Journal of Medical Virology 88:521–531 (2016)

Tax Secretion From Peripheral Blood Mononuclear Cells and Tax Detection in Plasma of Patients With Human T-Lymphotropic Virus-Type 1-Associated Myelopathy/Tropical Spastic Paraparesis and Asymptomatic Carriers

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Human T-lymphotropic virus-type 1 (HTLV-1) is the etiologic agent of the neurologic disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Tax viral protein plays a critical role in viral pathogenesis. Previous studies suggested that extracellular Tax might involve cytokine-like extracellular effects. We evaluated Tax secretion in 18 h-ex vivo peripheral blood mononuclear cells (PBMCs) cultures from 15 HAM/TSP patients and 15 asymptomatic carriers. Futhermore, Tax plasma level was evaluated from other 12 HAM/TSP patients and 10 asymptomatic carriers. Proviral load and mRNA encoding Tax were quantified by PCR and real-time RT-PCR, respectively. Intracellular Tax in CD4(+)CD25(+) cells occurred in 100% and 86.7% of HAM/TSP patients and asymptomatic carriers, respectively. Percentage of CD4(+)CD25(+) Tax+, proviral load and mRNA encoding Tax were significantly higher in HAM/TSP patients. Western blot analyses showed higher secretion levels of ubiquitinated Tax in HAM/TSP patients than in asymptomatic carriers. In HTLV-1-infected subjects, Western blot of plasma Tax showed higher levels in HAM/TSP patients than in asymptomatic carriers, whereas no Tax was found in non-infected subjects. Immunoprecipitated plasma Tax resolved on SDS–PAGE gave two major bands of 57 and 48 kDa allowing identification of Tax and Ubiquitin peptides by mass spectrometry. Relative percentage of either CD4(+) CD25(+) Tax+ cells, or Tax protein released from PBMCs, or plasma Tax, correlates neither with tax mRNA nor with proviral load. This fact could be explained by a complex regulation of Tax expression. Tax secreted from PBMCs or present in plasma could potentially become a biomarker to distinguish between HAM/TSP patients and asymptomatic carriers. ***J. Med. Virol. 88:521–531, 2016***.

KEY WORDS: HTLV-1; Tax secretion; HAM/TSP patients; asymptomatic carriers

INTRODUCTION

Human T-lymphotropic virus-type 1 (HTLV-1) infections are associated with adult T-cell leukemia (ATL) and with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP); however, most infected people remain asymptomatic carriers

Grant sponsor: Fondecyt; Grant number: 1080396; Grant sponsor: Comision Nacional de Investigacion Cientifica y Tecnologica (Conicyt) from Chile (Support Scholarship for Doctoral Thesis); Grant number: 24090150

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Accepted 19 July 2015

DOI 10.1002/jmv.24342
Published online 19 October 2015 in Wiley Online Library (wileyonlinelibrary.com).

[Bangham, 2000; Nagai and Osame, 2003; Verdonck et al., 2007; Yamano et al., 2009]. During cellular infection, HTLV-1 proviral DNA primarily integrates in CD4+ cells and to a lesser extent in $CD8^+$ T lymphocytes, B cells, monocytes, and dendritic cells [Nagai and Osame, 2003; Asquith et al., 2007; Pique and Jones, 2012].

Among HTLV-1 proteins, Tax oncoprotein acts on many viral and cellular processes, interfering with the cell cycle and with various other pathways by interaction with more than 100 cellular proteins [Boxus et al., 2008; Kfoury et al., 2012]. Tax up- or down- regulates more than 300 cellular genes, and affects the stability and activity of numerous cellular effectors [Lodewick et al., 2011; Romanelli et al., 2013]. HTLV-1 gene expression is activated by Tax through the interaction with members of the ATF/CREB family of transcription factors. This action increases the dimerization and the binding affinity of ATF/CREB proteins to Tax-responsive elements (TRE) present in the HTLV-1 5′ LTR [Okada and Jeang, 2002; Lodewick et al., 2011]. The homolog transcriptional coactivators CBP and p300 are then recruited to the transcription complexes through direct interaction with Tax, leading to nucleosomal histone acetylation and transcriptional activation [Nyborg et al., 2010].

In addition to the ATF/CREB pathway, Tax induces cellular gene expression involved in proliferation and differentiation of T lymphocytes by activation of the NF-κB pathway. Tax binds to IKKγ in the cytoplasm, causing NF-kB translocation to the nucleus [Harhaj and Harhaj, 2005; Journo et al., 2009; Shibata et al., 2011]. Tax recognizes IKKγ/NEMO—the regulatory subunit of the NF-κB inhibitor kinase (IKK) complex—resulting in activation of the IKKα and IKKβ catalytic subunits of the complex. This activation induces phosphorylation, polyubiquitination, and degradation of the NF-κB inhibitor (IκB) [Shembade and Harhaj, 2010]. As a consequence, NF-κB p50/RelA heterodimers are released from cytoplasmic sequestration by the IκB inhibitors, leading to active p50/RelA form accumulation in the nucleus, thus allowing activation of NF-κB controlled promoters [Harhaj and Harhaj, 2005; Harhaj et al., 2007].

Tax protein has been reported as a nuclear and cytoplasmic protein [Meertens et al., 2004; Alefantis et al., 2005a]. Post-translational modifications of Tax may control its cellular localization and function [Shirinian et al., 2013]. Nuclear Tax is usually reported as a SUMO modified protein, while cytoplasmic Tax is ubiquitinated, suggesting that SUMOylation is a nuclear localization signal, whereas ubiquitination is both a cytoplasmic localization and a secretion signal [Jain et al., 2007a; Lodewick et al., 2011; Kfoury et al., 2012; Bonnet et al., 2012; Xiao, 2012]. Stable ubiquitination of Tax C-terminal lysines proved to be an important regulator of Tax-mediated NF-κB activation [Chiari et al., 2004; Nasr et al., 2006; Journo et al., 2013]. Tax polyubiquitin chains are composed predominantly of K63-linked ubiquitin [Shembade et al., 2007; Xiao, 2012]. Although nuclear Tax is also found SUMOylated with either SUMO-1 or SUMO-2/3, its transcriptional role on Tax-induced NF-κB has been discarded [Pene et al., 2014]. Different Tax species could play differential roles during HAM/TSP progression. Nuclear Tax could exert the transactivator function on viral and cellular genes, producing T lymphocyte activation and cell transformation, while extracellular Tax could exert a cytokine-like action, and affect, among others, cell-to-cell communication processes.

Tax was demonstrated to co-localize with several cytoplasmic organelles associated with exocytosis, including the endoplasmic reticulum and the Golgi complex, suggesting migration through these two organelles during secretion [Alefantis et al., 2005b; Irish et al., 2009]. Tax protein has both a nuclear export signal and two putative secretory signals at the C-terminal portion [Alefantis et al., 2005a; Chevalier et al., 2005; Jain et al., 2007a]. Tax is also secreted from in vitro transformed cells by means of secretory-like vesicles [Alefantis et al., 2005a,b]. A previous report showed that ubiquitinated Tax is secreted via ER-Golgi pathway from PBMCs of HTLV-1 infected subjects [Medina et al., 2014].

Tax protein has been detected in culture medium of HTLV-1-transformed cell lines [Lindholm et al., 1990; Alefantis et al., 2005b]. In addition, we had found Tax in CSF of HAM/TSP patients [Cartier and Ramirez, 2005]. Tax secreted by PBMCs from infected subjects may account for the presence of this viral protein in CSF. In this study, we followed the content of tax mRNA and Tax viral protein in PBMCs from HTLV-1 infected subjects (HAM/TSP patients and asymptomatic carriers). In both cases, significantly higher values were determined in HAM/TSP patients together with a higher percentage of CD4(+) CD25(+) Tax(+) cells. Tax secreted from PBMCs was found ubiquitinated in both groups, being higher in HAM/TSP patients. Finally, increased amounts of Tax plasma levels were found in HAM/TSP patients compared with asymptomatic carriers.

MATERIALS AND METHODS

HAM/TSP Patients and Asymptomatic Carriers

All the experiments were performed in compliance with relevant laws and the University of Chile Ethics Committee guidelines in accordance with the ethical standards of the declaration of Helsinki. The informed consent was obtained from all individuals. Diagnosis of HTLV-1 infection was determined by serological tests (HTLV-I/II ELISA 4.0 by MP diagnostics, France, and indirect immunofluorescence assay with MT-2 cell line) and PCR analysis [Ramirez et al., 2004]. EDTA-treated blood was obtained from a total of 27 HAM/TSP patients, 25 asymptomatic carriers and 8 non-infected subjects. HAM/TSP patients fulfilled criteria of gait commitment

according to The World Health Organization, Osame's Motor Disability Score (OMDS) from 0 to 13 (0 normal gait and running, and 13 completely bedridden). The patient group composed by a male: female of 0.5:1 and a mean age of 57.8. The asymptomatic carrier group was characterized by absence of motor disability, a male:female ratio of 0.8:1 and a mean age of 41.7. Asymptomatic carriers were used as a reliable infected control group without neurological manifestations, and healthy donors were used as non-infected controls.

PBMCs Isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from 8 ml of EDTA-treated blood by Ficoll-Hypaque density gradient centrifugation; they were washed three times with phosphate-buffered saline (PBS). The number of PBMCs collected varied between 12.0×10^6 to 23.2×10^6.

Flow Cytometry

PBMCs were cultured 18 hr in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco) and 20 nM Concanamycin A (Sigma–Aldrich, St. Louis, MO) in order to inhibit the action of $CD8^+$ cytotoxic T lymphocytes [Alberti et al., 2011]. Cells were harvested and stained with fluorophore-conjugated antibodies against the following antigens: CD4-FITC (BD Biosciences, San Jose, CA), and Tax-APC, kindly provided by Dr. Yuetsu Tanaka. For nuclear Tax staining, cells were permeabilized with fixation and permeabilization reagents (eBiosciences, San Diego, CA). Matched isotype controls were used at the same concentration as the respective antibodies. A three-color flow cytometry in a FACS-CANTO instrument (Beckton Dickinson, San Jose, CA) was performed using WinMDI 2.9 software for data analysis. To calculate mean fluorescence intensity (MFI) the MFI software (University of Massachusetts) was used. Mean fluorescence of each marker over CD4+ subpopulation was employed to determine the level of Tax protein. Values of MFI plotted were divided by 100 to keep the same scale range of the mRNA data.

Isolation of CD4+CD25+ T Cells

CD4+ T cells were positively isolated from cryopreserved PBMCs with the Dynal CD4+ Positive Isolation Kit (Invitrogen Dynal, Oslo, Norway) according to manufacturer instructions. CD25+ cells were enriched with PE-anti-CD25 and anti-PE magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) using the Midi-MACS system (Miltenyi Biotech). The purity of the resulting CD4+CD25+ T cells was shown to be 90% by flow cytometry [Ramirez et al., 2010].

Tax Detection in Culture Medium From PBMCs by Western Blot

PBMCs (2×10^6 cells) were cultured 18 hr in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 20 nM concanamycin A (Sigma–Aldrich). Tax secreted from PBMCs was determined in 20 μl of the supernatant culture medium. Plasma Tax was evaluated from 1 μl of plasma further diluted 1:4 with distilled water. SDS/PAGE was performed with 10 or 12% polyacrylamide gels. Electrotransfer of proteins was performed with 600 mA at 4°C on nitrocellulose membrane (0.45 μm) using buffer with 25 mM Tris–HCl, 192 mM glycine and 20% (v/v) methanol. Primary antibodies were used diluted in TBS–T buffer (20 mM Tris–HCl, 137 mM NaCl, 0.1% [v/v] Tween-20, pH 7.6). Two anti-Tax antibodies were used: ascitic fluid from the HTLV-1 Tax hybridoma 168A51-2 (NIH, AIDS Reagent Program, Germantown, MD, Cat. 1316) diluted 1:1000 and a commercial monoclonal anti-Tax diluted 1:1000 (Covalab, Villeurbanne, France, Cat. Mab0022). Ubiquitin was detected with anti-ubiquitin monoclonal antibodies diluted 1:1000 (Upstate, Lake Placid, NY, Cat. 04-263). In all cases, primary antibodies were maintained with shaking for 12 hr at 4°C. After 5 washes with TBS–T (5 min each), membranes were incubated 1 hr with ImmunoPure Goat Anti-Mouse IgG (H+L) Peroxidase conjugated diluted 1:500,000 (Pierce Biotechnology, Rockford, IL, Cat. 31430), except for Tax determination in plasma, where donkey anti-mouse antibody HRP conjugated diluted 1:2500 (Thermo Fisher Scientific, Rockford, IL, Cat. SA1100, absorbed for several species) was used to prevent immunoglobulin interferences. Membranes were incubated with enhanced chemiluminiscence SuperSignal West Femto Chemiluminiscent substrate (Pierce Biotechnology). Finally, X-ray films (CL-Xposure film, Pierce Biotechnology) were exposed for different times. Blot quantification was carried out by scanning films using Uni-Scan-It Automated Digitizing System (Silk Scientific, Orem, UT).

After Tax analysis nitrocellulose membrane stripping was performed using a dehybridization buffer (200 mM glycine, 3.5 mM SDS, 10% Tween-20, pH 2.2) and rehybridized with antibodies against ubiquitin.

Real-Time PCR of Proviral DNA

DNA was extracted from PBMCs according to protocol described previously [Ehrlich et al., 1990]. The following genomic regions were amplified: 158 bp of HTLV-1 *tax* with primers SK43/SK44, and 268 bp of β-globin gene with primers PC04 and GH20 for internal calibration [Ehrlich et al., 1990]. DNA was amplified using Brilliant® II SYBR® Green master mix (Agilent Technologies Stratagene, La Jolla, CA, Cat. 600843). All assays were carried out in triplicate and the average value was used for calculations. The HTLV-1 proviral DNA load was calculated by the following formula: copy number of HTLV-1 *tax*/100 cells = (copy number of tax/[copy number of β-globin/2]) × 100.

Real-Time RT-PCR of Tax mRNA

RNA was extracted from PBMCs using Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was extracted from a different aliquot of the same samples used for DNA extraction. Primers for the amplification of HTLV-1 mRNA encoding Tax were previously described [Yamano et al., 2002]. The forward primer RPX-3 and the reverse primer RPX-4 + 3 were located upstream and downstream of the second splice junction site of HTLV-1 pX (tax/rex) mRNA. The amplified DNA fragment was 147 bp long. We used the human housekeeping gene hypoxanthine ribosyl transferase (HPRT) primers for internal calibration. The amplified control DNA was 80 bp [Specht et al., 2001]. cDNA was amplified using Brilliant® II SYBR® Green master mix (Agilent Technologies Stratagene, Cat. 600843). Amplifications were carried out in triplicates. Analysis of melting curves showed a single peak for each product amplified, matching with the size of PCR products analyzed in agarose gels. Relative quantitation was made with the comparative threshold cycle (ΔΔCT) formula with HPRT as endogenous housekeeping gene. Standard curves for the value of HTLV-1 tax mRNA and HPRT mRNA were generated using cDNA from MT-2 cells.

Tax Immunoprecipitation From Plasma

A pool of six HAM/TSP patients samples (165 μl of each plasma sample) was immunoprecipitated for mass spectrometry analysis. The pooled plasma sample was concentrated with 2 ml of ice-cold acetonitrile, maintained 2 hr at 4°C and then centrifuged at 12,000g for 20 min. The supernatant was removed and the remaining acetonitrile was eliminated by vacuum centrifugation for 15 min at room temperature. The pellet was resuspended in 1 ml of PBS and immunoprecipitated using "AminoLink Plus® Immobilization Kit" (Pierce Biotechnology, Rockford) according to manufacturer's instructions. Portions of 20 μl of two monoclonal anti-Tax antibodies (Covalab, Cat. mab0022, and the NIH hybridoma aforementioned) were linked to agarose matrix. The concentrated plasma sample was added and maintained with agitation overnight at 4°C. The volume of Tax eluate was reduced by vacuum centrifugation and resuspended in 30 μl of PBS. Salts were removed using ultracel ultrafiltration discs of 0.025 μm (Merck-Millipore, Darmstadt, Germany). Samples were submitted to SDS/PAGE and the gel was stained using GelCode™ Blue Stain Reagent (BioRad Laboratories, Hercules, CA) compatible with Mass spectrometry analysis.

Mass Spectrometry Analysis

SDS–PAGE bands were excised, destained, dithiothreitol and iodoacetamide treated, and proteolyzed in gel with trypsin. Peptides from proteolysis were extracted, mixed, and concentrated by vacuum centrifugation. Concentrated peptides were dissolved in 30 μl of 0.1% formic acid and 5% methanol. For LC-MS/MS analysis, 20 μl of the dissolved peptides were separated using a C18 column (Jupiter-Proteo 150 × 1.0 mm, 4 μm, and 90Å, Phenomenex, Torrance, CA) at room temperature using mobile phases A and B. Phase A contains 0.1% formic acid in water, and phase B 0.085% formic acid in 80% acetonitrile. The following conditions were employed: 0–10 min with phase A containing 2% of phase B, then 10–70 min with phase A under a gradient of phase B between 2% and 100% and 70–80 min with phase B. Chromatograms and spectra were acquired at positive mode on an Esquire 4000 LC-ESI IT MS/MS instrument (Bruker Daltonik, Bremen, Germany). Experimental data were examined on a local Mascot server against Tax (accession number BAB18052) and ubiquitin (accession number 225698038) sequences.

Statistics

Statistical analysis was made with GraphPad Prism version 6.01 software. Data were verified for Gaussian distribution. Data did not fit to a normal distribution according to the Shapiro–Wilks test, therefore, the non-parametric test of Kruskal–Wallis was used to determine differences between means of HAM/TSP patients with asymptomatic carriers and non-infected subjects. Significance was assumed at $P < 0.05$.

RESULTS

Ex Vivo Levels of Proviral Load and Tax mRNA in PBMCs, and Percentage of T Cells Expressing Tax From HAM/TSP Patients and Asymptomatic Carriers

To evaluate the ex vivo expression of tax mRNA and Tax protein in cells, we cultured PBMCs from HAM/TSP patients and asymptomatic carriers for 18 hr. Both viral mRNA and protein were detected in PBMCs and CD4(+)CD25(+) cells of all HAM/TSP patients (Table I). Thirteen asymptomatic carriers (86.7%) showed tax mRNA and Tax protein, in one subject neither the messenger nor the protein was detected, whereas another subject had an almost undetectable low tax mRNA and negative Tax protein (Table I). The levels of tax mRNA and the percentage of Tax(+) cells in HAM/TSP patients were significantly higher than in asymptomatic carriers ($P < 0.002$ and <0.0001, respectively). The average of tax mRNA and the percentage of CD4(+)CD25(+) Tax+ cells in HAM/TSP patients were 2.21 and 2.56, respectively; although the average of these parameters decreased to 1.49 and 1.39 in asymptomatic carriers, respectively. Accordingly, the average of proviral load was significantly higher in HAM/TSP patients than in asymptomatic carriers (Table I). Consequently, all these findings show that ex vivo

TABLE I. Levels of HTLV-1 Provirus (PVL) and Tax mRNA in PBMCs, and Percentage of CD4(+)CD25(+) Tax(+) Cells From HAM/TSP Patients and Asymptomatic Carriers

Category	Case	Age (year)	Gender	Provirus Tax[a]	mRNA Tax	% CD4CD25 Tax (+)
HAM/TSP	1	69	F	97.08	1.50	2.50
HAM/TSP	2	74	F	32.87	2.50	1.40
HAM/TSP	3	67	F	47.12	1.20	1.40
HAM/TSP	4	59	F	35.10	0.37	0.70
HAM/TSP	5	72	M	74.80	3.15	1.80
HAM/TSP	6	35	F	31.83	6.99	11.30
HAM/TSP	7	57	M	18.70	1.53	1.10
HAM/TSP	8	53	M	75.32	0.78	2.90
HAM/TSP	9	61	M	1.38	2.56	2.20
HAM/TSP	10	47	F	0.72	2.40	2.50
HAM/TSP	11	48	F	46.84	0.26	2.50
HAM/TSP	12	51	F	0.91	0.57	0.90
HAM/TSP	13	50	F	16.27	1.50	2.10
HAM/TSP	14	56	M	8.19	0.29	2.30
HAM/TSP	15	68	F	75.78	7.50	2.80
Average ± sd		57.8 ± 10.87		37.53 ± 31.32	2.21 ± 2.23	2.56 ± 2.51
Carrier	1	34	F	17.40	1.33	0.90
Carrier	2	35	M	11.26	1.37	1.10
Carrier	3	38	F	0.08	2.46	2.10
Carrier	4	36	F	14.30	1.17	1.30
Carrier	5	35	M	0.40	2.28	1.70
Carrier	6	21	F	6.20	2.88	2.30
Carrier	7	39	F	8.60	1.42	0.80
Carrier	8	33	F	0.06	2.23	2.10
Carrier	9	54	M	0.18	0.02	0.00
Carrier	10	59	F	0.20	2.89	1.80
Carrier	11	46	F	9.96	0.00	0.00
Carrier	12	30	M	14.40	1.00	1.50
Carrier	13	51	M	14.00	0.99	1.90
Carrier	14	58	F	5.20	1.12	1.90
Carrier	15	57	M	6.82	1.12	1.50
Average ± sd		41.7 ± 11.64		7.27 ± 6.15	1.49 ± 0.89	1.39 ± 0.71
P value				<0.0001	<0.002	<0.0001

[a]PVLs are reported as copy number Tax/100 cells.

cultured cells from HAM/TSP patients and asymptomatic carriers express both tax mRNA and intracellular Tax protein. The relative percentage of CD4(+)CD25(+) Tax+ cells does not correlate with tax mRNA ($P = 0.529$; $R^2 = 0.141$) nor proviral load ($P = 0.590$; $R^2 = 0.223$).

Ex Vivo Levels of Ubiquitined Tax Protein Secreted From PBMCs of HAM/TSP Patients and Asymptomatic Carriers

In our previous report, Tax was found to be secreted from PBMCs as a ubiquitinated form, probably with two ubiquitin subunits, inferred from the molecular mass of 57 kDa by Western blot, as Tax is a 40 kDa protein and ubiquitin a polypeptide of 8.5 kDa [Medina et al., 2014]. Figure 1A shows a representative Western blot HAM/TSP and carrier samples revealed against anti-Tax antibodies (Covalab) and with anti-ubiquitin after dehybridization. Tax measured in PBMCs supernatant represents secreted viral protein but not death of Tax-expressing HTLV-1 infected cells, because in the previous report we discarded cell death measuring LDH release using a cytotoxicity assay [Medina et al., 2014]. Tax was expressed as the relative amount per 20 μl of PBMCs supernatant, the use of loading control was difficult because PBMCs were cultured with 10% fetal bovine serum. Herein, densitometric data from Western blot of HAM/TSP patient and carrier culture media were expressed as relative units of Tax. Western blot normalization of different samples of HTLV-1 infected subjects was carried out using a common patient sample in all the blots as an internal control (P+ in Fig. 1A). The secretion of Tax from PBMCs of HAM/TSP patients were significantly higher than those of asymptomatic carriers ($P < 0.01$; Fig. 1B). The ranges varied from 0.60 to 14.2 in HAM/TSP patients and from 0.18 to 5.33 in asymptomatic carriers. Tax protein released from PBMCs correlate neither with tax mRNA nor with proviral load. No correlation was found between the secreted viral protein and, using OMDS, the motor dysfunction.

Plasma Tax Detected by Western Blot

Tax determination in plasma was carried out in a second group of HAM/TSP patients, as reported in Table II. Figure 2A shows a representative Western blot of Tax protein in plasma samples from HAM/TSP

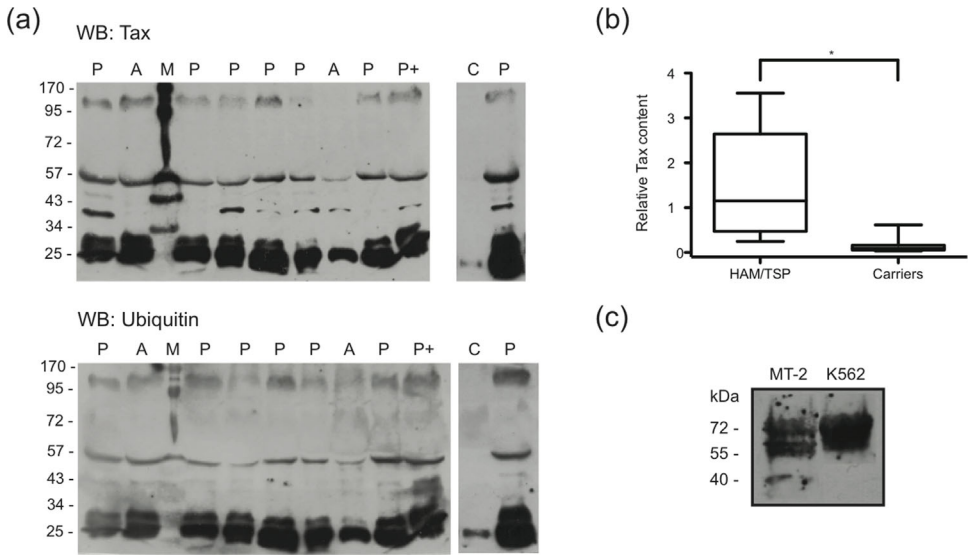

Fig. 1. Tax levels in culture medium of PBMCs from HAM/TSP patients and asymptomatic carriers. **A**: Representative Western blot with anti-ubiquitin monoclonal antibodies (upstate, dil 1:1000) and with anti-Tax monoclonal antibodies (NIH, dil 1:1000). Lanes are (from left to right), P: HAM/TSP patient N° 2, 6, 7, 10, 14, and 15 shown in Table I; A: Asymptomatic carrier N° 6 and 12 shown in Table I; M, molecular mass standard; P+, PBMCs culture medium from a single patient used for normalization purposes (HAM/TSP N° 1); C, negative control. Western blot sections correspond to a same dehybridized membrane. **B**: Densitometry of extracellular Tax levels from HAM/TPS patients and asymptomatic carriers. Statistical analysis include the mean values ± SEM ($^*P = 0.003$). **C**: Western blot of 40 kDa Tax protein from MT-2 cell culture medium as positive control from HTLV-1 infected cells using anti-Tax monoclonal antibodies (Covalab, dil 1:1000). K562 cell culture medium was used as non-infected control, showing non-specific signals on the blotting.

patients (P), asymptomatic carriers (A), controls (C, healthy subjects). The patient called P+ (patient 4) was used for Western blot normalization purposes of the densitometric analysis (Fig. 2A). The specific anti-Tax reactivity was detected at 57 kDa, which is the same to that found for PBMC secreted Tax. Tax was not detected in healthy subject samples (eight subjects). A significant increase in plasma Tax content was found in HAM/TSP patients compared with asymptomatic carriers ($P < 0.04$; Fig. 2B). No

TABLE II. Levels of HTLV-1 Provirus and Tax mRNA in PBMCs From HAM/TSP Patients and Asymptomatic Carriers Used for Plasma Tax Analysis

Category	Case	Age (year)	Gender	Provirus Tax[a]	mRNA Tax
HAM/TSP	1	48	F	15.67	3.22
HAM/TSP	2	80	F	11.85	4.80
HAM/TSP	3	35	F	23.61	6.99
HAM/TSP	4	71	M	14.65	1.29
HAM/TSP	5	72	F	17.03	1.21
HAM/TSP	6	69	F	17.74	1.2
HAM/TSP	7	59	M	9.54	1.84
HAM/TSP	8	59	F	19.49	0.001
HAM/TSP	9	51	F	4.35	0.57
HAM/TSP	10	42	F	22.15	2.21
HAM/TSP	11	67	F	17.92	7.5
HAM/TSP	12	73	F	4.00	10.22
Average ± sd		60.5 ± 13.93		14.83 ± 6.32	3.42 ± 3.24
Carrier	1	26	F	0.17	0.57
Carrier	2	36	F	2.15	0.48
Carrier	3	38	F	3.15	0.91
Carrier	4	57	M	1.83	0.19
Carrier	5	60	F	0.18	0.56
Carrier	6	47	M	9.91	0.23
Carrier	7	44	F	0.16	0.31
Carrier	8	63	M	8.92	0.01
Carrier	9	54	M	2.14	0.93
Carrier	10	58	F	4.40	0.47
Average ± sd		48.3 ± 12.15		3.30 ± 3.50	0.47 ± 0.29
P value				0.0001	0.0009

[a]PVLs are reported as copy number Tax/100 cells.

correlation was observed between plasma Tax and tax mRNA ($P = 0.3320$, $R^2 = 0.047$) or proviral load ($P = 0.0703$, $R^2 = 0.155$; Fig. 2C). Tax content in plasma, as Tax+ T cells, did not correlate with the clinical condition of patients using the OMDS ($P = 0.5284$, $R^2 = 0.041$; data not shown).

Mass Spectrometry Analysis of Plasma Tax

Immunoprecipitated Tax from a plasma pool was submitted to SDS/PAGE analysis, showing an intense band of 57 kDa and another faint band of 48 kDa. A band al 40 kDa, corresponding to non-ubiquitinated Tax was not visualized. Both bands would correspond to mono- and di-ubiquitinated Tax forms (Fig. 3), which were proteolyzed and analyzed by mass spectrometry. The non-ubiquitinated Tax was not detected. The analysis using Mascot local server exploring the presence of Tax according to the accession number BAB18052 showed the results summarized in Table III. As shown, both protein bands exhibited similar sequences between them and compared with databases using the corresponding Tax accession number. Tax protein was previously detected by the same mass spectrometry analysis for immunoprecipitated Tax from MT-2 cells, a chronically HTLV-1 infected cell line [Medina et al., 2014]. A similar approach was used for detection of ubiquitin bound to the 57 and 48 kDa forms of Tax, where one ubiquitin peptide was detected in both samples under accession number of ubiquitin conserved domain, N° 225698038 (Table III). These results are not conclusive for identifying the lysines mono-ubiquitinated or a single lysine di-ubiquitinated. However, this is the first approach for mass spectrometric determination of Tax on human plasma samples. Likewise, these results suggest the relationship of both 57 and 48 kDa bands with ubiquitinated Tax protein. Further studies are necessary to confirm our findings in this screening study.

DISCUSSION

In this experimental analysis, both tax mRNA and the viral protein were determined in 100% of the HAM/TSP patients (n = 15). These results are similar to a previous report that showed 93.8% tax mRNA expression in HAM/TSP patients [Yamano et al., 2002]. However, our findings do not agree with a research reporting viral mRNA only in 39.3% of Japanese HAM/TSP patients [Saito et al., 2009]. Although the experimental methods used in these

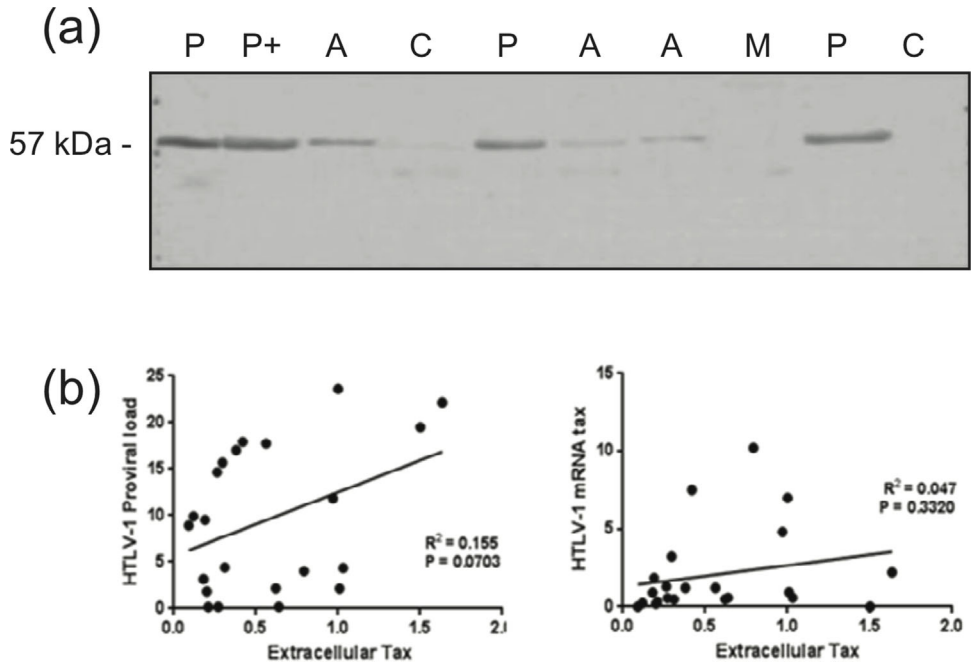

Fig. 2. Tax protein in plasma samples from HAM/TSP patients and asymptomatic carriers. **A**: Representative Western blot of plasma samples from HAM/TSP patients and asymptomatic carriers using anti-Tax antibodies diluted 1:1000 (NIH hybridoma) and secondary donkey anti-mouse antibodies, HRP conjugated, diluted 1:2500. Lanes are (from left to right) P, HAM/TSP patient N° 2, 9, and 10 shown in Table II; A, asymptomatic carrier N° 1, 6, and 10 shown in Table II; C, non infected control; M,,olecular mass standard; P+, plasma sample from a single patient used for normalization purposes. **B**: Relative amount of Tax in plasma samples from HAM/TSP patients and asymptomatic carriers refered to the densitometric analysis of a common HAM/TSP plasma sample (corresponding to patient 4 shown in Table II). Statistical analysis include the mean values ± SEM ($^*P = 0.04$). **C**: Correlation between the level of extracellular or plasmatic Tax and HTLV-1 proviral load, and mRNA tax. The levels of extracellular Tax were not significantly correlated with HTLV-1 proviral load ($P = 0.0703$, $R^2 = 0.155$), and HTLV-1 mRNA tax ($P = 0.3320$, $R^2 = 0.047$) in 12 HAM/TSP patients and 10 asymptomatic carriers.

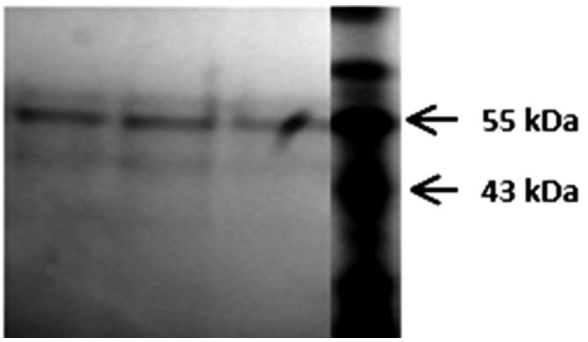

Fig. 3. SDS/PAGE of immunoprecipitated plasma samples. Lanes 1, 2, and 3 were loaded with 10 μl of the same eluted fraction bound to anti-Tax antibodies immovilized to the Amino-Link Plus® resin. Lane 4 correspond to molecular mass standard. The gel was stained using GelCode™ Blue Stain Reagent.

three studies were similar, genetic variability of the studied population could account for the differences in tax mRNA percentage. Furthermore, we simultaneously detected tax mRNA and intracellular Tax in 86.7% of asymptomatic carriers which closely agrees with a previous report exhibiting Tax in 75% of asymptomatic carriers [Yamano et al., 2002]. Ex vivo analyses gave significantly higher values in HAM/TSP patients compared with asymptomatic carriers in relation to tax mRNA expression in both PMBCs and CD4(+)CD25(+) cells, the percentage of Tax(+) cells, and the average proviral load. These results indicate that ex vivo Tax protein is frequently expressed in T cells from HTLV-1 infected people. Besides, Tax detection was possible in PBMCs culture medium, suggesting that this protein is mainly secreted from the cells. In HAM/TSP patients, ubiquitinated Tax levels secreted from cultured PBMCs was significantly higher compared with those from asymptomatic carriers ($P < 0.01$).

Follow-up studies with HTLV-1 infected subjects are necessary to analyze the temporal variation of cellular Tax in Treg cells. These studies would be very important to understand infection progression and pathogeny of HAM/TSP. In a previous longitudinal study, we followed the temporal Tax dynamics in PBMCs of nine patients [Ramirez et al., 2007]. We found a wide temporal variation in the proviral load and tax mRNA, which showed to be independent of the functional damage. These tax mRNA fluctuations in PBMCs probably account for the lack of correlation observed in the current study between viral protein and motor dysfunction—using OMDS. Although significantly higher levels of Tax—secreted from PBMCs and released to plasma—were observed in HAM/TSP patients than in asymptomatic carriers, the lack of correlation between Tax protein and proviral load or tax mRNA levels could be explained through a complex regulation of Tax protein expression. Previous findings of our group showed that Tax from culture media of PBMCs of HAM/TSP patients and of MT-2 cell line caused retraction of differentiated human neuroblastoma cells (SH-SY5Y) [Maldonado et al., 2011; Medina et al., 2014]. These observations indicate that the chronic Tax levels secreted from PBMCs could be sufficient for producing the neurotoxic effect on the long axons of corticospinal tracts involved in this progressive neurological disease. Thereby, these results suggest a new pathogenic HTLV-1 mechanism in HAM/TSP making more relevant monitoring Tax levels rather than mRNA or proviral load in both patients and carriers.

Protein sequence analysis showed that Tax protein contains all the features to be exported in vivo from PBMCs, including secretory signals at the carboxy-terminal portion [Jain et al., 2007a]. We have recently reported ubiquitinated Tax secreted from PBMCs of HTLV-1 infected subjects through the ER-Golgi pathway [Medina et al., 2014]. Tax secretion from infected PBMCs would account for the presence of this viral protein in plasma and CSF. Recently, it has also been reported exosomes derived from infected cells to contain Tax proteins and proinflammatory mediators [Jaworski et al., 2014].

Tax in plasma of HTLV-1 infected people was revealed by Western blot and Mass spectrometry. Two molecular mass forms, 57 and 48 kDa, suggest that Tax could be either mono- or di-ubiquitinated. According to the molecular mass by Western blot, Tax secreted from PMBCs was mainly a di-ubiquitinated protein. It has been suggested that this post-translational modification would represent a less active transcriptional form of Tax protein [Peloponese

TABLE III. Analysis of MS/MS Experimental Data Against Tax and Ubiquitin Sequences. Table Shown Peptides Matches in Mass Spectrometry Analysis of Tax Species Found at 57 and 48 kDa

	57 kDa band	48 kDa band
Tax Sequences	61-VIGSALQFLIPRLPSFPTQRTSKTLK-86	73-LPSFPTQRTSKTLK-86
	84-TLKVLTPPITHTTPNIPPSFLQAMRK-109	84-TLKVLTPPITHTTPNIPPSFLQAMR-108
	84-TLKVLTPPITHTTPNIPPSFLQAMRKYSPFR-114	84-TLKVLTPPITHTTPNIPPSFLQAMRK-109
	87-VLTPPITHTTPNIPPSFLQAMR-108	81-TSKTLKVLTPPITHTTPNIPPSFLQAMRK-109
	196-ISLTTGALIILPEDCLPTTLFQPAR-220	87-VLTPPITHTTPNIPPSFLQAMR-108
	262-DGQPSLVLQSSSFIFHKFQTK-282	87-VLTPPITHTTPNIPPSFLQAMRKYSPFR-114
Ubiquitin sequences	1-MQIFVKTLTGKTITLEVEPSDTIENVK-27	12-TITLEVEPSDTIENVKAKIQDKEGIPPDQQR-42

et al., 2004]. However, our previous results showed a similar effects on SH-SH5Y retraction in the presence of the 40 kDa Tax secreted from MT-2 cells [Maldonado et al., 2011] and the 57 kDa Tax form present in HAM/TSP PBMC supernatants [Medina et al., 2014], thus indicating an ubiquitin-independent neurotoxic effect of this secretable viral protein.

Extracellular Tax detection leads to new questions, namely, how extracellular Tax affects non-infected T cells; how selectively Tax causes neuron damage at CNS; if Tax plasma levels could be a reliable biomarker for HAM/TSP. Tax secreted from infected cells in vivo might function as an extracellular cytokine [Jain et al., 2007a]. This function could explain a Tax effect on non infected HTLV-1 cells, suggesting a new pathogenic mechanism of HTLV-1 mediated by Tax protein. Tax protein might stimulate PBMCs proliferation, cytokine synthesis by microglial cells and TNF-α secretion by neuronal cells [Marriott et al., 1991; Dhib-Jalbut et al., 1994; Cowan et al., 1997]. All these biological effects have been described in HAM/TSP patients during infection progress, leading us to associate HAM/TSP pathogenesis with them. Furthermore, soluble Tax has been reported to produce cytopathic alterations on muscle cells [Ozden et al., 2005]. This finding suggests that Tax could have a role in myopathies in the absence of muscle cells HTLV-1 infection. In addition, extracellular Tax immunomodulatory effect has been described on Tax uptake by dendritic cells (DCs) [Jain et al., 2007b]. DCs were activated once exposed to Tax, leading to T-cells proliferation and inflammatory response [Jain et al., 2007b]. Recent data showed that treatment of PBMCs with recombinant human Tax1 causes CCR5 downregulation and increase both in viability and in CC-chemokines levels [Barrios et al., 2011]. Furthermore, Tax-containing exosomes from infected cells were able to induce transcription and protection from apoptosis in non-infected PBMCs [Jaworski et al., 2014]. All these findings suggest that extracellular Tax would have important roles in extracellular signaling pathway on non-infected cells.

Various Tax forms may play differential roles during HAM/TSP progress. Nuclear Tax could exert the transactivator function on viral and cellular genes, producing T lymphocytes activation and a likely cell transformation [Boxus et al., 2008; Boxus and Willems, 2009]. Although cytoplasmic Tax is associated with organelles involved in secretory pathways and with the centrosome or microtubule organization center, Tax is additionally associated with the contact between cells in the inner side of the plasma membrane [Alefantis et al., 2005a; Nejmeddine and Bangham, 2010; Kfoury et al., 2012].

The mechanisms of Tax secretion in HAM/TSP patients, and the role of extracellular Tax on disease progression remain to be elucidated. Recently, we identified ubiquitinated Tax in the supernatant of PBMCs from HAM/TSP and asymptomatic carriers [Medina et al., 2014]. It was shown by using inhibitor (brefeldin A) and activators (ionomycin and phorbol myristate acetate) of protein transport that Tax secretion progresses through the endoplasmic reticulum–Golgi complex. In addition, an independient research has demonstrated that Tax can be released from HTLV-1-infected T-cell lines in exosomes [Jaworski et al., 2014]. Manumicyn A treatment inhibited exosome formation and consequently, the presence of Tax in exosomes. This finding shows that Tax release is associated to exosome production in cell lines. Therefore, the occurrence of extracellular Tax could involved two different mechanisms: free Tax by endoplasmic reticulum–Golgi complex and Tax associated with exosomes.

Tax in plasma indicates its secretion in vivo from infected lymphocytes, supporting a viral protein role in the pathogenesis of HAM/TSP. Since blood–brain barrier disruption in HAM/TSP patients facilitates lymphocyte infiltration in CNS, we suggest that Tax detected in CSF of HAM/TSP patients could be secreted from CNS infiltrating lymphocytes [Cartier and Ramirez, 2005; Cartier et al., 2007; Afonso et al., 2008] or may cross the blood–brain barrier bound to serum albumin or immunoglobulins [Reiber, 2003]. Even though Tax levels in CSF would have better predictive value than those in plasma, routine CSF sampling is not viable from neurological patients and not ethically permitted from asymptomatic carriers. In this context, follow-up studies of Tax secretion from PBMCs in culture or Tax measurement in plasma might become a more adequate marker to surveil the pathogenesis and development of HAM/TSP.

ACKNOWLEDGMENTS

We thank Professor Claudio Telha for his valuable input when reviewing the manuscript. We are grateful to Project Mecesup UCH 0115 that allowed the use of the Mass Spectrometry Instrument. We are also very grateful with the generous NIH donation of the HTLV-1 Tax hybridoma 168A51-2 (NIH, AIDS Reagent Program, Germantown, MD, Cat. 1316) that allowed us to carry out this research.

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