JOURNAL OF VIROLOGY, Sept. 1997, p. 6982–6989 Vol. 71, No. 9
0022-538X/97/$04.00+0

Induction of Tumor Necrosis Factor Alpha in Human Neuronal Cells by Extracellular Human T-Cell Lymphotropic Virus Type 1 Tax_1

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Received 24 January 1997/Accepted 30 May 1997

To examine the role of human T-lymphotropic virus type 1 (HTLV-1) Tax_1 in the development of neurological disease, we studied the effects of extracellular Tax_1 on gene expression in NT2-N cells, postmitotic cells that share morphologic, phenotypic, and functional features with mature human primary neurons. Treatment with soluble HTLV-1 Tax_1 resulted in the induction of tumor necrosis factor alpha (TNF-α) gene expression, as detected by reverse-transcribed PCR and by enzyme-linked immunosorbent assay. TNF-α induction was completely blocked by clearance with anti-Tax_1 monoclonal antibodies. Furthermore, cells treated with either a mock bacterial extract or with lipopolysaccharide produced no detectable TNF-α. Synthesis of TNF-α in response to soluble Tax_1 occurred in a dose-dependent fashion between 0.25 and 75 nM and peaked within 6 h of treatment. Interestingly, culturing NT2-N cells in the presence of soluble Tax_1 for as little as 5 min was sufficient to result in TNF-α production, indicating that the induction of TNF-α in NT2-N does not require Tax_1 to be continually present in the culture medium. Treatment of the undifferentiated parental embryonal carcinoma cell line NT2 with soluble Tax_1 did not result in TNF-α synthesis, suggesting that differentiation-dependent, neuron-specific factors may be required. These results provide the first experimental evidence that neuronal cells are sensitive to HTLV-1 Tax_1 as an extracellular cytokine, with a potential role in the pathology of HTLV-1-associated/tropical spastic paraparesis.

Human T-cell lymphotropic virus type 1 (HTLV-1) is associated with a number of disorders, the most common being adult T-cell leukemia and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is a chronic neurodegenerative disorder whose symptoms are primarily localized to functions associated with the lower spinal cord, including spastic paraplegia of the lower extremities, loss of bladder control, and sexual dysfunction (17, 34). Pathology is mainly limited to the lower and middle thoracic cords, where marked degeneration of the corticospinal tracts and demyelination are evident, accompanied by perivascular mononuclear infiltrates consisting primarily of $CD4^+$ T lymphocytes in early lesions, followed by the appearance of $CD8^+$ T lymphocytes in older lesions (17, 30). Another characteristic of patients with HAM/TSP is the observation of highly activated circulating cytotoxic T lymphocytes (CTLs) in both the blood and the cerebrospinal fluid (9, 18). Remarkably, the vast majority of this response is specific for the product of the HTLV-1 *pX* region, the transactivating protein referred to as Tax_1 (9, 19, 21).

These observations have led to a hypothesis that the demyelination and neuronal degeneration observed in HAM/TSP patients result from an immunopathological mechanism, in which HTLV-1 Tax_1-specific CTLs recognize and destroy infected resident cells in the central nervous system (CNS) (23, 30). However, a number of observations are not consistent with this hypothesis. First, although several reports have described the detection of HTLV-1 DNA in the CNS (15, 16, 24, 25, 37), it is unclear if CNS resident cells are actually infected, as opposed to cells in the infiltrate. One study identified some HTLV-1-infected cells as astrocytes (25), and another was unable to detect proviral DNA in either neuronal or glial cells in the spinal cord of a HAM/TSP patient (15). Second, high levels of HTLV-1 Tax_1-specific CTLs have been observed not only in HAM/TSP patients but also in symptom-free HTLV-1 carriers (9). In light of these findings, it has recently been suggested that the cellular infiltrates observed around spinal cord lesions in HAM/TSP are not effectors in an immunopathological response (1, 8). Rather, it is the efficiency with which those CTLs can limit viral spread that is the critical factor determining the course of infection with HTLV-1. It was proposed that tissue damage results from a bystander effect caused by the release of cytokines from these highly activated T cells that infiltrate the CNS.

The potent Tax_1-specific immunity in HTLV-1-infected individuals may reflect the possibilities either that Tax_1 is unusually immunogenic or that it is present in significant amounts in the host. HTLV-1 Tax_1 is a 40-kDa phosphoprotein whose primary function is the transactivation of viral transcription, but it also has the ability to influence the expression of a number of cellular genes (14). The influence of Tax_1 on cellular gene expression can be observed not only in HTLV-1-infected cells or transfectants expressing the Tax_1 gene, but also in cells treated with soluble Tax_1, conferring upon it the properties of an extracellular cytokine (2, 26–29). Furthermore, it has been observed that cells infected with HTLV-1 actively secrete functional Tax_1 (26, 28). Soluble Tax_1 treatment induces the nuclear accumulation of NF-κB (2, 26), as well as the expression of immunoglobulin κ light chain (2, 27),

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interleukin-2 receptor α (29), tumor necrosis factor alpha (TNF-α) (11), TNF-β (2), and interleukin-6 (11).

In considering a role for Tax_1 in the demyelination and axonal degeneration observed in HAM/TSP, TNF-α is of particular interest, since this cytokine is toxic for oligodendrocytes, the source of myelin (36). Two recent observations are relevant in this regard. First, in a rat model of HAM/TSP, it was observed that *Tax* gene expression was limited to the diseased spinal cord and correlated with expression of TNF-α (39). Second, it has been demonstrated that treatment of primary human microglia with soluble Tax_1 can induce TNF-α (11). This study is designed to investigate whether TNF-α gene expression in neuronal cells themselves could be induced by soluble HTLV-1 Tax_1, such that it would potentially play a role in the course of HAM/TSP.

MATERIALS AND METHODS

Cells and reagents. The NTera 2/cl.D1 (NT2) cell line was obtained from the American Type Culture Collection (Rockville, Md.) and was maintained in Opti-MEM I medium (Life Technologies, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (Life Technologies), 2 mM glutamine, and penicillin-streptomycin (NT2 culture medium). NT2-N cells were generated from the parent NT2 cells as described previously (32). Briefly, 4.5×10^6 NT2 cells were seeded in 175-cm^2 tissue culture flasks (Nunc, Naperville, Ill.) in 50-ml NT2 culture medium containing 10 μM *trans*-retinoic acid (Sigma, St. Louis, Mo.), the culture medium being changed twice per week. After 3 to 4 weeks of treatment with retinoic acid, the cells were removed with trypsin, replated at a sixfold-lower density in NT2 culture medium without retinoic acid, and cultured for an additional 2 days. The loosely adherent cells were then recovered in the culture medium by striking the flasks sharply. These cells were replated on eight-chamber glass culture slides (Nunc) that had been pretreated with a 1:60 dilution of Matrigel (Collaborative Research, Cambridge, Mass.), a basement membrane extract, according to the manufacturer's protocol. One hundred thousand cells were placed in each well in NT2 culture medium containing 1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, and 10 μM uridine as mitotic inhibitors. The cells were cultured for 1 week in the presence of these three inhibitors, followed by 2 to 3 weeks of culture with fluorodeoxyuridine and uridine, with the medium changed weekly. This resulted in a >95% pure population of NT2-N cells (32), with approximately 10^4 postmitotic cells remaining in each well. Before use in experiments, the cells were cultured for a minimum of 2 days in NT2 medium without mitotic inhibitors and then were used within 4 to 6 weeks thereafter. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by centrifugation through a Ficoll-Hypaque gradient (Pharmacia, Piscataway, N.J.). Lipopolysaccharide (LPS) was obtained from Sigma.

Recombinant HTLV-1 Tax_1. HTLV-1 Tax_1 protein was expressed in *Escherichia coli* (13) and purified by differential precipitation as described previously (12). Mock bacterial extracts were prepared in an identical fashion from *E. coli* cells not expressing the Tax_1 gene. For treatment of cells, recombinant Tax_1 solution was mixed with an equal volume of a solution containing 2 mM CHAPS {3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfate}, 2 mM EDTA, 2 mM dithiothreitol, and 20 mM β-mercaptoethanol (2× Tax_1 buffer) and incubated at 30°C for 30 min. Medium was then added, and the solution was passed through a 0.22 μm-pore-diameter Millex GV4 filter (Millipore, Bedford, Mass.). Two hundred microliters of the treated medium was added per well.

Immunofluorescence. NT2-N cells, cultured as described above in eight-chamber glass culture slides, and NT2 cells, cultured overnight in eight-chamber plastic culture slides (Nunc) (5×10^4 cells plated per well), were washed once with phosphate-buffered saline (PBS) and dried for 2 h at room temperature. The cells were then fixed for 10 min in acetone on ice (0.5 ml/well), dried at room temperature, and washed with PBS (0.5 ml/well). For indirect immunofluorescence, cells were treated with either SMI 35 (Sternberger Monoclonals, Baltimore, Md.) (monoclonal antibody to neurofilament H, ascites diluted 1,000-fold in PBS with 1% fetal bovine serum) or with Sp2/0 (control ascites diluted 1,000-fold in PBS with 1% fetal bovine serum) for 1 h at room temperature, followed by four washings for 15 min each with PBS. The cells were then treated with 0.1 ml of secondary antibody (fluorescein-labeled goat anti-mouse immunoglobulin G [IgG] [Kirkegaard and Perry Laboratories, Gaithersburg, Md.], diluted 100-fold in PBS with 1% fetal bovine serum) for 1 h at room temperature in the dark, and washed in PBS as described above. For direct immunofluorescence, cells were treated with 50 μl of fluorescein-labeled CAM 5.2 (antikeratin; Becton-Dickinson, San Jose, Calif.) or fluorescein-labeled mouse IgG1 (Becton Dickinson) for 1 h at room temperature in the dark and washed with PBS. Cells were mounted in Vectashield (Vector Laboratories, Burlingame, Calif.) prior to examination for immunofluorescence.

Detection of TNF-α. Culture supernatants (200 μl) were harvested and diluted with 250 μl of medium. TNF-α was detected with a high-sensitivity enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions. Each sample was assayed in duplicate.

RT-PCR. For reverse-transcribed PCR (RT-PCR), NT2-N cells were washed once with 0.4 ml of PBS/well, and mRNA was isolated with the QuickPrep Micro mRNA Purification Kit (Pharmacia) according to the manufacturer's protocol. First-strand cDNA was synthesized with a kit (Pharmacia) according to the manufacturer's protocol with random hexamers. The cDNA was then amplified by PCR with primer pairs for human TNF-α (5′ primr, CTTCTGCCTGCTG CACTTTGGA; 3′ primer, TCCCAAAGTAGACCTGCCCAGA), human nestin (5′ primer, AGAGGGGAATTCCTGGAG; 3′ primer, CTGAGGACCAGG ACTCTCTA) (7), human tau (5′ primer, TCCGCCAGGGACGTGGG; 3′ primer, GTGCAAATAGTCTACAA) (35), and human β-actin (5′ primer, TGAC GGGGTCACCCACACTGTGCCCATCTA; 3′ primer, CTAGAAGCATTTGC GGTGGACGATGGAGGG). The reactions were carried out in a volume of 100 μl, and the reaction mixture consisted of 50 pmol of primer; 1× PCR buffer II (Perkin-Elmer, Norwalk, Conn.); 2.5 mM $MgCl_2$; 50 μM dATP, dCTP, dGTP, and dUTP (Boehringer Mannheim, Indianapolis, Ind.); 1.25 U of *Taq* DNA polymerase (Perkin-Elmer); and 1 U of uracil-DNA glycosylase (Boehringer-Mannheim). The samples were initially treated for 10 min at 37°C to remove potentially contaminating amplimers by digestion with the uracil-DNA glycosylase and for 10 min at 95°C to inactivate the enzyme. Amplification of sequences was accomplished by 35 cycles of 15 s at 95°C, 15 s at 46 or 60°C (for nestin and tau primers or for TNF-α and β-actin primers, respectively), and 30 s at 72°C in a model 9600 thermal cycler (Perkin-Elmer).

Southern hybridization. Ten microliters of the amplification reaction was separated by electrophoresis on a 2% agarose gel. The DNA was then transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, N.H.) with the Turboblotter system (Schleicher and Schuell) according to the manufacturer's instructions, by the alkaline transfer protocol. The membranes were hybridized with end-labeled probes end labeled with [γ-^{32}P]ATP (6,000 Ci/mmol [New England Nuclear, Boston, Mass.]) as described previously (6). The following probes were used: nestin, GGAATGCGCTAGTCTCTG; tau, TCCAGGGACC CAATCTTC; TNF-α, ATCTCTCAGCTCCACGCCATTGGCCAGGAG; and β-actin, TCATCACCATTGGCAATGAGCGGT. Hybridization was performed at 60°C for TNF-α and β-actin probes or at 45°C for nestin and tau probes. After hybridization, the membranes were washed three times briefly with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, followed by two 30-min washes with 2× SSC–1% sodium dodecyl sulfate at the hybridization temperature for each probe. Radioactivity was visualized by exposure of the blots to phosphor screens and analysis on a PhosphorImager (model 445 SI; Molecular Dynamics, Sunnyvale, Calif.).

Immunodepletion of Tax_1 and Western blot analysis. One microgram of *E. coli* recombinant Tax_1 (100 ng/μl) was incubated with 1 μg (20 μl) of a cocktail of four monoclonal anti-Tax_1 antibodies (TAb 169, 170, 171, and 172 [1:1:1:1 ratio]) or 1 μg (10 μl) of p53 monoclonal antibody 1 (Oncogene Research Products, Cambridge, Mass.) for immunodepletion as described previously (27). Adsorbed complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting as described previously (27).

RESULTS

Postmitotic human neuronal cells treated with extracellular HTLV-1 Tax_1 protein produce TNF-α. It has not been demonstrated that HTLV-1 Tax works as an extracellular cytokine on neuronal cells. As a model system for human neurons, we utilized cells derived from NTera 2/cl.D1 (NT2), a human teratocarcinoma cell line (31, 32). When treated with retinoic acid, NT2 yields postmitotic cells (NT2-N) expressing a stable, terminally differentiated neuronal phenotype. As shown in Fig. 1a, NT2-N cells exist in clusters, each containing hundreds of cells, from which emanate a series of processes resembling dendrites and axons. This morphology contrasts dramatically with that of the undifferentiated NT2 cells (Fig. 1b). In addition, NT2-N cells express many neuronal cytoskeletal proteins, secretory markers, and surface markers not expressed by NT2 (31, 32, 41, 42). For example, NT2-N cells express neurofilament H (Fig. 1c) and tau (Fig. 2), the latter a marker for CNS neurons (32). Neither of these markers is expressed by the undifferentiated NT2 cells (Fig. 1d and 2). In contrast, NT2 cells express nestin, which is not expressed by NT2-N (Fig. 2). The morphology of these cells and the pattern of marker expression, including the expression of keratin by NT2 (Fig. 1g), are identical to those described previously (31, 32).

In our initial studies, NT2-N cells were cultured overnight in the presence of recombinant HTLV-1 Tax_1 protein at various

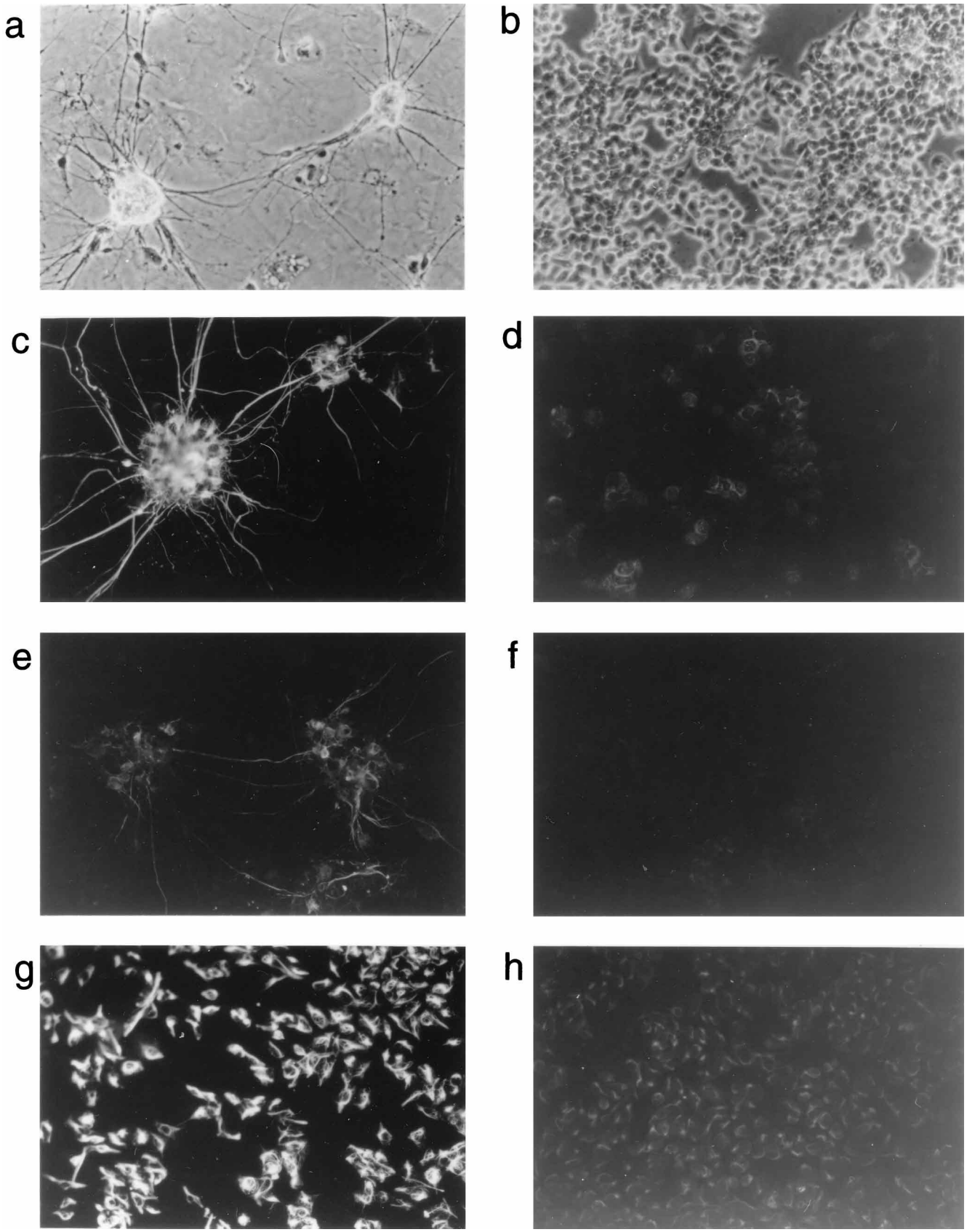


FIG. 1. Physical comparison of NT2-N and NT2. (a and b) Phase-contrast microscopy (×100) of NT2-N (a) and NT2 (b). NT2-N cells were generated as described in Material and Methods. (c to h) Immunofluorescence analysis of NT2-N (c and e) and NT2 (d, f, g, and h) was performed as described in Materials and Methods, with antineurofilament H (SMI 35) (c and d), control ascites (Sp2/0) (e and f), antikeratin (CAM 5.2) (g), and control IgG1 (h). All immunofluorescence photomicrographs and their corresponding negative controls were exposed identically at a magnification of ×200.

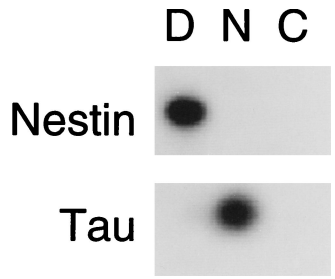


FIG. 2. RT-PCR detection of nestin and tau transcripts in NT2-N and NT2 cells. Cells maintained in eight-chamber slides were lysed directly on the slides, poly(A)$^+$ mRNA was isolated, and RT-PCR was performed with the appropriate primers. Products were analyzed by Southern blotting with oligonucleotide probes for nestin (top) and tau (bottom). D, NT2; N, NT2-N; C, control in which RNA was omitted from the cDNA synthesis after which RT-PCR was performed to monitor for contamination.

concentrations. Analysis of RNA derived from these cultures by RT-PCR demonstrated that TNF-α transcripts were induced in NT2-N cells treated with Tax_1 in a dose-responsive manner, with transcripts above background detectable down to 0.25 nM Tax_1 (Fig. 3, lanes 1 to 6 compared to lane 7).

Consistent with these results, assay of the culture supernatants from these cells revealed that whereas supernatants from cells treated with Tax_1 buffer alone did not contain detectable amounts of TNF-α (demonstrating that TNF-α is not constitutively secreted by NT2-N), TNF-α was produced by cells treated with Tax_1 in a dose-responsive manner (Fig. 4). A minimum of 0.25 nM Tax_1 was required to induce detectable levels of TNF-α in the NT2-N cultures.

Specificity of TNF-α production by NT2-N for HTLV-1 Tax_1. The requirement for the Tax_1 protein rather than a copurifying factor in the induction of TNF-α in NT2-N was demonstrated in several ways. First, the Tax_1 preparation was subjected to immunoprecipitation with Tax_1-specific monoclonal antibodies. This treatment completely removed the TNF-α-inducing activity, assayed both by RT-PCR (Fig. 5) and by ELISA (Fig. 6). In contrast, Tax_1 treated with a control antibody was able to induce TNF-α levels in NT2-N similar to those observed with untreated Tax_1 (Fig. 5 and 6). Western blot analysis of the adsorbed immune complexes confirmed that the majority of the Tax_1 was removed by immunoprecipitation with anti-Tax_1 antibodies, whereas a minimal amount of Tax_1 was depleted in samples treated with a control antibody (data not shown).

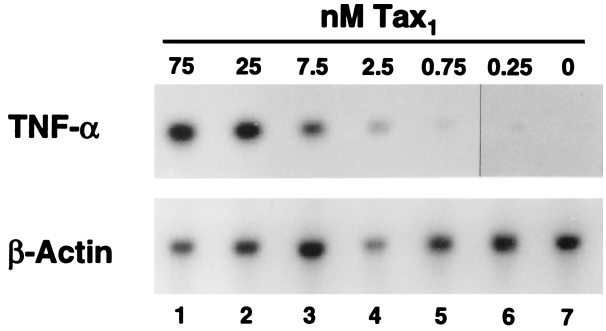


FIG. 3. RT-PCR detection of TNF-α transcripts in NT2-N cells treated with HTLV-1 Tax_1. NT2-N cells maintained in eight-chamber culture slides were cultured in the presence of various concentrations of soluble HTLV-1 Tax_1, as indicated, for 18 h, and RT-PCR was performed. The products were analyzed by Southern blotting with oligonucleotide probes for TNF-α (top) and β-actin (bottom). The data shown are representative of three independent experiments.

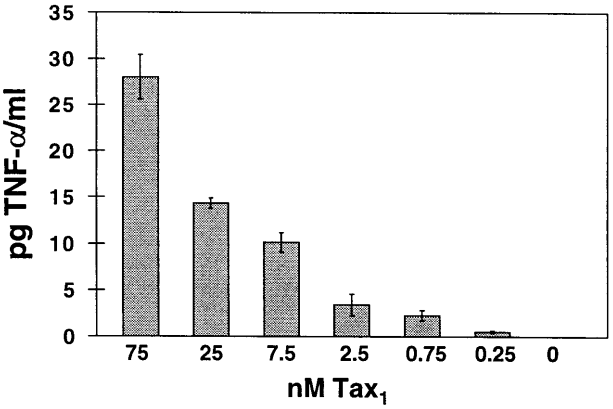


FIG. 4. Detection of TNF-α in culture supernatants of NT2-N cells treated with soluble HTLV-1 Tax_1. Culture supernatants from the experiment shown in Fig. 3 were assayed for the presence of TNF-α by ELISA. Each data point is the mean ± standard deviation for duplicates of each sample tested in the ELISA.

Second, because there was concern that endotoxin in the recombinant Tax_1 preparation could account for the TNF-α-inducing activity, NT2-N cells were treated with LPS at concentrations ranging from 10 ng/ml to 1 μg/ml. As shown in Fig. 7, no TNF-α was detectable in the cultures of any of the LPS-treated NT2-N cells. In marked contrast, 10-fold fewer PBMCs treated with even the lowest concentration of LPS produced maximal levels of TNF-α. Third, no TNF-α could be detected in culture supernatants from NT2-N treated with a mock bacterial extract that was subjected to procedures identical to those used for purification of the recombinant Tax_1 protein (Fig. 8). Taken together, these results demonstrate that the induction of TNF-α in NT2-N is dependent upon the HTLV-1 Tax_1 protein.

Kinetics of Tax_1-mediated TNF-α induction in NT2-N. The time required for NT2-N to produce TNF-α in response to treatment with soluble Tax_1 was examined by treating the cells with 25 nM Tax_1 for various periods. TNF-α could be detected in the culture medium at as early as 2 h of treatment (Fig. 9). Between 2 and 4 h of treatment, a large increase in TNF-α was typically observed, accounting for most of the TNF-α that

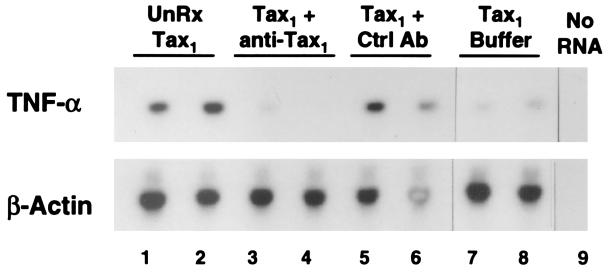


FIG. 5. Inhibition of Tax_1-mediated TNF-α synthesis in NT2-N by anti-Tax_1 antibodies as shown by RT-PCR analysis. RT-PCR was performed with RNA isolated from NT2-N cells cultured with a Tax_1 solution that was untreated (UnRx) (lanes 1 and 2) or immunodepleted with either anti-Tax_1 monoclonal antibodies (lanes 3 and 4) or a control monoclonal antibody (Ctrl Ab [anti-p53]) (lanes 5 and 6). Cell cultures were treated with a volume of Tax_1 solution that would give a concentration of 25 nM if Tax_1 were present. Cells were also cultured in the presence of Tax_1 buffer alone (lanes 7 and 8). PCR products were analyzed by Southern blotting with probes for TNF-α (top) and β-actin (bottom). Each lane represents an independent culture. Lane 9 is a control in which RNA was omitted from the cDNA synthesis, after which RT-PCR was performed to monitor for contamination.

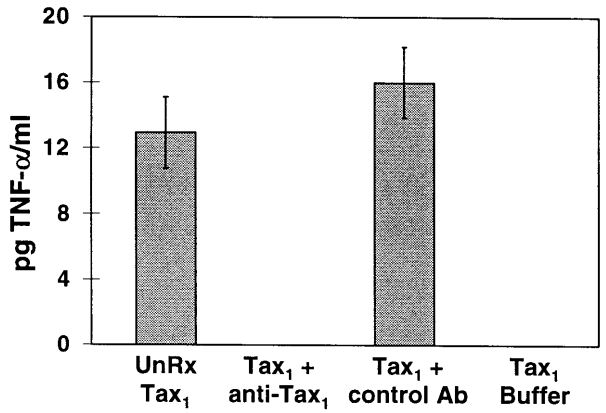


FIG. 6. Inhibition of Tax_1-mediated TNF-α synthesis in NT2-N by anti-Tax_1 antibodies as shown by ELISA. UnRx, untreated. The supernatants from the cultures described in the legend to Fig. 5 were analyzed for the presence of TNF-α by ELISA. Each data point is the mean ± standard deviation for duplicates of each sample tested in the ELISA.

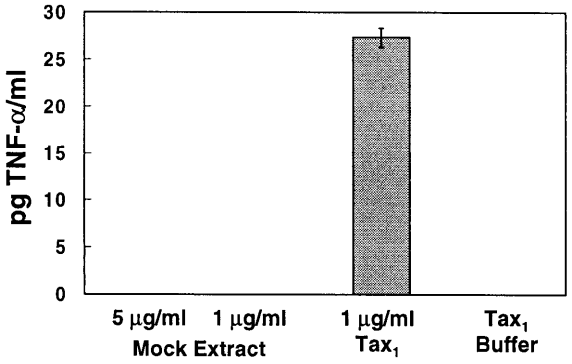


FIG. 8. Inability of a mock bacterial extract to induce TNF-α synthesis in NT2-N cells. NT2-N cells were treated for 18 h with extracts from bacteria with and without (mock) the Tax_1 expression construct. Concentration refers to micrograms of protein per milliliter, and 1 μg of Tax_1/ml is equivalent to a concentration of 25 nM. Culture supernatants were then assayed for TNF-α by ELISA. Each data point is the mean ± standard deviation for duplicates of each sample tested in the ELISA.

would appear in the culture medium. Maximal levels were observed by 6 h.

TNF-α synthesis in NT2-N pulsed with Tax_1. We next asked whether TNF-α synthesis by NT2-N required the presence of Tax_1 for the entire culture period. Cells were cultured for 2 days with Tax_1 continually or for only the first 2 h of culture, after which medium without Tax_1 was added. As shown in Fig. 10A, NT2-N cells pulsed with Tax_1 for only 2 h produced as much TNF-α as cells cultured with Tax_1 for the entire 2-day culture period. This observation is not likely explained by instability of Tax_1 in the culture, because addition of fresh Tax_1 after the initial 2-h pulsing did not result in a significant increase in TNF-α production (Fig. 10A).

To determine the minimal amount of time that NT2-N could be exposed to Tax_1 for stimulation of TNF-α synthesis, Tax_1 was added at the beginning of an 8-h culture (based on the results of the time course experiment indicating maximal induction of TNF-α by 6 h), with exposure of the cells to Tax_1 for periods of time ranging from 5 min to the full 8 h. As shown in Fig. 10B, culture of NT2-N cells with Tax_1 for as little as the first 5 min of the 8-h culture resulted in detectable TNF-α in the culture medium. Levels of TNF-α in the medium increased with longer exposure of the cells to Tax_1, with 1 h of treatment resulting in nearly the same level of TNF-α as if the Tax_1 were present during the entire 8-h culture. These results demonstrate that transient exposure of NT2-N cells to Tax_1 is sufficient to result in the synthesis of TNF-α.

Differentiation dependence of Tax_1-mediated TNF-α induction. Since NT2-N is a terminally differentiated product of the NT2 teratocarcinoma cell line, it was of interest to determine if the ability of HTLV-1 Tax_1 to induce TNF-α was restricted to the differentiated cells. As shown in Fig. 11, treatment of NT2 with up to 250 nM HTLV-1 Tax_1 did not result in the production of detectable TNF-α, in marked contrast to NT2-N. This finding suggests that neuron-specific factors are required to permit the synthesis of TNF-α in response to HTLV-1 Tax_1.

DISCUSSION

Studies of primary human neurons have been hampered by the ability to obtain pure populations of cells readily. NT2-N cells represent a nearly pure (>95%) population of terminally differentiated, postmitotic cells that bear a striking biochemi-

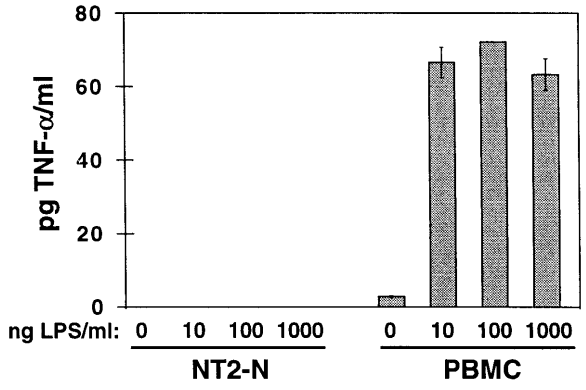


FIG. 7. Comparative influence of LPS on TNF-α synthesis by NT2-N cells and PBMCs. PBMCs were cultured in eight-well culture slides (1,000 cells/well) in NT2 culture medium, and NT2-N cells were cultured as usual (~10^4 cells/well). LPS was added to the cultures at the concentrations indicated for 16 h. Culture supernatants were then assayed for TNF-α by ELISA. Each data point is the mean ± standard deviation for duplicates of each sample tested in the ELISA.

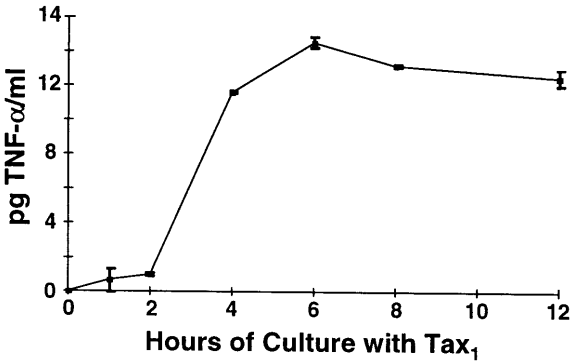
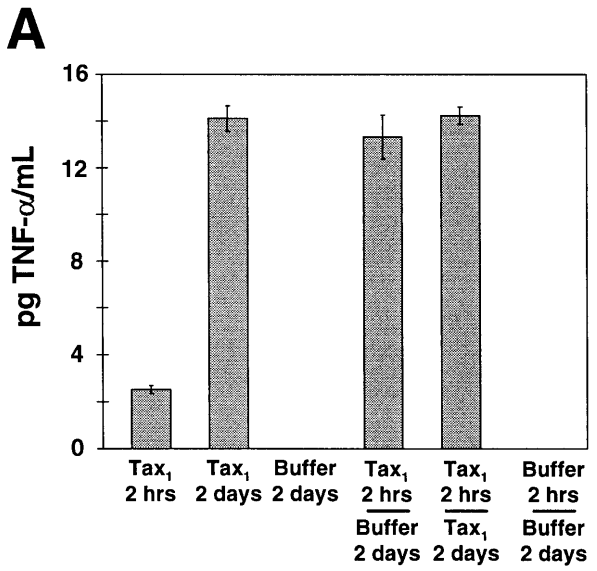


FIG. 9. Time course of TNF-α synthesis in NT2-N cells treated with extracellular HTLV-1 Tax_1. NT2-N cells were treated with 25 nM Tax_1 for the indicated times. Supernatants were removed and analyzed for the presence of TNF-α by ELISA. Each data point is the mean ± standard deviation for duplicates of each sample tested in the ELISA. The data shown are representative of three independent experiments.



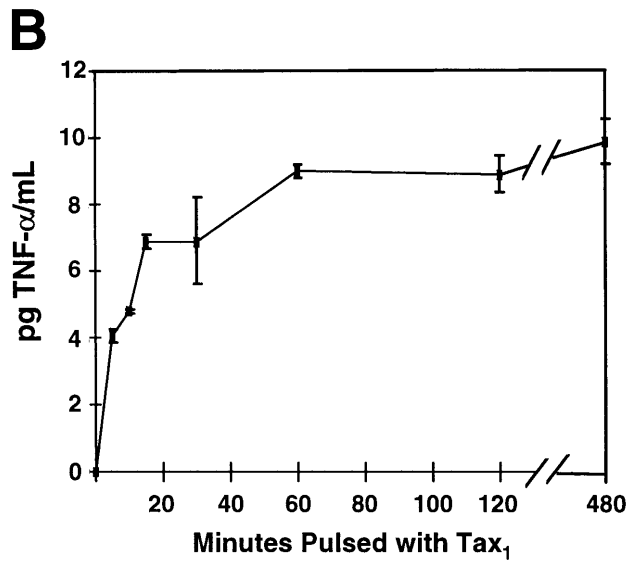


FIG. 10. TNF-α synthesis in NT2-N cells pulsed with soluble Tax_1. (A) NT2-N cells were cultured under the following conditions: continuously for 2 days in the presence or absence of 25 nM Tax_1, with Tax_1 for 2 h followed by 2 days of culture with or without 25 nM Tax_1, and with Tax_1 buffer for 2 h followed by 2 days with Tax_1 buffer. At the end of the culture, supernatants were assayed for the presence of TNF-α by ELISA. The first histogram represents an assay for TNF-α in the supernatant of a 2-h Tax_1-pulsed culture. (B) NT2-N cells were cultured for 8 h, with exposure of the cells to 25 nM Tax_1 at the beginning of the culture for the times indicated. The cells were then washed once with medium and cultured for the remainder of the 8 h in medium without Tax_1. At the end of the culture, supernatants were assayed for the presence of TNF-α by ELISA. No TNF-α was detected in the initial culture supernatants taken from the 5- to 120-min Tax_1-pulsed cells. Results shown are representative of three independent experiments. Each data point is the mean ± standard deviation for duplicates of each sample tested in the ELISA.

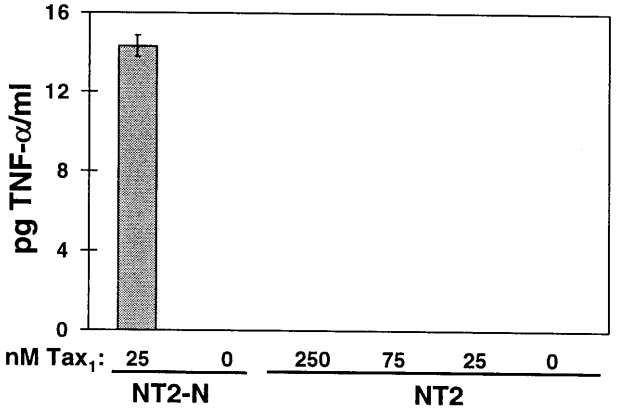


FIG. 11. Comparison of the influence of Tax_1 on TNF-α synthesis in NT2-N cells versus that in NT2 cells. NT2 cells were cultured in eight-well culture slides pretreated with Matrigel at 10^5 cells/well and cultured overnight. NT2-N cells were cultured as usual ($\sim 10^4$ NT2-N cells/well). All cells were then treated with Tax_1 for 18 h at the concentrations indicated. Culture supernatants were assayed for TNF-α by ELISA. Each data point is the mean ± standard deviation for duplicates of each sample tested in the ELISA.

cal, morphological, and functional resemblance to primary human CNS neurons (32, 41, 42). In addition, these cells have been valuable models for disease (10, 40, 41) and, upon transplantation into the brain, display characteristics expected of fully mature human neurons, including polarity (22). Taken together, these observations support the use of NT2-N as a useful model with which to examine the influence of HTLV-1 Tax_1 on primary human neurons.

We have demonstrated in NT2-N that a soluble form of the HTLV-1 Tax_1 protein is able to induce TNF-α gene transcription and the synthesis of TNF-α that can be detected in the culture medium. This effect has been observed with at least five independent purifications of the recombinant Tax_1 protein. TNF-α induction is dependent upon the Tax_1 protein itself and not a copurifying factor, based on the findings that anti-Tax_1 antibodies completely inhibited the response and that treatment with a mock protein extract was unable to induce TNF-α synthesis. Importantly, we have also ruled out any contribution from contaminating endotoxin, because direct treatment of NT2-N with LPS did not result in any detectable TNF-α synthesis. In contrast, PBMCs were exquisitely sensitive to the TNF-α-inducing effects of LPS. It should be noted in this regard that when considering the significance of TNF-α induction in adult human microglia (cells of macrophage origin in the CNS) by recombinant HTLV-1 Tax_1 (11), the influence of endotoxin can therefore not be ruled out.

The Tax_1-dependent synthesis of TNF-α in NT2-N occurred in a dose-dependent fashion, with a minimum of 0.25 nM Tax_1 required to result in the synthesis of detectable TNF-α. Interestingly, this is in the concentration range of Tax_1 observed in the culture medium of HTLV-1-infected cells (2). The induction of TNF-α by Tax_1 is relatively rapid in NT2-N, with a significant level of TNF-α appearing in the culture medium within 4 h of treatment with Tax_1 and a maximum reached by 6 h. The particular concentration dependence and kinetics of the response to Tax_1 are remarkably similar to those observed for induction of NF-κB binding activity by extracellular Tax_1 (2, 26). Indeed, evidence exists for the involvement of NF-κB in the regulation of TNF-α gene expression (20).

A remarkable and critical observation in this system was that NT2-N cells need not be exposed to Tax_1 for prolonged periods to induce TNF-α. Rather, exposure for only a few minutes is apparently necessary, based on the Tax_1-pulsing experiments described here in which treatment of NT2-N for as little as 5 min resulted in the synthesis of TNF-α. It is possible that even shorter exposures may be sufficient, depending upon the concentration of Tax_1 in the microenvironment of the neuron.

Whereas TNF-α could be readily induced by extracellular

Tax_1 in NT2-N, this was not the case in the parental cell line, NT2. Culturing NT2 in the presence of even 10-fold the concentration of Tax_1 typically used to induce TNF-α synthesis in NT2-N was unable to result in detectable levels of TNF-α in the culture medium. This observation suggests that differentiation-specific (neuron-specific?) factors may be involved in the ability to produce TNF-α in response to soluble Tax_1. The synthesis of TNF-α by neurons is not without precedent and has been observed in vivo. For example, TNF-α can be detected in neurons following injury (38), following systemic administration of LPS (3), or constitutively (4). This constitutive expression, detected at the RNA level, is consistent with our observations of low levels of TNF-α transcripts in untreated NT2-N cells. In these contexts, it has been proposed (5) that TNF-α functions in a neuroprotective manner and participates in regenerative events following injury, acting as a neuromodulator in the acute-phase response to inflammation and infection.

TNF-α in the CNS is associated with pathological events as well. Significantly, TNF-α is toxic for the myelin-producing cells of the CNS, the oligodendrocytes (36). Recently, Probert et al. (33) provided strong support for an in vivo role for TNF-α in demyelination. Transgenic mice that expressed a murine TNF-α transgene specifically in CNS neurons exhibited spontaneous focal demyelination, accompanied by a prominent lymphocytic infiltrate, astrogliosis, and microgliosis, all of which could be attributed to the expression of the neuronal TNF-α. These observations are of obvious interest in the context of the pathology observed in HAM/TSP and, considered together with the results presented in this study, suggest that induction of TNF-α in neurons by mere transient exposure to extracellular HTLV-1 Tax_1 may play a significant role.

ACKNOWLEDGMENTS

We thank Janet Duvall for excellent technical assistance, Subhash Dhawan for providing the PBMCs, and William E. Biddison and Suhayl Dhib-Jalbut for helpful discussions and critical reviews of the manuscript.

REFERENCES

1. **Bangham, C. R. M., A. G. Kermode, S. E. Hall, and S. Daenke.** 1996. The cytotoxic T-lymphocyte response to HTLV-I: the main determinant of disease? Semin. Virol. **7:**41–48.
2. **Brady, J. N.** 1992. Extracellular Tax_1 protein stimulates NF-κB and expression of NF-κB-responsive Ig kappa and TNF beta genes in lymphoid cells. AIDS Res. Hum. Retroviruses **8:**724–727.
3. **Breder, C. D., C. Hazuka, T. Ghayur, C. Klug, M. Huginin, K. Yasuda, M. Teng, and C. B. Saper.** 1994. Regional induction of tumor necrosis factor alpha expression in the mouse brain after systemic lipopolysaccharide administration. Proc. Natl. Acad. Sci. USA **91:**11393–11397.
4. **Breder, C. D., M. Tsujimoto, Y. Terano, D. W. Scott, and C. B. Saper.** 1993. Distribution and characterization of tumor necrosis factor-alpha-like immunoreactivity in the murine central nervous system. J. Comp. Neurol. **337:** 543–567.
5. **Cheng, B., S. Christakos, and M. P. Mattson.** 1994. Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. Neuron **12:**139–153.
6. **Cowan, E. P., and S. Dhib-Jalbut.** 1995. Molecular techniques for detection of gene expression in neuroimmunology, p. 41–60. *In* M. I. Phillips and E. Evans (ed.), Neuroimmunology. Academic Press, San Diego, Calif.
7. **Crino, P. B., M. A. Dichter, and J. Eberwine.** 1996. Embryonic neuronal markers in tuberous sclerosis: single-cell molecular pathology. Proc. Natl. Acad. Sci. USA **93:**14152–14157.
8. **Daenke, S., and C. R. Bangham.** 1994. Do T cells cause HTLV-1-associated disease? A taxing problem. Clin. Exp. Immunol. **96:**179–181. (Editorial.)
9. **Daenke, S., A. G. Kermode, S. E. Hall, G. Taylor, J. Weber, S. Nightingale, and C. R. Bangham.** 1996. High activated and memory cytotoxic T-cell responses to HTLV-1 in healthy carriers and patients with tropical spastic paraparesis. Virology **217:**139–146.
10. **Dewji, N. N., C. Do, and R. M. Bayney.** 1995. Transcriptional activation of Alzheimer's beta-amyloid precursor protein gene by stress. Brain Res. Mol. Brain Res. **33:**245–253.
11. **Dhib-Jalbut, S., P. M. Hoffman, T. Yamabe, D. Sun, J. Xia, H. Eisenberg, G. Bergey, and F. W. Ruscetti.** 1994. Extracellular human T-cell lymphotropic virus type I Tax protein induces cytokine production in adult human microglial cells. Ann. Neurol. **36:**787–790.
12. **Duvall, J. F., F. Kashanchi, A. Cvekl, M. F. Radonovich, G. Piras, and J. N. Brady.** 1995. Transactivation of the human T-cell lymphotropic virus type 1 Tax_1-responsive 21-base-pair repeats requires Holo-TFIID and TFIIA. J. Virol. **69:**5077–5086.
13. **Giam, C. Z., M. Nerenberg, G. Khoury, and G. Jay.** 1986. Expression of the complete human T-cell leukemia virus type I pX coding sequence as a functional protein in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83:**7192–7196.
14. **Gitlin, S. D., J. Dittmer, R. L. Reid, and J. N. Brady.** 1993. The molecular biology of human T-cell leukemia viruses, p. 159–192. *In* B. Cullen (ed.), Human retroviruses. IRL Press, New York, N.Y.
15. **Hara, H., M. Morita, T. Iwaki, T. Hatae, Y. Itoyama, T. Kitamoto, S. Akizuki, I. Goto, and T. Watanabe.** 1994. Detection of human T lymphotrophic virus type I (HTLV-I) proviral DNA and analysis of T cell receptor V beta CDR3 sequences in spinal cord lesions of HTLV-I-associated myelopathy/tropical spastic paraparesis. J. Exp. Med. **180:**831–839.
16. **Iannone, R., M. P. Sherman, P. E. Rodgers-Johnson, M. A. Beilke, C. A. Mora, R. M. Amin, S. R. Tinsley, L. D. Papsidero, B. J. Poiesz, and C. J. Gibbs, Jr.** 1992. HTLV-I DNA sequences in CNS tissue of a patient with tropical spastic paraparesis and HTLV-I-associated myelopathy. J. Acquir. Immune Defic. Syndr. **5:**810–816.
17. **Iwasaki, Y.** 1993. Human T cell leukemia virus type I infection and chronic myelopathy. Brain Pathol. **3:**1–10.
18. **Jacobson, S., D. E. McFarlin, S. Robinson, R. Voskuhl, R. Martin, A. Brewah, A. J. Newell, and S. Koenig.** 1992. HTLV-I-specific cytotoxic T lymphocytes in the cerebrospinal fluid of patients with HTLV-I-associated neurological disease. Ann. Neurol. **32:**651–657.
19. **Jacobson, S., H. Shida, D. E. McFarlin, A. S. Fauci, and S. Koenig.** 1990. Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. Nature **348:**245–248.
20. **Jongeneel, C. V.** 1995. Transcriptional regulation of the tumor necrosis factor alpha gene. Immunobiology **193:**210–216.
21. **Kannagi, M., S. Harada, I. Maruyama, H. Inoko, H. Igarashi, G. Kuwashima, S. Sato, M. Morita, M. Kidokoro, and M. Sugimoto.** 1991. Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells. Int. Immunol. **3:**761–767.
22. **Kleppner, S. R., K. A. Robinson, J. Q. Trojanowski, and V. M. Lee.** 1995. Transplanted human neurons derived from a teratocarcinoma cell line (NTera-2) mature, integrate, and survive for over 1 year in the nude mouse brain. J. Comp. Neurol. **357:**618–632.
23. **Koenig, S., and S. A. Jacobson.** 1992. Cytotoxic T cell response to HIV-1 and HTLV-I: issues of protection and pathogenesis, p. 49–59. *In* P. Racz, N. L. Letvin, and J. C. Gluckman (ed.), Cytotoxic T cells in HIV and other retroviral infections. Karger, Basel, Switzerland.
24. **Kuroda, Y., M. Matsui, M. Kikuchi, K. Kurohara, C. Endo, M. Yukitake, Y. Matsuda, O. Tokunaga, A. Komine-Sakaki, and R. Kawaguchi.** 1994. In situ demonstration of the HTLV-I genome in the spinal cord of a patient with HTLV-I-associated myelopathy. Neurology **44:**2295–2299.
25. **Lehky, T. J., C. H. Fox, S. Koenig, M. C. Levin, N. Flerlage, S. Izumo, E. Sato, C. S. Raine, M. Osame, and S. Jacobson.** 1995. Detection of human T-lymphotropic virus type I (HTLV-I) tax RNA in the central nervous system of HTLV-I-associated myelopathy/tropical spastic paraparesis patients by in situ hybridization. Ann. Neurol. **37:**167–175.
26. **Lindholm, P. F., S. J. Marriott, S. D. Gitlin, C. A. Bohan, and J. N. Brady.** 1990. Induction of nuclear NF-kappa B DNA binding activity after exposure of lymphoid cells to soluble tax_1 protein. New Biol. **2:**1034–1043.
27. **Lindholm, P. F., R. L. Reid, and J. N. Brady.** 1992. Extracellular Tax_1 protein stimulates tumor necrosis factor-β and immunoglobulin kappa light chain expression in lymphoid cells. J. Virol. **66:**1294–1302.
28. **Marriott, S. J., P. F. Lindholm, R. L. Reid, and J. N. Brady.** 1991. Soluble HTLV-I Tax_1 protein stimulates proliferation of human peripheral blood lymphocytes. New Biol. **3:**678–686.
29. **Marriott, S. J., D. Trinh, and J. N. Brady.** 1992. Activation of interleukin-2 receptor alpha expression by extracellular HTLV-I Tax_1 protein: a potential role in HTLV-I pathogenesis. Oncogene **7:**1749–1755.
30. **Moore, G. R., U. Traugott, L. C. Scheinberg, and C. S. Raine.** 1989. Tropical spastic paraparesis: a model of virus-induced, cytotoxic T-cell-mediated demyelination? Ann. Neurol. **26:**523–530.
31. **Pleasure, S. J., and V. M. Lee.** 1993. NTera 2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. J. Neurosci. Res. **35:**585–602.
32. **Pleasure, S. J., C. Page, and V. M. Lee.** 1992. Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. J. Neurosci. **12:** 1802–1815.
33. **Probert, L., K. Akassoglou, M. Pasparakis, G. Kontogeorgos, and G. Kollias.** 1995. Spontaneous inflammatory demyelinating disease in transgenic mice

showing central nervous system-specific expression of tumor necrosis factor alpha. Proc. Natl. Acad. Sci. USA **92:**11294–11298.
34. **Rodgers-Johnson, P. E.** 1994. Tropical spastic paraparesis/HTLV-I associated myelopathy. Etiology and clinical spectrum. Mol. Neurobiol. **8:**175–179.
35. **Sawa, S., F. Oyama, M. Matsushita, and Y. Ihara.** 1994. Molecular diversity at the carboxyl terminus of human and rat tau. Mol. Brain Res. **27:**111–117.
36. **Selmaj, K. W., and C. S. Raine.** 1988. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. Ann. Neurol. **23:**339–346.
37. **Tangy, F., J. C. Vernant, L. Coscoy, M. Ossondo, R. Bellance, V. Zaninovic, L. Cartier, M. Brahic, and S. Ozden.** 1995. A search for human T-cell leukemia virus type I in the lesions of patients with tropical spastic paraparesis and polymyositis. Ann. Neurol. **38:**454–460.
38. **Tchelingerian, J. L., J. Quinonero, J. Booss, and C. Jacque.** 1993. Localization of TNF alpha and IL-1 alpha immunoreactivities in striatal neurons after surgical injury to the hippocampus. Neuron **10:**213–224.
39. **Tomaru, U., H. Ikeda, O. Ohya, M. Abe, T. Kasai, I. Yamashita, K. Morita, A. Wakisaka, and T. Yoshiki.** 1996. Human T lymphocyte virus type I-induced myeloneuropathy in rats: implication of local activation of the pX and tumor necrosis factor-alpha genes in pathogenesis. J. Infect. Dis. **174:** 318–323.
40. **Turner, R. S., N. Suzuki, A. S. Chyung, S. G. Younkin, and V. M. Lee.** 1996. Amyloids beta40 and beta42 are generated intracellularly in cultured human neurons and their secretion increases with maturation. J. Biol. Chem. **271:** 8966–8970.
41. **Wolf, B. A., A. M. Wertkin, Y. C. Jolly, R. P. Yasuda, B. B. Wolfe, R. J. Konrad, D. Manning, S. Ravi, J. R. Williamson, and V. M. Lee.** 1995. Muscarinic regulation of Alzheimer's disease amyloid precursor protein secretion and amyloid beta-protein production in human neuronal NT2N cells. J. Biol. Chem. **270:**4916–4922.
42. **Younkin, D. P., C. M. Tang, M. Hardy, U. R. Reddy, Q. Y. Shi, S. J. Pleasure, V. M. Lee, and D. Pleasure.** 1993. Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line. Proc. Natl. Acad. Sci. USA **90:**2174–2178.