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Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia

(chemotaxis/central nervous system)

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ABSTRACT **Activated monocytes release a number of substances, including inflammatory cytokines and eicosanoids, that are highly toxic to cells of the central nervous system. Because monocytic infiltration of the central nervous system closely correlates with HIV-1-associated dementia, it has been suggested that monocyte-derived toxins mediate nervous system damage. In the present study, we show that the HIV-1 transactivator protein Tat significantly increases astrocytic expression and release of monocyte chemoattractant protein-1 (MCP-1). Astrocytic release of β-chemokines, which are relatively less selective for monocytes, including RANTES, macrophage inflammatory protein-1α, and macrophage inflammatory protein-1β, was not observed. We also show that MCP-1 is expressed in the brains of patients with HIV-1-associated dementia and that, of the β-chemokines tested, only MCP-1 could be detected in the cerebrospinal fluid of patients with this condition. Together, these data provide a potential link between the presence of HIV-1 in the brain and the monocytic infiltration that may substantially contribute to dementia.**

At present, our understanding of the pathogenesis of AIDS-related neurological damage is incomplete. However, there are much data to support the possibility that monocyte-derived cells, including macrophages and microglia, play a critical role in the genesis of this condition. Monocytic infiltration of the central nervous system (CNS) is a cardinal feature of AIDS-related neuropathology (1) and a significant correlate of dementia (2). Monocyte-derived cells are the prime targets for HIV-1 in the CNS (3–5). Moreover, such cells have been shown to release a number of substances that are highly toxic to neurons (6–11). These substances include the viral gene products Tat, glycoprotein 41 (gp41), and gp120, as well as the cellular products tumor necrosis factor-α, nitric oxide, platelet-activating factor, and quinolinate. *In vivo* studies have shown that many of these toxins are produced predominantly, if not exclusively, by monocyte-derived cells (12). Also, one study that examined brain tissues from pediatric patients has demonstrated that apoptotic neurons are frequently located in proximity to HIV-1-infected macrophages and microglia (13).

The mechanisms responsible for monocytic infiltration of the CNS remain to be elucidated. However, one possibility is that HIV-1-infected cells could stimulate the production of a relatively selective monocyte chemoattractant, such as monocyte chemoattractant protein-1 (MCP-1) (14–16). In one study (17), MCP-1 was found to be the most potent of a variety of monocyte chemoattractants, including RANTES, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MCP-1, MCP-2, and MCP-3. Furthermore, MCP-1 is inducible in astrocytes (18, 19), which are the most numerous cells in the brain.

We have previously shown that the HIV-1-encoded transactivator Tat, a soluble protein that is released from HIV-1-infected cells (20), can increase NF-κB binding in astrocytes (21). Because NF-κB can influence the expression of MCP-1 (22), in the present study we examined whether Tat could affect astrocytic expression and release of this chemoattractant. We have also examined the possibility that MCP-1 is elevated in the CNS of patients with AIDS dementia, a condition characterized not only by monocytic infiltration of the brain, but by an increase in Tat-encoding transcripts (23).

METHODS

Preparation of Tat Protein and Astrocytes. Highly purified recombinant Tat_{1-72} was prepared as described previously (7). Tat_{1-72} contains the epitope that increases NF-κB binding in astrocytes (21) and is similar to Tat_{1-86} (Intracel) in its ability to increase astrocytic expression of MCP-1.

Cultured Astrocytes. Brain tissue from 12- to 14-week-old human fetuses was obtained in accordance with National Institutes of Health guidelines. The tissue was mechanically disrupted by aspiration through a 19-gauge needle, washed in Eagle's minimal essential medium (EMEM), and then distributed into tissue culture flasks. Cells were maintained in EMEM containing 10% fetal bovine serum, 2 mM of L-glutamine and 5 μg/ml of gentamicin. Several days later, flasks were placed into an orbital incubator shaker set at 37°C and 210 rpm for 6 h. Non-adherent cells were removed. A portion of the adherent cells were later stained with an antibody to glial fibrillary acidic protein and only those cultures that were >95% positive were used.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted using RNAzol (Tel-Test) according to the manufacturer's instructions. Before RNA extraction, cells were maintained for 6 hr in serum-free media. Northern blot analysis was performed as described (24).

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; HIVD, HIV-1-associated dementia; HIV(N), HIV-1 positive patient without dementia; MCP-1, monocyte chemoattractant protein-1; MIP-1, macrophage inflammatory protein-1; MS, multiple sclerosis; NIN, non-inflammatory neurological; TPCK, *n*-tosyl-L-phenylalanine chloromethyl ketone; G6-PD, glucose 6-phosphate dehydrogenase.

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Experiments with gp41 and gp120. Astrocytes (10^6 per 1 ml medium) were stimulated with 100 nM of HIV-1_{IIIB} gp41 (Intracel) or 100 nM of HIV-1_{IIIB} gp120 (Intracel), and supernatants were assessed 24 hr later by immunoassay (R&D Systems).

Proliferation Assays. Astrocytes were grown in 96-well plates. At 70% confluency, cells were treated with varying concentrations of exogenous Tat, in media that contained 1 μCi (1 Ci = 37 Gbq) per well of tritiated thymidine (New England Nuclear). Twenty-four hours later, cells were washed and harvested onto glass fiber filters. Filters were then dried and placed into scintillation fluid for counting in a Betaplate Apparatus. Twenty hours after the administration of Tat, in doses ranging from 10 to 1000 nM, there was no measurable increase in astrocyte proliferation.

Trypsin Digestion. Trypsin digestion of Tat was performed by the addition of 25 μl of 0.25% trypsin per μg of Tat. The mixture was then incubated for 4 hr before the 1:1 addition of soybean trypsin inhibitor (Sigma).

Detection of MIP-1α, MIP-1β, and RANTES. MIP-1α, MIP-1β, and RANTES were detected by ELISA (R&D Systems). These ELISAs could detect concentrations as low as 10 pg/ml. Protein measurements were determined by comparison to a standard curve, run in duplicate with each assay.

Immunoabsorption. Immunoabsorption of Tat was performed as described previously (7). Briefly, a Tat-specific monoclonal antibody (Intracel) was bound to protein A–Sepharose (Pharmacia), washed, and then incubated with Tat for 60 min at room temperature, followed by centrifugation.

Cerebral Spinal Fluid (CSF) and Serum Studies. CSF was obtained from a prospectively characterized population of patients. Computerized tomography or magnetic resonance imaging scans were performed on all patients. Because opportunistic infections of the CNS may influence chemokine expression (25), those patients with such infections were excluded. Similarly excluded were patients with CNS lymphoma. CD4 count <200 and/or dementia were the AIDS-defining illnesses in those patients with HIV-1-associated dementia (HIVD). Of the HIV-1 positive patients without dementia [HIV(N)], five had a diagnosis of AIDS as defined by a CD4 count of less than 200 (n = 4) or non-CNS opportunistic infection (Candida, n = 1). The CD4 cell counts (number of cells per cubic millimeter) of the HIVD patients (n = 10, mean ± SE = 125 ± 38) were not significantly different from those of HIV(N) patients (n = 10, mean ± SE = 159 ± 60). The patients ages, in years, were as follows: HIVD, 46 ± SE = 9; HIV(N), 34 ± SE = 8; multiple sclerosis (MS), 40 ± SE = 11; non-inflammatory neurological (NIN) conditions, 39 ± SE = 8. Also, eight patients in each HIV positive group were on antiretroviral therapy at the time of lumbar puncture. Antiretroviral therapy consisted of azidothymidine only [n = 4 HIVD and 8 HIV(N)], azidothymidine + lamivudine (n = 1 HIVD), lamivudine + stavudine + viramine (n = 1 HIVD), or azidothymidine + lamivudine + saquinavir (n = 2 HIVD). In this limited sample size, we could not detect any correlation between triple therapy and CSF MCP-1 values among patients with HIVD. In fact, of the three patients on triple therapy, MCP-1 values were 4279, 1037, and 223 pg/ml, respectively.

HIV-1 positive patients were grouped as demented and nondemented according to criteria established by a task force of the American Academy of Neurology (26). CSF from 10 patients with clinically definite MS (27) and another 10 patients with noninflammatory conditions of the CNS (headaches or degenerative disc disease) were similarly stored and analyzed.

Analysis of matched serum samples showed that the MCP-1 levels were 2–6 times higher in the CSF than in the serum of patients with HIVD. In other groups, the ratio of CSF to serum levels of MCP-1 was between 0.2 and 1.0.

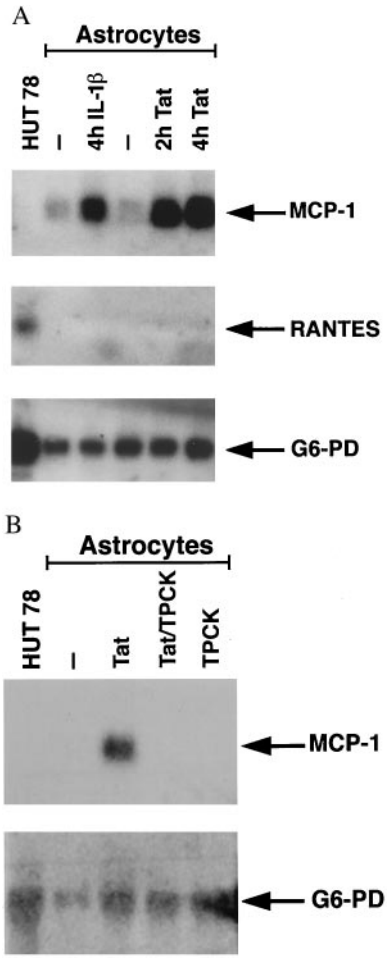

FIG. 1. (*A*) Total RNA was extracted from T lymphoblastoid HUT 78 cells (ATCC) and from variously treated human astrocytes. Ten micrograms per lane were then run on a 1% agarose–6% formaldehyde gel. After transfer of RNA to nitrocellulose, the blot was probed with the full-length cDNA for MCP-1 and, subsequently, RANTES. RNA from untreated astrocytes was run in lanes 2 and 4. Both 4 hr stimulation with 5 ng/ml of interleukin-1β (lane 3) and 2 or 4 hr stimulation with 100 nM of Tat (lanes 5 and 6) were associated with an increase in astrocytic MCP-1 expression. Astrocytic expression of RANTES was not detected. (*B*) Similar experiment except that RNA from HUT 78 cells (lane 1) was compared with RNA from astrocytes that were unstimulated (lane 2) or stimulated for 2 hr with 100 nM of Tat (lane 3), 25 μM of TPCK followed by 100nM of Tat (lane 4), or 25 μM of TPCK (lane 5). In *A* and *B*, the G6-PD probe was used as a control (24).

***In Situ* Hybridization Studies.** *In situ* hybridization was performed on paraffin-embedded brain tissue sections from two HIVD patients, one HIV(N) patient, two MS patients, and two normal controls. Patients were autopsied at similar postmortem times (12–18 hr). Cause of death was dementia (n = 2 HIVD) and interstitial pneumonia [n = 1 HIV(N)]. Sections from the frontal cortex, hippocampus, and brainstem were studied.

In situ hybridization was performed using a ^{33}P-labeled MCP-1 riboprobe (I.M.A.G.E. Consortium no. 488534, homologous to GenBank no. M37719). Before use, the probe was sequenced and tested by Northern blot analysis. Negative controls for *in situ* included hybridization with the MCP-1 sense strand.

RESULTS

MCP-1 Expression. To determine whether Tat could increase astrocytic expression of MCP-1, we stimulated astro-

cytes with 100 nM of Tat and then extracted total RNA 2 and 4 hr later. As demonstrated in Fig. 1, we observed an increase in MCP-1-encoding RNA as early as 2 hr after stimulation of astrocytes with Tat. Furthermore, consistent with the possibility that NF-κB is required for Tat's effect, we observed that this increase was inhibited by *n*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). This compound blocks the activation of NF-κB by interfering with the degradation of I-κBα (28) (Fig. 1*B*).

MCP-1 Protein. To determine whether increased expression of MCP-1 would correlate with increased protein synthesis or release, we next analyzed the supernatants of Tat-treated astrocytes by ELISA and Western blot. As demonstrated in Figs. 2*A* and 3, we found that exogenous Tat was associated with a dose-dependent increase in astrocytic release of MCP-1. This increase was specific in that astrocytic release of RANTES, MIP-1α, and MIP-1β was not observed. We also determined that neither gp120 nor gp41 had the same effect (data not shown). In addition, we found that release of MCP-1 was independent of proliferation, and could be inhibited by either pretreatment of Tat with trypsin or immunoabsorption of Tat with a specific antibody (Fig. 2*B*).

***In Vivo* Studies.** We next examined MCP-1 levels in the CSF of AIDS patients with and without dementia. Both groups of patients had significantly elevated levels of MCP-1 when compared with those patients with MS or NIN conditions. Additionally, patients with HIVD had significantly higher levels of MCP-1 than did HIV(N) patients (Fig. 4). Of note is that the levels of MCP-1 in the CSF of HIVD patients were within the range required to induce monocyte chemotaxis (16). Also, MCP-1 levels were substantially higher in the CSF as compared with the serum in HIVD patients. This suggests that MCP-1 was synthesized intrathecally. Simultaneously, we assayed all samples for RANTES, MIP-1α, and MIP-1β. RANTES was present in serum samples in concentrations of 1–2 ng/ml. However, these chemokines were below detectable limits (10 pg/ml) in the CSF.

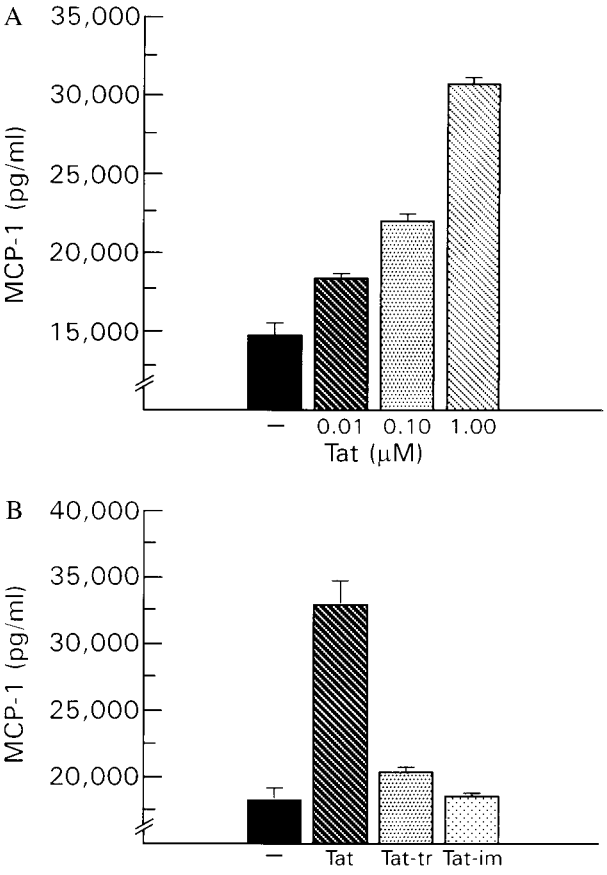

FIG. 2. MCP-1 ELISA analysis of supernatants from variously treated human astrocytes. (*A*) Astrocytes were grown to near confluency in 35-cm plates. Each well contained 10^6 cells in 1 ml of medium. The medium was then changed and astrocytes were stimulated with exogenous Tat in doses ranging from 0.01 to 1.0 μM. Twenty hours later, samples were taken for analysis by immunoassay (R&D Systems). As compared with untreated astrocytes (−) that, when grown in tissue culture, express MCP-1 in the absence of stimulation, Tat-stimulated astrocytes showed a significant increase in MCP-1 release. Data are shown as mean + SE for three replicates. (*B*) Similar experiment except that astrocytes were stimulated with 100 nM of Tat or with an equivalent amount of Tat that had first been either digested with trypsin (Tat-tr) or immunoabsorbed (Tat-im).

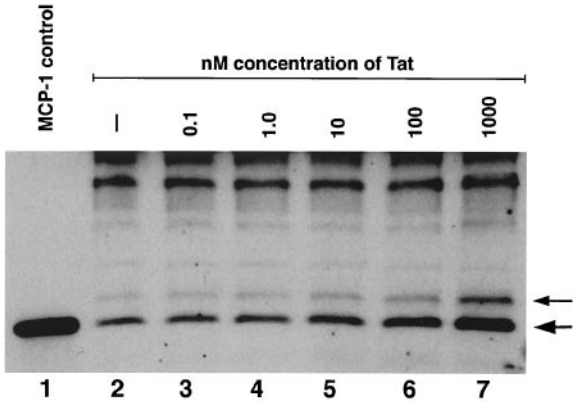

FIG. 3. Western blot analysis of MCP-1 in astrocyte supernatants. In lanes 2–7, 50 μg of protein from variously treated astrocyte supernatants were run on a 15% Tris·glycine denaturing gel. Three nanograms of non-glycosylated recombinant MCP-1 (R&D Systems) was run in lane 1 as a control. After protein transfer to nitrocellulose, the blot was probed with a polyclonal antibody that recognizes human MCP-1 (R&D Systems). After washing, an appropriate secondary antibody was applied [horseradish peroxidase conjugated anti-goat (Santa Cruz Biotechnology)] and electrochemiluminescence (Amersham) was used to visualize the bands. The two bands, which are specifically increased in association with Tat, are indicated by arrows. The lower arrow represents a band that runs with an apparent molecular mass of 9 kDa, whereas the upper band, of slightly higher molecular mass, is likely to represent MCP-1 that has been altered by the addition of O-linked carbohydrates. Both forms of MCP-1 are active *in vitro* (15).

The presence of MCP-1 in the brains of patients with HIVD was further supported by *in situ* hybridization. Strongly positive cells were noted in several brain regions, including CNS white matter (Fig. 5 *a* and *b*). Morphologically, cells that expressed MCP-1 included both astrocytes and neurons. Of interest, cells expressing MCP-1 RNA were often observed in perivascular regions (Fig. 5 *c*). In contrast, no positive cells were seen in normal brain tissue or in tissue from patients without dementia (Fig. 5*d*).

DISCUSSION

In summary, our results demonstrate that primary cultures of human astrocytes produce MCP-1, that this production is increased by the HIV-1 protein Tat, and that the production of MCP-1 is increased in the brains of AIDS patients with dementia.

One might speculate that altered MCP-1 expression could contribute to HIVD. Unlike RANTES, MIP-1α, or MIP-1β, MCP-1 does not have significant neutralizing activity against primary viral isolates (29) nor does it inhibit HIV-1 infection of microglia (30). In addition, although MCP-1 may have some antiviral activity under select *in vitro* conditions (31), in some experiments it has been associated with an increase in HIV-1 replication (32). Also, like other β-chemokines, MCP-1 stim-

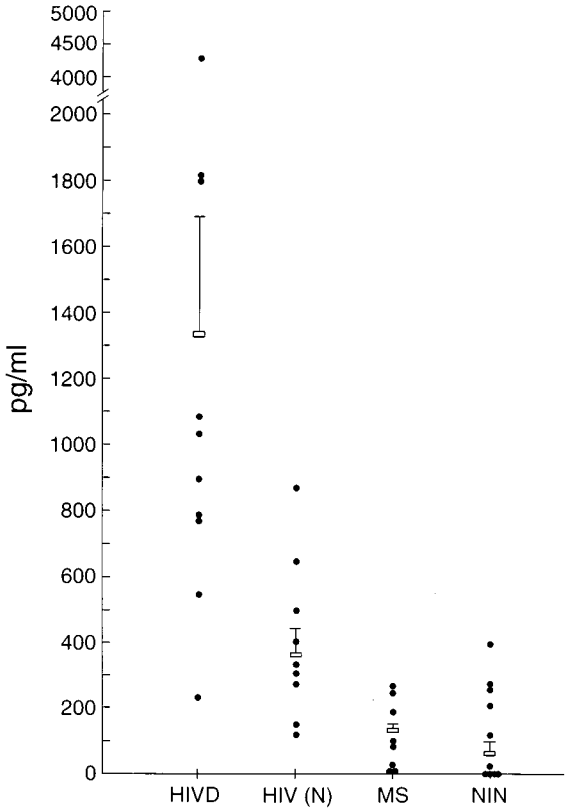

FIG. 4. MCP-1 levels in CSF. MCP-1 levels were analyzed by ELISA (R&D Systems) in CSF samples from HIVD patients (HIVD, $n = 10$), HIV(N) patients [HIV(N), $n = 10$], patients with MS (MS, $n = 10$) and patients with NIN conditions (NIN, $n = 10$). All data are represented as mean + SE and analyzed by non-parametric analysis using the Mann–Whitney test. The comparisons of HIVD to HIV(N), MS, and NIN were significant at $P < 0.002$, $P < 0.001$, and $P < 0.001$, respectively. The comparisons of HIV(N) to MS and NIN were significant at $P < 0.01$. There were no significant differences between the MS and the NIN groups.

ulation of select cell types has been associated with increased expression of proinflammatory substances such as interleukin-1β, interleukin-6, and arachidonate (33). Moreover, MCP-1 stimulation of monocytes has been associated with an increase in the release of superoxide (34).

Of additional importance, however, is the possibility that MCP-1 could contribute to the monocytic infiltration that has been observed to correlate with HIVD. Monocytic infiltration would in turn be associated with an increase in the release of neurotoxins. Furthermore, it could contribute to a positive feedback loop whereby more cells in the brain could be infected, leading to increased levels of Tat and hence more MCP-1.

The possibility that MCP-1 contributes to monocytic infiltration *in vivo* is supported by a number of studies. In transgenic mice, glial-specific expression of MCP-1 is associated with pronounced monocytic infiltration of the CNS (35, ††). MCP-1 injection into the murine hippocampus also leads to the selective recruitment of monocytes (14). Additionally, monocyte chemoattractant activity in the CSF of patients with viral meningitis can be inhibited with antibodies to MCP-1 (36).

The concentrations of MCP-1 in the CSF of patients with HIVD are sufficient to induce monocyte chemotaxis (16), and it is possible that local amounts in brain tissue are even higher.

††Tani, M. S., Rollins, B. J. & Ransohoff, R. M., 122nd Annual Meeting of the American Neurological Association, Sept. 28–Oct. 1, 1997, San Diego, CA, p. 97.

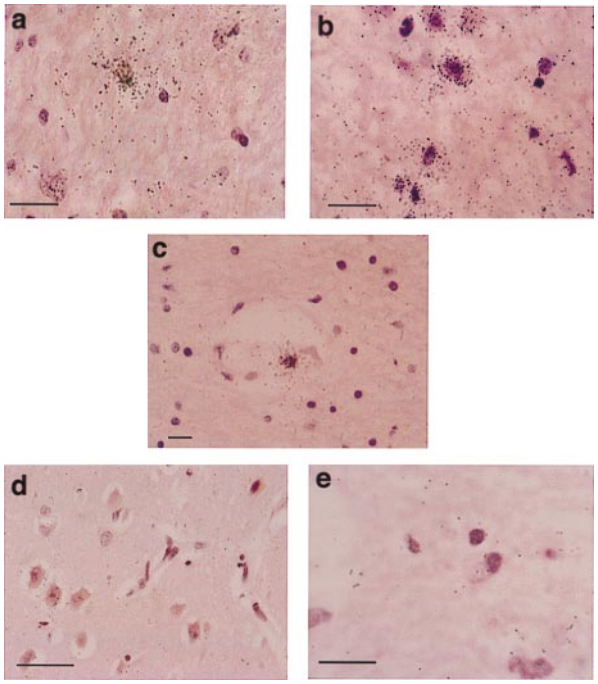

FIG. 5. Detection of MCP-1 RNA in brain tissue by *in situ* hybridization. Tissue sections were hybridized with an MCP-1 antisense probe. (*a* and *b*) Cells within the cerebral white matter of a HIVD patient show a strong signal for the presence of MCP-1 RNA. (*c*) Signal-positive cells are also seen in perivascular regions. A representative area from a normal patient shows absence of signal (*d*), as does a sample from an HIVD patient that was hybridized with an MCP-1 sense probe (*e*). (Scale bars represent 25 μm.)

Therefore, MCP-1 could play a significant role in the pathophysiology of AIDS dementia. Other chemokines, however, may also be present in association with this condition. For example, brain-derived cells can produce MIP-1α, MIP-1β, and RANTES *in vitro* (37). Also, whereas MIP-1α, MIP-1β, and RANTES were not detectable in the CSF samples assayed in this study, possibly because of their presence in amounts that were below the limits of detection, transcripts for these chemokines have been detected in HIVD brain tissues through the use of PCR techniques (38). However, it is not clear as to whether these chemokines are produced in sufficient quantities to induce chemotaxis.

The possibility that a viral protein such as Tat could up-regulate the expression of MCP-1 in the CNS of patients with HIVD is intriguing. Tat is essential for viral replication and, in comparison to HIV-1 structural proteins, is a relatively small diffusible molecule. Once released from infected cells (20), Tat could increase MCP-1 expression through a number of mechanisms. By its ability to increase NF-κB binding (21), it could directly effect MCP-1 expression. At later time points, Tat might also increase MCP-1 expression through indirect mechanisms. For example, Tat could stimulate the production of cytokines that can also induce MCP-1 expression (39, 40).

In the present study, low nanomolar concentrations of Tat were sufficient to increase astrocytic MCP-1 release. These concentrations are slightly lower than those required by gp41 to increase nitric oxide production in mixed neuroglial cultures (8). Because of the rapid degradation of extracellular protein in autopsy material and the cross reactivity of antisera to Tat with endogenous brain proteins (41), it is difficult to quantitate Tat protein *in vivo*. However, it has been shown that *tat* transcripts are elevated in the CNS of AIDS patients with both dementia (23) and encephalitis (42).

Although astrocytes may not be the only CNS cells to produce MCP-1, nor Tat the only stimulus for such production,

the ability of Tat to increase astrocytic expression of this chemokine is likely to be significant. Not only are astrocytes the most abundant cells in the brain, they are in intimate contact with the blood brain barrier. Such astrocytes could be expected to play an important role in the recruitment of monocytes to the CNS.

The finding that MCP-1 is significantly elevated in the CNS of patients with AIDS dementia could direct future therapies toward clinically effective inhibitors of MCP-1 or its principal receptor CCR-2 (43). These compounds might inhibit the attraction of peripheral monocytes to the CNS even in the presence of stimuli that could increase MCP-1 expression. Furthermore, these compounds might also inhibit the activation of monocyte-derived cells not only in the CNS but in the periphery. Of note is that MCP-1 antagonists are presently under investigation for the treatment of other inflammatory conditions, including rheumatoid arthritis (44).

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