Original Paper

NeuroImmunoModulation

Neuroimmunomodulation 2006;13:145–151
DOI: 10.1159/000097259

Received: June 2, 2006
Accepted: September 11, 2006
Published online: November 21, 2006

Modulation of T Cell Responses in HTLV-1 Carriers and in Patients with Myelopathy Associated with HTLV-1

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Key Words
Antagonists · Cytokines · HTLV-1 · Interferon-γ · Myelopathy · T cell responses

Abstract
Objective: Human T lymphotropic virus-type 1 (HTLV-1) activates the immune system leading to a persistent and exacerbated T-cell response with increased production of IFN-γ and TNF-α. Overproduction of pro-inflammatory cytokines is correlated with the development of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), although some HTLV-1 carriers also show high levels of these cytokines. In this study, the ability of regulatory cytokines and cytokine antagonists to inhibit spontaneous IFN-γ production was investigated. ***Method:*** IFN-γ levels were measured by ELISA before and after addition of cytokines or anti-cytokines. ***Results:*** Addition of IL-10 significantly reduced spontaneous IFN-γ synthesis in cell cultures from HTLV-1 carriers, while no differences were observed in HAM/TSP patients. There was also a tendency to decreased IFN-γ levels in cell cultures from HTLV-1 carriers with exogenous addition of TGF-β. In paired analysis, neutralization of IL-2 significantly decreased IFN-γ production in HTLV-1 carriers but not in HAM/TSP patients. Neutralization of IL-15 was less effective than neutralization of IL-2 in modulating IFN-γ production. In HTLV-1 carriers, anti-IL-2 and simultaneous addition of anti-IL-2 and anti-IL-15 decreased IFN-γ synthesis by 46 and 64%, respectively, whereas in patients with HAM/TSP simultaneous neutralization of both anti-cytokines only decrease IFN-γ levels by 27%. ***Conclusions:*** Although a large proportion of HTLV-1 carriers produced high levels of pro-inflammatory cytokines similar to those observed in HAM/TSP patients, immune response can be downregulated by cytokines or cytokine antagonists in most HTLV-1 carriers. This modulation can be an important step in the prevention of tissue damage and progression from the HTLV-1 carrier state to HAM/TSP.

Introduction

Human T-cell lymphotropic virus-type 1 (HTLV-1) infection modifies the cellular and humoral immune response. Activated T cells incorporate the virus in their genome, where regulatory proteins (Tax) alter activation and cell death pathways leading to a persistent activation

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and an exacerbated T-cell response. In a small percentage of infected individuals, HTLV-1 causes adult T-cell leukemia/lymphoma or a chronic inflammatory disease of the central nervous system (HTLV-1-associated myelopathy/tropical spastic paraparesis, HAM/TSP). Uveitis, polyarthritis and infective dermatitis in children have also been associated with HTLV-1 [1]. In vitro HTLV-1 infection induces spontaneous proliferation of lymphocytes [2], a persistent and high titer of anti-HTLV-1 antibodies [3], high HTLV-1 proviral load with an increased number of Tax-specific CD8+ T lymphocytes [4, 5], and also an increased expression of pro-inflammatory cytokines and chemokines in the peripheral blood and cerebral spinal fluid [6–8]. These immunological abnormalities are more pronounced in HAM/TSP patients, but evidence of enhanced T-cell activation is also detected in HTLV-1 carriers [8, 9]. HTLV-1 Tax activates interleukin (IL)-2 and IL-15 genes, the products of which participate in the spontaneous lymphoproliferation observed in HTLV-1-infected patients [10, 11]. Moreover, overproduction of pro-inflammatory cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-15 is related to the persistent inflammatory reaction observed in HAM/TSP patients [12, 13]. Therefore, the control of the exacerbated T-cell response in HTLV-1 infection is highly desirable in such patients. IL-10 and transforming growth factor β (TGF-β) have been recognized as important cytokines that downregulate the type 1 immune response. In this study, the ability of regulatory cytokines (IL-10 and TGF-β) and antagonists of cytokines (anti-IL-2, anti-IL-12 and anti-IL-15) to downregulate the high spontaneous IFN-γ production observed in unstimulated cell cultures of HTLV-1-infected patients was analyzed. Additionally, a comparative analysis of the role of these modulators that downregulate the immune response in HTLV-1 carriers and in HAM/TSP patients was performed. The overall data showed that IL-10, anti-IL-2 and anti-IL-2 plus anti-IL-15 significantly reduce IFN-γ production in HTLV-1 carriers but are not able to downregulate cells from HAM/TSP.

Patients and Methods

Study Subjects

The study population consisted of 28 HTLV-1 carriers and 25 patients with HAM/TSP. The HTLV-1 carriers were selected consecutively from blood bank donors referred to the HTLV-1 clinic of the Hospital Universitário Professor Edgard Santos, Federal University of Bahia, Brazil, from September 2003 to December 2005. Patients with HAM/TSP were matched by age (±5 years) and sex. The diagnosis of HTLV-1 infection was performed by enzyme-linked immunosorbent assay (Murex HTLV-1 + II, Abbot, Dartford, UK) and confirmed by Western blot analysis (Genelabs HTLV 2.3–2.4, Singapore). The diagnosis of HAM/TSP was made according to the World Health Organization guidelines. All of the HAM/TSP patients were independently observed by two neurologists and had HTLV-1 antibodies in their cerebral spinal fluid. Neurological and motor dysfunctions were measured by two scales: the Expanded Disability Status Scale [14] and Osame's Motor Disability Score [15]. All HAM/TSP patients had Osame's Motor Disability Score ≥1 and Expanded Disability Status Scale ≥3. Individuals who did not fulfill the criteria for HAM/TSP were classified as HTLV-1 carriers. All subjects were also screened for HIV-1 and -2 and hepatitis virus type B and C. Those who had any of these infections were excluded from the study. Samples were taken following informed consent was obtained, and the study was conducted with approval of the Ethical Committee of the Hospital Universitário Professor Edgard Santos.

Cell Cultures and IFN-γ Levels

Peripheral blood mononuclear cells (PBMCs) from 25 HAM/TSP patients and 28 HTLV-1 carriers were isolated from heparinized blood by density gradient centrifugation with Ficoll-Hypaque. The cells were cultured in RPMI 1640 (Life Technologies Gibco BRL, Grand Island, N.Y., USA), 10% human AB serum (Sigma, St. Louis, Mo., USA), glutamine, HEPES and antibiotics. A total of 3×10^6 cells/ml was plated in 24-well flat-bottom microtiter plates (Falcon, Becton Dickinson, Lincoln Park, N.J., USA). The cell cultures were kept only with media (media alone) or were supplemented with 100 ng/ml IL-10 (DNAX Institute, Palo Alto, Calif., USA) and 50 ng/ml TGF-β1 (R&D Systems, Minneapolis, Minn., USA). Anti-IL-2, anti-IL-12, and anti-IL-15 (R&D Systems) in a concentration of 20 μg/ml or a combination of both anti-IL-2 and anti-IL-15 were also used. Cell cultures were incubated at 37°C with 5% CO_2 and 95% air for 72 h. Supernatants of the cell cultures were collected and stored at –20°C until use. IFN-γ levels were determined using the ELISA sandwich technique following the instructions described by the manufacturers (BD Bioscience Pharmingen, San Jose, Calif., USA). Decreases in IFN-γ synthesis in the presence of each neutralizing antibody, IL-10 and TGF-β were calculated: [(spontaneous IFN-γ – treated IFN-γ/spontaneous IFN-γ) ×100]. The result was expressed as percent inhibition and the ability to suppress about 30% of IFN-γ synthesis was considered an efficient inhibition. The activity of the cytokines and cytokine antagonists was demonstrated by the inhibition of IFN-γ production in seronegative HTLV-1 cultures stimulated with an unrelated antigen (purified protein derivative of tuberculin – PPD – 2 μg/ml). Before the experiments, a dose-response curve, with concentrations ranging from 0.5 to 20 μg/ml, was performed to determine the best concentrations of monoclonal antibody to be used.

Statistical Analysis

The Mann-Whitney U test and Wilcoxon's matched-pair signed rank test were used to compare data. The GraphPad InStat program (San Diego, Calif., USA) was applied for statistical evaluation, and p values <0.05 were considered to indicate a significant difference.

Results

Demographic Data and IFN-γ Production

Twenty-five HAM/TSP patients and 28 HTLV-1 carrier subjects participated in the study. The age of the patients with myelopathy ranged from 39 to 72 years (mean ± SD: 51 ± 9 years); 14 were male and 11 female. The Osame's score of these patients ranged from 3 to 11 and the Expanded Disability Status Scale was >3 in all cases. The age of HTLV-1 carriers ranged from 22 to 66 years (mean ± SD: 48 ± 7 years); 14 were male and 14 female. In myelopathy patients, the mean spontaneous IFN-γ level was 2,624 (SD: 2,045 pg/ml; ranging from 364 to 8,215; median 2,363 pg/ml) was increased compared to HTLV-1 patients (1,425 ± 1,426 pg/ml; ranging from 62 to 4,575, median 796 pg/ml, p = 0.009, Mann-Whitney U test). IFN-γ levels in HTLV-1 carriers were quite variable, and ~40% of the HTLV-1 carriers had IFN-γ levels above the median observed in the patients with HAM/TSP.

Ability of IL-10 and TGF-β to Inhibit IFN-γ Production in PBMC Cultures of HTLV-1 Carriers and HAM/TSP Patients

IL-10 and TGF-β are the best-studied downregulatory cytokines. Both IL-10 and TGF-β were able to downregulate IFN-γ synthesis of PBMC from HTLV-1-negative healthy individuals stimulated with PPD (2 μg/ml). IL-10 (20 ng/ml) and TGF-β (20 ng/ml) suppressed IFN-γ synthesis by 97 and 47%, respectively, in PPD-stimulated cultures from HTLV-1 negative controls (data not shown). To evaluate the ability of these modulatory cytokines to decrease the high spontaneous IFN-γ production observed in HTLV-1-infected patients, PBMCs of HAM/TSP patients and HTLV-1 carriers were cultured in the presence of these cytokines (fig. 1). In unstimulated supernatants of HTLV-1 carriers and HAM/TSP patients, IFN-γ levels were 1,425 ± 1,426 and 2,624 ± 2,045 pg/ml, respectively (p = 0.0091). While the addition of 100 ng/ml of IL-10 reduced IFN-γ production to 496 ± 665 pg/ml in cell cultures from HTLV-1 carriers (p = 0.0018), showing a decrease in spontaneous IFN-γ synthesis of 64%, there was no significant difference in IFN-γ production after addition of IL-10 in HAM/TSP patients (1,877 ± 1,784 pg/ml, p = 0.1), with a decrease in IFN-γ production of only 28%. While TGF-β (50 ng/ml) suppressed IFN-γ by 46% in HTLV-1 carriers (765 ± 970 pg/ml, p = 0.08), no change in IFN-γ levels was observed in HAM/TSP patients (2,160 ± 1,809 pg/ml, p = 0.4).

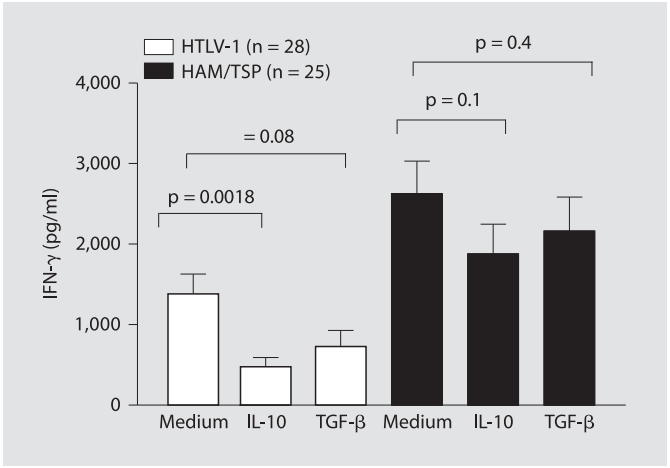

Fig. 1. Immunoregulatory effects of IL-10 and TGF-β on spontaneous IFN-γ production in HTLV-1 carriers (n = 28) and HAM/TSP patients (n = 25). PBMCs were cultured in the presence or absence of IL-10 (100 ng/ml) and TGF-β (50 ng/ml) for 72 h. IFN-γ levels were determined by ELISA; each bar represents the median and SD of IFN-γ levels from each group. Exogenous IL-10 suppressed spontaneous IFN-γ production by 64% in HTLV-1 carriers, and in HAM/TSP patients the decrease was only 28%. TGF-β decreased IFN-γ synthesis by 46% in HTLV-1 carriers and by 18% in HAM/TSP patients. Mann-Whitney U test.

Ability of Cytokine Antagonists (Anti-IL-2, Anti-IL-12 and Anti-IL-15) to Downregulate IFN-γ Production in PBMC of HTLV-1 Carriers and HAM/TSP Patients

IL-2 and IL-15 are cytokines participating in T-cell activation and proliferation, and both are involved in the spontaneous lymphoproliferation observed in HTLV-1-infected patients. In addition, IL-12 triggers type 1 immune responses and IFN-γ synthesis. A dose-response curve of the best concentration of neutralizing antibodies to downregulate IFN-γ synthesis was assayed. Thereafter, the effects of neutralizing antibodies against IL-2, IL-12 and IL-15 (20 μg/ml) were assayed in PBMC cultures of HTLV-1 carriers and myelopathy patients. Table 1 shows that anti-IL-2 antibody decreased IFN-γ production by 27% in HAM/TSP cultures and 46% in HTLV-1 carriers. After anti-IL-2 addition, IFN-γ levels in unstimulated cell cultures significantly decreased from 1,425 ± 1,426 to 767 ± 1,029 pg/ml in HTLV-1 carriers (p = 0.03) and from 2,624 ± 2,045 to 1,923 ± 1,721 pg/ml in HAM/TSP patients (p = 0.2). Anti-IL-15 reduced IFN-γ production by only 25% (1,066 ± 1,273 pg/ml, p = 0.1) in HTLV-1 carrier cultures. In HAM/TSP patients, a 27% reduction (1,925 ± 1,575 pg/ml, p = 0.3) was observed. To evaluate the role of anti-IL-2 plus anti-IL-15

Table 1. Inhibition of spontaneous IFN-γ production (means ± SD) after addition of anti-cytokines in PBMC cultures of HTLV-I carriers (n = 28) and HAM/TSP patients (n = 25)

Additive	HTLV-1 carrier			HAM/TSP patients		
	IFN-γ, pg/ml	suppression, %	p value	IFN-γ, pg/ml	suppression, %	p value
None	1,425 ± 1,426	–	–	2,624 ± 2,045	–	–
Anti-IL-2	767 ± 1,029	46	0.03	1,923 ± 1,721	27	0.2
Anti-IL-12	883 ± 844	38	0.3	2,395 ± 1,842	9	0.8
Anti-IL-15	1,066 ± 1,273	25	0.1	1,925 ± 1,575	27	0.3
Anti-IL-(2+15)	514 ± 720	64	0.003	1,914 ± 1,711	27	0.2

Mann-Whitney U test.

on IFN-γ production of HTLV-1 carriers and HAM/TSP, both anti-cytokine antibodies were added to PBMC cultures. No significant decrease in IFN-γ levels was observed in HAM/TSP cell cultures (1,914 ± 1,711 pg/ml, p = 0.2) after addition of anti-IL-2 plus anti-IL-15. However, in combination, both anti-cytokines significantly reduced IFN-γ levels in HTLV-1 carriers to 514 ± 720 pg/ml (p = 0.003). Addition of the antibodies against IL-2 and IL-15 inhibited IFN-γ synthesis in HTLV-1 carriers by approximately 64% while the decrease in IFN-γ production was only 27% in HAM/TSP cell cultures. There was a tendency to decreasing spontaneous IFN-γ production in HTLV-1 carriers after addition of anti-IL-12 (from 1,425 ± 1,426 to 883 ± 844 pg/ml, p = 0.3) while no significant change was observed in cell cultures from HAM/TSP patients (from 2,624 ± 2,045 to 2,395 ± 1,842 pg/ml, p = 0.8) in the presence of anti-IL-12.

Decrease in Spontaneous IFN-γ Production in HTLV-1 Carriers: Effect of Immunomodulators

As expected, individual variations in the modulation of T-cell responses were observed among HTLV-1 carriers. However, in the majority of these patients, a downmodulation of IFN-γ levels was observed with immunomodulators. Paired analysis of spontaneous IFN-γ production in the absence and presence of immunomodulators revealed that high concentrations (100 ng/ml) of IL-10 and anti-IL-2 (20 μg/ml) significantly decreased spontaneous IFN-γ levels in almost all HTLV-1 carriers ($p < 0.0001$, Wilcoxon's signed rank test). Only in 3 individuals in the HTLV-1 carrier group, IFN-γ levels did not change in the presence of IL-10 or anti-IL-2 (fig. 2a, b). After the addition of anti-IL-15, IFN-γ levels significantly decreased in 24 HTLV-1 carriers and remained at similar levels in 4 of the 28 subjects evaluated ($p < 0.0001$; fig. 2c). The same applied for spontaneous IFN-γ levels in the presence of anti-IL-2 plus anti-IL-15; T cells from 3 HTLV-1 carriers were not modulated after anti-IL-2 plus anti-IL-15 addition ($p < 0.0001$; fig. 2d). Additionally, we observed that cells from HTLV-1 carriers not sensible to IL-10 differed from those that were not able to be modulated by anti-cytokines. Only cells from 1 HTLV-1 carrier were not modulated by any immunomodulator.

Discussion

Activation of the host T cells by Tax protein coded by HTLV-1 may induce the production of cytokines [16], especially pro-inflammatory cytokines as IFN-γ and TNF-α [6, 17]. Although IFN-γ and TNF-α synthesis are important to control virus replication, they are also involved in the pathogenesis of HAM/TSP [18–20]. Herein we show that while in HAM/TSP a poor modulation of the immune response is observed, in HTLV-1 carriers, cytokines such as IL-10 and neutralization of IL-2 downmodulate IFN-γ production.

IL-10 is an important immunoregulatory cytokine and inhibits both type 1 and type 2 immune responses [21, 22]. The inability of IL-10 to downmodulate IFN-γ production in HAM/TSP may be related to an increase in activated/effector T cells. We have previously shown that cells expressing activated T-cell markers are increased in HAM/TSP and it is known that activated/effector T cells are less susceptible to immunomodulation [23, 24]. Inhibition of T cells by IL-10 has been shown to be related to downmodulation of antigen-presenting cells [25]. As T cells from HTLV-1-infected individuals proliferate and produce cytokines independent of accessory cells [1], the

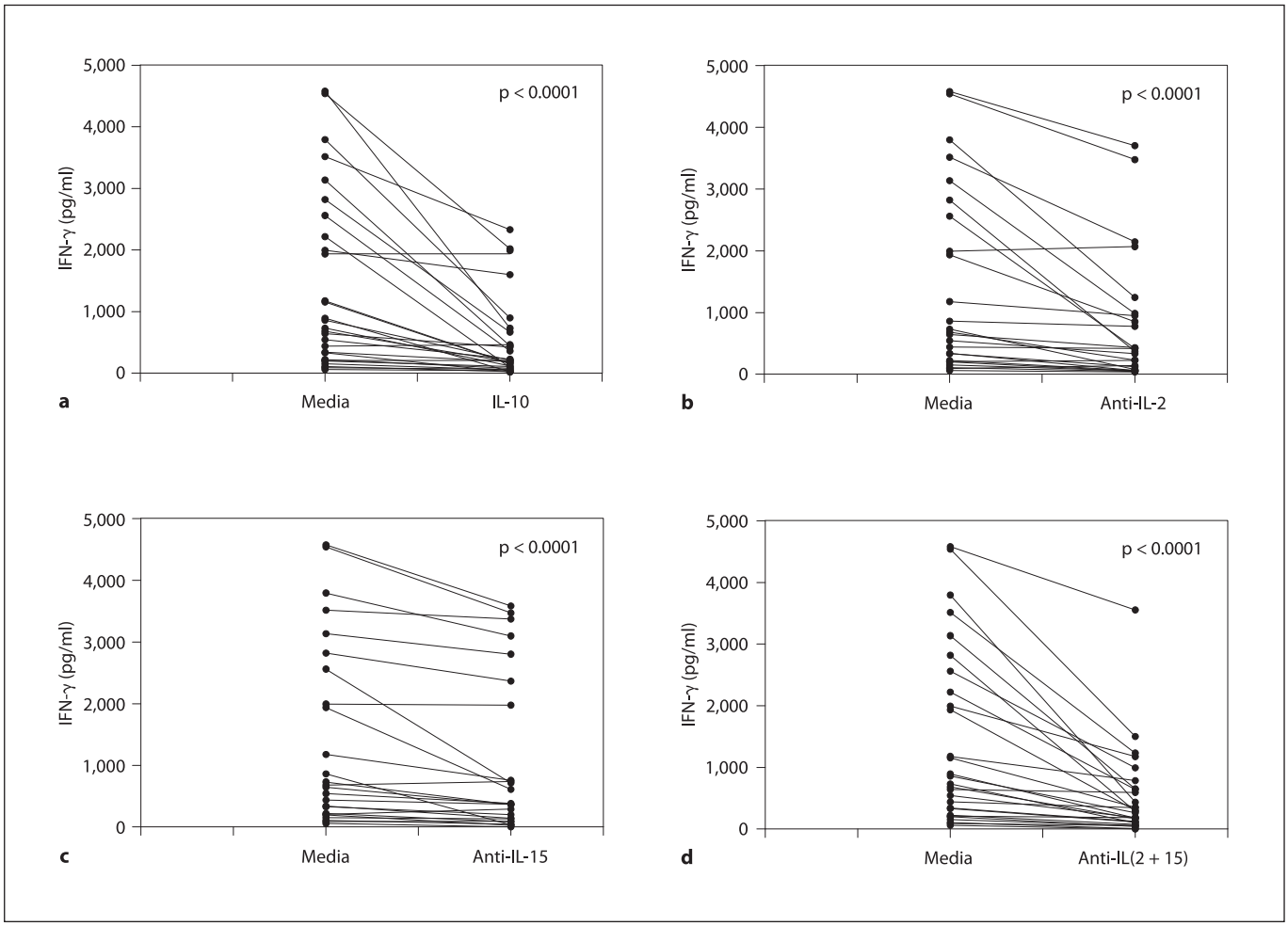

Fig. 2. Effect of IL-10 (**a**), anti-IL-2 (**b**), anti-IL-15 (**c**) and anti-IL-2 plus anti-IL-15 (**d**) on spontaneous IFN-γ production in PBMC cultures of HTLV-1 carriers (n = 28). The data represent the variation in spontaneous IFN-γ levels after the addition of cytokines and cytokine antagonists ($p < 0.05$, Wilcoxon matched-pair signed rank test).

downmodulation of the immune response mediated by IL-10 in HTLV-1 carriers indicates that this cytokine may directly modulate T-cell response. TGF-β has also potent immunoregulatory properties on lymphocyte functions. However, exogenous TGF-β had no effect in cultures of patients with HAM/TSP and downmodulates IFN-γ production in HTLV-1 carriers in a less effective way than IL-10. Previous studies have shown that the viral protein Tax inhibits the signal of TGF-β binding to nuclear regulatory proteins [26]. This may help HTLV-1-infected cells to escape TGF-β-mediated growth inhibition [27].

Molecules such as IL-2, IL-12 and IL-15 are important in the differentiation and proliferation of T cells. Because IL-12 induces IFN-γ production and promotes differentiation of type 1 T cells [28], the ability of IL-12 neutralization to decrease T-cell response was studied. The blockage of IL-12 failed to significantly decrease cytokine production in both HTLV-1 study groups, although there was a tendency to decreasing spontaneous IFN-γ levels in cells from HTLV-1 carriers. In HTLV-1 infection, one mechanism of the persistent T-cell activation is the existence of two autocrine loops producing IL-2 and IL-15 and the expression of their respective receptors (IL-2Rα and IL-15Rα), which are transcriptionally regulated by Tax [10, 11]. Upregulation of these cytokines is associated with adult T-cell leukemia/lymphoma and HAM/TSP [10, 13]. Previous studies have shown that the addition of blocking antibodies to IL-2 and IL-15 or their receptors

inhibited spontaneous lymphocyte proliferation in HAM/TSP patients [10, 11, 13]. This study demonstrates that anti-IL-2 and anti-IL-15 significantly decreased spontaneous IFN-γ production in HTLV-1 carriers, but they were not able to inhibit spontaneous IFN-γ synthesis in patients with myelopathy.

It is assumed that the cell damage observed in the central nervous system of HAM/TSP patients might be caused by pro-inflammatory cytokines such as IFN-γ and TNF-α released by HTLV-1-infected CD4+ T cells and HTLV-1-specific CD8+ T cells [12, 29, 30]. Moreover, a failure to modulate the immune response plays an important role in the pathogenesis of inflammatory diseases of the central nervous system, e.g. multiple sclerosis and experimental autoimmune encephalitis [31, 32]. We have previously shown that about 40% of the HTLV-1 carriers have a high lymphoproliferative response and also a high spontaneous production of IFN-γ and TNF-α [33]. Despite having immunological abnormalities similar to HAM/TSP, the majority of these individuals do not develop myelopathy. The fact that T cells from HTLV-1 carriers can be downmodulated raise the possibility that the absence of pathology seen in HTLV-1 carriers who have immunological abnormalities similar to HAM/TSP patients may be due to in vivo regulatory mechanisms that decrease T-cell proliferation and secretion of pro-inflammatory cytokines.

Therapeutic approaches based on the use of immunomodulators have been successful in the control of diseases associated with an exaggerated T-cell response [34, 35]. In our study, modulation of immune responses is impaired in HAM/TSP patients. However, as lymphocytes from HTLV-1 carriers can be downmodulated, modulation of T-cell responses may help to prevent the progression to myelopathy in HTLV-1 carriers.

Acknowledgments

This study was supported by the Brazilian National Research Council (CNPq), the Fundação de Amparo à Pesquisa do Estado da Bahia, NIH grant D43 TW007127-01 and NIH grant AI60830. We thank the members of the Hemocentro da Bahia (Brazil) and the Serviço de Transfusão de Sangue (Salvador, Brazil) for HTLV-1-positive blood donor referral. We also thank Elbe Myrtes Silva for the preparation of the manuscript. E.M.C. is a senior investigator of the CNPq.

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