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## Effect of TNF- $\alpha$ production inhibitors on the production of pro-inflammatory cytokines by peripheral blood mononuclear cells from HTLV-1-infected individuals

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# Effect of TNF- $\alpha$ production inhibitors on the production of pro-inflammatory cytokines by peripheral blood mononuclear cells from HTLV-1-infected individuals

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## Abstract

Human T lymphotropic virus type 1 (HTLV-1) is the causal agent of myelopathy/tropical spastic paraparesis (HAM/TSP), a disease mediated by the immune response. HTLV-1 induces a spontaneous proliferation and production of pro-inflammatory cytokines by T cells, and increasing interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels are potentially involved in tissue damage in diseases related to HTLV-1. This exaggerated immune response is also due to an inability of the natural regulatory mechanisms to down-modulate the immune response in this group of patients. TNF- $\alpha$  inhibitors reduce inflammation and have been shown to improve chronic inflammatory diseases in clinical trials. The aim of this study was to evaluate the ability of pentoxifylline, forskolin, rolipram, and thalidomide to decrease *in vitro* production of TNF- $\alpha$  and IFN- $\gamma$  in cells of HTLV-1-infected subjects. Participants of the study included 19 patients with HAM/TSP (mean age,  $53 \pm 11$ ; male:female ratio, 1:1) and 18 HTLV-1 carriers (mean age,  $47 \pm 11$ ; male:female ratio, 1:2.6). Cytokines were determined by ELISA in supernatants of mononuclear cell cultures. Pentoxifylline inhibited TNF- $\alpha$  and IFN- $\gamma$  synthesis with the minimum dose used (50  $\mu$ M). The results with forskolin were similar to those observed with pentoxifylline. The doses of rolipram used were 0.01-1  $\mu$ M and the best inhibition of TNF- $\alpha$  production was achieved with 1  $\mu$ M and for IFN- $\gamma$  production it was 0.01  $\mu$ M. The minimum dose of thalidomide used (1  $\mu$ M) inhibited TNF- $\alpha$  production but thalidomide did not inhibit IFN- $\gamma$  production even when the maximum dose (50  $\mu$ M) was used. All drugs had an *in vitro* inhibitory effect on TNF- $\alpha$  production and, with the exception of thalidomide, all of them also decreased IFN- $\gamma$  production.

Key words: HTLV-1; TNF- $\alpha$  inhibitors; Pentoxifylline; Immune response

## Introduction

Human lymphotropic virus type 1 (HTLV-1) infection is endemic in Brazil (1) and the city of Salvador has the highest prevalence among blood donors (1.35%) and in the population (1.76%) (2,3). HTLV-1 is the causal agent of adult T-cell leukemia-lymphoma (ATL), and of a chronic inflammatory disease of the central nervous system (HTLV-1 associated myelopathy/tropical spastic paraparesis, HAM/TSP) (4). HTLV-1 infection induces T-cell activation and *in vitro* spontaneous lymphocyte proliferation leading to the production of high levels of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in non-stimulated peripheral blood

mononuclear cells (PBMC) (5,6). HAM/TSP is characterized by progressive spastic paraparesis and occurs in less than 5% of HTLV-1-infected patients (7). High proviral load (8,9) and increased expression of the pro-inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  in PBMC as well as interleukin-1 (IL-1) and IL-6 in the cerebrospinal fluid (CSF) are associated with this disease (4,10). Although these cytokines are more pronounced in HAM/TSP patients, evidence of enhanced T-cell activation is also detected in HTLV-1 carriers (11). Moreover, a large proportion of HTLV-1 carriers, although they do not fulfill the criteria for HAM/TSP, have neurologi-

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cal symptoms such as erectile dysfunction and neurogenic bladder, features that may precede the development of HAM/TSP (12,13).

Due to the role of pro-inflammatory mediators in the pathogenesis of tissue damage associated with HTLV-1 infections, immunomodulatory agents are natural candidates to be used as adjuvant therapy in patients with HAM/TSP and in individuals who are at higher risk to develop myelopathy. Neutralization of TNF- $\alpha$  has been the most successful form of immunotherapy among the cytokine antagonists used in clinical practice (14-17). Inhibition of TNF- $\alpha$  synthesis can be achieved by several means: inhibition of transcription, decrease of mRNA half-life, and inhibition of translation (18). Although some substances act on more than one level, there are at least preferential modes of action. Pentoxifylline increases cAMP, blocks the transcription of TNF- $\alpha$  mRNA from macrophages (14) and down-regulates *in vivo* and *in vitro* the expression of intracellular adhesion molecule-1 (ICAM-1) in monocytes (19). Rolipram acts by blocking the transcription of TNF- $\alpha$  mRNA from macrophages; however, compared to pentoxifylline, it is more potent in suppressing TNF- $\alpha$  production from PBMC of patients with HAM/TSP (20). Thalidomide decreases the half-life of TNF- $\alpha$  mRNA (14,15,21). The role of TNF- $\alpha$  in the inflammatory process has been documented in several inflammatory and infectious diseases such as multiple sclerosis, inflammatory bowel diseases, rheumatoid arthritis, and mucosal leishmaniasis (14,16,17). The purpose of the present study was to evaluate the ability of TNF- $\alpha$  production inhibitors to decrease the *in vitro* production of pro-inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$ . The overall data showed that all drugs had an inhibitory effect on TNF- $\alpha$  production and, with the exception of thalidomide, all of them also decreased IFN- $\gamma$  production.

## Material and Methods

### Study design and patient selection

This was an experimental *in vitro* study evaluating the effect of four TNF- $\alpha$  inhibitors, i.e., pentoxifylline, forskolin, rolipram, and thalidomide, in PBMC of individuals infected with HTLV-1. Samples were taken under informed consent, and the study was conducted with the approval of the Ethics Committee of Hospital Universitário Professor Edgard Santos. The study subjects consisted of 37 patients who attended the HTLV-1 clinic of Hospital Universitário Professor Edgard Santos, Universidade Federal da Bahia, Brazil. All patients were evaluated by a neurologist. Motor and other neurological dysfunctions were measured with two scales: Expanded Disability Status Scale (EDSS) (22) and Osame's Motor Disability Score (OMDS). Exclusion criteria were positive serology for HIV-1 and -2, syphilis, and hepatitis virus B or C infection. Seven HTLV-1-negative individuals participated as controls. Nineteen ( $53 \pm 11$  years old) of the 37 HTLV-1-positive subjects had HAM/TSP defined by

neurological abnormalities (OMDS  $\geq 1$  EDSS  $\geq 2$ ) and the presence of anti-HTLV-1 antibody in CSF. The other 18 individuals ( $47 \pm 11$  years old) were HTLV-1 carriers and had OMDS = 0 and EDSS  $\leq 2$ . The diagnosis of HTLV-1 infection was based on a positive serology by ELISA (Murex HTLV-I + II, England), confirmed by Western blot analysis (Genelabs HTLV 2.3-2.4, Singapore).

### PBMC cultures and *in vitro* assays of drug effects

PBMC from HTLV-1-infected individuals and controls were isolated from heparinized blood by density gradient centrifugation with Ficoll-Hypaque as described (6). Briefly, PBMC were washed with saline and  $3 \times 10^6$  cells/mL per well were cultured on 24-well flat-bottom plates (Falcon, Becton Dickinson, USA), in RPMI 1640 (Life Technologies Gibco BRL, USA), supplemented with 10% human AB serum (Sigma, USA), glutamine, HEPES, and antibiotics. The cultures were kept without a stimulus for 72 h at 37°C, 5% CO<sub>2</sub> in the absence or presence of the TNF- $\alpha$  inhibitors. TNF- $\alpha$  and IFN- $\gamma$  levels were measured in cell supernatants by the ELISA sandwich technique according to manufacturer instructions (PharMingen, USA). The activity of TNF- $\alpha$  inhibitors and cell viability were tested by evaluating the best dose able to inhibit TNF- $\alpha$  and IFN- $\gamma$  while maintaining cell viability. Dose-response curves were constructed using non-stimulated PBMC from HAM/TSP patients and PBMC from healthy controls stimulated with phytohemagglutinin (PHA) at a final dilution of 1:10. The concentrations of the drugs tested were: 1, 10, 50, and 200  $\mu$ M for pentoxifylline, forskolin, and thalidomide, and 0.01, 0.1, 1, 10, 100  $\mu$ M for rolipram. Cell viability was measured by counting the number of live cells stained with Trypan blue after 72-h incubation with the drugs tested. The results are reported as pg/mL by interpolation in a standard curve with the recombinant cytokines.

### Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to detect IFN- $\gamma$

Total RNA was isolated from PBMC of 4 HAM/TSP and 8 HTLV-1 carriers with and without pentoxifylline (50  $\mu$ M). The RNA was extracted from PBMC using Trizol LS reagent (Invitrogen, USA). cDNA was synthesized using 3 mg total RNA and reverse-transcribed by M-MLV reverse transcriptase. PCR was then performed in a final volume of 50  $\mu$ L containing 2.0 mM MgCl<sub>2</sub>, 0.2 mM deoxyribonucleoside triphosphate (dNTP) mix (dATP, dCTP, dTTP, dGTP), 10X PCR buffer, 2.5 U *Taq* DNA polymerase recombinant (Invitrogen), and specific primers at 25-50 pmol using a Veriti Thermal Cycler (Applied Biosystems, USA). The human primer sequences were: HPRT forward: GCGTCGTGATTAGTGATGATGAAC and HPRT reverse: GGATTATACTGCCTGACCAAGG; IFN- $\gamma$  forward: CAGCTCTGCATCGTTTGGGTTCT and IFN- $\gamma$  reverse: TGCTCTTCGACCTCGAAACAGCAT. The PCR profiles used were: 30 cycles of 1 min at 95°C for denaturation, 1 min

at 60°C for annealing and extension for 1 min 30 s at 72°C, plus a final extension step of 10 min at 72°C. The PCR amplification products were visualized on 1.3% agarose gel stained with 0.2% ethidium bromide (Sigma). Band intensity was calculated using the Vision Works LS image acquisition system version 6.5.2 (UVP, UK). Results are reported as relative units (RU) corrected for HPRT expression.

### Statistical analysis

The nonparametric Mann-Whitney U-test was used to compare data between patients with HAM/TSP and HTLV-1 carriers. The nonparametric paired Wilcoxon signed rank test was used to compare the cytokine levels in PBMC culture supernatants without and with the drugs tested. These tests were performed using the Instat program (USA), and P values <0.05 were considered to be of statistical significance.

## Results

### Study subjects and IFN- $\gamma$ and TNF- $\alpha$ levels

The degree of neurological dysfunction and the concentrations of TNF- $\alpha$  and IFN- $\gamma$  in 19 patients with HAM/TSP and 18 HTLV-1 carriers are shown in Table 1. There was no difference in age between the two groups. As expected, EDSS and OMDS scores were higher in patients with HAM/TSP than in HTLV-1 carriers. There was wide variability in the levels of both cytokines. The median IFN- $\gamma$  level was 291 pg/mL, with values ranging from 0 to 1,877 pg/mL, in HAM/TSP patients, and 11 pg/mL, with values ranging from 0 to 1,950 pg/mL, in HTLV-1 carriers ( $P = 0.017$ ). The median level of TNF- $\alpha$  was 148 pg/mL in HAM/TSP patients, ranging from 0 to 1,334 pg/mL, and 5 pg/mL in HTLV-1 carriers, with values ranging from 0 to 926 pg/mL ( $P = 0.013$ ).

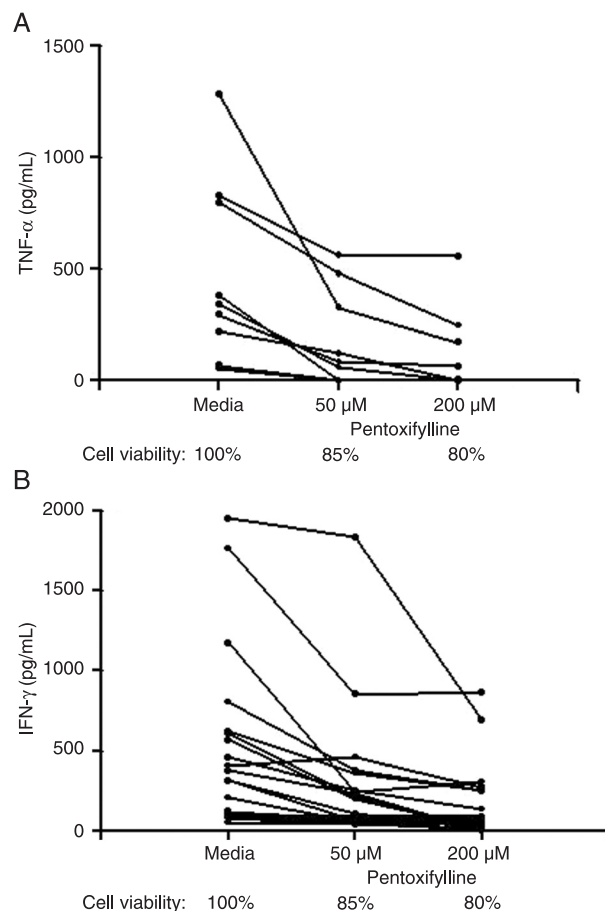
### Effect of TNF- $\alpha$ inhibitors

The effect of TNF- $\alpha$  inhibitors was determined in all infected subjects, independent of group, who had spontaneous TNF- $\alpha$  and IFN- $\gamma$  production >50 pg/mL. Since IFN- $\gamma$  levels were higher than TNF- $\alpha$  levels in HTLV-1 infection, there were more patients in the experiments evaluating IFN- $\gamma$  inhibition ( $N = 16$ ) than TNF- $\alpha$  inhibition ( $N = 9$ ). For each drug in culture the minimum and maximum doses able to suppress TNF- $\alpha$  and IFN- $\gamma$  in PHA-stimulated cultures and to maintain cell viability were used. Since rolipram was the least used of the three drugs tested, three concentrations of rolipram were used. Percent TNF- $\alpha$  and IFN- $\gamma$  inhibition by pentoxifylline is shown in Table 1 and the concentrations of these cytokines observed in cultures without and with TNF- $\alpha$  inhibitors are shown in Figures 1, 2, and 3. The percentage

**Table 1.** Demographic, clinical and immunological characteristics of HTLV-I carriers and HAM/TSP patients.

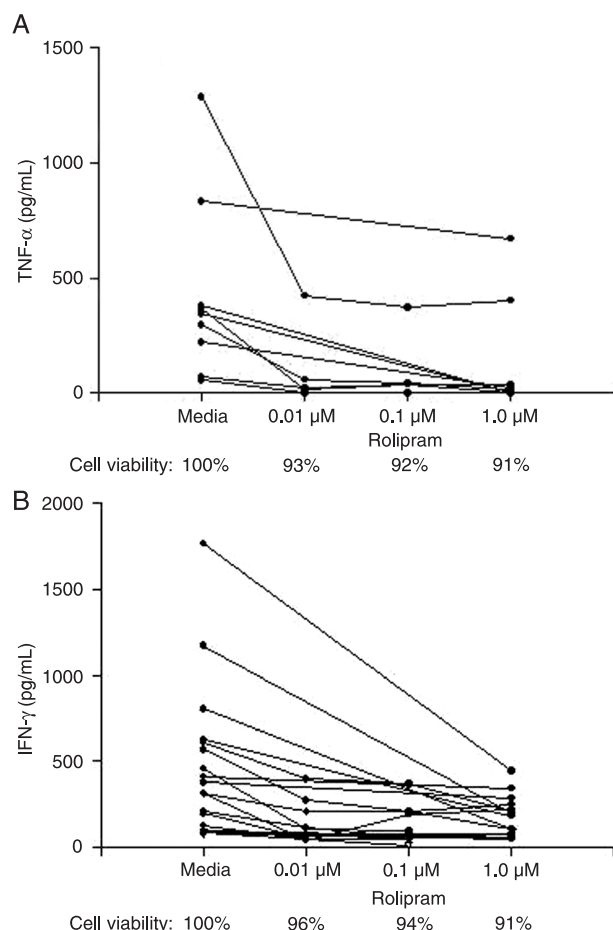
Clinical and immunological features	HAM/TSP (N = 19)	HTLV-I carriers (N = 18)
Age (years)	53 $\pm$ 11 (38-75)	47 $\pm$ 11 (27-66)
EDSS score	4.5 $\pm$ 1.8 (2.5-8.0)	1.5 $\pm$ 0.2 (1.0-1.5)*
OMDS score	4 $\pm$ 1.9 (1-10)	0
Spontaneous IFN- $\gamma$ (pg/mL)	513 $\pm$ 650 (0-1,877)	197 $\pm$ 470 (0-1,950)*
Spontaneous TNF- $\alpha$ (pg/mL)	332 $\pm$ 423 (0-1,334)	131 $\pm$ 277 (0-926)*

Data are reported as means  $\pm$  SD (range in parentheses). HAM/TSP = myelopathy/tropical spastic paraparesis; HTLV-1 = human T lymphotropic virus type 1; EDSS = Expanded Disability Status Scale; OMDS = Osame's Motor Disability Score. \* $P < 0.05$  compared to HAM/TSP (Mann-Whitney U-test).



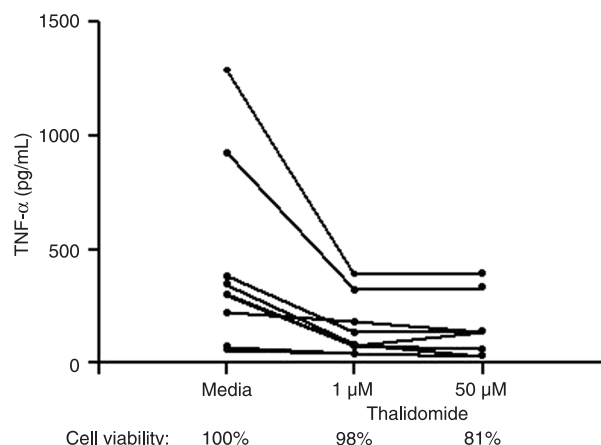
**Figure 1.** Effect of pentoxifylline on TNF- $\alpha$  and IFN- $\gamma$  levels. PBMC from HTLV-1-infected individuals ( $N = 9$  and  $N = 5$ ) were exposed to 50 and 200  $\mu$ M pentoxifylline and the levels of TNF- $\alpha$  (A) and IFN- $\gamma$  (B) were measured by ELISA. Cell viability was tested by counting the live and dead cells stained with Trypan blue after incubation with the drugs tested. TNF- $\alpha$  = tumor necrosis factor- $\alpha$ ; IFN- $\gamma$  = interferon- $\gamma$ ; PBMC = peripheral blood mononuclear cells; HTLV-1 = human T lymphotropic virus type 1.





**Figure 2.** Effect of rolipram on TNF- $\alpha$  and IFN- $\gamma$  levels. PBMC from HTLV-1-infected individuals (N = 8 and N = 10) were exposed to 0.01  $\mu$ M, 0.1  $\mu$ M, and 1.0  $\mu$ M rolipram and the levels of TNF- $\alpha$  (A) and IFN- $\gamma$  (B) were measured by ELISA. Cell viability was tested by counting the live and dead cells stained with Trypan blue after incubation with the drugs tested. For abbreviations, see legend to Figure 1.

of TNF- $\alpha$  inhibitor represents the data observed with the total number of individuals including HTLV-1 carriers and patients with HAM/TSP because the inhibition observed in these two groups was similar. For instance, the mean TNF- $\alpha$  inhibition with 50  $\mu$ M pentoxifylline in cells from the total group of 9 HTLV-1-positive subjects was  $71 \pm 26\%$ , being  $72 \pm 28\%$  in 6 HAM/TSP patients and  $70 \pm 30\%$  in 3 HTLV-1 carriers ( $P = 0.92$ ). At a concentration of 200  $\mu$ M, the inhibition of TNF- $\alpha$  observed in cell cultures of HTLV-1-positive subjects was  $84 \pm 25$ – $86 \pm 27\%$  in the HAM/TSP group and  $82 \pm 16\%$  in the HTLV-1 carriers ( $P = 0.85$ ). Pentoxifylline also decreases IFN- $\gamma$  synthesis. The inhibition of IFN- $\gamma$  production was  $46 \pm 24\%$  with 50  $\mu$ M pentoxifylline and  $65 \pm 29\%$  with 200  $\mu$ M. As shown in Figure 1A and B, cell viability at the highest concentration of the drug was 80%. The results with forskolin were similar to those observed with pentoxifylline.



**Figure 3.** Effect of 1 and 50  $\mu$ M thalidomide on TNF- $\alpha$  levels of PBMC from HTLV-1-infected individuals (N = 8). The levels of TNF- $\alpha$  were measured by ELISA. Cell viability was tested by counting the live and dead cells stained with Trypan blue after incubation with the drugs tested. For abbreviations, see legend to Figure 1.

The inhibition of TNF- $\alpha$  production was  $77 \pm 25\%$  with 50  $\mu$ M forskolin and  $73 \pm 29\%$  with 200  $\mu$ M. The inhibition of IFN- $\gamma$  synthesis was  $61 \pm 22$  and  $77 \pm 25\%$ , respectively. The cell viability at the highest concentration of the drug was 79%. The doses of rolipram used were 0.01, 0.1, and 1  $\mu$ M. The best inhibition of TNF- $\alpha$  production was achieved with 1  $\mu$ M, which caused  $80 \pm 22\%$  inhibition (Figure 2A). The minimum dose of rolipram (0.01  $\mu$ M) inhibited IFN- $\gamma$  production by  $47 \pm 20\%$  while the maximum dose (1.0  $\mu$ M) inhibited it by  $50 \pm 24\%$  (Figure 2B). The cell viability with 1  $\mu$ M rolipram was 91%. The minimum dose of thalidomide used (1  $\mu$ M) was able to inhibit TNF- $\alpha$  production by  $65 \pm 22\%$  (Figure 3). Moreover, IFN- $\gamma$  levels after the addition of thalidomide were assayed only in five experiments because thalidomide showed no inhibitory effect on IFN- $\gamma$  (less than 50% inhibition) when the maximum dose (50  $\mu$ M) was used. These results show that pentoxifylline, forskolin and rolipram were able to suppress *in vitro* TNF- $\alpha$  and IFN- $\gamma$  production. It was also observed that thalidomide was efficient only in suppressing TNF- $\alpha$  synthesis.

#### Detection of IFN- $\gamma$ mRNA by RT-PCR

Since the ability of these drugs to decrease IFN- $\gamma$  production represents new information, we evaluated whether pentoxifylline inhibited TNF- $\alpha$  RNA at the molecular level.

PBMC from HTLV-1-infected patients were cultured with and without 50  $\mu$ M pentoxifylline. After 72 h of culture the cells were harvested and IFN- $\gamma$  mRNA expression was analyzed by RT-PCR. IFN- $\gamma$  mRNA expression was detected in all cultures (with and without 50  $\mu$ M pentoxifylline) and no difference was observed between the HTLV-1-infected groups (data not shown).

## Discussion

HAM/TSP is a chronic immune-mediated neurologic disease characterized by leg weakness and spastic paraparesis that leads to physical disability (23,24). TNF- $\alpha$  plays an important role in the pathogenesis of chronic inflammatory diseases (18). In HAM/TSP, IFN- $\gamma$  and TNF- $\alpha$  may contribute to tissue damage by recruiting inflammatory cells and inducing apoptosis. Moreover, these cytokines are also increased in serum and CSF and also stimulate nitric oxide (NO) and metalloproteinase synthesis (18). We show here that several drugs known to decrease TNF- $\alpha$  synthesis inhibit not only TNF- $\alpha$  but also IFN- $\gamma$  production *in vitro* by PBMC of both HTLV-1 carriers and HAM/TSP patients.

In the present study, we compared the action of TNF- $\alpha$  inhibitors on spontaneous TNF- $\alpha$  and IFN- $\gamma$  production by PBMC of HTLV-1-infected individuals to evaluate the effective dose needed to suppress the production of these cytokines and to maintain cell viability. These *in vitro* tests are important due to the wide variability in the activation state or deregulation of the immune system during HTLV-1 infection, which may influence the effects of drugs, including enhancement of cellular toxicity. Our *in vitro* results show that rolipram suppresses both TNF- $\alpha$  and IFN- $\gamma$  production by cell cultures from HTLV-1-infected subjects even at very low concentrations.

Pentoxifylline has been extensively studied in terms of TNF-suppressing activity (25). Pentoxifylline has been effective in the treatment of erythema nodosum in leprosy (26,27) and, in combination with antimony therapy, it accelerates the healing time of cutaneous and mucosal leishmaniasis and the cure of patients refractory to antimony therapy (16,28-30). Our results show that pentoxifylline had a good suppressor effect on the spontaneous production of TNF- $\alpha$ , preserving cell viability. Additionally, it also decreased IFN- $\gamma$  production to a lesser extent. Forskolin had an effect similar to that of pentoxifylline; however, it was more efficient in inhibiting IFN- $\gamma$  than pentoxifylline. Thalidomide analogues have been considered to have a high therapeutic potential for TNF- $\alpha$  inhibition by increasing the degradation of TNF- $\alpha$  mRNA in macrophages (15). Our results showed that thalidomide inhibited TNF- $\alpha$  production by PBMC at a low dose (1  $\mu$ M). However, it did not inhibit IFN- $\gamma$  production. The specificity of thalidomide in inhibiting only TNF- $\alpha$ , preserving the production of IFN- $\gamma$ , can be important in situations where it is necessary to preserve the effector functions of this cytokine such as tumor cytotoxicity and antiviral activities, avoiding the induction of immunosuppression and predisposing to infections by intracellular agents and tumor development. Although IFN- $\gamma$  production in HTLV-1 infection can be decreased by these drugs, the molecular mechanisms leading to the suppression of this cytokine are still not fully understood. Here we showed that IFN- $\gamma$  mRNA expression was not altered by pentoxifylline, indicating that in such case the inhibition is

post-transcriptional, either regarding protein synthesis or protein secretion.

The drugs tested in the present study showed a high inhibitory effect on TNF- $\alpha$  production *in vitro* and, to a lesser extent, on IFN- $\gamma$  production by PBMC from HTLV-1-infected patients. IFN- $\gamma$  and TNF- $\alpha$  levels are very high in HTLV-1 infection and a possible role of these cytokines in both defense mechanisms but also in pathology associated with infection have been considered. IFN- $\gamma$  activates cytotoxic T cells and stimulates TNF- $\alpha$  secretion and could induce neuronal destruction mediated by HTLV-1-specific cytotoxic CD8 T cells and the production of mediators such as NO and metalloproteinases.

There is no effective therapy for HTLV-1. Anti-retroviral agents used for HIV associated with other drugs have improved the prognosis of ATL, but little effect has been shown on HAM/TSP (31). The pathogenesis of HAM/TSP is dependent on an exacerbated immunological response induced by the Tax gene of HTLV-1. However, although a large proportion of HTLV-1 carriers produce high levels of IFN- $\gamma$  and TNF- $\alpha$ , they do not develop the disease (6,32). We have observed that, in contrast to cells from patients with HAM/TSP, PBMC from HTLV-1 carriers can be down-modulated *in vitro* by regulatory cytokines such as IL-10 and TGF- $\beta$  and neutralizing antibodies against IL-2 and IL-15 (33). Other investigators have also shown a decrease in the frequency of regulatory T cells in patients with HAM/TSP (34). These observations suggest that drugs having as target the inhibition of IFN- $\gamma$  and TNF- $\alpha$  production are potential agents to be used in HAM/TSP.

Anti-TNF- $\alpha$  therapy has been effective in other chronic disorders associated with high TNF- $\alpha$  levels such as rheumatoid arthritis, Crohn's disease, multiple sclerosis, leprosy, and mucosal leishmaniasis (16,17,25,35,36). In HAM/TSP, open studies have also shown a beneficial effect of pentoxifylline (37,38).

Pentoxifylline, rolipram, forskolin, and thalidomide inhibited TNF- $\alpha$  production *in vitro* in patients infected with HTLV-1. As down-modulation of the inflammatory response is desirable to prevent neurological damage in HTLV-1 infection, this study gives support to the use of this type of drug alone or in combination with antiretroviral therapy in the treatment of HTLV-1-infected subjects at high risk to develop HAM/TSP.

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