

PRESENCE OF CIRCULATING LEVELS OF INTERFERON- γ , INTERLEUKIN-10 AND TUMOR NECROSIS FACTOR- α IN PATIENTS WITH VISCERAL LEISHMANIASIS

Iara Marques de MEDEIROS(1, 2), Aduato CASTELO(1) & Reinaldo SALOMÃO(1)

SUMMARY

Experimental murine *L. major* infection is characterized by the expansion of distinct CD4+ T cell subsets. The Th1 response is related to production of IFN- γ and resolution of infection, whereas Th-2 response with production of IL-4 and IL-10 and dissemination of infection. The objective of this study was to measure the circulating levels of IFN- γ , IL-10 and TNF- α in patients with visceral leishmaniasis (VL) before, during and at the end of therapy and to examine the association between cytokine levels and activity of VL. Fifteen patients with VL were evaluated. The cytokine determinations were done by using the enzyme-linked immunoassay (ELISA) before, during and at the end of therapy. At baseline, we detected circulating levels of IFN- γ in 13 of 15 patients (median = 60 pg/ml); IL-10 in 14 of 15 patients (median = 141.4 pg/ml); and TNF- α in 13 of 14 patients (median = 38.9 pg/ml). As patients improved, following antimonial therapy, circulating levels of IL-10 showed an exponential decay ($y = 82.34 e^{-0.10367x}$, $r = -0.659$; $p < 0.001$). IFN- γ was no longer detected after 7/14 days of therapy. On the other hand, circulating levels of TNF- α had a less pronounced decay with time on therapy, remaining detectable in most patients during the first seven days of therapy ($y = 36.99 - 0.933x$, $r = -0.31$; $p = 0.05$). Part of the expression of a successful response to therapy may, therefore, include reduction in secretion of inflammatory as well as suppressive cytokines. Since IL-10 and IFN- γ are both detected prior to therapy, the recognized cellular immune depression seen in these patients may be due to biological predominance of IL-10 (type 2 cytokine), rather than lack of IFN- γ (type 1 cytokine) production.

KEYWORDS: Cytokines; IFN- γ ; IL-10; TNF- α ; Visceral Leishmaniasis (Kala-Azar)

INTRODUCTION

The severity of disease produced by the diverse species of *Leishmania* that infect humans varies widely, ranging from cutaneous or mucosal involvement, that can be self-limited or destructive, to visceral infections that can be mild or fatal. The wide spectrum of clinical manifestations of leishmaniasis is related both to species of *Leishmania* involved and the immune status of the infected host¹³.

Leishmania are an example of a group of organisms that have adapted remarkably well in to their ability to infect, propagate and persist within mononuclear phagocytes. Visceral Leishmaniasis (VL) is a reticuloendothelial infection characterized by cell-mediated immune defects. Patients with active VL have negative intradermal skin tests, high levels of immunoglobulin, low or absence of lymphocyte proliferation,

and IFN- γ and IL-2 production upon *in vitro* stimulation with *Leishmania* antigens¹³. Lymphocytes from VL patients stimulated with *Leishmania* antigens do not activate macrophages to kill *Leishmania*. Such immunological abnormalities are reversed after successful therapy, suggesting that the parasite plays a key role in immunosuppression³.

Resistance or susceptibility to infection by *Leishmania* is associated with distinct patterns of cytokine production in murine models of *L. major* infection. Susceptibility is associated with the expansion of a subset of CD4+ T cells expressing a Th-2 phenotype, characterized by the production of IL-10 and IL-4. In contrast, resistance to infection is associated with the expansion of a subset of CD4+ T cells expressing a Th-1 phenotype, producing IL-2 and IFN- γ ⁶. IL-10 and IL-4,

Presented in part: 7th International Congress for Infectious Diseases, Hong Kong, June 1996.

This study was, in part, supported by Fundação de Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant 524088/96-9)

Iara Marques de Medeiros was supported by a grant from Fundação de Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Informed consent was obtained from patients or their parents or guardians before enrollment in this investigation.

(1) Division of Infectious Diseases. Escola Paulista de Medicina/Universidade Federal de São Paulo. Rua Botucatu, 740, 04023-062 São Paulo, SP, Brasil.

Fax: (011) 572-6348. e-mail: idipa@alphanet.com.br

(2) Division of Infectious Diseases. Universidade Federal do Rio Grande do Norte. Rua Conego Monte, s/n, 59037-170, Natal, RN, Brasil.

e-mail: iaramed@truenetn.com.br

Correspondence to: Reinaldo Salomão. Division of Infectious Diseases, Escola Paulista de Medicina/Universidade Federal de São Paulo. Rua Botucatu, 740, 04023-062 São Paulo, SP, Brasil. Fax: (011) 572-6348. e-mail: idipa@alphanet.com.br

however, may be produced by macrophages and B-cells, and IFN- γ may also come from CD8+ T cells or NK cell¹⁰. Indeed, in human beings, IL-10 is produced by both Th-1 and Th-2 T cells clones⁴. These cytokines will be referred as Th-1 and Th-2 types cytokines profiles¹⁷.

There is cross-regulation between Th-1 and Th-2 types cytokines profiles. In experimental leishmaniasis, resistance may be reversed by administration of IL-4¹⁵ and susceptibility may be reversed by administration of recombinant IFN- γ ¹². TNF- α , a cytokine produced mainly by macrophages, is critical for both resistance to *Leishmania* and resolution of infection in experimental leishmaniasis¹⁸.

IFN- γ is necessary for the activation of macrophages, the release of TNF- α and hence the killing of *Leishmania*. In contrast, IL-10 is a potent macrophage deactivator. It inhibits TNF- α production by activated macrophages, and is a strong inhibitor of the parasite-killing functions of IFN- γ -activated macrophages¹¹.

Understanding the cross-regulation of Th-1 and Th-2 types cytokines profiles and their effects on infected mononuclear phagocytes in patients with VL may contribute to a better comprehension of the immunobiology of this disease. Recently, marked elevation of both IL-10 and IFN- γ mRNA was shown in patients with VL^{5,9}. In this study, we measured the circulating levels of IFN- γ (Th-1 type cytokine profile), IL-10 (Th-2 type cytokine profile) and TNF- α (mainly produced by macrophage) in patients with VL before, during and at the end of therapy and examined the association between cytokine levels and clinical manifestation of VL.

MATERIALS AND METHODS

We evaluated 15 patients with VL admitted to Brazilian hospital (Natal, Northeast Brazil) between August 1995 and February 1996. Diagnoses were based on clinical manifestations and confirmed by the finding of *Leishmania* parasites in bone marrow (14 patients) or spleen aspirates (1 patient). All patients underwent daily clinical evaluation and a routine laboratory workup during hospitalization and were treated with meglumine antimoniate (Glucantime®), except one patient who received liposomal Amphotericin B (AmBisome®) as part of the protocol of another study. All patients had good response to therapy, with decrease in spleen and liver size, recovery of anemia and leucopenia, similar to previous results obtained in the same area¹⁶. Fifteen healthy individuals, without history of previous VL, leaving in the same area, were used as controls.

After obtaining informed consent, blood samples for cytokine determination were collected in heparinized tubes on days 0, 7, 14, 21, and 28 of antimonial therapy. Plasma was maintained at -70°C in aliquots and thawed just before use.

The plasma levels of TNF- α , IL-10, and IFN- γ were determined by a commercially available kit for enzyme-linked immunosorbent assay (ELISA-Genzyme-USA). The tests were

performed according to the manufacturer's instructions. The sensitivity of the tests were 5 pg/ml (IL-10), 3 pg/ml (IFN- γ) and 10 pg/ml (TNF- α).

The change of TNF-alpha with time on therapy was analyzed through linear regression analysis ($Y_i = \beta_0 + \beta_1 X_i$), whereas the change of IL-10 with time on therapy was better predicted by the model $Y_i = \beta_0 e^{-\beta_1 X_i}$, where X_i is day of treatment of the i th patient. Statistical programs 2D and 6D of the BMDP Statistical Software Inc (University of California, 1992, Los Angeles, California, USA) were used.

RESULTS

Except for one patient, who lived in the city of Natal, all came from of the state. Eleven were male and 4 were female, with a median age of 13 years (range 1-35 years). Fever, hepatosplenomegaly, anemia, leucopenia and hypoalbuminemia were observed in most patients, except for one who had undergone a splenectomy because of febrile hepatosplenomegaly. After therapy, all patients improved clinically and showed marked improvement of laboratory tests.

At baseline, we detected circulating levels of IFN- γ in 13 of 15 patients (median = 60 pg/ml, range 0-3000 pg/ml); IL-10 in 14 of 15 patients (median = 141.4 pg/ml, range 0-413.4); and TNF- α in 13 of 14 patients (median = 38.9 pg/ml, range 0-163). Circulating levels of IL-10 and TNF- α were absent in all individuals of control group, whereas IFN- γ was detected in only two of 15 controls (197.8 pg/ml and 41.7 pg/ml).

As patients improved, following antimonial therapy, circulating levels of IL-10 showed an exponential decay ($y = 82.34 e^{-0.10367x}$, $r = -0.659$; $p < 0.001$), being detectable in only 4 patients at day 7. IFN- γ was no longer detected after 7/14 days of therapy. On the other hand, circulating levels of TNF- α has a less pronounced decay with time on therapy, remaining detectable in most patients during the first seven days of therapy ($y = 36.99 - 0.933x$, $r = -0.31$; $p = 0.05$) (Figure 1a, b and c respectively).

DISCUSSION

Our results show that patients with active visceral leishmaniasis have raised levels of circulating Th-1 type (IFN- γ) and Th-2 type (IL-10) cytokines profiles, as well as the mainly macrophage-secreted TNF- α . A role for IL-10 in the immunosuppression associated with Kala-azar has been demonstrated. It has been shown that antibodies to IL-10 reversed the inhibitory effects of T cells (CD8+) obtained from patients with acute disease⁸ and restored IFN- γ production and lymphocyte proliferation in VL, an effect better obtained in combination with anti-IL-4 antibodies. On the other hand, addition of recombinant IL-10 to peripheral blood mononuclear cells (PBMC) obtained from patients cured of VL suppress IFN- γ production and lymphocyte proliferation³. The detection of circulating levels of this cytokine in all but one patient on pre-treatment samples adds evidence that it is associated with disease expression.

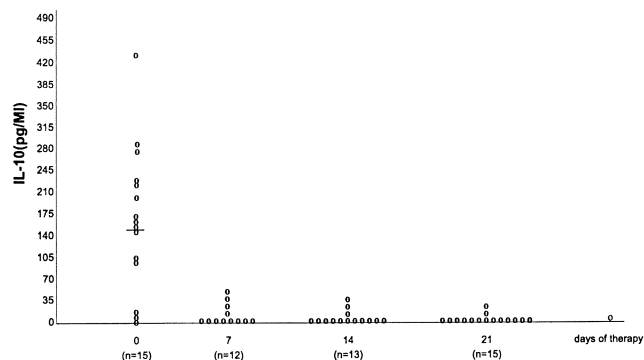


Fig. 1a – Plasma levels of IL-10 (pg/ml), measured by ELISA, in patients with visceral leishmaniasis before, during and after therapy
— = median.

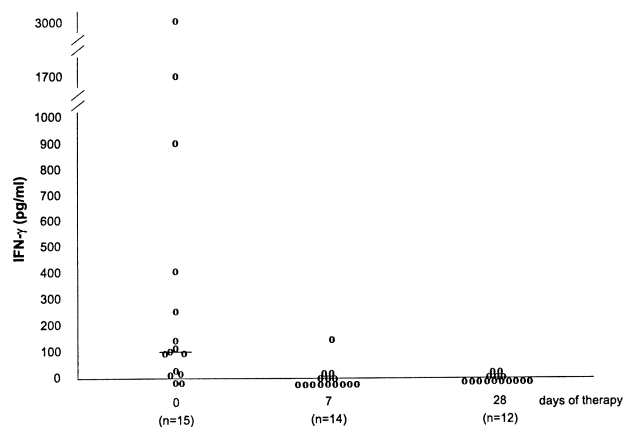


Fig. 1b – Plasma levels of IFN- γ (pg/ml), measured by ELISA, in patients with visceral leishmaniasis before, during and after therapy.
— = median.

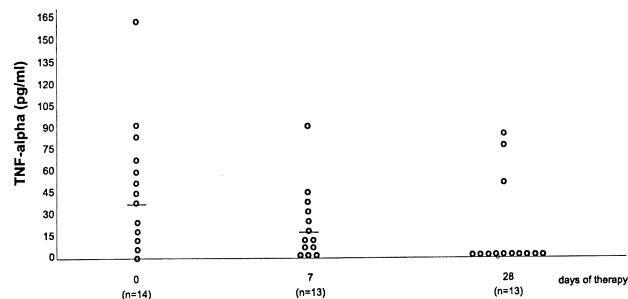


Fig. 1c – Plasma levels of TNF- α (pg/ml), measured by ELISA, in patients with visceral leishmaniasis before, during and after therapy.
— = median.

Detection of circulating IFN- γ prior to therapy was unexpected. Many reports have consistently linked active disease to an absence of IFN- γ production, as judged by PBMC stimulated and assayed *in vitro*^{2,8}. mRNA for both Th-1 and Th-2 types cytokines during active disease has been further described in PBMC, lymphoid tissue⁵ and bone marrow derived cells⁹, using a

reverse transcriptase PCR technique. Our demonstration of circulating levels of IFN- γ and IL-10 corroborates these results and provides further evidence that these cytokines are produced and released into circulation during active disease.

As previously shown^{1,14,16}, circulating levels of TNF- α were also detected during disease. It is interesting to consider that the higher levels of TNF- α are detected simultaneously with the highest levels of IL-10, a known inhibitor of TNF- α secretion.

Our results also show that following antimonial therapy and clinical remission, there is a rapid decline in circulating levels of IL-10. By the seventh day of therapy it was not detectable in 8 of 12 patients, and were detected in much lower levels on the remaining four patients. Circulating levels of IL-10 may be of value in following these patients, early after begin of therapy.

Decrease of IL-10 mRNA following therapy and detection of IFN- γ mRNA in acute and treated patients was previously reported. Thus, it was suggested that, the presence of IL-10, rather than the absence of IFN- γ , is characteristic of acute leishmaniasis⁵. However, in contrast with the results of these authors, who not detected differences in amounts of IFN- γ mRNA in VL lymph nodes before and after treatment, in our patients the circulating levels of IFN- γ also decline during therapy. It is possible that transcription of IFN- γ in lymph nodes remains detected by PCR, but circulating levels did not by ELISA.

We found that circulating levels of TNF- α declined as patients improved, but the slope of TNF- α decay was less pronounced than that of IL-10 and IFN- γ . In experimental *L. major* and *L. donovani* leishmaniasis, TNF- α has been shown to be a critical factor in disease control^{18,19}. In human VL the circulating levels of TNF- α have been related to activity of the disease^{1,16}.

PBMC obtained from individuals representing the spectrum of clinical responses to *L. chagasi* show different behavior regarding cytokines production *in vitro*. IL-10 is detected in supernatants during acute disease⁸, IFN- γ after recovery³ and TNF- α , as well as IL-1, is better induced after therapy⁷. Circulating levels evaluated in this report, however, show similar behavior for all three cytokines before, during and at the end of therapy, except perhaps for the slower decrease of TNF- α , that as previously described^{1,16} remains detectable during the first 7-14 days of therapy.

Part of the expression of a successful response to therapy may, therefore, include reduction in secretion of inflammatory as well as suppressive cytokines.

RESUMO

Presença de níveis circulantes de Interferon- γ , Interleucina-10 e Fator de Necrose Tumoral- α em pacientes com Leishmaniose Visceral

Infecção murina experimental por *L. major* caracteriza-se pela expansão de subpopulações distintas de células T CD4+. A

resposta Th-1 relaciona-se com a produção de IFN- γ e resolução da infecção, enquanto a resposta Th-2 com a produção de IL-4 e IL-10 e disseminação da infecção. O objetivo deste estudo foi de medir os níveis circulantes de IFN- γ , IL-10 e TNF- α em pacientes com leishmaniose visceral antes, durante e ao final do tratamento, e verificar a associação da presença destas citocinas com expressão da doença. Quinze pacientes com LV e quinze controles foram avaliados. As citocinas foram medidas por ensaio imunoenzimático (ELISA). Níveis circulantes de IFN- γ foram detectados em 13 dos 15 pacientes (mediana = 60 pg/ml; de IL-10 em 14 dos 15 pacientes (mediana = 141,4 pg/ml); e de TNF em 13 de 14 pacientes (mediana = 38,9 pg/ml) antes do início da terapêutica. Com a instituição da terapêutica e melhora clínica dos pacientes, os níveis circulantes de IL-10 declinaram exponencialmente ($y = 82,34 e^{-0,10367x}$, $r = -0,659$; $p < 0,001$) e, IFN- γ não foi mais detectado após 7/14 dias de terapêutica. Por outro lado, os níveis de TNF- α apresentaram queda menos acentuada, permanecendo detectável na maioria dos pacientes durante os primeiros dias de terapêutica ($y = 36,99 - 0,933x$, $r = -0,31$; $p = 0,05$). Parte da expressão de uma resposta terapêutica favorável pode, portanto, incluir redução da produção de citocinas tanto inflamatórias como supressoras. Como IL-10 e IFN- γ foram ambos detectados antes da terapia, a depressão imune celular presente em pacientes com LV pode ser devida à predominância de atividade biológica da IL-10 (citocina do tipo 2) e não à ausência de produção de IFN- γ (citocina do tipo 1).

ACKNOWLEDGMENTS

We are in debt to M. Burattini for helpful suggestions and to M.K.C. Brunialti for expert technical assistance.

REFERENCES

1. BARRAL-NETO, M.; BADARÓ, R.; BARRAL, A. et al. – Tumor necrosis factor (Cachectin) in human visceral leishmaniasis. *J. infect. Dis.*, **163**: 853-857, 1991.
2. CARVALHO, E. M.; BADARÓ, R.; REED, S. G. et al. – Absence of gamma-interferon and interleukin-production during active visceral leishmaniasis. *J. clin. Invest.*, **76**: 2066-2069, 1985.
3. CARVALHO, E. M.; BACELLAR, O.; BROWNELL, C. et al. – Restoration of IFN- γ production and lymphocyte proliferation in visceral leishmaniasis. *J. Immunol.*, **152**: 5949-5956, 1994.
4. DEL PRETE, G.; DE CARLI, M.; ALMERIGOGNA, F. et al. – Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J. Immunol.*, **150**: 353-360, 1993.
5. GHALIB, H. W.; PIUVEZAM, M. R.; SKEIKY, Y. A. W. et al. – Interleukin 10 production correlates with pathology in human *Leishmania donovani* infections. *J. clin. Invest.*, **92**: 324-329, 1993.
6. HEINZEL, F. P.; SADICK, M. D.; HOLADAY, B. J. et al. – Reciprocal expression of interferon-gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J. exp. Med.*, **169**: 59-72, 1989.
7. HO, J. L.; BADARÓ, R.; SCHWARTZ, A. et al. – Diminished in vitro production of interleukin-1 and tumor necrosis factor- α during acute visceral Leishmaniasis and recovery after therapy. *J. infect. Dis.*, **165**: 1094-1102, 1992.
8. HOLADAY, B. J.; POMPEU, M. M. L.; JERONIMO, S. et al. – Potencial role for interleukin-10 in the immunosuppression associated with Kala-azar. *J. clin. Invest.*, **92**: 2626-2632, 1993.
9. KARP, C. L.; EL-SAFI, S. H.; WYNN, T. A. et al. – In vitro cytokine profiles in patients with kala-azar: marked elevation of both interleukin-10 and interferon-gamma. *J. clin. Invest.*, **91**: 1644-1648, 1993.
10. LILES, W. C. & VAN VOORHIS, W. C. – Review: nomenclature and biologic significance of cytokines involved in inflammation and the host immune response. *J. infect. Dis.*, **172**: 1573-1580, 1995.
11. MOSMANN, T. R. – Properties and functions of interleukin-10. *Advanc. Immunol.*, **56**: 1-26, 1994.
12. MURRAY, H. W.; STERN, J. J.; WELTE, K. et al. – Experimental visceral leishmaniasis: production of interleukin 2 and interferon-gamma, tissue immune reaction and response to treatment with interleukin 2 and interferon-gamma. *J. Immunol.*, **138**: 2290-2297, 1987.
13. PEARSON, R. D. & SOUZA, A. Q. – Leishmania species: visceral (kala-azar), cutaneous and mucosal leishmaniasis. In: MANDELL, G. L.; DOUGLAS, R. G. & BENNETT, J. B. *Principles and practice of infectious diseases*. 3. ed. New York, Churchill Livingstone, 1990. p. 2066-2077.
14. PISA, P.; GENNENE, M.; SÖDER, O. et al. – Serum tumor necrosis factor levels and disease dissemination in leprosy and leishmaniasis. *J. infect. Dis.*, **161**: 988-991, 1990.
15. POWRIE, F.; MENON, S.; COFFMAN, R. L. – Interleukin 4 and interleukin 10 synergize to inhibit cell-mediated immunity in vivo. *Europ. J. Immunol.*, **23**: 3043-3049, 1993.
16. SALOMÃO, R.; CASTELO FILHO, A.; MEDEIROS, I. M. et al. – Plasma levels of tumor necrosis factor- α in patients with visceral leishmaniasis (kala-azar). Association with activity of the disease and clinical remission following antimonial therapy. *Rev. Inst. Med. trop. S. Paulo*, **38**: 113-118, 1996.
17. SKEIKY, Y. A. W.; GUDERIAN, J. A.; BENSON, D. R. et al. – A recombinant *Leishmania* antigen that stimulates human peripheral blood mononuclear cells express a Th1-type cytokine profile and to produce interleukin 12. *J. exp. Med.*, **181**: 1527-1537, 1995.
18. TITUS, R. G.; SHERRY, B. & CERAMI, A. – Tumor necrosis factor plays a protective role in experimental murine cutaneous leishmaniasis. *J. exp. Med.*, **170**: 2097-2104, 1989.
19. TUMANG, M. C. T.; KEOGH, C.; MOLDAWER, L. L. et al. – Role and effect of TNF- α in experimental visceral leishmaniasis. *J. Immunol.*, **153**: 768-775, 1994.

Recebido para publicação em 15/09/1997
Aceito para publicação em 28/01/1998