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Astrocyte-Specific Expression of Human T-Cell Lymphotropic Virus Type 1 (HTLV-1) Tax: Induction of Tumor Necrosis Factor Alpha and Susceptibility to Lysis by CD8$^+$ HTLV-1-Specific Cytotoxic T Cells

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Human T-cell lymphotropic virus type 1 (HTLV-1) is associated with a chronic neurological disease termed HTLV-1-associated myelopathy/tropical spastic paraperesis (HAM/TSP). Although the pathogenesis of this disease remains to be elucidated, the evidence suggests that immunopathological mechanisms are involved. Since HTLV-1 *tax* mRNA was colocalized with glial acidic fibrillary protein, a marker for astrocytes, we developed an in vitro model to assess whether HTLV-1 infection activates astrocytes to secrete cytokines or present viral immunodominant epitopes to virus-specific T cells. Two human astrocytic glioma cell lines, U251 and U373, were transfected with the 3′ portion of the HTLV-1 genome and with the HTLV-1 *tax* gene under astrocyte-specific promoter control. In this study, we report that Tax-expressing astrocytic glioma transfectants activate the expression of tumor necrosis factor alpha mRNA in vitro. Furthermore, these Tax-expressing glioma transfectants can serve as immunological targets for HTLV-1-specific cytotoxic T lymphocytes (CTL). We propose that these events could contribute to the neuropathology of HAM/TSP, since infected astrocytes can become a source for inflammatory cytokines upon HTLV-1 infection and serve as targets for HTLV-1-specific CTL, resulting in parenchymal damage by direct lysis and/or cytokine release.

Human T-cell lymphotropic virus type 1 (HTLV-1) was first isolated in 1980 (43) and is endemic in the southern regions of Japan, the Caribbean, the equatorial regions of Africa, South America (6, 33, 40), and the southeastern United States (17). HTLV-1 is the causative agent of adult T-cell leukemia (43, 49, 54) and a chronic progressive disease of the central nervous system (CNS) termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (6, 40). While the adult HTLV-1 seroprevalence rate in some of these areas of endemicity can be as high as 30%, only 1 to 5% develop HAM/TSP or adult T-cell leukemia; the remainder are asymptomatic carriers of the virus (10). Why one group of HTLV-1 seropositive individuals develop a neurologic disease, another develop leukemia, and the majority remain clinically asymptomatic is unknown. Possible explanations for these different disease outcomes include variations in virus strain (3, 38), human histocompatibility leukocyte antigen (HLA) associations (50), viral load (7, 18, 22, 44), and immune function (11, 12, 19, 35).

The pathogenesis of the HTLV-1-associated neurologic disease HAM/TSP is thought to involve the role of HTLV-1-specific immune responses, based on clinical, pathological, and immunological information (13, 14). Clinically, patients with HAM/TSP develop a progressive myelopathy in which corticospinal tract signs predominate (10, 36), and magnetic resonance imaging studies show spinal cord atrophy and subcortical white matter lesions (10, 32, 36). There is a mild pleocytosis with an elevated cerebrospinal fluid (CSF) immunoglobulin G index and oligoclonal bands (some directed towards HTLV-1 antigens) (19, 20, 31). Autopsy studies of the CNS correlate with neurologic findings. Histologically, there is evidence of demyelination with axonal loss in the posterior columns and corticospinal tracts. These lesions are associated with perivascular and lymphocytic infiltration, with foamy macrophages and fibrillary gliosis (1, 53). Early in disease, these lymphocytic infiltrations contain both CD4$^+$ and CD8$^+$ T cells, which become predominantly CD8$^+$ as the disease progresses. Immunologically, activated T cells have been detected in peripheral blood and CSF of HAM/TSP patients and have been shown to be CD8$^+$, HLA class I-restricted cytotoxic T lymphocytes (CTL) (14). Moreover, these cells are present in extraordinarily high frequencies, in which up to 1 in 100 CD8$^+$ T cells can be shown to be HTLV-1-specific CTL (4). These CTL predominantly recognize a product of the HTLV-1 *tax* gene, and immunodominant peptides of the HTLV-1 Tax protein which are presented in association with particular HLA class I alleles have been defined (4, 16, 21, 41).

Based on these findings, several models have been proposed for the immunopathogenesis of HAM/TSP. It has been hypothesized that activated HTLV-1-specific T cells cross the blood-brain barrier and recognize viral antigen in the CNS, where they may cause parenchymal damage by direct lysis and/or cytokine release. This model proposes that if resident cells in the CNS are infected with HTLV-1, they may present viral immunodominant epitopes in the presence of appropriate HLA molecules to infiltrating HTLV-1-specific immune cells. In support of this hypothesis, HTLV-1 *tax* mRNA has been colocalized to infiltrating CD4$^+$ cells and to cells in the CNS that express glial fibrillary acidic protein (GFAP), a marker for astrocytes (27). Moreover, HLA class I molecules have been

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demonstrated to be upregulated in HTLV-1-infected neuroglial cultures in vitro (28, 29) and in spinal cord autopsy material in situ (30). These observations, together with the demonstration of $CD8^+$ inflammatory cells in CNS tissue and the increased frequency of HTLV-1-specific $CD8^+$ CTL in peripheral blood lymphocytes (PBL) and CSF of HAM/TSP patients, strongly argue for an immunopathogenic role of HTLV-1-specific T cells in this disorder. Whether T cells directly lyse virus-infected resident glial cells or these infected cells release cytokines and cause bystander damage remains to be determined.

Elevated levels of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), gamma interferon, interleukin-1 (IL-1), IL-6, and granulocyte-macrophage colony-stimulating factor (23, 24, 37, 39), in the CSF of HAM/TSP patients support the involvement of cytokines in the pathogenesis of HAM/TSP and propose a role for cytokines as mediators of inflammation. In particular, TNF-α has been shown to cause direct damage to oligodendrocytes and myelin in vitro (2, 46). TNF-α has been associated with other inflammatory diseases of the CNS, including multiple sclerosis, encephalopathy secondary to AIDS (25, 34), and bacterial meningitis (52). In addition to its toxic effects, TNF-α is known to regulate the immune response through various mechanisms, such as activation of adhesion molecule expression on the endothelium and stimulation of HLA molecules on target cells. Immunohistochemical studies have shown that microglia, macrophages, astrocytes, and endothelia are capable of TNF-α secretion. Recently, the presence of TNF-α in the spinal cord tissue of HAM/TSP patients has been shown by in situ hybridization studies, although TNF-α expression failed to colocalize with CD45RO T cells, a marker for memory T cells, and CD-68 cells, a marker for microglia and/or macrophages (5).

T cells infected with HTLV-1 express constitutively high levels of TNF-α (48). In addition, microglial cell cultures derived from adult human brain tissue and subsequently infected with HTLV-1 also show detectable levels of TNF-α production (9). Since colocalization of HTLV-1 RNA with cells expressing GFAP from HAM/TSP CNS material has been demonstrated in situ, it was of interest to determine if astrocyte-specific expression of HTLV-1 Tax could also induce the expression of TNF-α. The 3′ portion of the HTLV-1 genome containing the gene for HTLV-I Tax protein downstream of a GFAP promoter was transfected in astroglioma cell lines. Stable transfectants that expressed HTLV-1 Tax were established, and the induction of TNF-α was correlated with levels of HTLV-1 Tax. Importantly, these astrocyte-specific HTLV-1 Tax-expressing transfectants were capable of presenting HTLV-1 Tax immunodominant epitopes serving as targets for $CD8^+$ HTLV-1-specific CTL. These findings suggest an immunologically active role for astrocytes in the pathogenesis of HAM/TSP.

MATERIALS AND METHODS

Generation of GFAP–HTLV-1 constructs (p103E and GFAP-tax). The *env-pX*-3′ long terminal repeat fragment from pKCROHS (45) containing a defective proviral HTLV-1 was excised with *Eco*RI and then ligated with a synthetic *Not*I linker. This fragment was then ligated to a *Not*I-digested pGEM11Z (Promega, Madison, Wis.), producing the pGEM11Z-OHS plasmid. A *Not*I-digested fragment of pGEM11Z-OHS was isolated and further subcloned into a *Not*I site at exon 1 of the astrocyte-specific murine GFAP gene of vector c445(−) (47), yielding p103E (Fig. 1).

The plasmid GFAP-tax was generated by insertion of the HTLV-1 *tax* cDNA into the c445(−) *Not*I site, obviating the need for a poly(A) signal. The HTLV-1 *tax* cDNA insert was generated by PCR amplification with high-fidelity thermostable Ultima polymerase (Perkin-Elmer, Madison, Wis.) by using primers with incorporated *Not*I restriction enzyme sites. The orientation and sequence of the PCR-amplified HTLV-1 *tax* cDNA insert were confirmed by restriction enzyme digestion and dideoxynucleotide sequence analysis (Sequenase; United States Biochemical, Cleveland, Ohio), respectively.

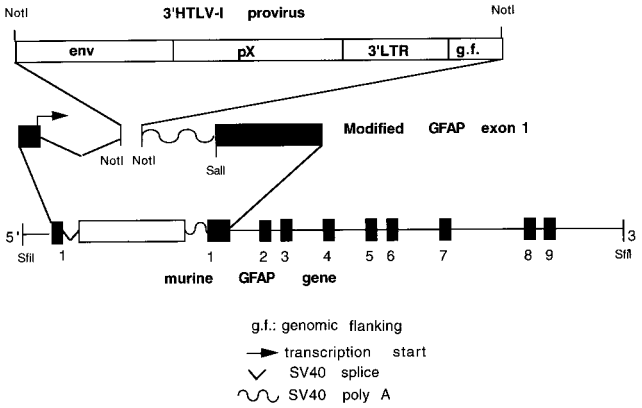

FIG. 1. Schematic representation of the p103E construct, which expresses HTLV-1 Tax protein under control of the GFAP promoter. The HTLV-1 *env-pX*-3′ long terminal repeat (LTR) fragment (4.3 kb) was inserted into exon 1 of the astrocyte-specific murine GFAP gene in the c445(−) vector (47).

Cell transfections. U251 and U373 glioma cell lines (the kind gift of Paul Drew) were transfected with two different constructs, p103E and GFAP-tax (Fig. 1), by using Lipofectin (Gibco/BRL, Gaithersburg, Md.) according to the manufacturer's recommendations. Briefly, 0.64 μg of pSV2neo (for antibiotic resistance) and 20 μg of the plasmid of interest (1:10 ratio) were diluted in 200 μl of serum-free medium (Opti-mem I) and combined with a solution containing 40 μl of Lipofectin added to 200 μl of Opti-mem I. The mixture was incubated at room temperature for 15 min and then added to 3×10^4 cells in 2 ml of Opti-mem I plated in 24-well plates. The transfection was incubated for 6 h at 37°C. After incubation, the cells were diluted with culture medium (440 ml of Dulbecco's modified Eagle's medium, 50 ml of fetal calf serum, 7 ml of 1 M HEPES, 5 ml of 200 mM L-glutamine, and 5 ml of penicillin or streptomycin) and plated at 37°C for 48 h. The cells were then replated with selection medium (culture medium with 0.6 mg of geneticin/ml). Every 5 to 7 days, the medium was replaced with fresh selection medium. After 2 to 3 weeks, the cells were replated in selection medium in six-well plates. The transfected cells were subsequently replated to culture flasks in culture medium.

Reverse-transcription (RT)-PCR. U251 and U373 cells growing in culture medium were washed with 0.5 ml of phosphate-buffered saline/80-cm^2 flask, and total RNA was isolated with RNAsol B (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer's specifications. First-strand cDNA was then synthesized from 5 μg of total RNA with the first-strand cDNA biosynthesis kit (Pharmacia Biotech, Alameda, Calif.) according to the manufacturer's specifications. cDNA was then amplified by PCR in a mixture containing 10× PCR buffer (Boehringer, Mannheim, Germany); 200 μM each dNTP; 1 μM each primer; and 2.5 U of *Taq* polymerase (Boehringer). For every PCR amplification, we used primer pairs that spanned introns. In this way, any genomic DNA contamination that could possibly be amplified would be distinguished from cDNA, since the amplified product would be an intron size larger than the cDNA of interest. The primer pairs used were the following: (i) human β-actin sense primer, 5′ GCATGGGTCAGAAGGATTCCTATG 3′, and human β-actin antisense primer, 5′ ACAGGGATAGCACAGCCTGGATAG 3′; (ii) HTLV-1 Tax sense primer, 5′ ACTCCTCAAGCGAGCTGCATG 3′, and HTLV-1 Tax antisense primer, 5′ AGAGGTTCTCTGGGTGGGGAAG 3′; (iii) human TNF-α sense primer, 5′ AGCCTCTTCTCCTTCCTGATCGTG 3′, and human TNF-α antisense primer, 5′ TATCTCTCAGCTCCACGCCATTGG 3′. Amplification conditions for the first cycle were 93°C for 5 min, 54°C for 40 s, and 72°C for 1 min. The next 34 cycles were run at 94°C for 40 s, 54°C for 40 s, and 72°C for 1 min. The PCR products were visualized on a 1% agarose gel by ethidium bromide.

TNF-α enzyme-linked immunosorbent assay. Cell-free supernatants were harvested from HTLV-1 *tax*-transfected astroglioma cells and assayed (Cytoscreen immunoassay TNF-α kit; BioSource International, Camarillo, Calif.) for the detection of biologically active TNF-α, as described by the manufacturer.

Southern blot. To denature the amplified DNA, the 1% agarose gel was soaked in a denaturing solution containing 1.5 M NaCl and 0.5 N NaOH for 15 min with gentle agitation. The agarose gel was then neutralized with 0.4 M Tris acetate–0.02 M disodium EDTA (TAE) buffer (pH 7.5) for 10 min. The DNA was then transferred to a nylon membrane (maximum-strength Nytran Plus; Schleicher & Schuell, Keene, N.H.) with an electroblotter (model TE 50; Hoefer Scientific Instruments, San Francisco, Calif.) at 10 V for 80 min. The DNA was cross-linked on UV light with the 1800 UV Stratalinker (Stratagene, Inc., La Jolla, Calif.). Prehybridization was then carried out for 1 h at 42°C in a solution

containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 6% sodium dodecyl sulfate (SDS), 8.3× Denhardt's solution; 20 mM $NaPO_4$ (39 monobasic:61 dibasic $NaPO_4$), and 100 μg of salmon sperm DNA/ml. For radioactive labeling, a 20-μl reaction mix containing 10 μM probe, 10 U of T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, Mass.), 1× T4 polynucleotide kinase buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM dithiothreitol [pH 7.6] at 25°C), and 0.4 μM [γ-^{32}P]ATP (6,000 Ci/mmol) (New England Nuclear, Boston, Mass.) was incubated at 37°C for 1 h. The unincorporated [γ-^{32}P]ATP was removed by using STE select −D G-25 columns (5 Prime → 3 Prime, Inc., Boulder, Colo.) according to the manufacturer's specifications. The membranes were then hybridized overnight under the same prehybridization conditions, with end-labeled probes. The following probes were used: (i) human β-actin, 5′ ATGTTTGAGACCTTCAACACCCCAGCCATG 3′; (ii) HTLV-1 Tax, 5′ GGACAGACTCTTCTTTTCGGATACCCAGTCTACGTG 3′; (iii) human TNF-α, 5′ ATCTTCTCGAACCCCGAGTGACAAGCCTGTA 3′. After hybridization, the membranes were washed twice for 5 min each time at 50°C with 2× SSC. A high-stringency wash with a solution containing 3× SSC, 10 mM $NaPO_4$, 10× Denhardt's solution, and 5% SDS was performed for 45 min at 50°C, followed by a third wash with 1× SSC and 1% SDS for 50 min at 50°C. Finally, the radioactivity was detected by exposing the blots to film (scientific imaging film; Eastman Kodak Co., Rochester, N.Y.) overnight at −70°C.

Immunoprecipitation and immunoblot analysis. Cells were lysed in TNN buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol, 100 μg of phenylmethylsulfonyl fluoride/ml, 20 μg of aprotinin/ml, and 10 μg of leupeptin/ml) for 30 min on ice, and cell debris was removed by centrifugation at 10,000 × *g* for 10 min (8). One microliter of anti-Tax monoclonal antibody (a mix of four monoclonal antibodies) (26) was added to 350 μg of protein extract and rotated for 1 h at 4°C. The immune complexes were precipitated following 1 h of rotation at 4°C in the presence of 20 μl of protein A- or protein G-Sepharose (1 mg/ml) complexed with mouse immunoglobulin G. Immune complexes were washed four times with 500 μl of cold TNN buffer to remove nonspecifically bound proteins. Immune complexes were eluted from the Sepharose beads by boiling for 5 min in Laemmli buffer and then separated on a SDS–10% polyacrylamide gel. The proteins were transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) at 100 V for 1 h at 4°C with Western transfer buffer (0.1 M glycine, 12.5 mM Tris [pH 8.3]). Membranes were blocked with 5% milk powder in TNET buffer (10 mM Tris [pH 7.5], 2.5 mM EDTA, 50 mM NaCl, 0.1% Tween 20) for 1 h, washed in TNET without milk, and incubated for 1 h with primary antibody (1:1,000 dilution of anti-Tax monoclonal mix) at room temperature. After extensive washing, the immunoprecipitated proteins were detected with the anti-mouse horseradish peroxidase-linked secondary antibody, followed by enhanced chemiluminescence (Amersham Life Sciences Inc., Arlington Heights, Ill.).

CTL assay. Cytotoxicity assays were performed, and percent specific lysis was calculated as described previously (13); results are presented as the means of triplicate assays. Standard deviations were less than 5%. Spontaneous release ranged between 15 and 25%. As targets, 10^6 HTLV-1-expressing $CD4^+$ T cells, autologous Epstein-Barr virus-transformed B cells pulsed with 1 μg of the immunodominant HTLV-1 Tax peptide, LLFGYPVYV (41), or a control non-HTLV-1 CMV peptide, IAGNSAYEYV (51)/ml, and U251 cell lines transfected with the p103E construct expressing varying amounts of HTLV-1 Tax were incubated with 0.2 mCi of $Na_4{}^{51}Cr$ overnight. As effectors, long-term HLA A2-restricted, $CD8^+$ HTLV-1-specific CTL lines were used at the indicated effector:target ratio in a 4-h ^{51}Cr release assay. Two $CD8^+$ CTL lines were generated as described previously (21, 30) from the PBL and one from the CSF of an HLA A2 HAM/TSP patient.

RESULTS

Detection of HTLV-1 Tax expression in GFAP–3′ HTLV-1-transfected astrocytic glioma cell lines. (i) RT-PCR analysis for HTLV-1 *tax* mRNA. To express HTLV-1 Tax in an astrocyte-specific fashion, the 3′ portion of the HTLV-1 virus carrying an SV40 poly(A) tail was spliced into the GFAP gene exon 1 (47) (Fig. 1). The resulting construct (p103E) was then transfected into two human astrocytic glioma cell lines which express GFAP, U251 and U373. To study the effects of HTLV-1 Tax protein on these cell lines, we established two systems of gene transfection (see Materials and Methods). Initially, a transient transfectant system was used, which offered a quick method for gene expression analysis. As shown in Fig. 2, both the U251 and U373 astrocytic cell lines transfected with the p103E construct expressed the appropriate HTLV-1 *tax* mRNA signal (Fig. 2A, lanes 2 and 5) as seen in the control HTLV-1-infected T-cell line, Hut-102 (lane 8), as detected by RT-PCR and Southern blot analysis. These observations were confirmed with an additional HTLV-1 *tax* construct (designated GFAP-tax) which only contained the HTLV-1 *tax* cDNA driven by the GFAP promoter. Transient transfections of the U251 and U373 cell lines with this construct also induced the expression of HTLV-1 *tax* mRNA (Fig. 2A, lanes 3 and 6). Untransfected controls do not show HTLV-1 *tax* mRNA expression (Fig. 2A, lanes 1 and 4).

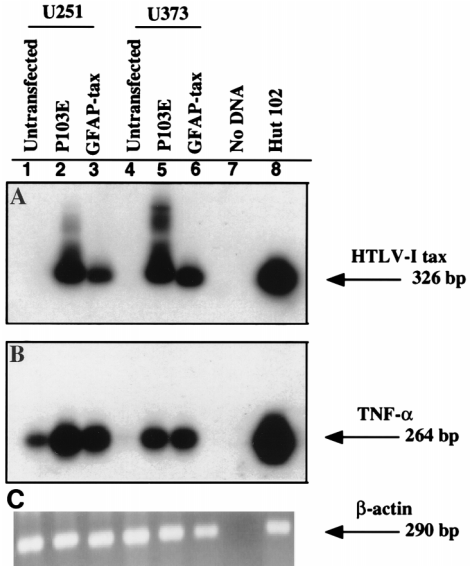

FIG. 2. Expression of HTLV-1 *tax* and TNF-α gene mRNAs in transiently transfected human glioma cell lines. (A) Southern blot with an HTLV-1 *tax* DNA probe labeled with ^{32}P. The HTLV-1 *tax* probe recognizes an amplified 326-bp PCR product from HTLV-1 *tax* cDNA in transiently transfected U251 and U373 glioma cell lines. (B) Southern blot with a TNF-α gene DNA probe labeled with ^{32}P. The TNF-α gene probe recognizes an amplified 264-bp PCR product from TNF-α gene cDNA in U251 and U373 cell lines. (C) Ethidium bromide staining for a 290-bp PCR product of β-actin gene cDNA in U251 and U373 cell lines.

Stable U251 and U373 p103E transfectants were also generated and analyzed for HTLV-1 *tax* mRNA expression. As shown in Fig. 3A, clones of these transfectants differentially

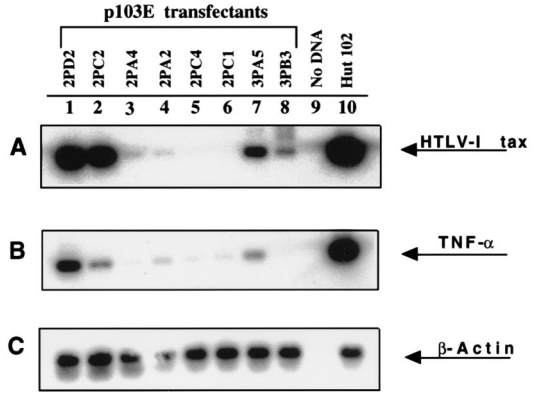

FIG. 3. Expression of HTLV-1 *tax* and TNF-α gene mRNA in stable p103E-transfected human glioma cell clones. (A) Southern blot with an HTLV-1 *tax* DNA probe labeled with ^{32}P. The HTLV-1 *tax* probe recognizes an amplified 326-bp PCR product from HTLV-1 *tax* cDNA in stable transfected U251 and U373 glioma cell lines. (B) Southern blot with a TNF-α gene DNA probe labeled with ^{32}P. The TNF-α gene probe recognizes an amplified 264-bp PCR product from TNF-α gene cDNA in U251 and U373 glioma cell lines. (C) Southern blot with a β-actin gene probe, which recognizes a 290-bp PCR product from β-actin gene cDNA in U251 and U373 glioma cell lines.

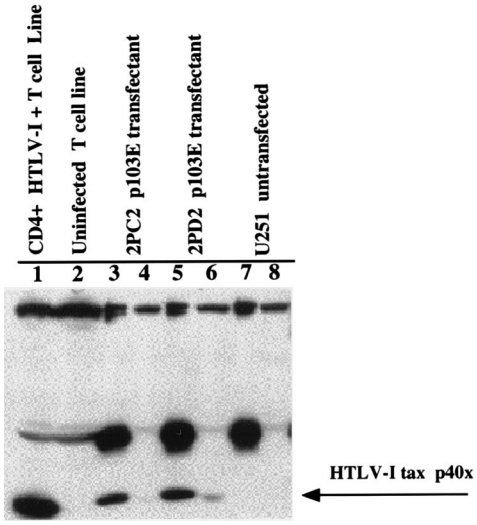

FIG. 4. Expression of HTLV-1 Tax protein from p103E-transfected human glioma cell clones. HTLV-1 Tax protein was detected by immunoprecipitation and immunoblot analysis with anti-Tax monoclonal antibodies (a mix of four monoclonal antibodies) from the p103E stable transfectants expressing HTLV-1 *tax* mRNA. Lane 1, control HTLV-1-expressing $CD4^+$ cell line; lane 2, non-HTLV-1-expressing T-cell line control; lanes 3 and 4, serial 10-fold dilution of protein from the p103E-transfected 2PC2 glioma cell transfectant clone; lanes 5 and 6, serial 10-fold dilution of protein from the p103E-transfected 2PD2 glioma cell transfectant clone; lanes 7 and 8, serial 10-fold dilution of protein from the untransfected U251 glioma cell line.

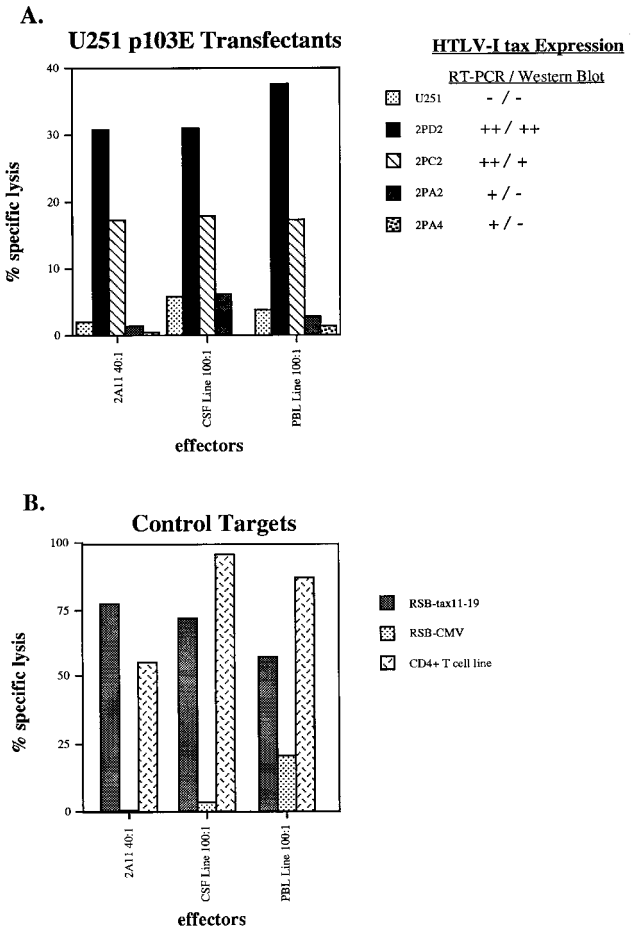

FIG. 5. CTL assay showing specific lysis of U251 transfected lines expressing HTLV-1 Tax protein. Three HLA A2-restricted, HTLV-1 Tax11-19 peptide (LLFGYPVYV)-specific, $CD8^+$ CTL effectors were used (one CTL clone, 2A11, and two CTL lines). (A) CTL effectors specifically lysed U251 transfectants (2PD2 and 2PC2) previously shown (Fig. 3 and 4) to express HTLV-1 Tax protein, as detected by immunoprecipitation assay. No lysis was observed with the U251 untransfected control or with the 2PA2 and 2PA4 transfectant targets, which contained low levels of HTLV-1 *tax* mRNA (Fig. 3) and did not express HTLV-1 Tax p40x protein, as shown by Western blotting. (B) As controls, CTL lysed an HTLV-1 expressing $CD4^+$ T-cell line target and the autologous B-cell line (RSB) pulsed with the antigenic peptide but not with an irrelevant CMV peptide.

expressed HTLV-1 *tax* mRNA. For example, clones 2PD2 and 2PC2 had a higher level of HTLV-1 *tax* mRNA signal (Fig. 3A, lanes 1 and 2) than did the other p103E-transfected U251 clones. Similarly, the p103E-transfected U373 clone, 3PA5, had a higher level of HTLV-1 *tax* mRNA expression than clone 3PB3 (Fig. 3A, lanes 7 and 8). All lanes had comparable levels of β-actin expression (Fig. 3C).

(ii) Immunoprecipitation of HTLV-1 Tax p40x protein from p103E stable glioma transfectants. In addition to the demonstration of HTLV-1 *tax* mRNA in the GFAP–3′ HTLV-1-transfected astrocytic glioma cell lines (Fig. 2A and 3A), HTLV-1 Tax protein was also detected in a Western blot assay from the p103E stable transfectants expressing HTLV-1 *tax* mRNA. The two U251 transfectants expressing high levels of HTLV-1 *tax* mRNA, 2PC2 and 2PD2 (Fig. 3A, lanes 1 and 2), were selected for HTLV-1 Tax protein immunoprecipitation with a mixture of monoclonal antibodies specific for HTLV-1 Tax (26). As shown in Fig. 4, the HTLV-1 Tax p40x protein was immunoprecipitated from the 2PC2 and 2PD2 transfected cell lines (Fig. 4, lanes 3 and 5) with a band at the same molecular weight as that from a control $CD4^+$ HTLV-1-infected T-cell line known to express high levels of HTLV-1 Tax protein (Fig. 4, lane 1). HTLV-1 Tax p40 protein was not detected in either an uninfected T-cell line or an untransfected U251 cell line (Fig. 4, lanes 2 and 7). Importantly, these results parallel the HTLV-1 *tax* mRNA expression in these transfectants. By RT-PCR, 2PD2 had a stronger signal than 2PC2 for HTLV-1 Tax message (Fig. 3A, lanes 1 and 2), and by immunoprecipitation HTLV-1 Tax p40x protein could be detected in a 10-fold greater dilution of 2PD2 than 2PC2 (Fig. 4, lanes 4 and 6).

HTLV-1 Tax-expressing astrocytic glioma transfectants serve as immunological targets for HTLV-1-specific $CD8^+$ cytotoxic cells. Since it had been shown that the GFAP–3′ HTLV-1-transfected astroglial cells express HTLV-1 *tax* mRNA (Fig. 2 and 3) and produce HTLV-1 Tax p40x protein (Fig. 4), it was of interest to evaluate whether these Tax-expressing glioma transfectants were susceptible to lysis by HTLV-1-specific $CD8^+$ cytotoxic cells. Four different U251 p103E-transfected glioma cell lines showing varying levels of HTLV-1 *tax* mRNA and protein expression (Fig. 5) were used as targets in CTL assays with two HTLV-1-specific CTL lines and one HTLV-1-specific CTL clone. All CTL effectors were $CD8^+$ and specific for the HTLV-1 Tax11-19 peptide (LLF GYPVYV) in association with HLA A2. These CTL lysed a control HTLV-1-expressing $CD4^+$ T-cell line target and the autologous B-cell line pulsed with the antigenic peptide but not with an irrelevant CMV peptide (Fig. 5B). Since the parent U251 astroglioma cell line was derived from an HLA A2 donor and expressed high levels of HLA A2 on the cell surface (data not shown), p103E transfectants of this line could be used as targets for these HTLV-1 Tax peptide-specific CTL. As demonstrated in Fig. 5, all three CTL effectors lysed the two HTLV-1 *tax*-transfected glioma cell lines, 2PD2 and 2PC2,

which correlated with the level of HTLV-1 *tax* mRNA and Tax p40x protein expression (Fig. 3A, lanes 1 and 2, and Fig. 4). No lysis was observed above the U251 untransfected control with the 2PA2 and 2PA4 transfected targets (Fig. 5A), which contained low levels of HTLV-1 *tax* mRNA (Fig. 3, lanes 3 and 4) and did not express HTLV-1 Tax p40x protein by Western blotting (data not shown). These results demonstrate that GFAP–3′ HTLV-1-transfected astroglial cells known to express HTLV-1 *tax* mRNA and to produce HTLV-1 Tax p40x protein can appropriately process this protein and present a known immunodominant HTLV-1 Tax peptide to HTLV-1 Tax peptide-specific CTL.

Detection of TNF-α expression in GFAP–3′ HTLV-1 (p103E)-transfected astrocytic glioma cell lines. GFAP–3′ HTLV-1-transfected astroglial cells expressing HTLV-1 *tax* mRNA were also capable of TNF-α gene expression compared to untransfected controls. As shown in a transient transfection system (Fig. 2), transfection with the 3′ portion of HTLV-1 (p103E construct) or the HTLV-1 *tax* cDNA (GFAP-tax construct) activated TNF-α gene expression in both U251 and U373 cell lines (Fig. 2B, lanes 2 and 3 and lanes 5 and 6). While the U251 untransfected control showed basal levels of TNF-α gene mRNA expression (Fig. 2B, lane 1), clear upregulation of TNF-α was demonstrated by transfection with the p103E or GFAP-tax constructs. In addition to detection of TNF-α message, biologically active TNF-α protein was unregulated in the P103E transfectant. Supernatants from the p103E U251 transfectant (Fig 2B) contained a 10-fold increase in TNF-α protein (825 pg/ml) compared to that in the untransfected U251 cell line, as demonstrated by an enzyme-linked immunosorbent assay specific for human TNF-α. TNF-α was not constitutively expressed in the U373 astroglial cell line (Fig. 2B, lane 4), and the HTLV-1-infected T-cell line had high expression of this cytokine (Fig. 2, lane 8). Since the GFAP-tax construct induced levels of TNF-α comparable to those induced by the p103E construct, this would suggest that HTLV-1 Tax protein, and not other proteins expressed from the 3′ portion of the HTLV-1 genome, was the gene product responsible for the transactivation of TNF-α in these transfected astroglial cell lines (Fig. 2B, lanes 3 and 6).

Similarly to what was observed for GFAP–3′ HTLV-1 transiently transfected astroglial cells (Fig. 2), stable p103E U251 transfectants expressing HTLV-1 *tax* mRNA also induced the expression of TNF-α (Fig. 3B), which correlated with the level of HTLV-1 *tax* mRNA expression (Fig. 3A). The two U251 transfectants expressing high levels of *tax* mRNA, 2PC2 and 2PD2, also expressed TNF-α, with higher levels of TNF-α in 2PD2 than in 2PC2, again correlating with the higher levels of HTLV-1 *tax* mRNA in 2PD2, as detected by RT-PCR (Fig. 3A, lanes 1 and 2). Similarly, the U373 p103E-transfected clone, 3PA5, expressed more TNF-α than the clone 3PB3 (Fig. 3B, lanes 7 and 8), which again correlated with a higher level of HTLV-1 *tax* mRNA expression (Fig. 3A, lanes 7 and 8). Glioma transfectants carrying the p103E construct, but not showing HTLV-1 *tax* mRNA expression (for example, 2PC4), failed to activate TNF-α gene mRNA expression above basal levels (Fig. 3A and B, lane 5). Comparable levels of β-actin were demonstrated in all lanes (Fig. 3C).

DISCUSSION

The mechanisms implicated in the pathogenesis of HAM/TSP suggest a role for HTLV-1-specific immune responses, although it is unclear how these immunological responses contribute to disease pathogenesis. One possibility is that the activated HTLV-1-specific CTL detected in such high frequency in peripheral blood (4) are able to cross the blood-brain barrier and recognize cells within the CNS potentially infected with HTLV-1, thereby causing parenchymal damage by direct lysis and/or cytokine release. Recently, the colocalization of HTLV-1 mRNA with GFAP-producing cells (a marker for astrocytes) from the spinal cord of a HAM/TSP patient has also been shown (28). The demonstration that a resident glial cell may be infected with HTLV-1 raises the possibility that an HTLV-1-infected astrocyte can functionally present HTLV-1 viral epitopes to HTLV-1-specific T cells and/or release proinflammatory cytokines that may be associated with HAM/TSP pathogenesis. These hypotheses were tested in vitro by expressing HTLV-1 Tax in an astrocyte-specific fashion by cloning the 3′ portion of the HTLV-1 virus carrying an SV40 poly(A) tail into the GFAP gene exon 1 (Fig. 1) (47). The resulting construct (p103E) was then transfected into human GFAP-positive astrocytic glioma cell lines to determine if and to what extent HTLV-1 Tax could be expressed.

The results presented in this report demonstrate that astrocytic glioma cell lines transfected with the HTLV-1 p103E construct, in either a transient or a stable transfection system, express HTLV-1 *tax* mRNA, as detected by RT-PCR assays, and HTLV-1 Tax protein, as determined by immunoprecipitation with HTLV-1 Tax-specific monoclonal antibodies. As expected, the level of Tax protein expression correlated with the amount of HTLV-1 *tax* message. Importantly, these HTLV-1 Tax-expressing astrocytic cell lines were capable of appropriately processing HTLV-1 Tax into peptides that can associate with HLA and be recognized by HTLV-1 Tax peptide-specific $CD8^+$ CTL. These HTLV-1 Tax peptide-specific CTL have been shown previously to recognize an immunodominant HTLV-1 Tax epitope, LLFGYPVYV, in association with HLA A2 (4, 16, 21, 41) and have been detected directly from PBL and CSF of HLA A2 HAM/TSP patients at a frequency that can be as a high as 1 in 100 $CD8^+$ cells (4). As discussed, since $CD8^+$ cells are associated with HAM/TSP spinal cord lesions, it has been suggested that these T cells may be functional CTL and contribute to disease pathogenesis by recognizing HTLV-1-infected cells in the CNS (15). Our in vitro experiments suggest that astroglial cell lines expressing HTLV-1 Tax in association with HLA A2 can act as targets for HTLV-1 Tax peptide-specific CTL. It remains to be determined if HTLV-1-infected primary astrocyte cultures rather than glioma cell lines can express and present HTLV-1 Tax and if these in vitro observations can be extended in vivo. Towards this end, we have developed transgenic mice carrying the 3′ portion of the HTLV-1 genome under GFAP promoter control. This model could allow a mechanistic approach to determine the effects of astrocyte-specific Tax expression in vivo and whether astrocytes serve as targets for activated Tax-specific CTL upon immunization with Tax.

The observation that HTLV-1 Tax upregulates the expression of TNF-α in astrocytes is of particular interest in our understanding of the pathogenesis of HAM/TSP. TNF-α is thought to be an important mediator of inflammation and is produced by a number of cell types, including activated macrophages (42). Locally, TNF-α has effects on a variety of targets to enhance an inflammatory process and indirectly regulates other inflammatory events through the activation of cytokines, such as gamma interferon, IL-1, IL-6, and granulocyte-macrophage colony-stimulating factor, all of which have been demonstrated to be elevated in HAM/TSP CSF (23, 24, 37, 39). In previous studies with in situ hybridization, we have demonstrated expression of TNF-α gene mRNA on spinal cord autopsy material from chronic HAM/TSP patients (5). However, TNF-α gene mRNA could not be colocalized with

cells expressing CD45RO (a marker for memory T cells) nor with cells expressing CD-68 (a marker for microglial cells and macrophages). In this report, we show that HTLV-1 Tax-expressing astrocytes promote TNF-α gene mRNA in vitro. Since HTLV-1 sequences have been colocalized with GFAP-expressing cells in the spinal cord material of HAM/TSP patients, we suggest that HTLV-1-infected astrocytes could be a possible source of TNF-α at a lesion site. Astrocyte expression of TNF-α may be directly involved in the pathogenesis of HAM/TSP by its effects on antigen presentation and lymphocyte activation and migration, as well as by its toxic effects on oligodendrocytes and myelin, or indirectly by the regulation of other inflammatory cytokines. In addition, we are investigating whether HTLV-1 Tax is able to activate other proinflammatory cytokines, such as IL-1 and IL-6, in p103E-transfected astroglioma cell lines.

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