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Tumor necrosis factor alpha expression in the spinal cord of human T-cell lymphotrophic virus type I associated myelopathy/tropical spastic paraparesis patients

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HTLV-I Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP) is a chronic degenerative disease mainly affecting the spinal cord. The pathogenesis of HAM/TSP is unknown, but is thought to involve immunopathogenic mechanisms. Several reports have detected inflammatory cytokines such as tumor necrosis factor alpha (TNFα) in HAM/TSP patients. In this study, we used *in situ* hybridization (ISH) to examine the expression of TNFα RNA in spinal cord autopsy specimens from three chronic HAM/TSP patients with long-term disease (10–20 years duration). ISH identified many TNFα-expressing cells throughout all three patient's spinal cord tissues. Two patient's spinal cord tissue showed inflammatory cells, however double labeling by ISH for TNFα RNA with immunohisto-chemistry for CD45RO (a marker for memory T-cells) or CD-68 (a marker for microglia/macrophages) did not colocalize TNFα RNA with either CD45RO or CD-68 positive cells. Therefore, TNFα is expressed in the spinal cord of chronic HAM/TSP patients compared to normal controls and TNFα-expressing cells do not appear to be memory T-cells, microglia or macrophages.

Keywords: tumor necrosis factor alpha; cytokine; *in situ* hybridization; HTLV-I associated myelopathy/tropical spastic paraparesis

Introduction

Human T-cell Leukemia Virus I (HTLV-I) is associated with the neurologic disease HTLV-I Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP), a chronic progressive disease mainly affecting the spinal cord. The pathogenesis of HAM/TSP has remained elusive, but as dysregulation of the immune system is suspected. Cerebrospinal fluid (CSF) of HAM/TSP patients show increased numbers of activated T lymphocytes (Ijichi *et al*, 1989), $CD8^+$ HTLV-I-specific cytotoxic T-lymphocytes (Jacobson *et al*, 1990), and elevated levels of inflammatory cytokines including tumor necrosis factor alpha (TNFα), interferon gamma, interleukin (IL)-1, IL-6 and granulocyte-macrophage colony stimulating factor (Nishimoto *et al*, 1990; Ohbo *et al*, 1991; Kuroda *et al*, 1993; Kurada and Matsui, 1993). Also, inflammatory cells are present in the parenchyma and leptomeninges of spinal cord autopsy specimens from HAM/TSP patients (Moore *et al*, 1989; Wu *et al*, 1993). These results suggest that an inflammatory reaction is present in the central nervous system (CNS) of HAM/TSP patients, and this inflammation may be related to the pathogenesis of HAM/TSP. Although the exact inflammatory mechanism of HAM/TSP remains unclear, cytokines including TNFα appear to be important mediators.

TNFα is an inflammatory cytokine produced by many different cells, both in peripheral blood and CNS. TNFα is a pleiotropic mediator of many inflammatory responses, and high levels of TNFα is damaging to oligodendrocytes and myelin sheaths *in vitro* (Selmaj and Raine, 1988; Brosnan *et al*, 1988). Furthermore, TNFα is associated with several inflammatory diseases of the CNS, including multiple sclerosis, encephalopathy secondary to Acquired Immunodeficiency Syndrome (AIDS)

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(Lahdevirta *et al*, 1988, Mintz *et al*, 1989) and bacterial meningitis (Waage *et al*, 1989). Immunohistochemical studies demonstrated TNFα in microglia, macrophages, astrocytes and blood vessel endothelial cells from multiple sclerosis patients (Selmaj *et al*, 1991; Cannella and Raine, 1995) and in macrophages, microglia and endothelial cells in spinal cord specimens from Human Immunodeficiency Virus (HIV)-infected patients (Tyor *et al*, 1993). In addition, TNFα was localized to astrocytes and macrophages from patients with subacute sclerosing panencephalitis (Hofman *et al*, 1989).

Although the role of TNFα in HAM/TSP has been extensively investigated, results are inconclusive. Serum levels of TNFα protein (as measured by enzyme-linked immunoassay (ELISA)) is increased in HAM/TSP patients relative to controls (Tendler *et al*, 1991), although some investigators found no elevation in TNFα levels (Kuroda *et al*, 1993). Expression of TNFα RNA in peripheral blood leukocytes (PBLs) (as measured by reverse-transcriptase-polymerase chain reaction (RTPCR)) is increased in HAM/TSP patients relative to normal controls, however TNFα RNA expression in HAM/TSP PBLs is not increased relative to HTLV-I seropositive, asymptomatics or HTLV-I seronegative, neurologically-diseased controls. (Tendler *et al*, 1991). However, in another study, TNFα RNA expression was detected in PBLs from an increased number of HAM/TSP patients relative to both HTLV-I seropositive, asymptomatic controls and HTLV-I seronegative, normal controls (Watanabe *et al*, 1995).

TNFα has also been examined in the CNS of HAM/TSP patients. CSF cells stained positive for TNFα by immunocytochemistry in six of 12 HAM/TSP patients examined, while CSF cells from patients with non-inflammatory neurological disease were negative (Nakamura *et al*, 1993). In contrast, Kuroda and colleagues found undetectable TNFα protein levels in CSF (as measured by ELISA) (Kuroda *et al*, 1993). Studies using immunohistochemistry to examine HAM/TSP CNS tissues have observed TNFα staining on perivascular monocytes (Umehara *et al*, 1994) and microglia (Wu *et al*, 1993) in spinal cord specimens from HAM/TSP patients.

Although TNFα is a cytokine normally secreted during inflammation, there is increasing evidence that cells infected with HTLV-I also can secrete TNFα. Tschachler and colleagues (1989) found that nine of nine T-cells lines transfected with HTLV-I constitutively express high levels of TNFα mRNA. In addition, six of six HTLV-I infected T-cell lines directly established from patients with acute T-cell leukemia (an HTLV-I-associated lymphoproliferative disorder), also expressed high levels of TNFα mRNA (Tschachler *et al*, 1989). The constitutive production of TNFα in HTLV-I infected T-cells has been reported by several others (Lal and Rudolph, 1991; Kobayashi *et al*, 1990). Subsequent studies have shown that isolated microglial cell cultures, but not astrocytes or oligodendroglial cells, derived from adult human brain and subsequently infected with HTLV-I had detectable TNFα production, as measure by ELISA assay for protein and RT–PCR assay for mRNA (Hoffman *et al*, 1992). Similar observations were made for monocytes, a cell that is readily detected in HAM/TSP brains. Observations in our laboratory have indicated that astrocytes are infected with HTLV-I RNA (Lehky *et al*, 1995), but the ability of astrocytes to produce TNFα in response to HTLV-I infection was not determined.

In this study we used *in situ* hybridization (ISH) to examine the expression of TNFα in spinal cord specimens from three patients with chronic HAM/TSP (disease duration of at least 10 years). ISH allows fine sensitivity and specificity in detecting gene expression, and this is the first study to detect TNFα expression within the spinal cord tissues of patients with chronic HAM/TSP. We attempted to identify the immunologic phenotype of the cells expressing TNFα in spinal cord autopsy specimens by combining ISH for TNFα with immunohistochemistry for CD45RO (a marker for memory T-cells) and CD-68 (a marker for microglia/macrophages) and could not colocalize TNFα expression with any of these cell markers.

Results

ISH using the TNFα antisense RNA probe detected strong TNFα signal (brightfield microscopy, silver grain appear black) in autopsy thoracic/lumbar spinal cord specimens from all three HAM/TSP patients (Figure 1a–c, arrows). The specificity of this signal is demonstrated by the absence of signal when the TNFα sense probe was used on adjacent sections from HAM/TSP patients (Figure 1d, a representative section) and when the TNFα antisense RNA probe on sections of normal spinal cord detected no TNFα expression (Figure 1e, a representative section).

TNFα-positive cells were observed using the antisense RNA probe in all areas of the thoracic/lumbar spinal cord, including posterior column, anterior column, lateral column, gray matter, blood vessel wall, and nerve root (Figure 2a–f, data from Patient #1). Spinal cord specimens from Patients #1 were further studied for co-localization of TNFα RNA by ISH and immune cell markers by immunohistochemistry. Co-localization of TNFα RNA expression (arrows, black silver grains) with immunohistochemistry staining for the memory T-cell marker CD45RO (arrowheads, brown staining cells) revealed CD45RO-staining cells and TNFα-positive cells within the same section (Figure 3a).

However, no CD45RO positive cells co-localized with TNFα-RNA (Figure 3a and b). Similarly, co-localization of TNFα RNA expression (arrows, black silver grains) with staining for the microglia/macrophage marker CD-68 (arrowheads, brown staining cells) revealed many CD-68 staining cells and TNFα-positive cells within the same section (Figure 3c). However, there were no cells for which CD-68 staining co-localized with a TNFα-positive cell.

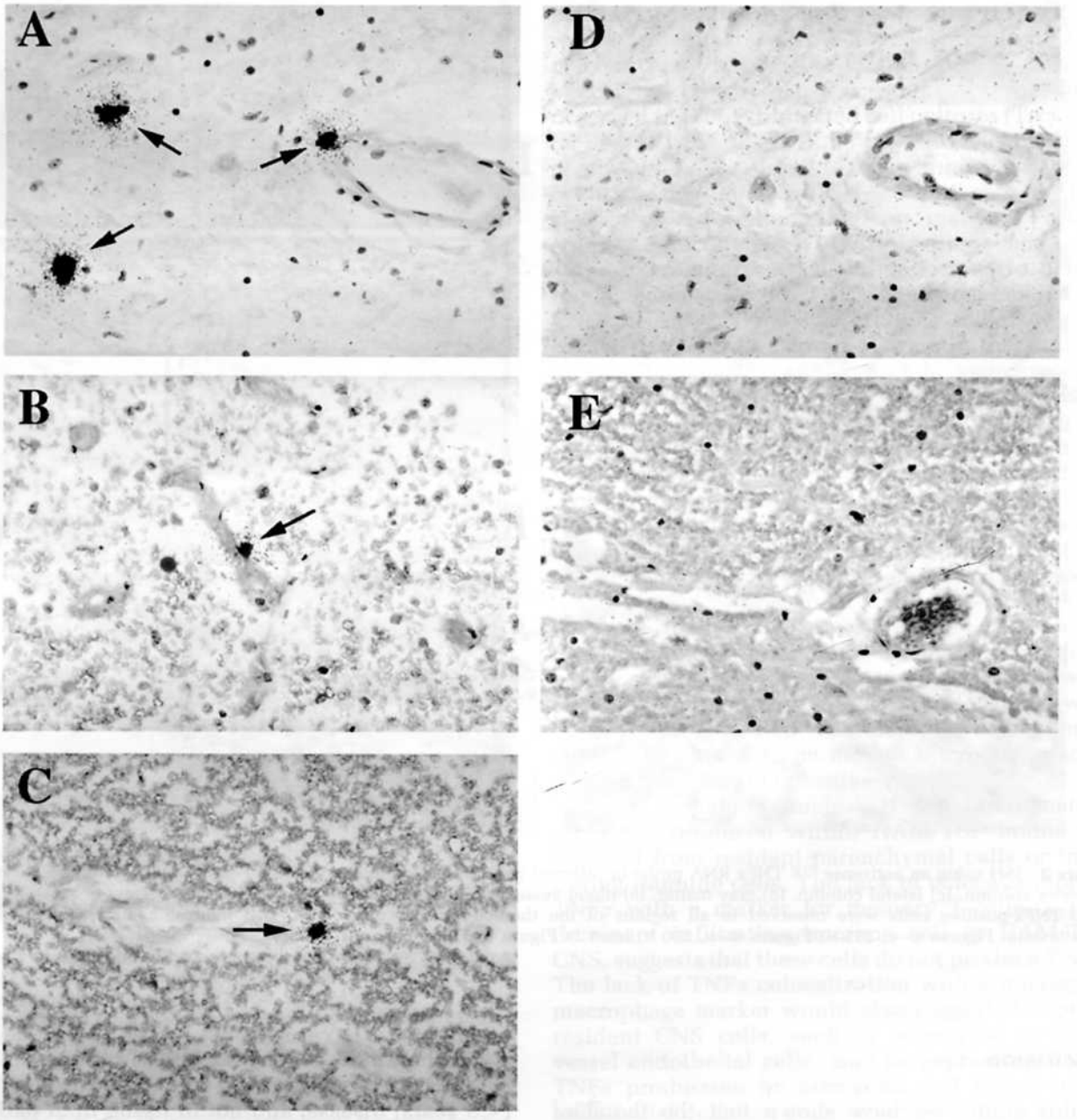

Figure 1 ISH using an antisense and sense ^{35}S TNFα RNA probe. The hybridized RNA is detected as silver grains (black dots) over TNFα-expressing cells when the antisense probe is used. The sense probe to TNFα mRNA does not anneal, and is used as a negative control. (a–c) HAM/TSP spinal cord from the thoracic/lumbar region of Patient #1, #2, and #3, respectively, using the antisense TNFα RNA probe, reveals several TNFα-positive cells (arrows). (d) an adjacent section to (a), using the sense TNFα probe did not label any cells. (e) normal spinal cord, using the antisense TNFα RNA probe, did not detect TNFα-positive cells (all figures: hematoxylin and eosin counterstain, 200×).

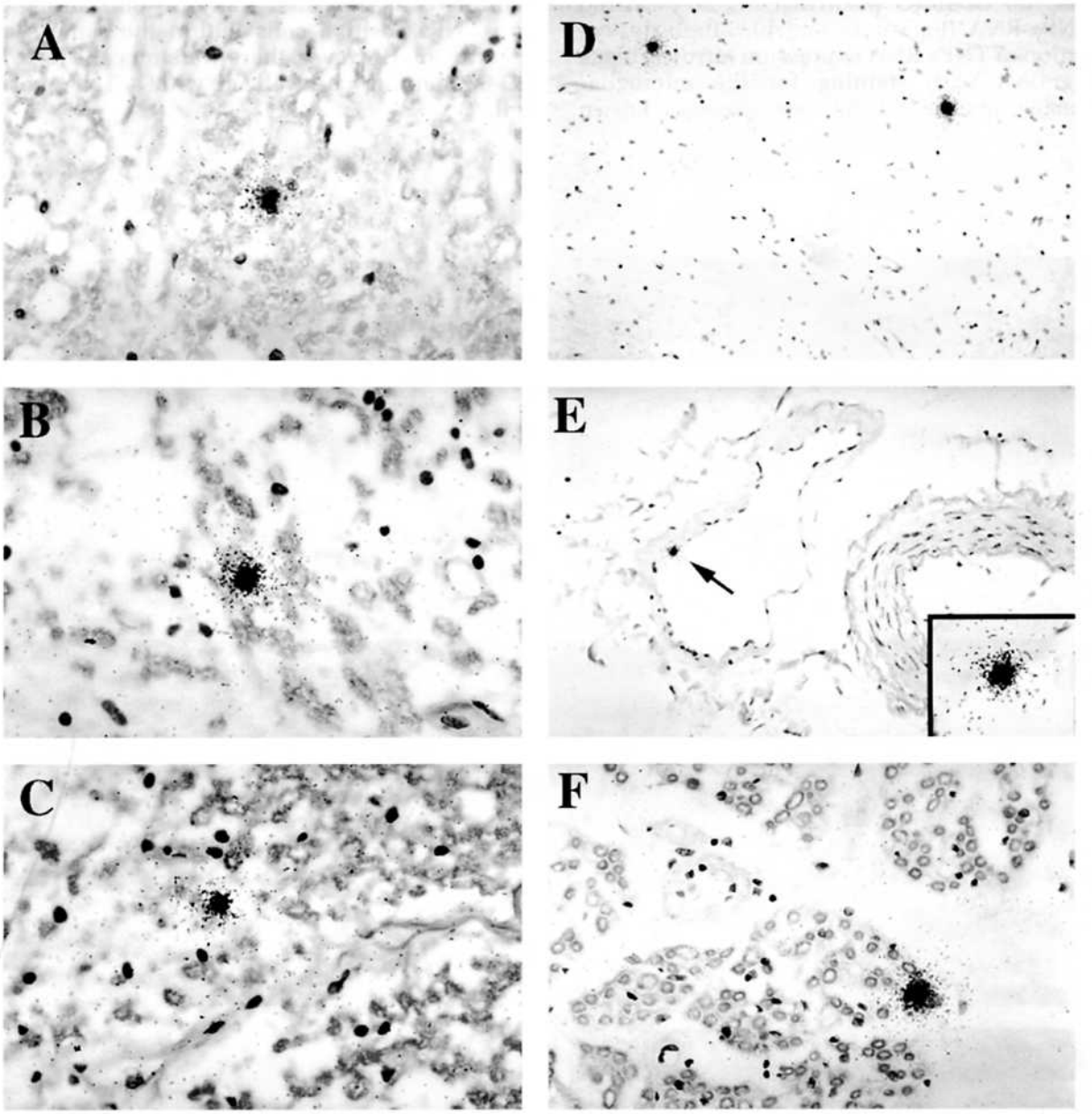

Figure 2 ISH using an antisense ^{35}S TNFα RNA probe in different regions of the spinal cord from Patient #1. (a) anterior column. (b) posterior column. (c) lateral column. (d) gray matter. (e) blood vessel wall (arrow, insert contains high power view of area). (f) nerve root. TNFα-positive cells were observed in all regions of the thoraco-lumbar spinal cord. (All figures: hematoxylin and eosin counterstain. Figures a–c, 315×; Figures d–e, 100×, inset in Figure e, 315×; Figure f, 200×.

Discussion

In this study, we have shown that the thoracic/lumbar spinal cords of three chronic HAM/TSP patients contain TNFα-expressing cells. TNFα expression was observed in all areas of the spinal cord, including the posterior column, anterior column, lateral column, gray matter, blood vessel wall and nerve root.

Umehara and colleagues (1994) observed TNFα staining only in tissue from patients with short-term (<5 years) disease, and not in tissue from patients with long-term disease. The patients in this study had a disease duration of 10–25 years and TNFα expression was detected in all three specimens. These differences may be attributed to the increased sensitivity of ISH compared to immunohistochemistry. Alternatively, ISH detects gene expression

(mRNA), but does not necessarily represent protein production. It is possible that TNFα mRNA is present without concomitant protein production to a level than can be detected by immunohistochemistry.

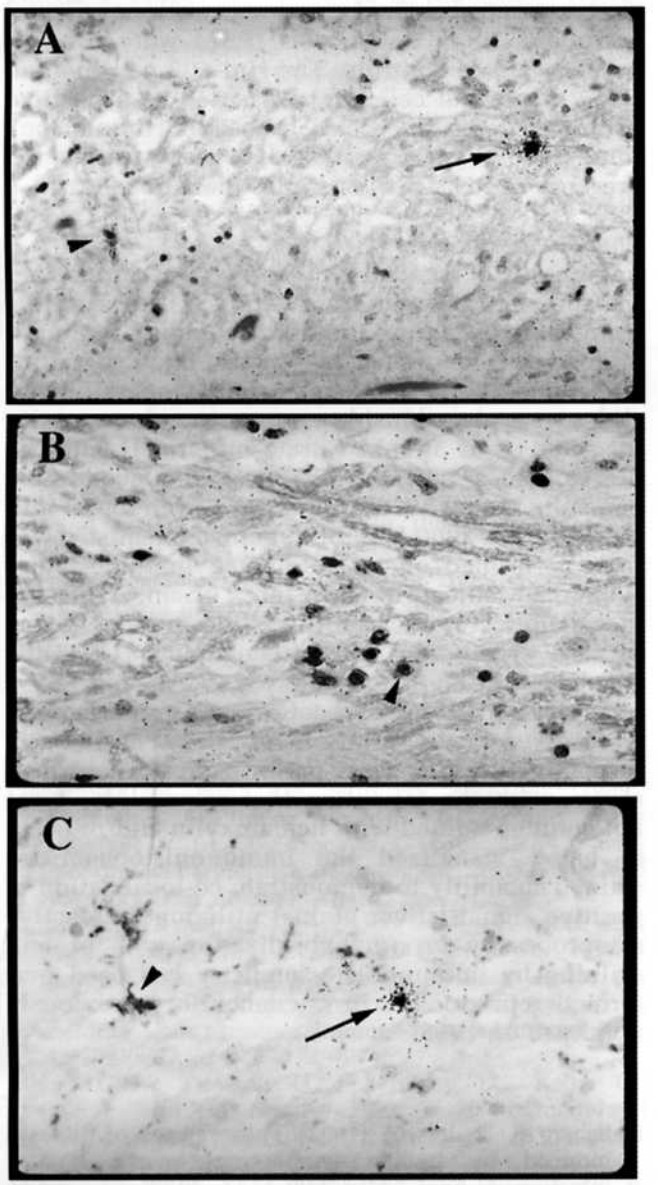

Figure 3 Immunohistochemistry with an antibody recognizing CD45RO (a marker for memory T-cells) or CD68 (a marker for microglia/macrophages) plus TNFα ISH using an ^{35}S TNFα antisense probe on spinal cord specimens from Patient #1. The CD45RO-positive cells (a and b) and the CD68-positive cells (c) are pigmented with DAB (brown staining cells) and the TNFα mRNA is detected as black silver grains. (a) TNFα-positive cell on the right side of the field (arrow), with a CD45RO-positive cell on the left side of the field (arrowhead) (hematoxylin and eosin counterstain, 200×). (b) Several CD45RO-positive cells (arrowhead, an example) with no TNFα signal (hematoxylin and eosin counterstain, 315×). (c) TNFα-positive cell is seen on the right side of the field (arrow), and several CD-68-positive cells and cell processes are seen to the left (arrowhead, an example) (hematoxylin counterstain, 315×). The TNFα-positive cells do not co-localize with either CD45RO or CD-68 staining.

In two patients from our series (Patients #1 and #3), TNFα-positive cells were present in the spinal cord with only minimal inflammatory infiltrate in the same region. This contrasts with previous immunohistochemistry studies that observed TNFα staining within inflammatory lesions (Umehara *et al*, 1994). Although these TNFα-positive cells could still represent inflammatory cells such as lymphocytes occasionally seen in these tissues, the lack of a robust inflammatory infiltrate suggests that other mechanisms of TNFα production may be present. Viral transactivation of TNFα expression has been observed in HTLV-I-infected cell cultures (Tschachler *et al*, 1989; Lal and Rudolph, 1991; Kobayashi *et al*, 1990) and it is possible that a similar process is occurring in the CNS of HAM/TSP patients (see Hollsberg and Hafler, 1993 for review). Further studies colocalizing HTLV-I positive cells with TNFα expression need to be performed to definitively demonstrate that HTLV-I infected cells in the CNS of HAM/TSP patients produce TNFα.

CNS tissues from Patient #1 were further studied by simultaneous ISH and immunohistochemistry to determine the phenotype of the cells expressing TNFα. Our laboratory has previously co-localized ISH signal (silver grains) with protein detection by immunohistochemistry in CNS autopsy specimens (Lehky *et al*, 1995). We combined ISH with immunohistochemistry for a memory T-cell marker (CD45RO) and a microglia/macrophage marker (CD-68). Double-labeling for TNFα mRNA and CD45RO demonstrated several positive cells of both types in the same section, but none colocalized. A similar observation was made with the marker for microglia/macrophages: many CD-68-positive cells were observed, but none of these cells co-localized with TNFα RNA expression. Therefore, the cells expressing TNFα appear to be neither microglia, macrophages, nor CD45RO positive T-cells.

It has remained unclear if the inflammatory cytokines produced within HAM/TSP brains are secreted from resident parenchymal cells or infiltrating immune cells. The lack of colocalization of TNFα with a marker for memory T-cells, a predominant infiltrating immune cell in HAM/TSP CNS, suggests that these cells do not produce TNFα. The lack of TNFα colocalization with a microglia/macrophage marker would also suggest that other resident CNS cells, such as astrocytes or blood vessel endothelial cells, may be expressing TNFα. TNFα production by astroyctes and blood vessel endothelial cells has been observed both in culture and in the CNS inflammatory disease multiple sclerosis. (Lieberman *et al*, 1989; Cannella and Raine, 1995).

The results of this study indicate that TNFα is expressed in all areas of the spinal cord of three patients with chronic HAM/TSP. This expression

can be observed in the absence of an inflammatory infiltrate, and does not appear to be in infiltrating memory T-cells, microglia, or macrophages. Further studies should examine TNFα expression in astrocytes and TNFα expression in HTLV-I infected cells within the CNS of HAM/TSP patients.

Materials and methods

Autopsy specimens

Thoracolumbar spinal cord specimens were obtained from three patients with HAM/TSP. Patient #1 was a 64 year old Black male from the United States with a 12-year history of HAM/TSP who died secondary to occlusive coronary atherosclerosis. Histological examination revealed meningeal, perivascular, and parenchymal inflammation, consisting of lymphocytes positive for the T-cell marker A6 (CD45RO). In addition, there was leptomeningeal thickening and vascular fibrosis, as well as focal pallor of the corticospinal tracts. Patient #2 and #3 are a 68 year old Hispanic female from the United States with a 25-year history of HAM/TSP, and a 73 year old Japanese male with a 10-year history of HAM/TSP, respectively. Pathological and immunological studies on specimens from Patients #2 and #3 have been described previously and have neuropathological findings consistent with the diagnosis of HAM/TSP (Wu *et al*, 1993; Umehara *et al*, 1993, 1994). Control CNS material was obtained from one patient who died of non-neurologic causes. CNS tissue specimens were fixed in 10% formalin and embedded into paraffin.

In situ *hybridization*

Five-micron thick paraffin sections were placed onto sialinized slides (American Histolab, Gaithersburg, MD). ISH was performed as described previously (Levin *et al*, 1996; Lehky *et al*, 1995) with a 5 day incubation for emulsion autoradiography. Slides were counterstained with hematoxylin and eosin. The ^{35}S-labeled RNA probe was a 1.2 kb fragment transcribed from the TNFα cDNA (bases 274 to 1007, Wang *et al*, 1985). Each specimen was tested with the antisense and sense TNFα RNA probes, the positive and negative probes, respectively. Control spinal cord as well as normal peripheral blood leukocytes (PBL) were used as negative tissue controls. The Hut-102 cell line (an HTLV-I infected cell line) was used as a positive control. A β-actin RNA probe (Lofstrand Inc., Gaithersburg, MD) was used to demonstrate the presence of RNA in all samples (data not shown).

Phenotypic analysis

Immunohistochemical studies were performed on the spinal cord tissue from Patient #1 prior to ISH using a modified avidin-biotin complex immunoperoxidase technique on formalin-fixed, paraffin-embedded tissue sections. Briefly deparaffinized slides were placed inside a pressure cooker containing 1500 cc of 50 mM Citrate buffer, pH 6.0. The pressure cooker and slides were then placed into a Samsung Model 5620T microwave oven for 40 min at full power (900 W). Immunohistochemistry was then performed on an automated immunostainer (Ventana Medical Systems, Inc, Tucson, AZ) using the manufacturer's paraffin slide protocol. The primary antibodies A6 (recognizing CD45RO, Zymed, South San Francisco, CA) and KP-1 (recognizing CD-68, Dako Corp, Carpinteria, CA) were used at a dilution of 1:50 and 1:80, respectively. ISH was performed as described above, with the exception that some slides were not counter-stained with hematoxylin and/or eosin to better visualized the immunohistochemistry stain. The ability to demonstrate co-localization of positive signal (silver grains) utilizing radioactive riboprobes by *in situ* hybridization with protein staining by immunohistochemistry has been performed reproducibly in our laboratory previously (Lehky *et al*, 1995).

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