock (PBS)-treated cells and cells that were treated with solutions from which Tat had been immunoadsorbed as described previously (18).

Quantitative Immunoassays for Cytokines—Cell culture supernatants were analyzed for IL-1β, IL-6, or TNF-α. Additionally, cell extracts of U373 cells were analyzed for IL-1β. In each case, ELISA kits were used from R&D systems, and the procedure was followed as per the manufacturer's instructions. Briefly, 200 μl of standard or sample was added to each well, which had been precoated with a murine monoclonal antibody (2 μg/ml) against the appropriate cytokine. Following a 2-h incubation with the test sample and three washes, 200 μl of a rabbit polyclonal antibody (diluted 1:1000) directed against the appropriate cytokine was added. One hour later, the plates were washed, color developed, and analyzed using a microtiter plate reader. A standard curve was generated on each microtiter plate, which was used for quantifying the amount of cytokine in each sample. The sensitivity of detection for IL-1β was 4 pg/ml, while that of IL-6 and TNF-α was 5 pg/ml.

RNA Extraction, RT-PCR, and Southern Blot Analysis—First strand cDNA was prepared from total cellular RNA as per the manufacturer's protocol (Amersham Pharmacia Biotech). PCR was conducted using published primers for IL-1β, IL-6, and β-actin (19). β-Actin primers served as internal controls in each reaction. PCR products were resolved in a 1.5% agarose gel and transferred to a nylon membrane and probed with [32P]ATP end-labeled oligonucleotide probes. IL-β, IL-6, and β-actin oligonucleotide probes were designed based on products amplified using the above primers (IL-1β, 5’-CTG CAC GCT CCG GGA CTC ACA CCA-3’; IL-6, 5’ AAT CGG GTA CAT CCT GGA CGG CAT CTG-3’; β-actin, 5’ GAG ACC TCC ACC CCC ATC AG-3’). Additionally, cell extracts of astrocytes (D) were treated with Tat (100 ng/ml) for either 5, 30, or 60 min, and cytokine levels were measured in the culture supernatants 3 h later. A 5-min exposure was sufficient to induce maximal amounts of cytokine production. Sets of cultures were also pretreated with antisera to TNF-α for 30 min followed by Tat exposure. The antisera was maintained in the medium for the duration of the experiment. IL-1 or IL-6 production by monocytes (A, B) or IL-6 production by astrocytes (D) was not inhibited by antisera to TNF-α.

RESULTS

To determine the effect of a transient exposure of Tat on cytokine production, we treated monocytes and astrocytes with concentrations of Tat (100 ng/ml) previously shown to induce cytokine production in these cells (18, 20–22). We found that a 5-min exposure to Tat was sufficient to induce maximal amounts of proinflammatory cytokines IL-1β, IL-6, or TNF-α in the monocytes (Fig. 1A–C) and IL-6 in the astrocytes (Fig. 1D). IL-1β or TNF-α could not be detected in the astrocyte culture supernatants at all time points tested. To determine whether the IL-1β or IL-6 production in these cells was regulated via TNF-α, we analyzed the ability of TNF antisera to inhibit IL-1β or IL-6 production. IL-1β and IL-6 induction by Tat was independent of TNF-α production (Fig. 1, A, B, and D).

Even though we used highly purified cultures of monocytes and astrocytes, we could not exclude the possibility of small amounts of other contaminating cell types in these cultures. Hence we used a human monocytoid (THP-1) and astrocytic (U373) cells for further experiments. We have previously characterized Tat-induced TNF-α in these cells (18). Hence, to determine the effect of Tat on the production of cytokines IL-1β and IL-6, we treated THP-1 and U373 cells with Tat and measured IL-1β and IL-6 in the culture supernatants. Increasing levels of IL-1β were produced by THP-1 cells in a dose-dependent manner. Significant increases were noted with concentrations of 100 ng/ml Tat (Fig. 2A).

To determine whether the induction of IL-1β occurred at the level of transcription or translation, we estimated IL-1β mRNA levels in THP-1 cells by RT-PCR. Tat induced IL-1β mRNA expression in a dose-dependent manner. The RT-PCR was much more sensitive and was able to detect IL-1β mRNA induction with 10 ng/ml Tat (Fig. 2D).

The U373 cells did not produce detectable levels of IL-1β in the culture supernatants, even with 1 μg/ml Tat. Since stimuli such as IL-1α and IL-2 result in production of cell associated IL-1β (23, 24), we tested cell extracts for the presence of IL-1β but were still unable to detect any IL-1β protein (data not shown). To determine whether the block in IL-1β production
was at the level of transcription or translation, we measured mRNA levels in Tat treated U373 cells. IL-1β mRNA levels in U373 cells were comparable with that of THP-1 cells (Fig. 2, D and E). Hence there was a translation block in the U373 cells.

Significant amounts of IL-6 were produced in the culture supernatants of THP-1 cells and the U373 cells (Fig. 2, B and C). Comparatively, however, the U373 cells produced nearly 20-fold more IL-6 than the THP-1 cells at both 100 ng/ml and 1 μg/ml concentrations of Tat. IL-6 mRNA production paralleled the production of IL-6 protein in U373 cells (Fig. 2, C and F) and THP-1 cells (data not shown).

We next conducted experiments to determine whether a transient exposure to Tat would be sufficient to induce cytokine production in U373 and THP-1 cells. A 5-min incubation was insufficient for inducing TNF-α or IL-1β in THP-1 cells, but was sufficient to induce IL-6 production in U373 cells at 180 min post Tat exposure (Fig. 3). Following a 30- or 60-min exposure to Tat, all three cytokines could be induced. A 60-min exposure led to higher levels and earlier release of cytokines (Fig. 3).

We used highly purified Tat protein in all our experiments and have previously shown the specificity of Tat action (18, 25). However, to further determine whether there were any contaminating substances that might result in IL-1β or IL-6 production, we immunoabsorbed Tat and used the remaining solution for treating the cells. The cell extracts were analyzed for IL-1β and IL-6 mRNA levels, since this was a much more sensitive technique for detection of Tat effects as compared with cytokine detection in culture supernatants. A complete block in IL-1β and IL-6 mRNA production was noted as shown in the U373 cells (Fig. 4), demonstrating that our Tat preparations are devoid of other bioactive substances.

Previous studies have shown that Tat induces NFκB activation (26) and that Tat-induced TNF-α production is NFκB-dependent (18). Hence, we pretreated the cells with TLCK, an inhibitor of NFκB activation followed by incubation with Tat. A complete block in IL-1β and IL-6 production was noted in the U373 cells (Fig. 5, A and B) and the THP-1 cells (data not shown). We examined the role of extracellular divalent cations including calcium in Tat-induced cytokine production. Neither EGTA nor BAPTA was able to block cytokine production. Fig. 5C shows the effect on IL-1β production in U373 cells. A similar lack of response was seen for IL-6 production in U373 cells and IL-1β and IL-6 production in THP-1 cells (data not shown).

**DISCUSSION**

Our studies show that cytokine expression in monocytes and astrocytes are differentially regulated by Tat. While monocytes could be induced to produce all three cytokines, i.e. IL-1β, IL-6, and TNF-α, astrocytes produced only IL-6. The levels of IL-6 produced by astrocytic cells were nearly 20-fold greater than those produced by monocytes. The astrocytic cells did not produce measurable amounts of IL-1β, even though mRNA for IL-1β could be induced. Similarly, we have shown previously that Tat could induce only small amounts of TNF-α in the astrocytic cells (18). Furthermore, we were unable to inhibit the production of IL-6 with antisera to TNF-α in astrocytes or monocytes. This is an interesting observation, since IL-1β and

![Fig. 2. Dose dependence of Tat-mediated IL-1β and IL-6 production. THP-1 or U373 cells were treated with Tat as described under “Experimental Procedures.” Cell culture supernatants were analyzed by ELISA (A–C). Each value represents the mean ± S.D. of three experiments done in triplicates. mRNA levels were determined in cell extracts by RT-PCR followed by Southern blot analysis and compared with that of β-actin (D–F). A and D show induction of IL-1β in THP-1 cells. B shows IL-6 induction in THP-1 cells. C and F show strong induction of IL-6 protein and mRNA in U373 cells. D shows IL-1β mRNA induction in THP-1 cells. E shows IL-1β mRNA induction in U373 cells. In each case, mRNA induction was noted with 10 ng/ml of Tat, while significant (p < 0.05) protein production occurred at 100 ng/ml Tat.](image-url)
TNF-α have been shown to induce IL-6 gene expression in astrocytes (27). This suggests that the effect of Tat on IL-6 production is specific.

Within the brain, IL-1β is primarily produced by activated microglia (brain macrophages) (28). HIV-infected macrophages also release high levels of IL-1β (29). Our studies show that Tat protein may, at least in part, contribute to the elevation of IL-1β levels in the brain of patients with HIV dementia. Tat-induced IL-1β is independent of TNF-α production. The increase in IL-1β may induce astrogliosis (30), promote HIV-1 replication (31), and induce other cytokines such as TNF-α (28), resulting in further brain injury.

Tat-treated astrocytic cells not only produced large amounts of IL-6, but the elevated levels were present for prolonged periods. Several studies have shown that astrocytes are an important source of IL-6 (32). IL-6 has prominent effects on the brain, which include activation of the hypothalamic pituitary-adrenal axis, decreased appetite, and neuronal growth (33). Furthermore, IL-6 has been implicated in neuronal degeneration. Transgenic mice with IL-6 develop severe neurologic disease accompanied with neurodegeneration and astrogliosis (34). IL-6 has also been implicated in pathogenesis of neuronal injury in Alzheimer’s disease (35).

Tat has been shown to induce changes in intracellular calcium in neurons through an influx of extracellular calcium (36). We hence examined the role of extracellular calcium in Tat-induced cytokine production. Removal of extracellular calcium...
had no effect on cytokine production. These observations are interesting since a recent study showed that calcium channel antagonists do not significantly alter the course of HIV dementia (37).

Previous studies have shown NF-κB activation in the brains of patients with HIV infection (38). Further Tat induces NF-κB activation in glial cells (26). We now show that Tat-induced cytokine production is likely NF-κB-dependent. Interestingly, the same cytokines have also been shown to activate NF-κB itself (39). Thus cytokine production once initiated by Tat could result in a positive feedback loop between NF-κB and cytokine production. This process may therefore lead to an amplification of cytokine production without requiring the continued presence of Tat.

Importantly, in this study we show that exposure to Tat for even a few minutes is sufficient to induce cytokine production in monocytes and astrocytes for prolonged periods of time. These findings are consistent with our previous observations that, following a single intraventricular injection of Tat in rats, progressive glial activation and macrophage infiltration could be seen for several days, even though Tat itself could not be detected in the brain after a few hours (12). Furthermore, an exposure of Tat in the order of only milliseconds is sufficient to induce prolonged depolarization in neurons (25, 40). Together, these studies suggest that a transient exposure to HIV-Tat protein results in a cascade of events leading to glial cell activation and neuronal degeneration. We thus propose that a “hit and run” phenomenon may be operative in neuropathogenesis of HIV infection, which may also explain why cross-sectional measurements of viral load in the brain at autopsy do not always correlate with neuronal degeneration and dementia.

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