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Short Communication

Long-term effects of HTLV-1 on brain astrocytes: sustained expression of Tax-1 associated with synthesis of inflammatory mediators

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HTLV-1 is the causative agent of a chronic neurological disease, TSP/HAM. The persistently activated CTL response to the viral protein Tax-1 suggests the existence of persistent viral replication with continuous expression of Tax-1. Although CD4+ T-cell is the main target for HTLV-1, previous observations have indicated that the astrocyte, the major neural cell in close contact with blood vessel and thus with HTLV-1-infected T-cells infiltrating the CNS, may also be infected. We tested *in vitro* the hypothesis of persistent/restricted infection in human and rat astrocytes after transient contact with an infectious T-cell line (C91PL). Long-term analysis showed prolonged expression of Tax-1 in astrocytes, associated with secretion of inflammatory mediators (TNFα, IL1α, MMP-2, and MMP-9). These data suggest a possible contribution of Tax-1-expressing astrocytes to TSP/HAM pathogenesis. *Journal of NeuroVirology* (2000) **6,** 350–357.

Keywords: retrovirus; astrocyte activation; inflammation; cytokine; metalloproteinase; zymography

Introduction

Human T lymphotropic virus type I (HTLV-1) is the causative agent of a slowly progressing neurological disease termed tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) (Gessain *et al*, 1985; Osame *et al*, 1986). The risk of TSP/HAM is associated with a remarkably high proviral load in circulating T lymphocytes (Nagai *et al*, 1998) and a genetic background that controls the efficiency of the anti-HTLV-1 cytotoxic T lymphocyte (CTL) response (Jeffery *et al*, 1999). An activated cellular and antibody immune response is characteristic of TSP/HAM (Jacobson *et al*, 1990; Kira *et al*, 1992). The persistently activated CTL response to the Tax-1 protein of HTLV-1 (Jacobson, 1996; Parker *et al*, 1992) strongly suggests that there is persistent viral replication in HTLV-1 infected cells, with continuous expression of certain HTLV-1 genes, at least that coding for Tax-1 protein (Wodarz *et al*, 1999). The oligoclonal expansion of CTL directed against HTLV-1 Tax-1(11–19) peptide, detected in the cerebrospinal fluid of TSP/HAM patients (Hoger *et al*, 1997), and the diversity of intrathecal anti-HTLV-1 antibody synthesis (Kitze *et al*, 1996) support the hypothesis of a specific immune response elicited by antigen presenting cells (APC) in the central nervous system (CNS) compartment, the expansion of activated T cells being further maintained by the cell-cycle regulatory protein, Tax-1. The nature of the cells involved in the processing and presentation of viral peptides which mediate HTLV-1-specific T cell activation in the CNS is not known. Cells of immune and neural origin, such as macrophage/microglia and astrocytes, may be suspected, since they have the potential to propagate the immune response (Hall *et al*, 1999; Soos *et al*, 1998). Classically, the CD4+ T lymphocyte is the main target for HTLV-1 (Richardson *et al*, 1990), and the CD4+ T cells that infiltrate the CNS parenchyma and cerebrospinal fluid harbor provirus and express Tax-1 mRNA and protein (Moritoyo *et al*, 1996, 1999). Whether neural cells can be infected by HTLV-1, then turn into potential APC for HTLV-1 peptides remains controversial. Some investigators

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have reported that the sole viral reservoir within the CNS is the T cell (Kubota *et al*, 1994; Matsuoka *et al*, 1998), while others have also detected provirus and Tax-1 mRNA in cells of neural origin, including astrocytes (Kira *et al*, 1992; Kuroda *et al*, 1994; Lehky *et al*, 1995). This discrepancy may be explained, at least in part, by the fact that HTLV-1 establishes a persistent, then latent, infection in neural cells, with progressive restriction and extinction of viral gene expression, as observed for HIV infection (Petito *et al*, 1999) Establishment of viral persistence or latency in the CNS following viral infection is common (Dubois-Dalcq, 1995) and implies pathways that reduce or abolish the expression of certain viral genes, impairing the productive and lytic phases of viral replication (Ahmed *et al*, 1996; Oldstone, 1993). Astrocytes may be a preferential target by which HTLV-1 establishes persistent/latent infection in the CNS as astrocytes are the glial cells which come into closest contact with peripheral blood via astrocytic feet on blood vessels and can be infected, at least *in vitro*, by HTLV-1 (Watabe *et al*, 1989; Yamada *et al*, 1991).

Results

The hypothesis of such persistent/latent HTLV-1 infection of astrocytes was tested using transient coculture (20 h) of human and rat astrocytes with γ-irradiated CD4+ HTLV-1-producing human T lymphocytes (cell line C91PL (Poiesz *et al*, 1980)), as previously reported (Giraudon *et al*, 1995). We analyzed the time course of viral protein expression and the possible production of viral particles. In addition, phenotypic changes associated with infection were also investigated. HTLV-1 replication was demonstrated by the co-expression of structural (p24 and p19) and regulatory proteins (Tax-1), which accumulated in 2–5% of rat astrocytes (primary cultures) and in 20 or 90% of human astrocytes (the Dev-Ast cell line, which resembles a mature human astrocyte, and primary cultures from the cortex of the 16-week human fetus, respectively). Similar data were consistently observed in experiments performed over a period of 4 years. As shown in Figure 1a–d, during the first week post-infection, Tax-1 and p24 were detected by immunofluorescence in multinucleated cells, subsequently identified as astrocytes (glial fibrillary acidic protein-positive cells, not shown). The few microglial cells (ED-1 positive) present in primary cultures never exhibited viral antigens. At the same time, RT–PCR analysis revealed the presence of tax mRNA in the infected human and rat cultures (Figure 3a) which was mainly present in infected astrocytes, as residual mRNA from HTLV-1 infected-T lymphocytes could not be detected after 4–5 days post-contact (negative RT–PCR for hu-

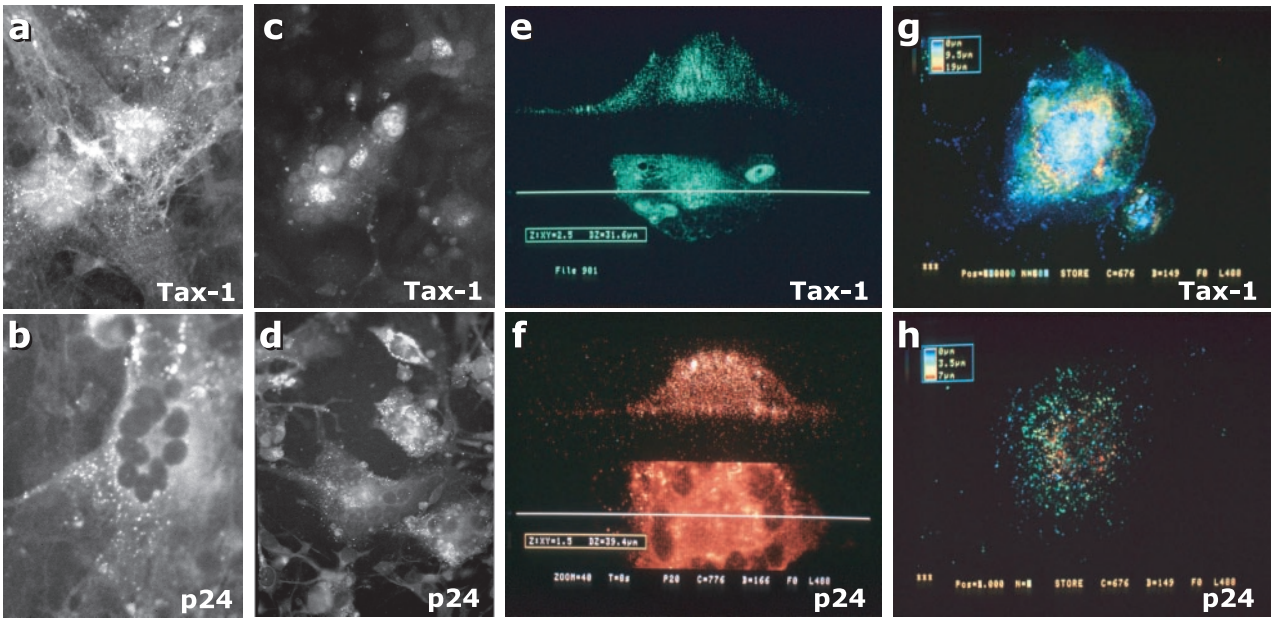

Figure 1 Tax-1 and p24 are expressed in 1-week-infected astrocytes. (a–d). Immunodetection of Tax-1 (Ab NIH#467) and p24 (Ab NIH#AF) in rat astrocytes (**a,b**) and in the human cell line Dev (**c,d**). (e-h). Subcellular localization of Tax-1 and p24 in primary cultures of human astrocytes using confocal laser microscopy (CLSM). The three-dimensional fluorescent distribution inside the cells (overlays in **g,h**) was obtained by the combination of a stack of serial sections taken at 0.5 μm intervals along the optical axis (0–19 μm), each section representing an average of 16 measurements so as to improve the signal to noise ratio. In **e** and **f** the phi-z section was parallel to the optical axis and the cut axis was defined by the x/z-line selected to pass through the cytoplasm, the nucleus and the nucleolus.

man GAPDH and TNFα in the infected rat primary cultures, data not shown). The subcellular localization of the immunodetected proteins Tax-1 and p24 was determined by confocal microscopy (Figure 1e–h). In the human astrocytes, p24 was present in the cytoplasm and nucleus and on the apical membrane, while Tax-1 protein was present in the cytoplasm, including the processes, and in the nucleus and, at to a lesser extent, on the apical cell membrane. In the rat astrocytes, Tax-1 and p24 showed a more diffuse cytoplasmic localization and were not found in the nucleus (data not shown). We then examined whether HTLV-1 replication in astrocytes led to the production of infectious viral particles. This infection was non-productive in human and rat astrocytes because (i) treatment of naive astrocytes with the supernatant from cultures of infected astrocytes or contact with infected astrocytes themselves never led to new infection of the culture, (ii) electron microscopic examination of infected rat and human astrocytes never evidenced of virus budding on the membrane of astrocyte contrarily to that of HTLV-1-producing T lymphocytes (C91PL) (Figure 2). Pronounced morphological changes were seen in multinucleated cells, with the presence of a lobulated nucleus, numerous nuclear pores associated with condensed heterochromatin, and an enlarged nucleolus. Cell lysis or nuclear fragmentation were not observed in infected astrocyte cultures. Taken together, these observations suggest that a persistent/latent infection was progressively established in the astrocytes.

The time-course of infection was then monitored for up to 6 weeks in infected human and rat astrocytes. Previous PCR analysis (Giraudon, *et al*, 1995) had indicated that HTLV-1 provirus is present in infected human astrocytic cell line for at least 4 weeks. Tax-1 and p24 expression was monitored by immunocytochemistry (ICC) and tax mRNA level was determined by RT–PCR using primers previously designed by Kinoshita *et al* (1989). As shown in Figure 3a, tax mRNA was expressed in human and rat cultures for at least 30 and 22 days post-infection, respectively. Tax-1 protein was detected in the same period (28 and 32 days p.i, respectively–Figure 3b). ICC performed during 7th

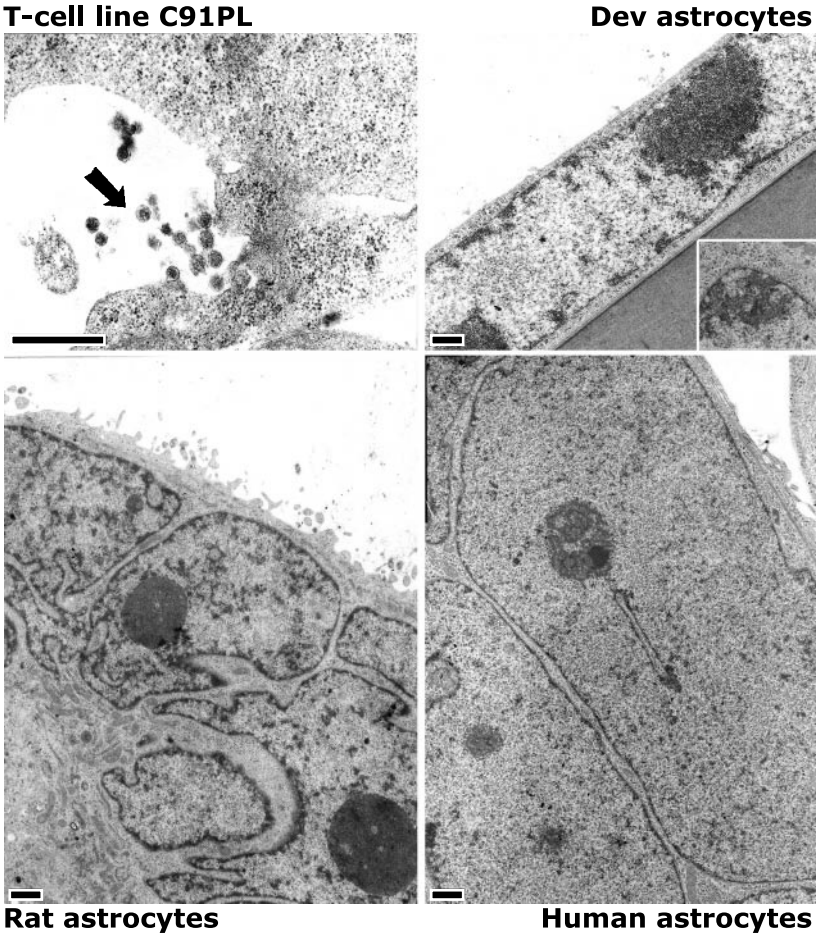

Figure 2 Morphological changes, (multinucleated cells, lobulated nucleus, numerous nuclear pores, condensed heterochromatin, enlarged nucleolus) but no virus budding, are seen in HTLV-1-infected astrocytes by electron microscopy (×12 000). In the T-cell line C91PL (×36 000), the arrow indicates the budding of viral particles. Scale bars represent 1 μm.

to 8th week post-infection showed that Tax-1 protein progressively became undetectable (data not shown). In contrast, p24 expression was detected by ICC only during the first 2 weeks in infected rat (Figure 3b) and human astrocytes (not shown). These results corroborate those of a

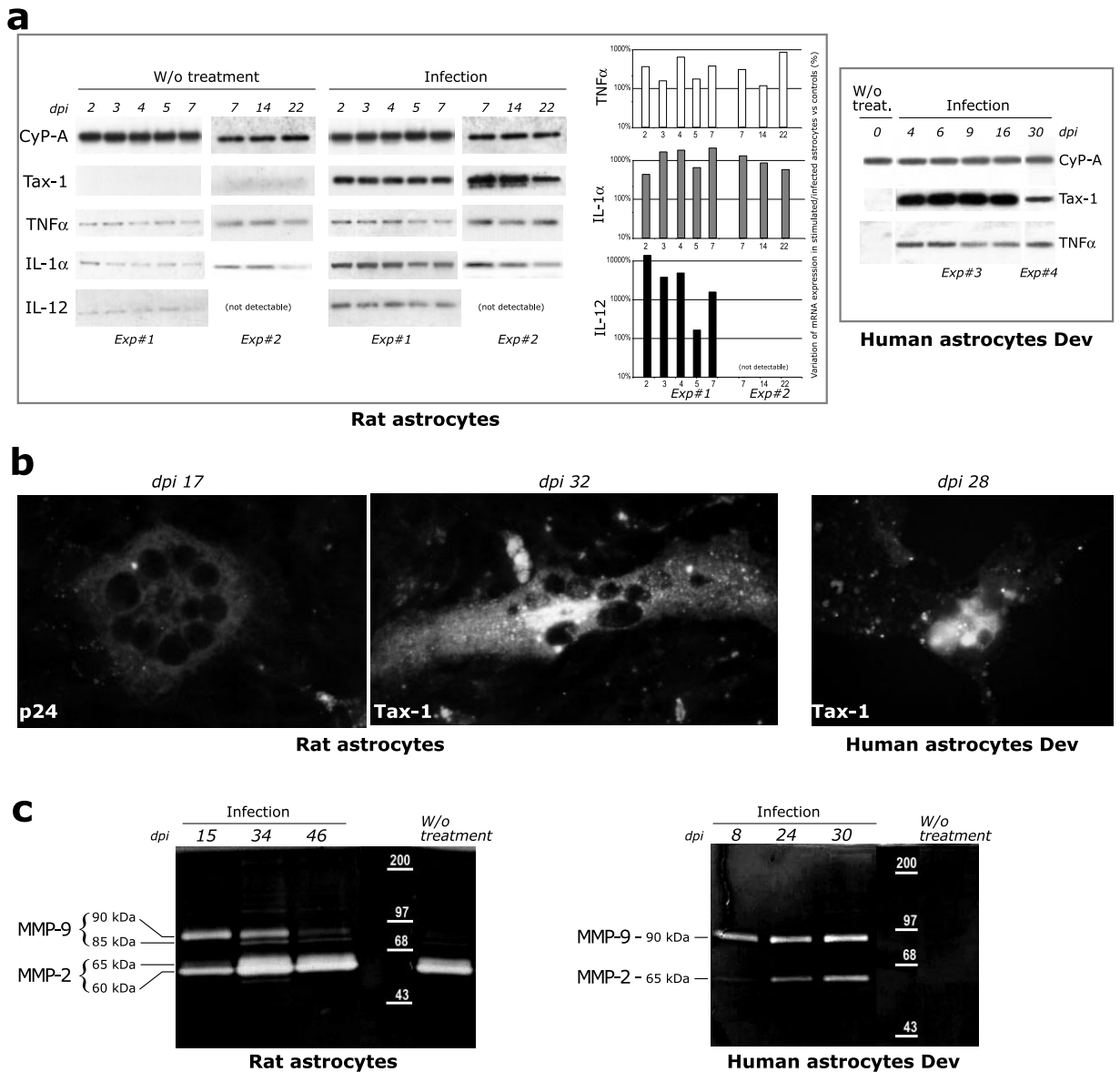

Figure 3 Sustained expression of Tax-1 and inflammatory mediators in HTLV-1-infected astrocytes. (**a**) RT–PCR analysis. The following primers were used: rat/human Cyclophilin-A (CyP-A), GenBank #M19533, p5=135..155, p3=410..392, probe=229..208; rat TNFα, GenBank #X66539, p5=259..277, p3=443..424, probe=366..349; human TNFα, GenBank #X01394, p5=293..314, p3=840..819, probe=515..486; rat IL-1α, GenBank #D00403, p5=759..783, p3=1381..1357, probe=969..9 48; rat IL-12, GenBank#U16674, p5=311..328, p3=590..573, probe=456..427. Primers for HTLV-1 tax], RPX3, RPX4 and RPX-PR1 the probe encompassing the splice junction for *tax-rex* mRNA, were defined by Kinoshita *et al* (1989) and located at p5=5118..5137, p3=7379..7360, probe=join [7334..7325,5206..5197] GenBank #J02029. The specificity of RT–PCR products was assessed by Southern blotting using an internal radiolabeled probe specific for each cDNA. In rat astrocytes, the upregulation of inflammatory cytokines was demonstrated by comparison of cDNA levels in infected *versus* control cells (normalized to that for CyP-A cDNA), after counting each band in a liquid scintillation counter (see graph). In human astrocytes, TNFα was undetectable in untreated but induced in infected cells expressing Tax-1 mRNA. (**b**) Immunodetection of Tax-1 and p24 in long-term infected rat and human astrocytes. (**c**) Zymography (gelatin-substrate PAGE) detected MMP-2 and MMP-9 (precursor and cleaved forms, as reported by Fridman *et al* (1995)) in conditioned media from infected rat and human astrocytes (15–46 dpi or 8–30 d.p.i., respectively). The lane with conditioned media from cultures without treatment (control) represents the basal secretion. The enzyme activity is revealed by substrate degradation which results in a clear band at the expected molecular weigth. Induction of MMP-9 secretion was detected in infected rat and human astrocytes. MMP-2 secretion was induced in infected human astrocytes and upregulated in infected rat astrocytes as evidenced by comparing the optical density of MMP-2 bands in infected *versus* control cells using an image analysis software (+29% and +10% at 34 and 46 d.p.i., respectively).

previous analysis using a radio-immunoprecipitation assay which indicated that p24 synthesis was halted 2 weeks after infection (Giraudon, *et al*, 1995). Thus, the replication of HTLV-1 in astrocytes is characterized by a progressive restriction of viral protein expression.

The continuous expression of the multifunctional protein Tax-1 for at least 1 month suggests that astrocytes in infected cultures may be profoundly affected. Tax-1 is a secreted protein that activates the expression of several molecules, including inflammatory mediators (Lindholm *et al.*, 1992; Buckle *et al*, 1996). The production of cytokines and matrix metalloproteinases, known to be involved in the inflammatory process, was investigated over a period of 1 month post-infection. Expression of mRNAs coding for the inflammatory cytokines TNFα, IL1α, and IL12 was monitored by RT–PCR in infected rat astrocytes (the rat cytokine primers did not amplify their human counterparts). As shown in Figure 3a, cytokine expression was globally upregulated in infected cells compared with control cells. In particular, TNFα and IL1α were continuously expressed at elevated level and their expression paralleled that of Tax-1. Up-regulation of TNFα was confirmed at the protein level by ELISA (72 ng/ml on day 8 post-infection compared with 3 ng/ml in control cells–Biosource International, Camarillo). In human astrocytes, TNFα was undetectable in untreated cells but induced in cells expressing Tax-1 mRNA. TNFα mRNA was continuously expressed for at least 30 days p.i (Figure 3a). This corroborates our previous study reporting secretion of TNFα by infected/stimulated Dev cells (Giraudon *et al*, 1995, 2000) and another study indicating that Tax-1 transfected-astrocytes promote TNFα expression *in vitro* (Mendez *et al*, 1997). As inflammatory cytokines are known to up-regulate the secretion of matrix metalloproteinases (MMPs) (Borden and Heller, 1997), MMP-2 and MMP-9 secretion was evaluated by gelatin-substrate zymography as previously reported (Giraudon *et al*, 1995). As shown in Figure 3c, MMP-2 and MMP-9 were nearly undetectable in cell supernatants from untreated human astrocyte cultures while MMP-2 was constitutively expressed in rat cells. MMP-9 expression was induced in infected rat and human astrocytes as early as 8 days post-infection and persisted for at least 30 days. MMP-9 modulation coincided with the expression of Tax-1 and TNFα in astrocytes, suggesting the involvement of Tax-1-induced TNFα. In contrast, expression of MMP-2 was changed (induction in human astrocytes, upregulation in rat astrocytes) with much slower kinetics, begining from day 34 or 24 post-infection in rat or human astrocytes, respectively. This indicates the existence of distinct regulatory mechanisms for these proteases in neural cells. These data show that continuous activation of astrocytes is associated with longterm expression of Tax-1.

Discussion

The present results indicate that HTLV-1 replication takes place in astrocytes, but rapidly acquires all features of persistent/latent viral infection as formulated by Ahmed and Steven (Ahmed, *et al*, 1996), with (i) a progressive and selective decrease in the synthesis of viral proteins, (ii) no lytic phase of viral replication, and (iii) no virus production and an inability to infect neighboring naive cells. Such progressive restriction probably stems from the multiple specific interactions between the virus and host cell components on which every step of the HTLV-1 life cycle (entry, reverse transcription, integration, transcription, and assembly) depends. The interaction of cellular factors with the 21 bp Tax-1-responsive elements in the HTLV-1 long terminal repeat (LTR, viral promoter) directly affects the regulation of productive viral replication and the establishment and maintenance of latent viral infection within targeted cell populations (Zhao and Giam, 1991; Suzuki *et al*, 1993). Similar interactions may occur within astrocytes, as cell-specific DNA-protein complexes can be formed between the HTLV-1 LTR and cellular factors from astrocytic cell lines (Tillmann *et al*, 1994). CREB-ATF-related cellular factors of neural origin, distinct from those found in immune cells (Tillmann, *et al*, 1994), are suspected of influencing basal and Tax-1-mediated viral transcription and affecting Tax-1-mediated *trans*-activation within glial cells. These observations may explain the unique outcome of HTLV-1 infection in astrocytes, as reported in this study. In addition, restriction of viral gene expression could explain the difficulty in detecting viral product in postmortem CNS specimen from TSP/HAM patients.

The prolonged Tax-1 expression in long-term infected astrocytes is closely associated with the prolonged secretion of TNFα and MMPs, molecules often found in other neurological diseases (Eddleston and Mucke, 1993; Yong *et al*, 1998; Loddick and Rothwell, 1999). The elevated expression of MMP-9 in the cerebrospinal fluid of the majority of TSP/HAM patients (Giraudon *et al*, 1996, 1998; Lezin *et al*, 2000) and the detection of TNFα at lesions in the spinal cord (Umehara *et al*, 1994; Fox *et al.*, 1996) consistently support the concept of bystander damage of nervous tissue in TSP/HAM (Ijichi *et al*, 1993; Giraudon *et al*, 1996). The present study suggests that Tax-1-expressing astrocytes, although rare may be present in the early phase of HTLV-1 infection and play a significant role in TSP/HAM pathogenesis by consolidating the similar bystander effects presumably produced by Tax-1-expressing T lymphocytes. Indeed, inflammatory cytokines released from Tax-1-expressing astrocytes could affect astrocytic function and thus neuron survival, since we have recently demonstrated that Tax-1-induced TNFα alters astrocytic

uptake and catabolism of glutamate in astrocytes (submitted). Tax-1-expressing astrocytes may be also effective APCs for T cell-mediated immune responses. Several reports indicate that cytokines increase the expression of major histocompatibility complex antigens and co-stimulatory molecules (ICAM-1, LFA-1α, B7 isoforms) which mediate astrocyte-T cell interactions (Soos *et al*, 1999; Massa and Wu, 1995) and stimulate the proliferation of Th lymphocytes (Tan *et al*, 1998; Nikcevich *et al*, 1997). Transfected astrocytes expressing Tax-1 are able to appropriately process HTLV-1 Tax-1 into peptides, which, once associated with HLA, can be recognized by HTLV-1 Tax-1-specific T cells (Mendez *et al*, 1997). We and others have shown that HTLV-1 infection induces HLA class I and II expression on neural cells (Lehky *et al*, 1995b; Giraudon *et al*, 1995). Infected astrocytes expressing Tax-1 could thus play a role in the local activation of HTLV-1 specific T cells.

In conclusion, the prolonged expression of Tax-1 in astrocytes, resulting from contact with HTLV-1-infected T lymphocytes, and the subsequent secretion of inflammatory mediators from astrocytes raise an important question as to the possible contribution of Tax-1-expressing astrocytes in the pathogenesis of TSP/HAM, in particular during the early stage of the disease.

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