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The Elevated Interferon Gamma Production is an Important Immunological Marker in HAM/TSP Pathogenesis

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Abstract

Human T-lymphotropic virus type 1 (HTLV-1) is the agent of the HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), which may occur in >5% of patients during their lifetime. HTLV-1-infection causes disturbances in the immune system, and the viral load may also play an important role in the pathogenesis of HAM/TSP. Some cytokines are involved in the pathogenesis of this disorder. We have determined IL-2, IL-4, IL-10, IL-12 p70, IFN-γ and TNF-α production among HTLV-1-infected subjects from our HTLV-out Clinic in Institute of Infectious 'Emílio Ribas' in Sao Paulo city, Brazil. PBMC obtained from healthy controls (n = 32), asymptomatic HTLV-1 carriers (n = 68) and HAM/TSP patients (n = 44) were grown in the absence and in the presence of phytohaemagglutinin (PHA), and the supernatants' fluids were measured for cytokines production. IL-2 levels were increased in the asymptomatic HTLV-1 carriers, and IFN-γ was increased in both groups of patients (asymptomatic HTLV-1 carriers and more significantly among HAM/TSP patients). IL-4, IL-10, TNF-α and IL-12 p70 levels were not significantly increased on both groups of patients, as compared with controls. The major finding of this study is that IFN-γ was an important cytokine for the HAM/TSP pathogenesis. Therefore, immune modulation of IFN-γ may be critical to treat of HAM/TSP patients.

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1), the first defined pathogenic human retrovirus, is the agent of the HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1, 2], and of the adult T-cell leukaemia [3–5]. Others diseases are associated with HTLV-1, such as multiorgan inflammatory disorders including myositis, uveitis, arthritis, dermatitis and alveolitis [6–10]. HTLV-1 Infection is silent, persistent and >5% of patients develop a clinical condition three decades after infection. Brazil is considered an endemic area for HTLV-1 infection, particularly the Northeast, where city of Salvador and Sao Luis city, account with 1% of the blood donors are positive for the virus [11].

Human T-cell lymphotropic virus type 1 infection causes disturbances in the immune system and the viral load may also play an important role in the pathogenesis of HAM/TSP [12, 13]. Some cytokines are involved in the pathogenesis of that disorder, such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-2 (IL-2) and interleukin-1 (IL-1), which are produced by Th1 cells and play a critical role in cellular immunity. IL-4 and IL-10 are involved in humoral immunity and overnaïve T CD4 cells [14, 15]. Classically, HTLV-1 Infection can induce spontaneous T-cell proliferation and increased interleukin-2 (IL-2) secretion and IL-2R expression. The activation mediated by HTLV-1 in the T cell may be induced by the viral protein Tax, leading to transcriptional activation, transduction signal pathways, cell cycle control and apoptosis. These Tax protein properties may explain the ability of HTLV-1 to immortalize T cells [16, 17]. However, the pathway through which HTLV-1 induces T-cell transformation and IL-2 independence is not clear [18].

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Thus, we measured IL-2, IL-4, IL-10, IL-12 p70, IFN-γ and TNF-α levels in the supernatants of cultured PBMC, both from asymptomatic HTLV-1 carriers and patients with HAM/TSP. This study aims to determine the complete cytokine profile on a large number of HTLV-1-infected subjects.

Material and methods

Patients and controls

Human T-cell lymphotropic virus type 1-infected subjects were recruited in the HTLV outpatient clinic of the 'Emílio Ribas' Institute of Infectious Diseases, Sao Paulo, Brazil. The control group was made up of individuals seronegative for HTLV-1/2 1I and for human immunodeficiency virus type 1 (HIV-1) both by ELISA and Western blot methods. Informed consent was obtained from all subjects, and the Ethical Committee Board approved the research protocol. Thus, on this study, 144 subjects were studied; they were divided into three groups: group I was made up of 32 HTLV-1 and HIV-1-seronegative subjects (control group), group II comprised 68 asymptomatic HTLV-1-infected carriers (HTLV-1 group), and group III was made up of 44 HAM/TSP patients (HAM/TSP group), diagnosed by Kagoshima criteria. HIV-1-infected subjects were excluded for this analysis, as well HCV-infected individuals.

All serum samples were screened for the presence of antibodies to HTLV-1 and -2 using two commercially available EIA assays: HTLV-1/HTLV-2 Ab Capture ELISA (Ortho Diagnostics, Raritan, NJ, USA) which contains recombinant HTLV-1 and HTLV-2 envelope and core proteins for both coating and detection, and GE 80/81 Assay (Murex Diagnostics, Dartford, UK). Tests were performed according to the directions of the manufacturer. All specimens that were reactive for either EIA were confirmed by Western-blot (HTLV Blot 2.4, Diagnostic Biotechnology, Singapore) and/or PCR.

Peripheral blood mononuclear cells culture

PBMCs were collected in heparinized tubes and isolated using Ficoll–Hypaque density gradients (Amersham Pharmacia, Piscataway, NJ, USA). The cells were washed, adjusted to 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% foetal calf serum, and grown with or without 2.5 μg/ml phytohaemagutinin (PHA) at 37 °C, 5% CO_2 for 24 h. The supernatant fluids were harvested and stored at −70 °C for cytokines assays.

Measurement of cytokines

Enzyme immunoassays (EIA) were used to measure the concentration of the cytokines IL-2, IL-4, IL-10, IL-12 p70, IFN-γ and TNF-α. Antibody matched pairs and respective standards were purchased from R&D Systems (Duoset ELISA development kit; Minneapolis, MN, USA) and used according to the manufacturer's recommendation. The detection limit was 10 pg/ml for both cytokines. Optical density was measured with a 450 nm filter (BioRad, Hercules, CA, USA), and the concentration was determined using a standard curve developed with the GraphPrism software (GraphPrism Soft Inc., San Diego, CA, USA).

HTLV-1 proviral load by real time PCR

The forward and reverse primers used for HTLV-1 DNA quantification were F 5′-CAATCACTCATACAACCCCCAA-3′, R 5′-TCTGGAAAAGACAGGGTTGGG-3′ and the TaqMan probe was 5′ FAM-TCCTCCAGGCCATGCGAAATACTC-3′ TAMRA for the Tax region. For quantitation of the human albumin gene, the primers Alb-S (5′-GCTGTCATCTCTTGTGGGCTGT-3′) and Alb-AS (5′-AAACTCATGGGAGCTGCT GGTT-3′) and the Alb TaqMan probe (5′-FAM-CCTGTCATGCCCACACAAATCTC TCC-TAMRA-3′) were used. Albumin DNA quantification was performed in parallel on all samples to determine the amount of cellular DNA present and was used as an endogenous reference to normalize variations because of differences in PBMC count or DNA extraction, the sensitivity of the assay was 10 copies/10^4 PBMC [18].

Statistical analysis

Differences in patients' characteristics or laboratory values among the three groups were evaluated with the two-way Mann–Whitney test. In both cases P-values < 0.05 were considered statistically significant, and the $\chi 2$ test with Yates's correction was used to analyze and correlate cytokines production.

Results

Table 1 shows the demographical and immunological data from controls, asymptomatic HTLV-1 carriers and HAM/TSP patients. Controls were 20 women and 12 men with a mean age of 41 years, the asymptomatic HTLV-1 group had 46 women and 22 men with a mean age of 42 years, and the HAM/TSP group was made up of 23 women and 21 men with a mean age of 47 years. The time of known HAM/TSP development ranged from 5 to 18 years. The median T $CD4^+$ and T $CD8^+$ (respectively) count was 798 and 985 for the control group, 980 and 600 for the asymptomatic HTLV-1-infected carriers, 1662 and 740 cells/mm^3 for the HAM/TSP patients. The HTLV-1 proviral was higher in HAM/TSP patients than asymptomatic (18 versus 352 copies/10^4 PBMC, median, $P < 0.0001$).

Table 1 Demographical and immunological characteristics of the control group, asymptomatic HTLV-1 carriers and HAM/TSP patients.

Variable	Control (n = 32)	Asymptomatic HTLV-1 carriers (n = 68)	HAM/TSP patients (n = 44)	P-value
Gender				
Women	20	46	23	–
Men	12	22	21	–
Age (years)				
Mean	41 ± 9	42 ± 12	47 ± 10	ns
T CD4				
Median, cells/mm^3	798	980*	1662	*0.03
Percentile (25–75%)	472–1169	668–1189	771–1462	–
T CD8				
Median, cells/mm^3	985	600	740	–
Percentile (25–75%)	644–1354	356–805	506–840	ns
Time of HAM/TSP disease				
Median in years	–	–	10.2	–
Range	–	–	5.2–18.2	–
HTLV-1 proviral load, (copies/10^4 PBMC)				
Median	–	18	352	<0.0001
Percentile (25–75%)	–	0–104	136–783	–

HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; ns = non significant.
*P-value when the asymptomatic subjects were compared with control group.

Quantification of IL-2, IL-4, IL-10, IL-12p70, IFN-g and TNF-a by ELISA

Figure 1 shows the IL-2, IL-4, IL-10, IL-12p70, IFN-g and TNF-α concentrations as determined by ELISA in the 24 h supernatants from PBMC cultures.

IL-2 levels were thrice higher among the asymptomatic HTLV-I carriers as compared with the control group in the spontaneous release (P = 0.03). The addition of PHA did not increase the IL-2 production.

IL-4 levels were increased in the HTLV-1 group as compared with the controls after PHA stimulus (P = 0.05). There was no statistically significant difference among groups for the IL-10 and IL-12 p70 levels.

The IFN-γ levels were seven times higher among the asymptomatic HTLV-1 carriers when compared with the control group, both spontaneously and PHA stimulated (P = 0.0005 and P = 0.006 respectively). The HAM/TSP group had IFN-γ levels that were twice higher than those from the HTLV-1 asymptomatic carriers (spontaneous, P = 0.008), and also than the control group, both spontaneously and after PHA stimulus (P < 0.0001 and P = 0.02 respectively). We concluded that HAM/TSP individuals presented higher IFN-γ levels release than asymptomatic carriers. However, two HAM/TSP patients had undetectable IFN-γ levels, both spontaneously and after PHA stimulation.

The TNF-α levels were increased among the HTLV-1 asymptomatic carriers, both spontaneously and after PHA stimulus (P = 0.02 and P = 0.04 respectively), as compared with controls. These levels were similar between HAM/TSP patients and asymptomatic HTLV-1 asymptomatic carriers.

Discussion

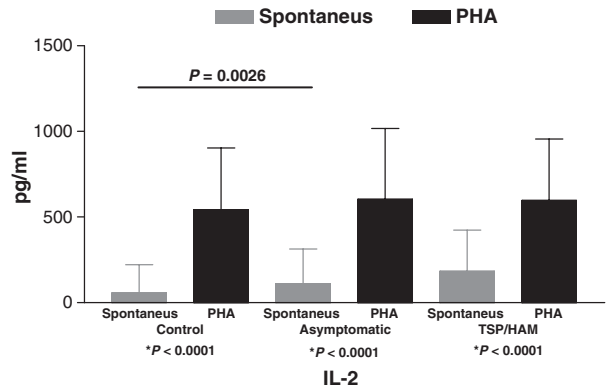
The complex interaction between HTLV-1 and host immune cells results in a state of cell activation, generating a chronic inflammatory process in the spinal cord in some infected subjects after a long incubation time. In long term follow-up, this process usually results in neuronal and glial damage with demyelinization, which is the final outcome for appearance of the neurological symptoms of HAM/TSP. The role of cytokines in the pathogenesis of this process is still obscure.

The IL-2 levels were slightly increased among asymptomatic HTLV-1-carriers (20%), indicating that IL-2 levels were not responsible for a higher spontaneous proliferation of lymphocytes noted mainly in HAM/TSP patients. These results indicate that spontaneous proliferation of T cells is intimately related to HTLV-1 infection and is probably because of autocrine or paracrine pathways which are not involved with IL-2 and the IL-2 receptor system [19]. In fact, it is possible that T cell proliferation is indeed related to some IL-2 independent pathway, as previously shown [15].

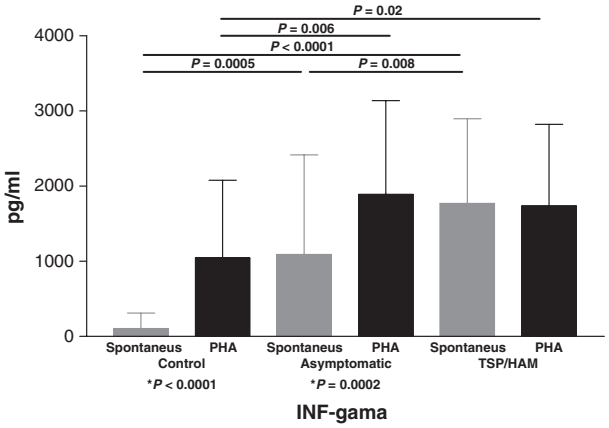
Our results agree with the evidence that the Tax protein may promote G1- to S-phase transition, although this may involve additional proteins [19]. A role for other viral proteins that may constitutively activate the IL-2 receptor pathway has also been suggested. By virtue of their activated state, HTLV-1-infected T cells can non-specifically activate resting, uninfected T cells via virus-mediated up-regulation of adhesion molecules [19].

IL-10 and IL-4 levels were not modified on the different groups. Thus, it is possible to infer that normal or decreased levels of these cytokines may not affect the IFN-γ production. In addition, IL-12 p70 levels also were not altered, indicating a downregulation by the high IFN-γ levels.

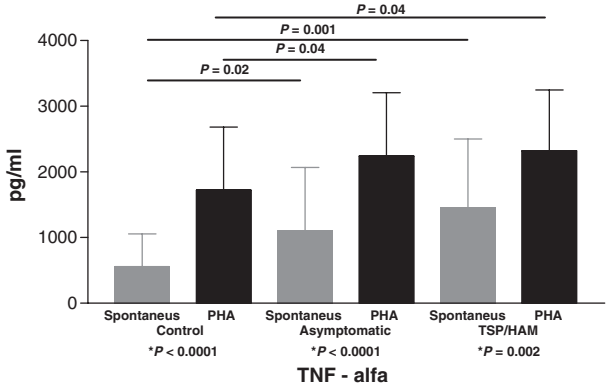
Surprisingly, the TNF-α levels were not modified on the different groups. TNF-α mRNA expression was reduced from controls to HAM/TSP patients, but the difference was not statistically significant. Only production of TNF-α was increased among the HTLV-1-infected subjects (either asymptomatic HTLV-1 or HAM/TSP patients), when paired with controls. By contrast, the spontaneous IFN-γ expression was increased when compared with the asymptomatic carriers and with controls. Similar results were obtained in the supernatants; in fact,



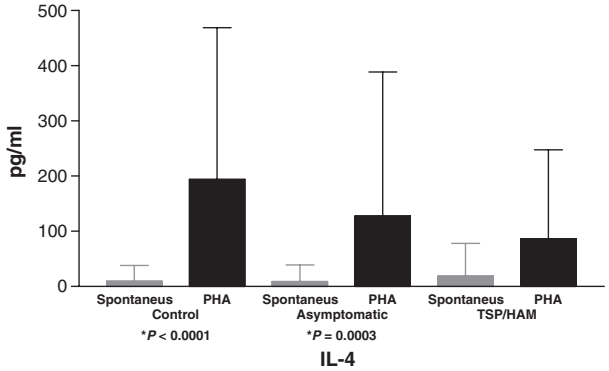
Group	Spontaneus (pg/ml)	PHA (pg/ml)
Control	25 (0 – 9.5)	526 (167 – 919)
Asymptomatic HTLV-1	**69** (0 – 70)	600 (243 – 965)
HAM/TSP	**166** (3.5 – 169)	563 (221 – 851)



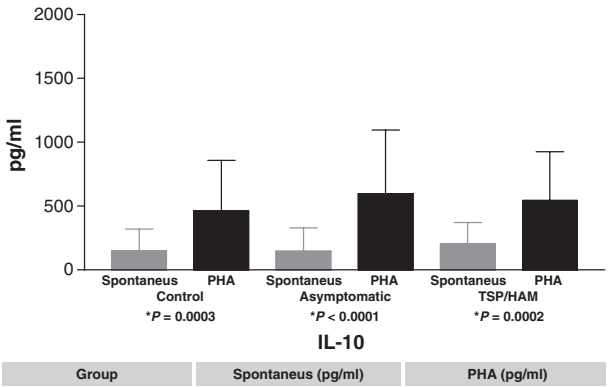
Group	Spontaneus (pg/ml)	PHA (pg/ml)
Control	106 (0 – 177)	1044 (264 – 1044)
HTLV-1 asymptomatic	**1089** (9 – 1989)	**1888** (895 – 2681)
HAM/TSP	**1764** (805 – 2681)	1734 (717 – 1734)



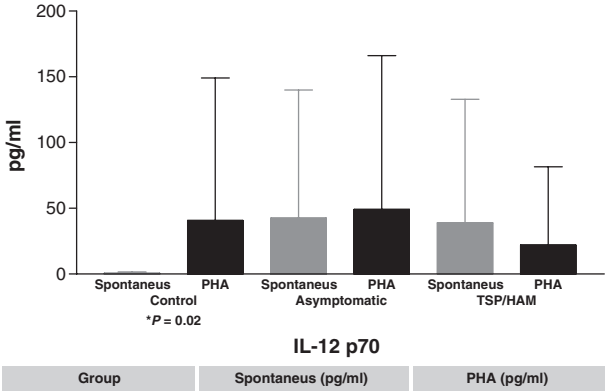
Group	Spontaneus (pg/ml)	PHA (pg/ml)
Control	559 (146 – 915)	1721 (820 – 2445)
HTLV-1 asymptomatic	1099 (337 –1562)	2243 (1522 – 2920)
HAM/TSP	1456 (559 – 2250)	2321 (2040 – 3082)



Group	Spontaneus (pg/ml)	PHA (pg/ml)
Control	9 (0 – 3.5)	194 (0 – 250)
Asymptomatic HTLV-1	9 (0 – 2)	127 (0 – 101)
HAM/TSP	18 (0 – 17)	86 (0 – 95)



Group	Spontaneus (pg/ml)	PHA (pg/ml)
Control	151 (0 – 305)	463 (108 – 723)
Asymptomatic HTLV-1	146 (0 – 274)	596 (261 – 869)
HAM/TSP	205 (10 – 321)	544 (261 – 838)



Group	Spontaneus (pg/ml)	PHA (pg/ml)
Control	0.5 (0 – 0.5)	40 (0 – 39)
Asymptomatic HTLV-1	42 (0 – 15)	49 (0 – 15)
HAM/TSP	38 (0 – 6)	22 (0 – 6.5)

Figure 1 Detection of IL-2, IL-10, IL-4, IL-12 p70, IFN-γ and TNF-α from the control group, asymptomatic HTLV-1 carriers and HAM/TSP patients.

IFN production was high, both on the asymptomatic carriers, but more prominent on the HAM/TSP patients.

IFN-γ production was higher on asymptomatic HTLV-1 carriers and HAM/TSP patients than on healthy controls. One possible explanation could be that the Tax protein induces IFN-γ production and T-cell proliferation [20]. It may also be that HTLV-1-infected subjects possess a higher number of activated T $CD4^+$ cells, resulting in upregulation of the cells and increased IFN-γ levels [20]. Theoretically, a higher HTLV-1 viral load in HAM/TSP patients could be an alternative explanation in this population, as seen in this population [18]. Similar results were noted by another study from Brazil [21] regarding the high production of IFN-γ, but not for IL-10, TNF-α and IL-12 production. In fact, the latter discrepancies probably were because of different methodologies used in the studies.

Finally, the major antiviral factor was IFN-γ, possibly the main responsible for the immune system activation seen among asymptomatic HTLV-1 carriers and even more evident among HAM/TSP patients. In fact, the latter showed higher levels of that cytokine, even spontaneously, showing that HTLV-1 *per se* may induce a strong immune activation that may be responsible for the very low plasma viral load or cellular replication. However, this ineffective response, drive by different co-stimulatory molecule expression, perforin and granzyme content in HAM/TSP patients by the different state of activation of the CTLs [22], probably is the main mechanism for tissue damage in the spinal cord, which is the remarkable picture of HAM/TSP pathogenesis. Our findings should be seen with caution, as we did use a specific T cell mitogen (PHA), but HTLV-1 antigens, such as tax, may be more accurate to study the immune response among HTLV-1-infected subjects.

Concluding the major finding of this study points to the importance of IFN-γ on the HAM/TSP pathogenesis. Therefore, immune modulation of this cytokine may be critical for HAM/TSP treatment, as recently showed by our group [23]. However, further studies are warranted to test this hypothesis.

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