Induction of Tumor Necrosis Factor Alpha in Human Neuronal Cells by Extracellular Human T-Cell Lymphotropic Virus Type 1 Tax1

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To examine the role of human T-lymphotropic virus type 1 (HTLV-1) Tax1 in the development of neurological disease, we studied the effects of extracellular Tax1 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 Tax1 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-Tax1 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 Tax1 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 Tax1 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 Tax1 to be continually present in the culture medium. Treatment of the undifferentiated parental embryonal carcinoma cell line NT2 with soluble Tax1 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 Tax1, 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 Tax1 (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 Tax1-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 Tax1-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 Tax1-specific immunity in HTLV-1-infected individuals may reflect the possibilities either that Tax1 is unusually immunogenic or that it is present in significant amounts in the host. HTLV-1 Tax1 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 Tax1 on cellular gene expression can be observed not only in HTLV-1-infected cells or transfectants expressing the Tax1 gene, but also in cells treated with soluble Tax1, 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 Tax1 (26, 28). Soluble Tax1 treatment induces the nuclear accumulation of NF-κB (2, 26), as well as the expression of immunoglobulin κ light chain (2, 27).
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, 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 can induce TNF-α expression (39). This study is designed to investigate whether TNF-α gene expression in neuronal cells themselves could be induced by soluble HTLV-1 Tax, such that it would potentially play a role in the course of HAM/TSP.

MATERIALS AND METHODS

Cells and reagents. The NTERa 2/c1D1 (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 10% 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 × 10⁸ NT2 cells were seeded in 175-cm² tissue culture flasks (Nunc, Naperville, Ill.) in 50 ml NT2 culture medium containing 10 μM retinoic acid (Sigma, St. Louis, Mo.). This 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 1.60 dilution of Matrigel (Collaborative Research, Cambridge, Mass.), a basement membrane product, 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 10⁷ postmitotic cells remaining in each well. Through 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 (St. Louis, Mo.).

Postmitotic human neuronal cells treated with extracellular HTLV-1 Tax protein was expressed in E. coli recombinant Tax (30 ng/μl) was incubated with 1 μg (20 μl) of a cocktail of four monoclonal anti-Tax antibodies (EAB 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).

Immunodepletion of Tax and Western blot analysis. One microgram of E. coli recombinant Tax, (10 ng/μl) was incubated with 1 μg (20 μl) of a cocktail of four monoclonal anti-Tax antibodies (EAB 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 recombinant HTLV-1 Tax protein induce 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/c1D1 (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 heavy, nestin (5 microns), and α-tubulin, which is not expressed by NT2. The morphology of these cells and the pattern of marker expression, including the expression of keratin by NT2 (Fig. 1a), is 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 protein at various concentrations and immunodepleted as described above. 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 with random hexamers. The cDNA was then amplified by PCR with primers for human TNF-α (5′ primer, CTCCTGGCTGCT CTTTGGA; 3′ primer, TCCGAGTACGACGCGCAG), human beta-actin (5′ primer, IGAC GGGTCCATTCACTTGA; 3′ primer, GAGCCTCAATGGTTG), and p53 (5′ primer, AGAGG GGAATCTCTGGAG; 3′ primer, CTGAGGACCGAAGCATTCT). The reactions were carried out in a volume of 100 μl, and the reaction mixture consisted of 50 pmol of primer; 1/10 PCR buffer II (Perkin-Elmer, Norwalk, Conn.); 2.5 mM MgCl₂, 50 μM dATP, dCTP, dGTP, and dUTP (Boehringer Mannheim, Indianapolis, Ind.); 1.25 U of Taq DNA polymerase (Perkin-Elmer); and 1 μl of uracil-DNA glycosylase (Boehringer-Mannheim). The samples were initially treated for 10 min at 37°C to potentially contaminating amplifiers 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°C 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).

**Immunodepletion of Tax and Western blot analysis.** One microgram of E. coli recombinant Tax, (10 ng/μl) was incubated with 1 μg (20 μl) of a cocktail of four monoclonal anti-Tax antibodies (EAB 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).
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.
untreated Tax1 (Fig. 5 and 6). Western blot analysis of the induced TNF-α levels in NT2-N similar to those observed with the Tax1 was removed by immunoprecipitation with anti-Tax1 adsorbed immune complexes confirmed that the majority of immunoprecipitated Tax1-specific monoclonal antibodies, whereas a minimal amount of Tax1 was depleted in samples treated with a control antibody (data not shown).

Consistent with these results, assay of the culture supernatants from these cells revealed that whereas supernatants from cells treated with Tax1 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 Tax1 in a dose-responsive manner (Fig. 4). A minimum of 0.25 nM Tax1 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 Tax1. The requirement for the Tax1 protein rather than a copurifying factor in the induction of TNF-α in NT2-N was demonstrated in several ways. First, the Tax1 preparation was subjected to immunoprecipitation with Tax1-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, Tax1 treated with a control antibody was able to induce TNF-α levels in NT2-N similar to those observed with untreated Tax1 (Fig. 5 and 6). Western blot analysis of the adsorbed immune complexes confirmed that the majority of the Tax1 was removed by immunoprecipitation with anti-Tax1 antibodies, whereas a minimal amount of Tax1 was depleted in samples treated with a control antibody (data not shown).

Second, because there was concern that endotoxin in the recombinant Tax1 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 Tax1 protein (Fig. 8). Taken together, these results demonstrate that the induction of TNF-α in NT2-N is dependent upon the HTLV-1 Tax1 protein.

Kinetics of Tax1-mediated TNF-α induction in NT2-N. The time required for NT2-N to produce TNF-α in response to treatment with soluble Tax1 was examined by treating the cells with 25 nM Tax1 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 could be detected by RT-PCR analysis. RT-PCR was performed with RNA isolated from NT2-N cells cultured with a Tax1 solution that was untreated (UnRx) (lanes 1 and 2) or immunodepleted with either anti-Tax1 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 Tax1 solution that would give a concentration of 25 nM if Tax1 were present. Cells were also cultured in the presence of Tax1 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.

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

**TNF-α synthesis in NT2-N pulsed with Tax₁.** We next asked whether TNF-α synthesis by NT2-N required the presence of Tax₁ for the entire culture period. Cells were cultured for 2 days with Tax₁ continually or for only the first 2 h of culture, after which medium without Tax₁ was added. As shown in Fig. 10A, NT2-N cells pulsed with Tax₁ for only 2 h produced as much TNF-α as cells cultured with Tax₁ for the entire 2-day culture period. This observation is not likely explained by instability of Tax₁ in the culture, because addition of fresh Tax₁ 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 Tax₁ for stimulation of TNF-α synthesis, Tax₁ 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 Tax₁ for periods of time ranging from 5 min to the full 8 h. As shown in Fig. 10B, culture of NT2-N cells with Tax₁ 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 Tax₁, with 1 h of treatment resulting in nearly the same level of TNF-α as if the Tax₁ were present during the entire 8-h culture. These results demonstrate that transient exposure of NT2-N cells to Tax₁ is sufficient to result in the synthesis of TNF-α.

**Differentiation dependence of Tax₁-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 Tax₁ 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 Tax₁ 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 Tax₁.

**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-
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 Tax1 on primary human neurons.

We have demonstrated in NT2-N that a soluble form of the HTLV-1 Tax1 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 Tax1 protein. TNF-α induction is dependent upon the Tax1 protein itself and not a copurifying factor, based on the findings that anti-Tax1 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 Tax1 (11), the influence of endotoxin can therefore not be ruled out.

The Tax1-dependent synthesis of TNF-α in NT2-N occurred in a dose-dependent fashion, with a minimum of 0.25 nM Tax1 required to result in the synthesis of detectable TNF-α. Interestingly, this is in the concentration range of Tax1 observed in the culture medium of HTLV-1-infected cells (2). The induction of TNF-α by Tax1 is relatively rapid in NT2-N, with a significant level of TNF-α appearing in the culture medium within 4 h of treatment with Tax1 and a maximum reached by 6 h. The particular concentration dependence and kinetics of the response to Tax1 are remarkably similar to those observed for induction of NF-κB binding activity by extracellular Tax1 (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 Tax1 for prolonged periods to induce TNF-α. Rather, exposure for only a few minutes is apparently necessary, based on the Tax1-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 Tax1 in the microenvironment of the neuron.

Whereas TNF-α could be readily induced by extracellular...
Taxα 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 Taxα, 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 Taxα. 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, astrogliaisis, and microgliaosis, 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 myelin-specific protein in extracellular HTLV-1 Taxα may play a significant role.

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