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Human T-cell leukemia virus type I infection of monocytes and microglial cells in primary human cultures

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ABSTRACT **The pathogenesis of progressive spastic paraparesis [HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP)], a serious consequence of human T-cell leukemia virus type I (HTLV-I) infection, is unclear. T and B lymphocytes can be naturally infected by HTLV-I, but the susceptibility to HTLV-I infection of other cell types that could contribute to the pathogenesis of HAM/TSP has not been determined. We found that a human monocyte cell line (THP-1), primary human peripheral blood monocytes, and isolated microglial cells but not astrocytes or oligodendroglial cells derived from adult human brain were infected by HTLV-I *in vitro*. Infection with HTLV-I enhanced the secretion of interleukin 6 in human microglial cell-enriched cultures but did not stimulate the release of interleukin 1 from monocytes or microglial cells. Tumor necrosis factor α production was stimulated by HTLV-I infection of monocytes and microglial cells and could be enhanced by suboptimal amounts of lipopolysaccharide. Since both tumor necrosis factor α and interleukin 6 have been implicated in inflammatory demyelination and gliosis, our findings suggest that human microglial cells and monocytes infected with and activated by HTLV-I could play a role in the pathogenesis of HAM/TSP.**

Human T-cell leukemia virus type I (HTLV-I) and human immunodeficiency virus (HIV) are predominantly tropic for cells of the immune system yet both are capable of causing central nervous system (CNS) pathology. Lesions associated with HTLV-I consist of gliosis, axonal injury, demyelination, and perivascular mononuclear cell infiltrates (1, 2). In both HTLV-I and HIV-I infections, reactive gliosis is prominent but there is little evidence for direct astroglial infection (2, 3). The close proximity of mononuclear cells to these lesions could allow viral- or cellular-derived products of infected mononuclear cells to play a direct role in neuropathogenesis. Indeed, recent studies showed that neurotoxins were released from HIV-infected monocytes but not from HIV-infected T lymphocytes (4, 5). Furthermore, neurological disease in simian immunodeficiency virus (SIV)-infected rhesus monkeys is caused only by SIV strains that replicate in monocytes (6, 7).

Therefore, we studied the ability of HTLV-I to infect cells isolated from primary human brain cultures. We used a method for cell-free infection by HTLV-I (8) to show that HTLV-I can productively infect peripheral blood monocytes and brain-derived microglial cells *in vitro*. Furthermore, tumor necrosis factor α (TNF-α) and interleukin (IL) 6 production are stimulated by HTLV-I infection of microglial cells and monocytes, suggesting that cytokine release from HTLV-I-infected or -activated cells could be involved in the neuropathogenesis of HTLV-I infection.

MATERIALS AND METHODS

Cell Lines. THP-1 and MT-2 cells were grown in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and glutamine (300 μg/ml) and subcultured every 4–5 days. T cells activated for 48 h with phytohemagglutinin were cultured in the same medium with 200 units of IL-2 (Cellular Products).

Primary Cell Cultures. Human brain tissue was obtained from patients undergoing partial temporal lobe resection for the treatment of intractable epilepsy. Histologically, the tissue was normal or showed slight gliosis but no inflammation. Finely minced brain tissue was dissociated by digestion with trypsin and DNase, passed through nylon mesh, and separated into populations of nonadherent oligodendrocytes (>90%) and adherent microglia and astrocytes by Percoll-gradient separation and differential adherence (9). After shaking the mixed microglia/astrocyte culture at 150 rpm for 5 h, the adherent cells were enriched for c-fms$^+$, Leu-M3$^+$, Leu-M5$^+$ microglia (>90%). The nonadherent cells contained both microglia (40%) and astrocytes (GFAP$^+$ GC$^-$, 60%; where GFAP is glial fibrillary acidic protein). Fifty thousand cells were plated in 100-μl drops on coverslips pretreated with poly(L-lysine) (10 μg/ml) and cultured in 24-well plates. The cultures were fed every 3–4 days and maintained in Dulbecco's modified Eagle's medium containing 5% heat-inactivated human AB sera (Irvine Scientific) supplemented with antibiotics and glutamine. Monocytes were recovered from the peripheral blood of HIV and hepatitis B seronegative donors after leukopheresis and purification by counter-current centrifugal elutriation as described (10). After overnight adherence, elutriated monocytes (10^6 cells per culture) formed a monolayer that contained >98% monocytes as determined by cell morphology, nonspecific esterase staining, and cell surface expression of Leu-M3 and c-fms, but not CD3. Adherent monocyte monolayers were maintained in RPMI 1640 medium containing 5% human AB serum and medium was changed weekly.

HTLV-I Infections. Cell-free infections with HTLV-I were performed as described by Fan *et al.* (8). Concentrated DNase-treated MT-2 supernatants containing HTLV-I had no detectable endotoxin levels as measured by the limulus assay.

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Abbreviations: GFAP, glial fibrillary acidic protein; HTLV-I, human T-cell leukemia virus type I; LPS, lipopolysaccharide; RT, reverse transcriptase; TNF-α, tumor necrosis factor α; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; IL, interleukin; HIV, human immunodeficiency virus; CNS, central nervous system; FITC, fluorescein isothiocyanate.

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Adherent monocyte monolayers or glial cell cultures were infected with 100 μl of viral stock for 2 h at 37°C. The THP-1 monocyte cell line was infected by incubating 10^7 cells in logarithmic-phase growth in 1 ml of concentrated HTLV-I for 1–2 h at 37°C in a shaking water bath. All HTLV-I-exposed cells were washed twice to remove unabsorbed virus, and supernatant aliquots were examined for viral antigen several times after exposure. Supernatants from HTLV-I-infected monocyte cultures were used to infect phytohemagglutinin-stimulated human T cells as described (8).

Virus Detection. Levels of HTLV-I p19 antigen were determined in tissue culture supernatants or in cell pellets lysed with 1% Triton X-100 by ELISA (Cellular Products).

Immunofluorescence. Single- and dual-label immunofluorescence staining was performed as described (11). A mouse monoclonal antibody to HTLV-I p24 (DuPont) was used to detect HTLV-I-infected cells. Antibodies to cellular antigens included Leu-1 (CD3), Leu-M3 (CD16), and Leu-M5 (CD11) antibodies from Becton Dickinson, a rat monoclonal antibody to human c-fms from Oncor (Long Island, NY), and a rabbit anti-GFAP antibody from Dako Laboratories (Miami, FL). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-rat IgG (Dako Laboratories) and FITC- and rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories) were obtained commercially. A mouse monoclonal antibody to galactocerebroside (GalC) was kindly provided by Monique Dubois-Dalcq (National Institute of Neurological Disorders and Stroke, Bethesda, MD).

Ultrastructured Analysis. Cultures containing HTLV-I-infected macrophages were harvested 21 days after infection, pelleted, fixed in 1.25% (vol/vol) glutaraldehyde, and post-fixed in 1% osmium tetroxide. Sections were prepared and viewed as described (12).

Polymerase Chain Reactions (PCRs). DNA or cDNA (1 μg) from infected or uninfected cells was amplified in a thermal cycler (Perkin–Elmer/Cetus) as described (13, 14). Primer pairs were specific to the tax region of the HTLV-I genome (15) or for the TNF-α and glyceraldehyde-3-phosphate dehydrogenase genes (16) and crossed splice junctions to allow detection of RNA. The PCR consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 55 or 57°C for 1 min, and extension at 72°C for 2 min. Portions of the PCR amplification products were subjected to gel electrophoresis as described (13–15).

Cytokine Detection. TNF-α and IL-1α, -1β, and -6 were assayed in tissue culture supernatants by an ELISA [Endogen (Boston), Genzyme, and R & D Systems (Minneapolis), respectively]. Standard curves for TNF-α and IL-1 production by uninfected monocytes were determined by adding lipopolysaccharide (LPS) at 0.1 pg/ml to 10 ng/ml to the adherent cell cultures and collecting supernatants 24 h later. IL-1 and TNF-α (100 pg/ml) were detected after stimulation with LPS at 1 or 10 pg/ml; optimal levels of TNF-α (1500 pg/ml) and IL-1 (2000 pg/ml) were induced with LPS at 10 ng/ml. IL-6 levels were determined in culture supernatants 24 h after the medium was changed.

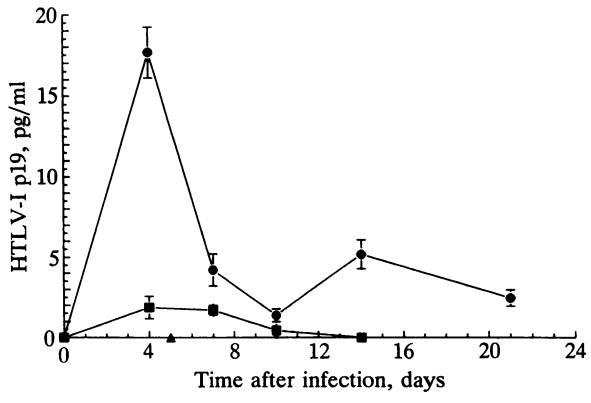

FIG. 1. HTLV-I infection of primary human glial cell cultures. Supernatants from microglial-cell-enriched cultures (●), mixed-glial cultures (■), and oligodendroglial cell cultures (▲) were collected and assayed for HTLV-I p19 by ELISA. Data represent the mean ± SEM for three infections of each cell population.

RESULTS

Seven- to 14-day-old mixed-glial-cell and enriched microglial-cell cultures became infected by HTLV-I 4–21 days after *in vitro* exposure (Fig. 1). Extracellular HTLV-I p19 production was higher in the microglia-cell-enriched cultures than in mixed-glial-cell cultures. In contrast, cultures enriched for oligodendrocytes produced no detectable virus. Microglial cells were not lysed by HTLV-I and did not form syncytia. However, the appearance of a honeycomb-like cytoplasm in microglial cells in HTLV-I-infected cultures suggested that these cells had been activated (Fig. 2*A*). Uninfected mixed

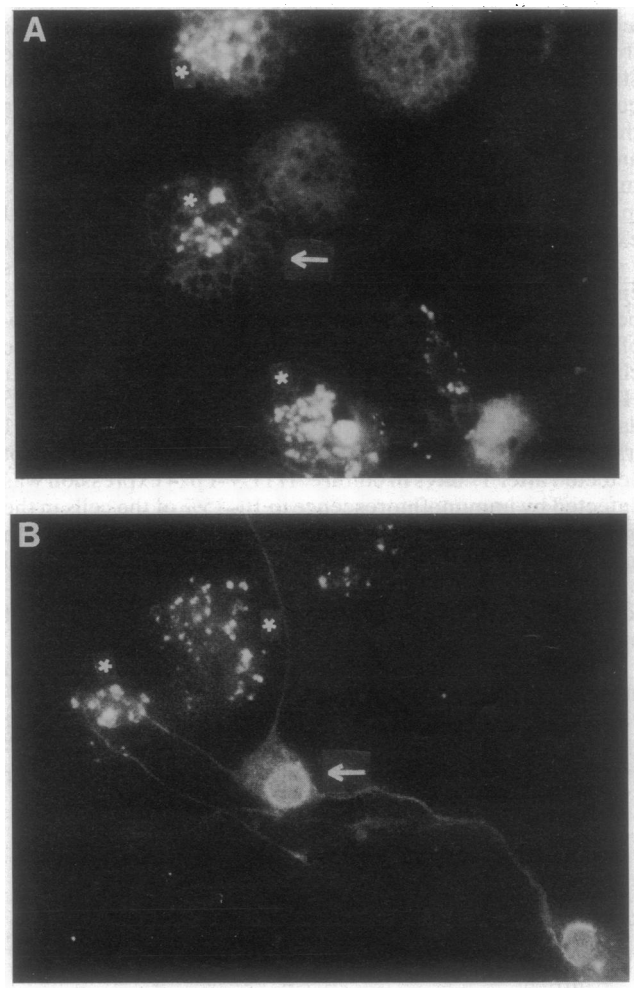

FIG. 2. Dual-label immunofluorescence on adult human brain cultures in the absence of HTLV-I and 10 days after HTLV-I infection. (*A*) Antibody to c-fms and a FITC-labeled anti-IgG conjugate outline the cytoplasm of activated microglial cells (→). Antibody to HTLV-I p24 and a rhodamine-labeled anti-IgG conjugate brightly stain cytoplasmic aggregates (*), indicating expressing HTLV-I p24 cells. (*B*) Antibody to GFAP and a FITC-labeled anti-IgG conjugate identify a process-bearing cell as an astrocyte (→). Antibody to HTLV-I p24 and a rhodamine-labeled anti-IgG conjugate brightly stain cytoplasmic aggregates (*), indicating HTLV-I expression in cells that do not express GFAP. Similar studies were performed on three other infections at several times after infection.

glial and microglial-cell-enriched cultures had no HTLV-I p24 expression or evidence of activation (data not shown). Fourteen days after infection and after four complete medium changes, cell-free supernatants from these HTLV-I-exposed cultures were tested for infectivity in normal human T-cell cultures. HTLV-I p19 was detected in T-cell culture supernatants from 14 days (3.8 ng/ml) to 28 days (8.7 ng/ml) after exposure. These results confirmed that the HTLV-I p19 reactivity in microglial cell culture supernatants was indicative of infectious HTLV-I.

To determine which cell type(s) were expressing viral antigens, dual-label immunofluorescence staining was performed 1–3 weeks after infection. Microglial cells were identified by staining with antibodies to either Leu-M3 or c-fms, astrocytes were identified with antibody to GFAP, and intracellular HTLV-I antigens were identified by staining with antibodies to HTLV-I p24. Antibody to c-fms and HTLV-I p24 identified HTLV-I-infected microglia in three infections (Fig. 2*A*). Similar results were obtained when antibodies to Leu-M3 were used to identify microglia (data not shown). The number of HTLV-I-positive microglia varied from 8 to 16%. No HTLV-I p24 staining was seen in cells from uninfected cultures and anti-CD3 antibody failed to stain cells in mixed-glial-cell and microglial-cell-enriched cultures, indicating that human T cells were not present in these cultures (data not shown). Astrocytes were stained by anti-GFAP antibody in HTLV-I-infected mixed-glial-cell cultures but failed to express HTLV-I p24 (Fig. 2*B*). Thus, microglia were the only cells in primary brain cultures that expressed HTLV-I p24 detectable by immunofluorescence.

Since HTLV-I p19 and p24 expression persisted in cultures enriched for microglial cells for several weeks, parallel experiments were performed on monocytes and on the macrophage cell line THP-1 to determine their susceptibility to chronic infection *in vitro*. In monocytes, extracellular HTLV-I p19 was detected from 3 days after infection to 65 days after infection (Fig. 3). In contrast, the cell line THP-1 transiently produced extracellular HTLV-I p19 that was not detected after 17 days in culture. HTLV-I p24 expression was detected by immunofluorescence in 10–15% of the cells in the monocyte cultures. This p24 expression was associated with virion budding from the monocyte cell surface as determined by electron microscopy (Fig. 4). Therefore, while phagocytosis of HTLV-I could be occurring, a productive infection of monocytes was also demonstrated.

To confirm the findings of productive HTLV-I infection in monocytes and microglial cells, RNA was analyzed by reverse transcriptase (RT)–PCR using a primer to *tax* that crossed a splice junction (15). Both HTLV-I-infected monocytes 45 days after infection and microglial cells 14 days after

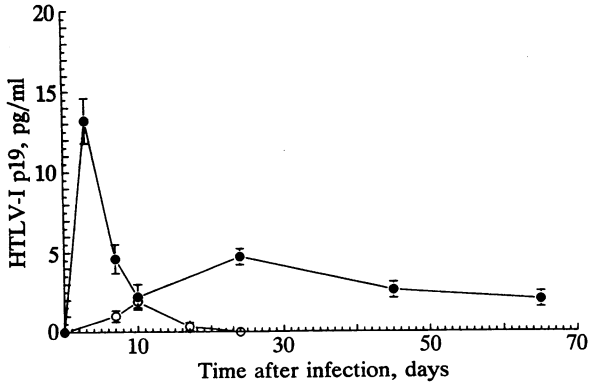

FIG. 3. HTLV-I infection of peripheral blood monoclear cells (●) and the human monocyte cell line THP-1 (○). Supernatants were collected and assayed for p19 by ELISA. Data represent the mean ± SEM for three infections of each cell type.

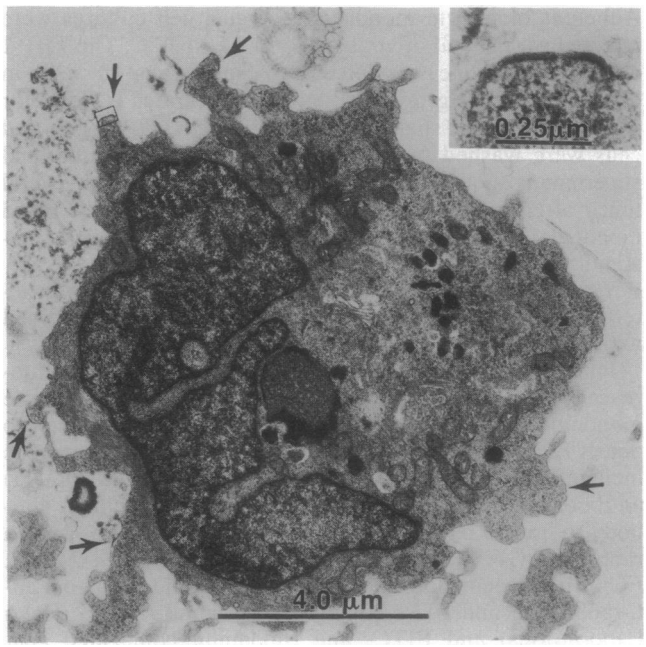

FIG. 4. Electron micrograph of a peripheral blood monocyte 21 days after exposure to cell-free supernatant from an MT-2 cell culture (17). Multiple budding sites of the virus are indicated (→). (*Inset*) Higher magnification of an area of the cell membrane with budding HTLV-I.

infection expressed a correctly spliced *tax* gene product (Fig. 5). Oligodendrocyte cultures remained negative for *tax* gene product expression throughout the culture period (data not shown). These data indicate that HTLV-I infection and viral production at low levels can be persistent in brain-derived microglia and blood-derived macrophages.

Since the *tax* gene product transactivates cellular genes (20) and stimulates cytokine production in T cells (21), we determined IL-1, IL-6, and TNF-α production in HTLV-I-infected monocytes and microglia. Uninfected monocytes (Table 1) and microglia (Table 2) failed to secrete IL-1 or TNF-α as detected by ELISA. However, from 4 to 19 days after HTLV-I infection, TNF-α was detected in supernatants from microglial cell cultures (Table 2). Fourteen days after HTLV-I infection, suboptimal amounts of LPS, a cytokine inducer, increased TNF-α production by HTLV-I-infected monocytes 2- to 3-fold (Table 1). This increase in TNF-α production correlated with an increase in TNF-α mRNA as detected by RT–PCR (Fig. 6). As reported (23), little or no TNF-α mRNA was detected in cultured mononuclear cells; however, it was expressed after

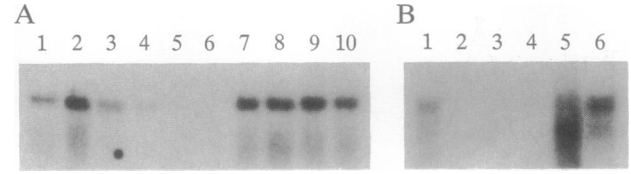

FIG. 5. Detection of HTLV-I tax/rex mRNA in cell lines by RT–PCR. Each RNA (1 μg) was subjected to RT–PCR and analyzed by liquid hybridization using an oligonucleotide probe surrounding the second splice junction site of tax/rex mRNA (18, 19) yielding a 154-base-pair band. (*A*) Lanes: 1–5, MT-2 RNA at 1 μg, 10 ng, 100 pg, 1 pg, and 10 fg, respectively; 6, RNA from uninfected peripheral blood monocytes on day 21 of culture; 7, RNA from the same monocytes infected with HTLV-I 7 days after infection; 8, 14 days after infection; 9, 21 days after infection; 10, 45 days after infection. (*B*) Lanes: 1, enriched brain microglial cells 14 days after HTLV-I infection; 2, same as lane 1 minus RT; 3, microglial cells 14 days in culture; 4, same as lane 3 minus RT; 5, MT-2 RNA at 1 μg; 6, MT-2 RNA at 10 ng.

Table 1. Cytokine production by peripheral blood monocytes exposed to HTLV-I and LPS

Time after infection, days	Condition(s)	Cytokine, pg/ml	
		TNF-α	IL-1
14	Unexposed	0	0
	LPS	106 ± 8	100 ± 8
	HTLV-I	143 ± 47	0
	HTLV-I + LPS	519 ± 31	107 ± 12
21	HTLV-I	107 ± 15	0
	HTLV-I + LPS	497 ± 41	104 ± 9

Suboptimal dose of LPS (10 pg/ml) was determined from standard curves for both IL-1 and TNF-α induction in human peripheral blood macrophage cultures. Data represent the mean ± SEM from three or more determinations.

HTLV-I infection of either microglial cells or monocytes (Fig. 6 and Table 1). Infection of microglial cells with HTLV-I also produced a 3- to 4-fold increase in IL-6 secretion from 4 to 19 days after infection (Table 2). Neither IL-1α nor IL-1β was detected after HTLV-I infection of monocytes (Table 1) or microglial cells (data not shown).

DISCUSSION

The pathogenesis of HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is unknown, but several lines of evidence suggest that HTLV-I replication and cellular activation are involved. HTLV-I is recovered from HAM/TSP patients more easily than from acute T-lymphocyte leukemia patients or HTLV-I-seropositive asymptomatic patients (24). The number of infected peripheral blood mononuclear cells is increased in HAM/TSP patients (24, 25). The presence of virus-specific responses such as HTLV-I-specific cytotoxic T lymphocytes (17) and oligoclonal immunoglobulin in spinal fluid reactive with HTLV-I (26) suggests that the immune system is reacting to a persistent viral infection (27, 28).

The major site of HTLV-I replication *in vivo* appears to be $CD4^+$ T cells (29) although less commonly B cells (30) and some $CD8^+$ T cells (31) can be infected. Infected macrophages have not been identified *in vivo*. In this study we have demonstrated that a monocyte cell line (THP-1), primary human monocytes derived from peripheral blood, and microglial cells derived from adult human brain were all infectable *in vitro*. This extended host-cell range is consistent with previous studies demonstrating transmission of infection from lymphoid cells to other cell types (18, 19, 32) and with recent studies demonstrating cell-free infection of numerous cell lines *in vitro* (8).

HTLV-I sequences are present either directly within or in cells migrating into the brain and spinal cord of HAM/TSP patients (2, 22). However, HTLV-I antigens have not been localized to specific cell types in the nervous system by immunofluorescence (2). Our results suggest that monocytes and microglia are candidate cells for HTLV-I infections in the CNS. The detection of HTLV-I proviral DNA by PCR in the spinal cord of a HAM/TSP patient suggested that infection of resident CNS cells is occurring at very low levels (22). In HAM/TSP patients, T cells are the major site of productive HTLV-I replication. However, as we have shown, monocytes and resident brain microglia are infectable and produce low levels of virus over several weeks in culture. Therefore, it is possible that the low-level expression in the CNS of HAM/TSP patients results from chronic infection of microglia or macrophages.

Table 2. Cytokine production by human microglial cells exposed to HTLV-I

Time after infection, days	Cytokine, pg/ml	
	TNF-α	IL-6
Unexposed	0	1600 ± 160
4	90 ± 20	6100 ± 200
7	260 ± 40	6830 ± 180
12	310 ± 40	5200 ± 370
19	240 ± 40	4800 ± 130

Unexposed cultures were sampled at the same days as HTLV-I-infected cultures. Data represent the mean ± SEM of triplicate determinations from two experiments (HTLV-I infected) or four time points (unexposed).

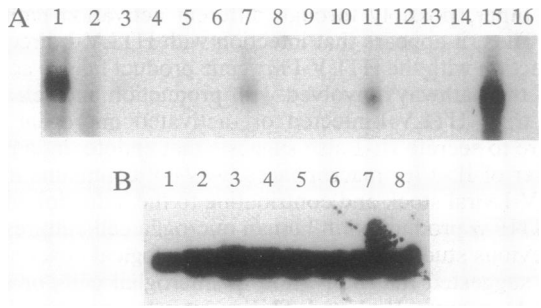

FIG. 6. Detection of TNF-α mRNA in HTLV-I-infected monocytes. Monocytes and brain microglial cells were cultured 7 days as adherent cells and then infected with HTLV-I. Fourteen days after infection, RNA was extracted from cell lysates. Each RNA (1 μg) was subjected to RT–PCR and analyzed by liquid hybridization using an oligonucleotide probe as described (22) yielding a 568-base-pair band. Lanes: 1, infected brain microglial cells day 21 of culture; 2, minus RT; 3, same microglial cells uninfected; 4, minus RT; 5, peripheral blood monocytes 7 days of culture; 6, minus RT; 7, same monocytes infected with HTLV-I 14 days after infection; 8, minus RT; 9, same monocytes exposed to heat-inactivated virus; 10, minus RT; 11, enriched microglial infected with HTLV-I 14 days after infection; 10, minus RT; 11, same uninfected monocytes treated for 24 h with LPS (10 pg/ml); 12, minus RT; 13, same monocytes day 21 of culture; 14, minus RT; 15, same monocytes infected with HTLV-I 14 days after infection and then treated for 24 h with LPS (10 pg/ml); 16, minus RT. (*B*) Detection of glyceraldehyde-3-phosphate dehydrogenase expression by RT–PCR. All eight RNAs (1 μg) used in *A* were subjected to RT–PCR. The band is 195 base pairs.

The transregulatory protein of HTLV-I (tax) activates not only the HTLV-I long terminal repeat promoter (33, 34) but also promoters of cellular genes such as IL-2 (35), IL-2 receptor α chain (36), c-fos (37), and granulocyte-macrophage colony-stimulating factor (38). Therefore, during the course of HTLV-I infection, the *tax* gene product may also affect cellular proliferation and differentiation.

HAM/TSP lesions could be caused directly by cytokine toxicity or indirectly by cytokine-induced cell-mediated cytotoxic reactions resulting from the effects of tax on cellular activation and transcription. In this regard, we found that HTLV-I like HIV-1 is not only capable of infecting microglial cells (39) but also activates them to secrete IL-6 and TNF-α. However, in contrast to the ability of HIV-I to rapidly induce IL-1 and TNF-α in rat glial cells (40), we detected TNF-α and IL-6 but not IL-1 in the supernatants of cultures containing HTLV-I-infected human microglia.

The reasons for this difference in the IL-1 and TNF-α response to HTLV-I are unclear. Human microglia have the capacity to produce IL-1 since IL-1 RNA can be detected in uninfected microglia (S.D.-J., unpublished data). IL-1 was detected in supernatants of HTLV-I-infected and uninfected monocyte cultures after LPS stimulation (Table 1). Rat microglia secrete IL-1 after stimulation with LPS or interaction with HIV-1 (40). However, both IL-1 and TNF-α have membrane-bound forms (41, 42) and there are several examples of induction of IL-1 transcription without translation (43). Furthermore, IL-1 and TNF-α can be induced independently of

each other, possibly through different activation pathways (40). Thus, it appears that infection with HTLV-I directly or interaction with the HTLV-I *tax* gene product fails to activate all of the pathways involved with production and release of IL-1 from HTLV-I-infected or -activated microglial cells. Failure to secrete IL-1 also suggests that endotoxin, a potent inducer of IL-1 for macrophages, was not contaminating the HTLV-I viral stock and contributing to the induction of IL-6 and TNF-α production in human microglia cell cultures.

Previous studies based on neuropathological observations have suggested that activation of microglial cells or astrocytes to secrete IL-1 and TNF-α may be involved in the pathogenesis of HAM/TSP (2). Our studies support this hypothesis and suggest that brain macrophages are involved either directly by infection with HTLV-I or indirectly through activation resulting from HTLV-I binding or the release of soluble tax protein (44). Additional *in vitro* studies identifying cytokine production by individual infected and uninfected but activated cells in these cultures will be required to clarify the roles of direct infection by HTLV-I and indirect activation by the *tax* gene product or secreted cytokines.

In the lesions causing HAM/TSP, infected monocytes trafficking into the CNS or resident microglia infected or activated by contact with HTLV-I-infected T cells could be the source of IL-6 and TNF-α produced in the CNS. The demyelination and axonal injury occurring in these lesions may be related to TNF-α release, which can directly injure oligodendroglia (45, 46). IL-6 on the other hand may be stimulating astrocytes to release more TNF-α or other cytotoxic factors (46) leading to further cell injury. Thus, the pathologic mechanisms involved in HAM/TSP could include both active viral replication and secretion of potentially toxic cytokines by glial cells.

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