

Evaluation of IFN- γ and TNF- α as immunological markers of clinical outcome in cutaneous leishmaniasis

IFN- γ e TNF- α como marcadores da resposta clínica na leishmaniose cutânea

Argemiro D'Oliveira Junior¹, Paulo Machado¹, Olívia Bacellar¹, Lay Har Cheng¹,
Roque P. Almeida¹ and Edgar M. Carvalho¹

Resumo Para avaliar se os níveis séricos de IFN- γ e TNF- α podem ser usados como marcadores da resposta terapêutica na leishmaniose cutânea, 54 pacientes com história de uma única lesão ulcerada com duração de até 30 dias foram arrolados para o estudo. IFN- γ e TNF- α foram mensurados por ELISA no sobrenadante de cultura de linfócitos, antes e 60 dias após início da terapia. Cicatrização completa da lesão 60 dias após o tratamento foi considerada critério de cura. Os níveis de IFN- γ e TNF- α foram similares em ambos os grupos antes da terapia. Houve tendência de aumento dos níveis de IFN- γ nos pacientes curados aos 60 dias, os valores não alcançam significância estatística. Em ambos os grupos de pacientes, os níveis iniciais de TNF- α foram semelhantes antes da terapia e caíram significativamente após tratamento independente da cura ou manutenção da lesão ativa.

Palavras-chaves: IFN- γ . TNF- α . Citocinas. Resposta clínica. Leishmaniose cutânea.

Abstract To evaluate if IFN- γ and TNF- α levels could be used as markers of therapeutic response in cutaneous leishmaniasis, 54 patients with history of one ulcerated cutaneous lesion, with up to 30 days onset, were enrolled in the study. IFN- γ and TNF- α were measured by ELISA in lymphocyte cultures supernatant before and 60 days after initiating therapy. Cure was considered to be a complete healing of lesion 60 days after treatment. IFN- γ and TNF- α levels were similar in both groups of patients before therapy. There was a tendency to increase IFN- γ levels in patients that were cured in 60 days, however the values did not reach statistical significance. In both groups of patients, TNF- α levels were similar before therapy and fell significantly after treatment, irrespective of cure or maintenance of active lesion.

Key-words: IFN- γ . TNF- α . Cytokine. Clinical outcome. Cutaneous leishmaniasis.

Progression from infection to disease in experimental models of leishmaniasis has been associated with impairment of cell mediated immune response¹⁶. Intact T cell response and TNF- α and IFN- γ secretion have been demonstrated to play an important role in the control of infection^{14,17}. In human leishmaniasis, absence of cellular immune response is associated with parasite multiplication and severe disease as observed in diffuse cutaneous leishmaniasis and visceral leishmaniasis^{4,7}. These diseases are characterized by the presence of a great number of parasites in macrophages and the absence of IFN- γ production³.

Unlike in diffuse cutaneous and visceral forms of the disease, lymphocytes from cutaneous leishmaniasis patients are able to proliferate and produce IFN- γ upon stimulation with leishmania antigen^{5,6}. In spite of the T cell activation and evidence that macrophages from these patients are able to kill leishmania¹², clinical manifestations characterized by single or multiple cutaneous ulcers are observed. Antimonials are the drug of choice for treatment of leishmaniasis but, in areas of *L. braziliensis* transmission, therapeutic failure has been observed in up to 30% of the patients¹⁸. There is no clinical or laboratory test that would predict which patients

1. Serviço de Imunologia do Hospital Universitário Prof. Edgar Santos da Universidade Federal da Bahia Salvador-Bahia, Brazil.

Financial support: This work was supported by National Institute of Health grant AI-30639 and by Financiadora de Estudos e Projetos, Centro de Apoio ao Desenvolvimento da Ciência e Tecnologia and Programa de Apoio aos Núcleos de Excelência.

Address to: Dr. Edgar M. Carvalho. Serviço de Imunologia/Hospital Universitário Prof. Edgard Santos/UFBA. R. João das Botas s/n, 5º andar, Canela 40110-160 Salvador, BA, Brazil.

Fax: 55 71 245-7110

E-mail: imuno@ufba.br/edgar@ufba.br

Recebido para publicação em 05/3/2001

will respond or fail treatment. Since IFN- γ and TNF- α activate macrophages to produce oxygenated products and nitric oxide, components important for killing of leishmania, it is likely that patients who produce high levels of these cytokines may have a better chance to be cured of *L. braziliensis*

infection. In this study IFN- γ and TNF- α levels were evaluated in supernatants of leishmania antigen-stimulated lymphocyte cultures before and after antimonial therapy to determine if secretion of these cytokines could be used as markers of therapeutic response.

MATERIAL AND METHODS

Fifty-four patients with localized cutaneous leishmaniasis (LCL) from Corte de Pedra, an endemic area of *L. braziliensis* transmission, in the State of Bahia, Brazil, were selected from March to December of 1998 and enrolled in the study. The patients were diagnosed based on presentation with typical cutaneous leishmaniasis ulcer and positive leishmania antigen skin test. Inclusion criteria in the study were: presence of only one ulcerated lesion (maximum diameter of 30mm), illness duration up to 30 days, and no previous treatment. Exclusion criteria included pregnancy and previous use of antimonial or other drugs used to treat leishmaniasis.

Soluble leishmania antigen was prepared as previously described⁵. IFN- γ and TNF- α levels were measured in supernatant of lymphocyte cultures before and 60 days after treatment initiation. In 11 patients these cytokine were also evaluated on day 120 after cure. Mononuclear cells were separated by density gradient centrifugation using lymphocyte separation medium. After washing with saline, the cells were adjusted to 3×10^6 in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% AB serum, stimulated with

leishmania antigen (10 μ g/ml) and cultured for 48 hours at 37°C and 5% CO₂. IFN- γ and TNF- α production were measured in supernatants of lymphocyte culture by an ELISA sandwich technique.

All patients were treated with 20mg/Sb^v/kg/day (Glucantime, Rhodia), intravenously for 20 days. Based on the clinical response at day 60, the patients were divided into two groups: Group 1 - complete healing of the lesion within 60 days and persistence of cure until one year after treatment; and Group 2 - activity of lesion after 60 days and need of one or more courses of antimonial treatment within the next year.

The study was approved by the Ethical Committee of Hospital Universitário Prof. Edgard Santos. Informed consent was obtained from all subjects after the nature of the procedures had been fully explained to them.

Statistical analysis. Mann Whitney test was used to compare values between groups and Student's T-test was used to compare values within groups, using Epi Info 6.04 (Center for Disease Control and Prevention, Atlanta, GA). A P \leq 0,05 was considered statistically significant.

RESULTS

Twenty patients out of 54 were cured of cutaneous lesion after 60 days of treatment. The groups did not differ in terms of the duration of illness, size of the lesion, localization above or beyond the waist, regional lymphadenopathy and median age.

The levels of IFN- γ before therapy in patients of group 1 were similar to that observed for group 2 (Table 1). IFN- γ production also did not differ significantly between

groups after therapy. When IFN- γ levels before and after therapy were compared, within each group, there was noticeable enhancement of IFN- γ levels in group 1. IFN- γ increased from 1,914pg/ml (range = [99-3,807]) to 4,734pg/ml (0-5,916). This increase was not found to be statistically significant, but was based only on the available observation of eight patients. No such trend was observed for IFN- γ levels within group 2.

Table 1 - Level of IFN- γ (pg/ml) in cured (Group 1) and not cured (Group 2) patients with cutaneous leishmaniasis.

Groups	Before treatment median (range)	After treatment median (range)	P value*
1	1,914 (99-3,807)	4,734 (0-5,916)	0.07
2	1,896 (10-5,337)	1,795 (42-6,380)	0.9
P value**	0.94	0.21	

* Compares levels before and after treatment, subjects with missing values excluded.

** Compares groups, all available values used.

TNF- α levels in group 1 before treatment were similar to those observed in group 2 (Table 2). TNF- α after therapy in group 1 was also not significantly different from that in group 2. In spite of a small number of patients evaluated after treatment (12 patients), there were significant decreases in the level of TNF- α after therapy in both, group 1 (650pg/ml [0-2,810] to 80pg/ml [0-360]) and group 2 (545pg/ml [0-1,366] to 146pg/ml [0-944]).

There was no statistically significant correlation between IFN- γ or TNF- α production and the presence of regional lymphadenopathy (data not show). In eleven patients that were cured at day 60 or day 90 TNF- α and IFN- γ levels were evaluated at day 120. In those patients IFN- γ levels before therapy (1423 \pm 855pg/ml) did not differ from that observed after therapy (1591 \pm 1048pg/ml). In contrast there was a significant drop (p<0.05) in the TNF- α levels (584 \pm 244pg/ml) before therapy to (95 \pm 86pg/ml) after therapy.

Table 2 - Level of TNF- α (pg/ml) in cured (Group 1) and not cured (Group 2) patients with cutaneous leishmaniasis.

Groups	Before treatment median(range)	After treatment median (range)	P value*
1	650 (0-2,810)	80 (0-360)	0.02
2	545 (0-1,366)	146 (0-944)	0.03
P value**	0.22	0.29	

* Compares levels before and after treatment, subjects with missing values excluded.

** Compares groups, all available values used.

DISCUSSION

The magnitude of the immune response in cutaneous leishmaniasis depends upon the duration of the illness¹, clinical form of the disease⁸, parasite species and vector species². In this study, in order to avoid clinical and epidemiological heterogeneity, patients were selected for ulcer duration of 30 days or less, were selected from the same endemic area and evenly enrolled over a 10-month period. When the group of patients who healed after 60 days was compared with patients in which the ulcer remained active, no significant differences regarding age, presence of lymphadenopathy, localization and size of the lesion, and illness duration were found. In order to evaluate the immunological aspects of these patients we chose to measure levels of IFN- γ and TNF- α . IFN- γ is the major cytokine involved in macrophage activation, and IFN- γ and TNF- α stimulate the synthesis of nitric oxide, considered be the major product involved in killing leishmania^{13 14 20}. In experimental models of leishmaniasis, IFN- γ or TNF- α knock out mice exhibited more severe disease and inability to control parasite growth^{15 19}. In humans, a decrease in TNF- α and IFN- γ levels after cure of mucosal disease has been observed^{8 10 12}.

In the present study, high levels of IFN- γ and TNF- α were observed in both groups of patients before therapy. Although there was an enhancement of IFN- γ production in patients who were healed by 60 days after therapy initiation, there was also a tendency for increasing IFN- γ in patients who failed therapy. Thus, it is unlikely that IFN- γ increase at day 60 after therapy could be a marker of later response to therapy. TNF- α levels were also high in both groups of patients. In patients who cured within 60 days, both the TNF- α levels before therapy and subsequent reduction after therapy were associated with clinical cure. However although

patients who failed treatment had lower initial levels of TNF- α , they also showed decreased levels post treatment. Considering that the interval between the evaluations were to short the levels of these cytokine were determined after 120 days in 11 patients that healed their lesions with 60 or 90 days. Again in these later evaluation there was no significant difference between IFN- γ levels before and 120 days after therapy, but there was a maintenance of the difference between TNF- α levels before and after cure.

Considering that both, those who were cured and those who failed treatment, had high IFN- γ and TNF- α levels before therapy, therapeutic failure was not dependent on the inability to produce these two cytokines early in the infection. In fact, T cell response in cutaneous leishmaniasis has been shown to be preserved in previous studies^{5 9}. Since these patients have a strong Th1 type of immune response, an intense inflammatory reaction, and very few parasites at the site of the lesion, it cannot be ruled out that a strong and uncontrolled inflammatory reaction may be involved in the pathogenesis of the skin ulcer. In favour of these hypotheses was the absence of association between IFN- γ and TNF- α levels before therapy and early after cure of the disease. Although it is well known that IFN- γ up regulates TNF- α , while there was an increase in IFN- γ after therapy in patients who were cured at 60 days, there was a decrease in TNF- α level in these patients. It's then possible that the clinical outcome may be related more to the ability of the host to modulate the inflammatory response than with the magnitude of the immune response before therapy. Patients that are able to modulate the response and decrease their TNF- α levels after therapy in spite increase in IFN- γ production are those more likely to be cured.

REFERENCES

- Almeida RP, Rocha P, Ribeiro de Jesus A, Costa J, Carvalho EM. Evaluation of cellular immune response in patients with different clinical form of tegumentary leishmaniasis. *Allergy and Immunology* 14:11-19, 1995.
- Berman JD. Human Leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last ten years. *Clinical Infectious Diseases* 24:684-703, 1997.
- Bomfim G, Nascimento C, Costa J, Carvalho EM, Barral-Netto M, Barral A. Variation of cytokine patterns related to therapeutic response in diffuse cutaneous leishmaniasis. *Experimental Parasitology* 84:188-194, 1996.
- Carvalho EM, Barral A, Pedral-Sampaio D, Barral-Netto M, Badaró R, Rocha H, Johnson WD. Immunological markers of clinical evolution in children recently infected with *Leishmania donovani chagasi*. *Journal of Infectious Diseases* 165:535-540, 1992.
- Carvalho EM, Johnson WD, Barreto E, Marsden PD, Costa JML, Reed SG, Rocha H. Cell-mediated immunity in american cutaneous and mucosal leishmaniasis. *Journal of Immunology* 135:4144-4148, 1985.
- Castes M, Agnelli A, Rondon AJ. Mechanisms associated with immunoregulation in human cutaneous leishmaniasis. *Clinical Experimental Immunology* 57:279-286, 1984.

7. Castes M, Moros Z, Martinez A, Trujillo D, Castellanos PL, Rondon AJ, Convit J. Cell-mediated immunity in localized cutaneous leishmaniasis patients before and after treatment with immunotherapy or chemotherapy. *Parasite Immunology* 11:211-222, 1989.
8. Castes M, Trujillo D, Rojas ME, Fernandez CT, Araya L, Crabera M, Blackwell J, Convit J. Serum level of tumor necrosis factor in patient with American cutaneous leishmaniasis. *Brazilian Journal of Medical and Biological Research* 26:233-238, 1993.
9. Coutinho SG, Pirmez C, Mendonça SCF, Conceição-Silva F, Dórea RCC. Pathogenesis and immunopathology of leishmaniasis. *Memórias do Instituto Oswaldo Cruz* 82:214-228, 1987.
10. Da-Cruz AM, Oliveira MP, De Luca PM, Mendonça SCF, Coutinho SG. Tumor necrosis factor- α in human American tegumentary leishmaniasis. *Memórias do Instituto Oswaldo Cruz* 91:220:225-229, 1996.
11. Grimaldi G, Tesh RB. Leishmaniasis of the New World: current concepts and implications for future research. *Clinical Microbiology Research* 6:230-250, 1993.
12. Jesus AR, Almeida RP, Lessa H, Bacellar O, Carvalho EM. Cytokine profile and pathology in human leishmaniasis. *Brazilian Journal of Medical and Biology Research* 31:143-148, 1998.
13. Liew FY. Regulation of nitric oxide synthesis in infectious and autoimmune disease. *Immunology Letter* 43:95-98, 1994.
14. Liew FY, Parkinson C, Millor S, Severn A, Carrier M. Tumor necrosis factor (TNF- α) in leishmaniasis. 1. TNF- α mediates host protection against cutaneous leishmaniasis. *Immunology* 69:570-573, 1990.
15. Nashleanas M, Kanaly S, Scott P. Control of *Leishmania major* infection in mice lacking TNF receptors. *Journal of Immunology* 160:5506-5513, 1998.
16. Reiner SL, Locksley RM. The regulation of immunity of *Leishmania major*. *Annual Review of Immunology* 13:161-177, 1995.
17. Scott P, Natovitz P, Coffman RL, Pearce E, Sher A. Immunoregulation of cutaneous leishmaniasis. T cell line that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *Journal of Experimental Medicine* 168:1675-1684, 1988.
18. Soto-Mancipe J, Grogil M, Berman JD. Evaluation of pentamidine for the treatment of cutaneous leishmaniasis in Colombia. *Clinical Infectious Diseases* 16:417-425, 1993.
19. Wang ZE, Reiner SL, Zheng S, Dalton DK, Locksley RM. CD4+ effector cells default to the Th2 pathway in interferon gamma deficient mice infected with *Leishmania major*. *Journal of Experimental Medicine* 179:1367-71, 1994.
20. Wei XQ, Charles IG, Smith A, Feng GJ, Ure J, Huang FP, Xu D, Muller W, Moncada S, Liew FY. Altered immune response in mice lacking nitric oxide synthase. *Nature* 375:408-411, 1995.