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Presence of HTLV-I Tax protein in cerebrospinal fluid from HAM/TSP patients

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Summary. Infection with human T-cell lymphotropic virus type I (HTLV-I) have been associated with the development of the HTLV-I-associated myelopathy/ Tropical Spastic Paraparesis (HAM/TSP). The disease affects the pyramidal tract at the distal segments of spinal cord, generating a spastic paraparesis. We studied the presence of Tax protein in cerebrospinal fluid cells and spinal fluid (CSF) of 35 Chilean patients: 22 HAM/TSP patients (15 HTLV-I-seropositives, and 7 seronegatives), and 13 controls (9 PSP and 4 CJD non-infected patients). Tax antigens were evaluated with monoclonal antibodies reacting with Tax by immunofluorescence and ELISA assays in cerebrospinal fluid cells and CSF, respectively. Proviral was evaluated by PCR of *tax* gene in cerebrospinal fluid cells. Tax antigen was detected in CSF and lymphocytes of CSF from 4 and 12 HAM/TSP patients, respectively. Lymphocytes of CSF of 8 HAM/TSP (6 seropositives and 2 seronegatives) showed the presence of *tax* gene. These results show that cells of CSF from HAM/TSP patients are able to express and export Tax protein towards the CSF. This is the first report of the presence of Tax protein in cerebrospinal fluid cells and CSF from HAM/TSP HTLV-I seronegative patients.

Introduction

Human T lymphotropic virus type I (HTLV-I) is the retrovirus causing adult T-cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [15, 31]. HAM/TSP is a neurological disease defined by a progressive slowly spastic paraparesis that begins at fifth decade of life and it predominates

in women. A generalized hyperreflexia Babynski signs, spastic hypertonus, clinically characterize it; the weakness involves the lower limbs and their shows uncommonly sensitive symptoms. Neuropathological studies show degeneration of cortico-spinal tract in all of HAM/STP patients, and also compromise the cervical segments of the posterior columns in some cases [9].

The molecular mechanisms of the CNS tissue damage in HAM/TSP has not been fully understood. Many investigators support the hypothesis that the immune response against Tax or other viral proteins is implicated in the pathogenesis of HAM/TSP, but a direct role of these viral proteins can not be ruled out [16, 17, 24]. It has been suggested that HAM/TSP progression might be mediated by the ability of HTLV-I to activate T-cell lymphocytes. This cell activation can produce Tax to function as an intracellular molecule [26]. As soon as, the increase of several cytokines, α-TNF, vascular and cellular adhesion factors associated to lymphocytes would be pathogenetic factors [39, 40]. The activated CTL response to the Tax protein suggests that there is persistent viral replication in HTLV-I infected cells, with continuous expression of *tax* gene [16, 33, 42]. CD4+ T cells form cuffings in the perivascular areas of the CNS parenchyma and meninges (CSF) fluid harbor provirus and express Tax mRNA and protein [28]. Some investigators have reported that the sole viral reservoir within the CNS is the T cell [19], while other scientists have suggested that the effect of the virus on the induction and progression of HAM/TSP might be mediated by an indirect mechanism, such as the ability of Tax to function as an extracellular molecule [26].

Up to date, there are few studies showing the presence of HTLV-I or viral proteins in CSF or CSF's cells. Takenouchi et al. reported that the proviral loads in CSF cells of HAM/TSP patients were higher than those in PBMCs [38]. They suggested that the ratio of proviral loads in CSF cells/PBMCs would be associated with clinically progressive disease and with recent onset of HAM/TSP. These findings could indicate that clinical progression of HAM/TSP would be associated with increased proliferation or immigration of HTLV-I-infected lymphocytes to the central nervous system. Nagai et al. also reported that HTLV-I proviral load in cerebrospinal fluid cells were significantly higher than that of the matched peripheral blood mononuclear cells, and a high ratio of human T-cell lymphotropic virus type I proviral load in cerebrospinal fluid cells to peripheral blood mononuclear cells were observed in patients with short duration of illness [29].

Here, we studied the presence of Tax protein in both CSF and CSF's cells from 35 Chilean patients (22 HAM/TSP and 13 neurological controls) with the goal to progress the understanding of the molecular mechanisms associated with the CNS tissue damage in the HAM/TSP disease.

Materials and methods

Patients

We studied Tax protein in CSF from 35 neurological patients: 22 HAM/TSP (15 HTLV-I seropositives and 7 seronegatives but PBMC-tax positive by PCR) and 13 controls (9 progressive spastic paraparesis [PSP] and 4 Creutzfeldt-Jakob disease [CJD]) non-infected patients.

All cases were patients from the Neurology Service, of El Salvador Hospital, Medicine School of University of Chile. All cases had Spanish ethnic background. Other causes of progressive spastic paraparesis were excluded through clinical presentation according to neurophysiological, radiological, immunological, hematological, and cytochemical analysis [7]. Associated pathologies like as dacryosialadenitis by Shirmer's test and biopsy of minor salivary glands were studied in all HAM/TSP patients [8]. Serology of hepatitis A and B and abdominal ecography were studied in all cases. Bone densitometry were performed in some patients. Skin biopsies were carried out in patients with cutaneous lesions to define cutaneous lymphoproliferative activity. Hematological analyses were performed to detect leucemoid lymphocytes [5, 37].

Samples

10 ml of blood and 5 ml of CSF were obtained from each patient. Serum and CSF were used for antibody determinations. PBMC and CSF cells were used for DNA extraction. CSF cells were used for Tax protein determination by IFA test. All cases were selected with an Informed Consent previously accepted. The study was carried out with an approbation of Committee of Ethic from the El Salvador Hospital.

Determination of viral antigens in CSF cells by indirect immunofluorescence assay

Five hundred to one thousand of cells were washed twice with phosphate-buffered saline and allowed to dry on a microscope spot-slide for IFA testing. The cells were stained using a 1:1,000 dilution of mouse monoclonal anti-Tax, according to method previously described [13]. We judged cells to be positive for HTLV-I antigen if we observed punctuate nuclear and cytoplasmic staining in the presence of anti-HTLV-I antibody. Three different determinations with duplicate samples were performed for each patient.

Determination of viral antigens in CSF by ELISA

An indirect ELISA with 1:1,500 dilution of mouse monoclonal anti-Tax and one hundred microliters of sample was used to determine viral antigens in CSF [13, 22]. Three different determinations with triplicate samples were performed for each patient.

Gene amplification of Tax by Polymerase Chain Reaction

DNA was extracted from purified cells from CSF. An extraction control used identical procedures without PBMC. Protective clothing, separate equipment, newly prepared reagents, ultraviolet irradiation and others measures to prevent contamination were routinely used. We amplified 158 bp of *tax* with primers SK43/SK44. Primers were used at a final concentration of 1 μM in a reaction mixture of 50 μl containing 1–2 μg of each DNA sample, 2.5 units of *Thermus aquaticus* DNA polymerase and comprised of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.2 mM each dNTP. Reaction mixtures were amplified for 35 cycles in a Perkin Elmer GeneAmp PCR System 2400 using the following conditions: denaturation at 94 °C for 20 s, annealing at 50 °C for 20 s, and extension at 72 °C for 20 s. Mixtures were finally heated at 72 °C for 8 min. Amplified DNA for HTLV-I was analyzed by electrophoresis on 7.5% polyacrylamide gel followed by silver nitrate staining [34].

Results

Twenty-two HAM/TSP patients were studied: 13 women and 9 men. They had an average age of 56.9 (20–74 years) and the average time of paraparesis of 7.1

Table 1. Clinical features of 22 Chilean HAM/TSP patients

Patient	Serology	Sex	Age (years)	Evolution (years)	Motor	Sensitive	Bladder	Associated pathology
1	seropositive	M	55	11	1	−	6	dacryosialadenitis
2	seropositive	M	67	8	2	−	7	cutaneous lymphoma
3	seropositive	F	67	6	2	−	6	dacryosialadenitis
4	seropositive	F	73	28	3	+	8	dacryosialadenitis
5	seropositive	F	68	12	1	+	7	dacryosialadenitis
6	seropositive	M	42	4	1	+	7	cutaneous lymphoma osteoporosis
7	seropositive	M	64	8	1	−	7	dacryosialadenitis chronic lymphoma
8	seropositive	F	52	3	1	−	6	hepatic cirrhosis
9	seropositive	F	58	5	2	+	7	dacryosialadenitis
10	seropositive	M	49	6	1	−	6	hepatic cirrhosis
11	seropositive	F	20	6	1	−	6	cognitive imperment, osteoporosis
12	seropositive	F	55	2	1	+	7	osteoporosis
13	seropositive	F	73	7	4	+	8	dacryosialadenitis
14	seropositive	F	61	8	1	+	6	artritis
15	seropositive	F	48	10	1	−	7	dacryosialadenitis
16	seronegative	M	52	3	1	+	6	none
17	seronegative	M	46	10	1	+	6	none
18	seronegative	M	74	5	1	−	6	none
19	seronegative	F	55	2	1	−	6	cutaneous lymphoma
20	seronegative	F	48	3	3	+	8	dacryosialadenitis hepatic cirrhosis
21	seronegative	M	73	4	1	−	5	none
22	seronegative	F	52	6	1	+	6	dacryosialadenitis

Motor involvement: Spastic gait without support (1), spastic gait with support (2), wheelchair (3), bedridden (4)

Bladder involvement: Normal (5), frequency (6), urgency (7), incontinence (8)

(2–28 years). All patients had a normal CSF. Cytochemical analysis showed 1–15 cells in CSF. Table 1 shows the principal clinical features of HAM/TSP patients: sex and age, evolution, motor function, sensitive compromise, bladder performance, and the different associated pathologies: dacryosialadenitis, hepatic cirrhosis, chronic lymphoma, cutaneous lymphoma and osteoporosis.

We have also studied the blood and CSF of a control group composed by 9 Progressive Spastic Paraparesis (PSP) non-associated to HTLV-I infection, and 4 Creutzfeldt-Jakob (CJ) patients. These cases were sex and age matched with HAM/TSP patients. All PSP patients had a normal CSF and they were clinically similar to HAM/TSP cases. Cytochemical analysis of CSF samples from CJ patients showed normal results.

Detection of Tax protein in CSF cells from TSP/HAM patients

The percentage of HAM/TSP patients with Tax positive CSF cells was evaluated by IFA (Fig. 1). Tax antigen in CSF cells was detected in 54.5% (12/22) of HAM/TSP patients: 8 seropositives and 4 seronegatives (Table 2). The percentage of Tax protein-expressing cells ranged from 0.20% to 0.67%. The percentage of Tax positive cells in the seropositive HAM/TSP patients was higher than seronegative HAM/TSP patients. As demonstrated in Table 2, the percentage of Tax-expressing CSF cells in seropositive and seronegative HAM/TSP cases ranged from 0.50 to 0.67 and 0.20 to 0.43, respectively. Tax antigen was not detected in cells of CSF from any control patient. Average percentage of Tax expressing CSF cells in seropositive TSP/HAM patients (0.576 ± 0.059) was higher than seronegative TSP/HAM patients (0.298 ± 0.104).

Detection of Tax protein in CSF from TSP/HAM patients

To evaluate if there was a correlation between the presence of Tax protein in CSF cells and the level of free Tax in the CSF, a Tax Enzyme immunoassay was used to measure the Tax in CSF from all (12) patients with positive Tax expressing CSF cells. Tax antigen in CSF was detected in four HAM/TSP patients: 3 seropositive (cases 4, 9, and 14) and 1 seronegative (case 19) (Table 2). Thus, Tax antigen was

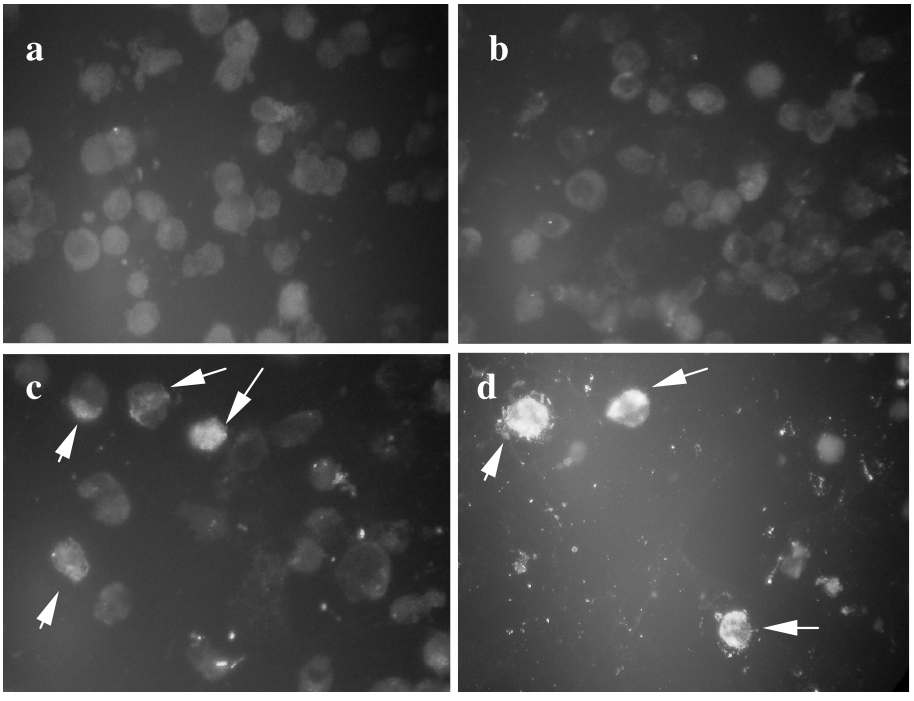

Fig. 1. Detection of Tax protein in CSF cells by IFA. **a**, H9 cells; **b**, CSF cells from a CJD patient; **c**, CSF cells from a seronegative patients with HAM/TSP; **d**, CSF cells from a seropositive patients with HAM/TSP. Arrows: CSF cells expressing Tax protein

Table 2. Detection of Tax free in CSF by ELISA, Tax antigen and tax gene in CSF's cells by immunofluorescence assay and PCR from 12 TSP/HAM patients

Patient	ELISA Average DO ± SD	ELISA Result[1]	Immunofluorescence Average percentage of positive cells ± SD	PCR tax gene
1	0.240 ± 0.024	(−)	0.57 ± 0.0577	(+)
3	0.207 ± 0.039	(−)	0.50 ± 0.1	(+)
4	0.543 ± 0.039	(+)	0.63 ± 0.0577	(+)
5	0.189 ± 0.019	(−)	0.50	(−)
7	0.199 ± 0.027	(−)	0.57 ± 0.1528	(−)
9	0.677 ± 0.043	(+)	0.67 ± 0.0577	(+)
13	0.253 ± 0.035	(−)	0.60	(+)
14	0.711 ± 0.043	(+)	0.57 ± 0.0577	(+)
18	0.217 ± 0.031	(−)	0.43 ± 0.0577	(−)
19	0.469 ± 0.038	(+)	0.33 ± 0.0577	(+)
21	0.182 ± 0.014	(−)	0.23 ± 0.0577	(−)
22	0.274 ± 0.020	(−)	0.20	(+)

Note 1: Cut-off 0.327 ± 0.015

simultaneously detected in CSF and CSF cells from these four HAM/TSP cases. Tax in CSF was detected in three seropositive patients with high percentage of Tax expressing cells. However, free Tax was found in a seronegative patient with low percentage of expressing cells among seronegative cases. Average absorbance of Tax expressing CSF from the 3 seropositive TSP/HAM patients (0.643 ± 0.089) was higher than seronegative TSP/HAM patient (0.469 ± 0.038). We did not find any relation among level of Tax antigen and percentage of CSF cells that express Tax protein in these four patients (Table 2).

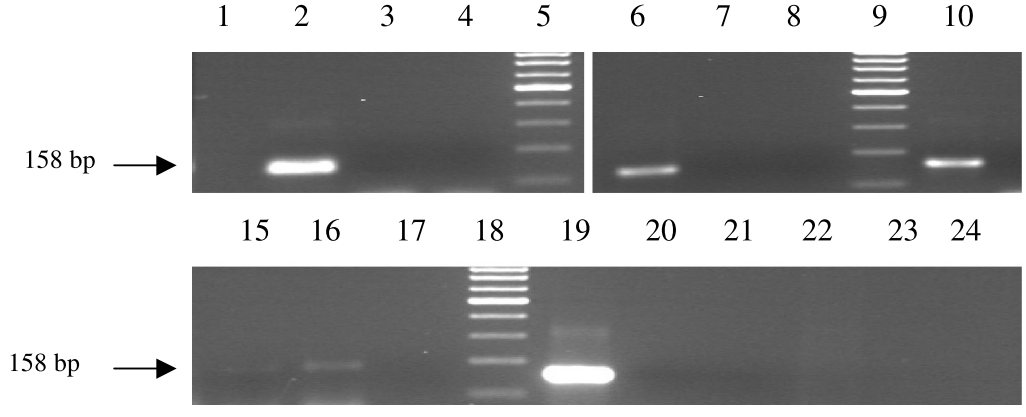

Fig. 2. Detection of *tax* of HTLV-I in CSF cells of patients with HAM/TSP by PCR. *2*, and *19*, positive control (MT2 cells); *1*, and *17*, negative control (H9 cells); *6*, *7*, *8*, and *10*, cells from seropositive patients with HAM/TSP; *15*, *16*, *20*, *21*, *22*, *23*, and *24*, cells from seronegative patients with HAM/TSP; *3*, and *4*, Creutzfeldt-Jakob disease patients; *5*, *9*, and *19*, molecular weight marker (100 bp ladder). Arrows: amplified product of *tax* gene (fragment of 158 bp)

Presence of tax gene in CSF cells from TSP/HAM patients

To study the presence of HTLV-I provirus and specifically tax gene in CSF cells, a PCR assay was performed to detect tax gene in CSF cells from all (12) patients with positive Tax expressing CSF cells (Fig. 2). Amplified genetic product of tax gene was detected in cerebrospinal fluid cells from eight patients: six HTLV-I seropositive HAM/TSP, and two HTLV-I seronegative HAM/TSP patients (Table 2). Tax gene was detected by PCR in 3 seropositive cases expressing free Tax antigen in CSF (patients 4, 9, and 14) and three seropositive patients non-expressing Tax antigen (patients 1, 3, and 13). Tax gene was detected in CSF cells from one HTLV-I seronegative HAM/TSP expressing Tax antigen in CSF (patient 19), and one HTLV-I seronegative HAM/TSP non-expressing Tax in CSF (patient 22).

Discussion

Results shows cerebrospinal fluid cells from HAM/TSP patients are able to express and export Tax protein in cerebrospinal fluid. Tax expressing CSF cells were detected in 54.5% from HAM/TSP cases. Also, free Tax in CSF was found in 18.2% of them. Tax protein in CSF was detected from patients at small (2 years), medium (5 and 8 years) and great (28 years) period of time of duration of illness of disease (patients 4, 9, 14, and 19). This finding confirms the presence of Tax protein in CSF during all stages of evolution of HAM/TSP. These results suggest that Tax antigen may be detected before the onset of symptoms. Further longitudinal studies enrolling a large number of patients must be conducted.

The presence of Tax in CSF would give to this protein the chance to keep in directly contact with central nervous tissue. Tax is a phosphoprotein can bind and activate several cellular kinases, e.g. cdK4, cdK6, and PKc [21]. Also, this viral protein can inhibit the catalytic sub-units of PP-2α phosphatase. Fu showed that Tax could establish tertiary complexes, Tax-Kinase-Phosphorilase, to maintain the catalytic activity of kinases [13]. The equilibrium among level of phosphorilation and desphosphorilation is crucial to the axonal transport and associated proteins [23]. A possible action of Tax on axonal transport could alter this system affecting the distal segments of longest axons.

The HAM/TSP neuropathology shows a symmetrical degeneration of corticospinal tracts expressed specially at dorsal and lumbar segments and a compromise of Goll tracts at cervical levels [9]. This special form of lesions could explain the relation between the failure of the axonal transport and the neuropathological injuries. This relationship makes possible to understand the clinical evolution of this progressive papaparesis, and the electrophysiologic, radiologic and hystopathologic findings that define a clinical and structural form of central daying-back degenerations [1, 6, 9, 10, 30]. This phenomena is resembling to observed in familiar spastic paraplegias where a similar damage of the axonal transport and central daying-back compromise of the longest axons is found [3, 36].

The longest axons have great metabolic requirements and they are very susceptible to injuries of the axonal transport. It may easily affect by isquemy,

oxidative stress or turn over of proteins [11]. The degenerative damage of the longest axons could be explained by the constant presence of Tax at the extracellular space of CNS and the capacity of Tax to bind with kinase or phosphatases enzymes. Electronic microscopy studies of HAM/TSP have showed the presence of abnormal accumulation of neurofilaments and Hirano bodies in some axons. These cytoskeleton alterations could affect the axonal transport suggesting a degenerative process [25]. These changes are similar those seen in Amiotrophic Lateral Sclerosis where it had been showed an inhibition of retrograde transport of growth factor by hyperphosphorilation of microfilaments [3].

The increase of lymphocytes at CSF has been described in 25 to 35% of the HAM/TSP [7, 32]. The HAM/TSP patients without increase of cells at CSF generally maintain this condition, but those cases with increase of number of cells usually maintain this tendency. Also it is possible to detect lymphocytes bind to meninges and spinal cord in different cases by hystopathology [27]. In this study it was observed the increase of lymphocytes at 31.8% of CSF. Although the patients had a low number of CSF cells, we detected intracellular Tax protein in 54.5% of them.

It has been described the increase of several cytokines, vascular and cellular adhesion factors associated to lymphocytes as pathogenetic factors [39, 40]. These facts would only explain by the activation given rise in the infected lymphocytes [33].

The presence of infected cells in the extracellular space of CNS in patients with HAM/TSP has suggested a pathogenic inflammatory disease. These lymphocytes have been the target to modify by different therapies. Feng found that α-INF administration reduce significantly the number of CD4 at CSF [12]. This decrease did not show a relationship with the health improvement of patients. On the other side, Kubota reported an infected HTLV-I patient with a great expansion of CD8 at CSF, but these lymphocytes had a clearly low reactivity against Tax antigen [20].

Tax is an oncoprotein interacting with different proteins during the cell cycle. Tax modulates the expression of many cellular genes through the CREB/ATF-, SRF- and NF-κB-associated pathways [2]. Tax can interact with a precursor of interleukin 16 [41]. This ability suggests that Tax could be able to interact with different regulatory proteins by direct protein–protein interaction. Thus, the pathogenic hypothesis based in a sole inflammatory disease is not enough to explain the degenerative damage found in patients with HAM/TSP.

To our knowledge, this is the first detection of Tax protein at CSF from seronegative patients with HAM/TSP. In addition, this is the first report of the presence of Tax protein in cerebrospinal fluid cells from these patients. The inflammatory or immune hypothesis does not permit to explain the pathogeny of HAM/TSP in these seronegative patients because they do not have immune response against HTLV-I [34]. Also it was previously detected tax gene in PBMC from seronegative patients with HAM/TSP [35], and now we report the presence of Tax protein at CSF and tax gene in CSF cells from these patients. Seronegative status with stable HTLV-I infection has been also demonstrated in animal model. Koya reported seronegative rats with lymphocytes that exclusively expressed

Tax [18]. Zucker-Franklin detected the sole presence of tax in PBMC of 8% from HTLV-I seronegative blood donors, and it was showed the transmission of tax to rabbits using PBMC from these blood donors [43]. This finding and our results supports the hypothesis that Tax protein is related with the development of a chronic, progressive, and no remitting paraparesis that affect the longest axons of motor and sensitive tracts of the spinal cord in patients with HAM/TSP.

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