

Downregulation of IFN- γ production in patients with recurrent vaginal candidiasis

Lucas P. Carvalho, BSc, Olivia Bacellar, PhD, Nilma Neves, MD,
Amélia R. de Jesus, MD, PhD, and Edgar M. Carvalho, MD, PhD *Salvador, Brazil*

Background: Recurrent vaginal candidiasis (RVC) is an important health problem with unknown pathogenesis. Although impairment of the T-cell response is associated with persistent or recurrent candidiasis, data on immunologic responses in patients with RVC are controversial.

Objectives: To evaluate the T-cell response in patients with RVC and the ability of cytokines and cytokine antagonists to modulate IFN- γ production in cultures stimulated with *Candida albicans* antigens.

Methods: Participants in the study included 13 patients with RVC and 7 control women with sporadic candidiasis. Cytokines were determined by ELISA in supernatants of mononuclear cells with *C. albicans*, purified protein derivative, or tetanus toxoid antigen.

Results: IFN- γ production was absent or low in 11 of 13 women (84.6%) with RVC. Absent or low IFN- γ production was specific to *C. albicans* antigens (189 ± 389 pg/mL), because high IFN- γ levels were found in cultures stimulated with purified protein derivative (739 ± 774 pg/mL) or tetanus toxoid antigens (1085 ± 546 pg/mL). Monoclonal antibody anti-IL-10 enhanced IFN- γ levels (750 ± 753 pg/mL), and IL-10 suppressed this cytokine production in patients with sporadic candidiasis.

Conclusions: Mononuclear cells from patients with RVC stimulated with *C. albicans* antigen have low or absent IFN- γ production. IL-10 plays an important role in downregulation of the T-cell response in these patients. (*J Allergy Clin Immunol* 2002;109:102-5.)

Key words: Recurrent vaginal candidiasis, cytokines, immune regulation

Recurrent vaginal candidiasis (RVC) is an important health problem and has been observed in up to 5% of women who have vulvovaginal infection caused by *Candida* species.^{1,2} Although several species of *Candida* can cause disease in humans, RVC is predominantly associated with *Candida albicans*.³⁻⁵ Several factors such as use of antibiotics, pregnancy, uncontrolled diabetes mel-

Abbreviations used

PPD: Purified protein derivative
RVC: Recurrent vaginal candidiasis
TT: Tetanus toxoid

litus, and use of estrogens are associated with development of candidiasis. Additionally, it has been shown that impairment of cell-mediated immunity and neutrophil dysfunction can increase susceptibility to *C. albicans* infection.⁶ In experimental models of candidiasis, CD4+ T (T_H1 or T_H2) cells have been determined to play a central role in the outcome of infection.⁷ Protection correlates with the occurrence of T_H1-type responses,^{8,9} whereas T_H2-like responses are associated with disease exacerbation and pathology.^{10,11} Evidence for the role of human T-cell responses in the control of candidiasis includes high frequency and severity of this fungal infection in patients with HIV infection^{12,13} and documentation of an impaired cell-mediated immune response in patients with mucocutaneous candidiasis.¹⁴⁻¹⁶ In spite of the strong association between severe candidiasis and an abnormal T_H1-type immune response, the pathogenesis of RVC is not well established. Although absent or low lymphoproliferative responses to *C. albicans* antigen have been noted,¹⁷⁻¹⁹ similar levels of IL-2 and IFN- γ have been detected in women with RVC and control patients with sporadic candidiasis.²⁰ Additionally, expression of T_H1 cytokines has been documented in cells recovered from vaginal fluid in patients with RVC.²¹ Other studies of patients with RVC have documented increased levels of *C. albicans*-specific IgE in vaginal fluid,²² as well as an association between respiratory allergies and vaginitis.²³⁻²⁵ The aims of this study were to characterize the cytokine profile in the supernatant of mononuclear cells obtained from patients with RVC and stimulated with *C. albicans* antigens and to determine the ability of IL-4 and IL-10 to modulate T-cell immunologic responses to *C. albicans* antigen.

METHODS

Subjects

Participants in this study included 13 women who were referred to the immunology service of Hospital Universitário Professor Edgard Santos because of recurrence of vaginal candidiasis despite

From Serviço de Imunologia do Hospital Universitário Professor Edgard Santos, Universidade Federal da Bahia, Salvador, Bahia, Brazil.

Received for publication April 9, 2001; revised September 6, 2001; accepted for publication September 25, 2001.

Reprint requests: Edgar M. Carvalho, MD, PhD, Hospital Universitário Professor Edgard Santos, Serviço de Imunologia, 5º andar, Rua João das Botas s/nº, Canela 40110-160, Salvador, Bahia, Brazil.

Copyright © 2002 by Mosby, Inc.

0091-6749/2002 \$35.00 + 0 1/84/120555

doi:10.1067/mai.2002.120555

TABLE I. Age, duration of illness, and levels of IFN- γ in 13 women with recurrent vaginal candidiasis in cultures stimulated with *C albicans* antigen or PHA

Patient No.	Age (y)	Duration of disease (y)	Levels of IFN- γ (pg/mL)		
			Media	<i>C albicans</i>	PHA
1	30	2	0	0	2150
2	39	2	0	0	2901
3	30	2	0	75	ND
4	39	3	0	0	1448
5	37	3	0	0	1662
6	25	3	0	148	2288
7	25	5	18	1132	1572
8	42	5	0	0	3358
9	26	6	39	997	1673
10	24	6	0	58	1700
11	29	10	0	0	2214
12	32	13	0	175	1997
13	32	14	0	0	1766

ND, Not done.

antimycotic therapy. The diagnosis of candidiasis was based on identification of *Candida* organisms on cytologic examination. In the 7 patients for whom the *Candida* species was characterized, *C albicans* was the causal agent. All the patients had negative serology to HIV infection, and none had diabetes or renal or liver failure. Control patients were 7 women with sporadic candidiasis. These patients had no history of vulvovaginal candidiasis and no evidence of *C albicans* infection during a 1-year follow-up period. The study followed the guidelines of the ethical committee of the Hospital Universitário Professor Edgard Santos, and informed consent was obtained from all patients.

C albicans antigen preparation

C albicans isolate from a patient with RVC was cultured in Sabouraud's media. The cultures were centrifuged at 3000g for 30 minutes, and the pellet was washed 3 times with PBS. The pellet was resuspended in 0.5N sodium hydroxide and then frozen (-70°C) and thawed at 37°C about 20 times. After the suspension was centrifuged at 3000g for 30 minutes, the supernatant was collected and the pH was adjusted to 7.3. The protein content was determined by using the method of Lowry et al.²⁶

Delayed-type hypersensitivity response

Intradermal skin testing was performed by inoculation with 0.1 mL of *C albicans* antigen (Alergofar, Rio de Janeiro, Brazil) on the forearm. The responses were evaluated after 48 hours, and results were considered positive when induration was greater than 5 mm.

Cell culture and cytokine assay

PBMCs were isolated from heparin-treated venous blood by Ficoll-Hypaque gradient centrifugation (LSM; Organon Teknika Corporation, Durham, NC). After PBMCs were washed 3 times in 0.9% sodium chloride, they were resuspended in RPMI-1640 culture medium (Gibco BRL, Grand Island, NY) supplemented with 10% human AB serum, 100 IU/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. Cells were adjusted to 3×10^6 cells/mL, put in 24-well plates, and stimulated with *C albicans* antigen (0.05 $\mu\text{g/mL}$), tetanus toxoid (TT) (5 LF/mL), purified protein derivative (PPD) (2 $\mu\text{g/mL}$), or PHA in a final dilution of 1:100. In the cultures of PBMCs from some of these patients, monoclonal antibodies anti-IL-4 and anti-IL-10 or isotype controls (kindly provided by Dr Coffman, DNAX Institute, Palo Alto, Calif) were added in a concentration of 200 $\mu\text{g/mL}$. In a few cultures of PBMCs obtained from women with sporadic candidiasis and stimulated with *C albicans* antigen, recombinant

human IL-10 was added (200 ng/mL). After incubation for 72 hours at 37°C in an atmosphere of 5% CO_2 , supernatants were collected and stored at -70°C . The levels of IL-5, IL-10, and IFN- γ were measured by an ELISA (R&D Systems, Minneapolis, Minn) sandwich method, and the results are expressed as picograms per millimeter.

Statistical analysis

The statistical comparisons were performed by using the Mann-Whitney *U* test.

RESULTS

The age, duration of illness, and the IFN- γ production in cultures stimulated with *C albicans* antigen and PHA for the patients studied are shown in Table I. The age of the patients ranged from 24 to 42 years (32 ± 6 years); the median illness duration was 5 years (range, 2-14 years). Although high levels of IFN- γ (526 ± 329 pg/mL) were observed in the supernatants of lymphocyte cultures obtained from women with sporadic candidiasis and stimulated with *C albicans* antigen, absent or low production of IFN- γ was observed in 11 of 13 women (84.6%) with RVC. However, high levels of IFN- γ were observed in the cultures of all 12 patients with RVC whose cells were stimulated with PHA (2094 ± 545 pg/mL). Results of intradermal skin tests with *C albicans* antigen were negative in all patients with RVC and positive in 5 of 7 patients with sporadic candidiasis. The immunosuppression observed in patients with RVC was specific to *C albicans* antigens, because cells from these patients secreted high levels of IFN- γ when stimulated with PPD or TT (Fig 1). The production of IFN- γ in lymphocyte cultures from patients with RVC when stimulated with PPD was 739 ± 774 pg/mL, and when stimulated with TT, 1085 ± 546 pg/mL. These values were higher ($P < .05$) than those observed in cultures stimulated with *C albicans* antigen (198 ± 389 pg/mL).

Levels of IL-5 and IL-10 were measured in supernatants of lymphocyte culture to evaluate the possibility that the decrease in IFN- γ production was related to a strong $\text{T}_\text{H}2$ -like immune response. Low levels of both IL-5 (39 ± 60 pg/mL) and IL-10 (16 ± 32 pg/mL) were detected in cultures stimulated with *C albicans* antigen (Fig 2).

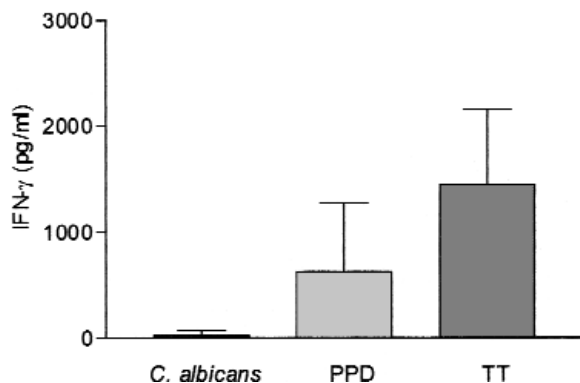


FIG 1. Levels of IFN- γ in supernatants of PBMCs from patients with RVC stimulated with *C. albicans* (n = 13), PPD (n = 9), and TT (n = 4). Data represent mean and SD of IFN- γ production in cultures stimulated with *C. albicans* antigen (0.05 μ g/mL), PPD (2 μ g/mL), or TT (5 LF/mL).

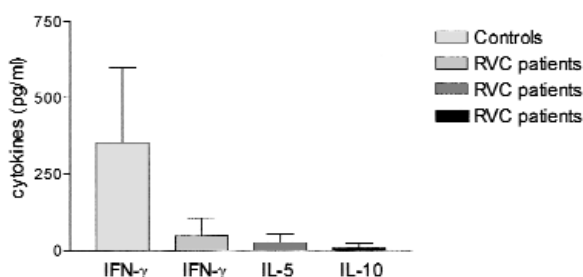


FIG 2. Cytokine profile in patients with RVC (n = 13) and control patients (n = 7). Data represent mean and SD of IFN- γ , IL-5, and IL-10 production in supernatants of PBMCs stimulated with *C. albicans* antigen (0.05 μ g/mL).

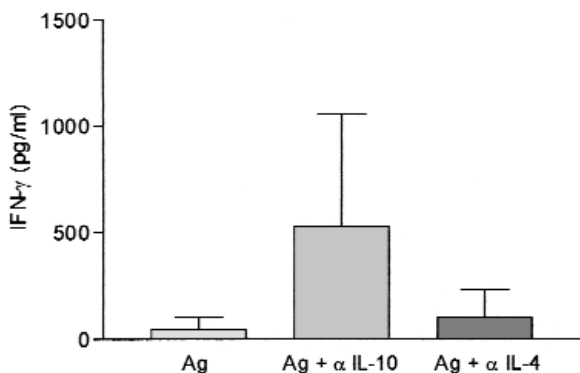


FIG 3. Evaluation of the ability of monoclonal antibodies against IL-4 and IL-10 to restore IFN- γ production in patients with RVC (n = 6). Cultures were stimulated with *C. albicans* antigen (0.05 μ g/mL) in the absence or presence of monoclonal antibodies anti-IL-4 (200 μ g/mL) and anti-IL-10 (200 μ g/mL). Ag, Antigen.

Monoclonal antibodies anti-IL-4 and anti-IL-10 were added to the cultures stimulated with *C. albicans* antigens to determine whether the inability to produce IFN- γ in patients with RVC was due to suppressive effects of regulatory cytokines (Fig 3). IFN- γ production in cultures of PBMCs from 6 women with RVC stimulated with *C. albicans* anti-

gens was 64 ± 70 pg/mL. Addition of monoclonal anti-IL-4 antibody had modest effects on IFN- γ production (100 ± 81 pg/mL). In contrast, neutralization of IL-10 enhanced IFN- γ production 10-fold (750 ± 753 pg/mL). Although it was variable, enhancement of IFN- γ production after IL-10 neutralization was observed in all 6 patients. Isotype controls had no effect on IFN- γ production. IL-10 was added to lymphocyte cultures of patients with sporadic candidiasis to evaluate this cytokine's ability to downregulate the immune response to *C. albicans* antigen. The IFN- γ levels after stimulation with *C. albicans* antigen were 353 ± 248 pg/mL but were reduced significantly to 10 ± 12 pg/mL after the addition of recombinant human IL-10 (200 μ g/mL).

DISCUSSION

The main findings of this study are that the majority of the women with RVC had an antigen-specific impairment of IFN- γ production and that neutralization of IL-10 enhanced the production of this cytokine. All patients studied were young non-malnourished women without a history of debilitating disease and with negative HIV serology. We chose to evaluate IFN- γ production because this cytokine is produced by natural killer and T_H1 cells and is associated with a host defense mechanism against *C. albicans*.³ Although there was great variability in levels of IFN- γ production, it was clear that in the majority of the women with RVC, IFN- γ production was absent or low.

Factors involved in the impairment of T-cell responses to *C. albicans* antigen include the nature of the antigen preparation, the phase of the menstrual cycle during which the evaluation is performed,²⁷ increased synthesis of PGE₂,¹⁷ and an imbalance in T_H1 and T_H2 subpopulations of cells. The antigen used was the same throughout the study, and reproducibility of the results was documented. Additionally, although polysaccharidic antigens may suppress the T-cell response,^{28,29} our antigen was prepared in such a way that it should predominantly contain protein, and based on Lilic studies, it should induce more IFN- γ and lymphocyte proliferation.¹⁴ Although we did not inquire about the phase of the menstrual cycle, this variable would not be expected to affect our results, because the chance of being in the same phase of the menstrual cycle would be equal for both groups of patients (those with sporadic candidiasis and those with RVC). High activity of T_H2 cells suppress IFN- γ production,³⁰ but because of the difficulty in detecting IL-4 in supernatants of human lymphocyte cultures, IL-5 was used as a marker of T_H2 activation. The observation in the present study of low levels of IL-5 in supernatants of PBMCs from these patients argues against the possibility that a strong T_H2 response was involved in the downregulation of IFN- γ production. Although IL-10 levels were not elevated, the effect of neutralization of this cytokine in IFN- γ production was evident. The observation that anti-IL-10 antibody restores immune response in patients, in spite of the very small amount of this cytokine in supernatants, may be explained in two ways: (1) IL-10 can be used quickly by the cells and (2) IL-10 production can be

autoregulated.³¹ In such cases, the low levels of IL-10 did not rule out that this cytokine had been produced in great amounts. We have observed similar findings in patients with visceral leishmaniasis³² and schistosomiasis.³³

Our data indicate that patients with RVC have an immunosuppression specific to *C albicans* antigens, because normal T-cell responses were documented in cultures with unrelated antigens such as TT and PPD. Considering that a predominant T_H2 response was not observed, it is possible that the unresponsiveness to *C albicans* antigen may be related to anergy and that IL-10 may play an important role in the modulation of the immune response of these patients. IL-10, one of the most important regulatory cytokines, decreases both lymphocyte proliferation³⁴ and cytokine secretion by both macrophages and lymphocytes.^{30,35} The potential role of IL-10 in the pathogenesis of infectious diseases such as visceral leishmaniasis,³² schistosomiasis,³³ and diffuse cutaneous leishmaniasis³⁶ has been documented.

Considering that downregulation of the T_H1-type immune response was not observed in all patients with RVC, the absence of IFN- γ production and the downregulation mediated by IL-10 only may explain in part the pathogenesis of RVC. It is possible that although a T-cell response is observed in peripheral blood, an impairment in T-cell function may occur in the vaginal fluid or vaginal mucosa. Alternatively, it can not be ruled out that other suppressor factors may act at the neutrophil and macrophage levels, decreasing the killing of *C albicans*.

We thank Dr Robert L. Coffman (DNAX Institute, Palo Alto, Calif) for providing the monoclonal antibody anti-human IL-4 and anti-human IL-10. We also thank Elbe Myrtes de S. Silva for secretarial assistance.

REFERENCES

- Hurley R. Candidal vaginitis. *Proc R Soc Med* 1977;70(Suppl 4):1-2.
- Sobel JD. Pathogenesis and epidemiology of vulvovaginal candidiasis. *Ann N Y Acad Sci* 1988;544:547-57.
- Odds FC. *Candida* and candidosis. Baltimore: University Park Press; 1979. p. 104-10.
- Fleury FJ. Adult vaginitis. *Clin Obstet Gynecol* 1981;24:407-38.
- Morton RS, Rashid S. Candidal vaginitis: natural history, predisposing factors and prevention. *Proc R Soc Med* 1977;70(Suppl 4):3-6.
- Fulurija A, Ashman RB, Papadimitriou JM. Neutrophil depletion increases susceptibility to systemic and vaginal candidiasis in mice, and reveals differences between brain and kidney in mechanisms of host resistance. *Microbiology* 1996;142:3487-96.
- Puccetti P, Romani L, Bistoni F. A TH1-TH2-like switch in candidiasis: new perspectives for therapy. *Trends Microbiol* 1995;3:237-40.
- Romani L, Mencacci A, Grohmann U, Moccis S, Mosci P, Puccetti P, et al. Neutralizing antibody to interleukin 4 induces systemic protection and T helper type 1-associated immunity in murine candidiasis. *J Exp Med* 1992;176:19-25.
- Romani L, Mencacci A, Cenci E, Spaccapelo R, Mosci P, Puccetti P, et al. CD4⁺ subset expression in murine candidiasis. Th responses correlate directly with genetically determined susceptibility or vaccine-induced resistance. *J Immunol* 1993;150:925-31.
- Romani L, Puccetti P, Mencacci A, Cenci E, Spaccapelo R, Tonnetti L, et al. Neutralization of IL-10 up-regulates nitric oxide production and protects susceptible mice from challenge with *Candida albicans*. *J Immunol* 1994;152:3514-21.
- Spaccapelo R, Romani L, Tonnetti L, Cenci E, Menacchi A, Del Sero G, et al. TGF-beta is important in determining the in vivo patterns of susceptibility or resistance in mice infected with *Candida albicans*. *J Immunol* 1995;155:1349-60.
- Dahl KM, Keath EJ, Fraser VJ, Powderly WG. Molecular epidemiology of mucosal candidiasis in HIV-positive women. *AIDS Res Hum Retroviruses* 1997;13:485-91.
- Duerr A, Sierra MF, Feldman J, Clarke LM, Ehrlich I, DeHovitz J. Immune compromise and prevalence of *Candida* vulvovaginitis in human immunodeficiency virus-infected women. *Obstet Gynecol* 1997;90:252-6.
- Lilic D, Cant AJ, Abinun M, Calvert JE, Spickett GP. Chronic mucocutaneous candidiasis. I. Altered antigen-stimulated IL-2, IL-4, IL-6 and interferon-gamma (IFN-gamma) production. *Clin Exp Immunol* 1996;105:205-12.
- Valdimarsson H, Higgs JM, Wells RS, Yamamura M, Hobbs JR, Holt PJ. Immune abnormalities associated with chronic mucocutaneous candidiasis. *Cell Immunol* 1973;6:348-61.
- Kirkpatrick CH. Chronic mucocutaneous candidiasis. *Eur J Clin Microbiol Infect Dis* 1989;8:448-56.
- Witkin SS, Hirsch J, Ledger WJ. A macrophage defect in women with recurrent *Candida* vaginitis and its reversal in vitro by prostaglandin inhibitors. *Am J Obstet Gynecol* 1986;155:790-5.
- Witkin SS. Inhibition of *Candida*-induced lymphocyte proliferation by antibody to *Candida albicans*. *Obstet Gynecol* 1986;68:696-9.
- Hobbs JR, Brigden D, Davidson F, Kahan M, Oates JK. Immunological aspects of candidal vaginitis. *Proc R Soc Med* 1977;70(Suppl 4):11-4.
- Fidel PL Jr, Lynch ME, Redondo-Lopez V, Sobel JD, Robinson R. Systemic cell-mediated immune reactivity in women with recurrent vulvovaginal candidiasis. *J Infect Dis* 1993;168:1458-65.
- Fidel PL Jr, Ginsburg KA, Cutright JL, Wolf NA, Leaman D, Dunlap K, et al. Vaginal-associated immunity in women with recurrent vulvovaginal candidiasis: evidence for vaginal Th1-type responses following intravaginal challenge with *Candida* antigen. *J Infect Dis* 1997;176:728-39.
- Witkin SS, Jeremias J, Ledger WJ. Vaginal eosinophils and IgE antibodies to *Candida albicans* in women with recurrent vaginitis. *J Med Vet Mycol* 1989;27:57-8.
- Kudelko NM. Allergy in chronic monilial vaginitis. *Ann Allergy* 1971;29:266-7.
- Palacios HJ. Hypersensitivity as a cause of dermatologic and vaginal moniliasis resistant to topical therapy. *Ann Allergy* 1976;37:110-3.
- Moraes PS. Recurrent vaginal candidiasis and allergic rhinitis: a common association. *Ann Allergy Asthma Immunol* 1998;81:165-9.
- Lowry OH, Rousembrough NJ, Farr AL, Randal RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-75.
- Corrigan EM, Clancy RL, Dunkley ML, Evers FM, Beagley KW. Cellular immunity in recurrent vulvovaginal candidiasis. *Clin Exp Immunol* 1998;111:574-8.
- Fischer A, Ballet JJ, Griscelli C. Specific inhibition of in vitro *Candida*-induced lymphocyte proliferation by polysaccharidic antigens present in the serum of patients with chronic mucocutaneous candidiasis. *J Clin Invest* 1978;62:1005-13.
- Lee WM, Holley HP Jr, Stewart J, Galbraith GM. Refractory esophageal candidiasis associated with a low molecular weight plasma inhibitor of T-lymphocyte function. *Am J Med Sci* 1986;292:47-52.
- Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 1989;170:2081-95.
- de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991;174:1209-20.
- Carvalho EM, Bacellar O, Brownell C, Regis T, Coffman RL, Reed SG. Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. *J Immunol* 1994;152:5949-56.
- Araujo MI, de Jesus AR, Bacellar O, Sabin E, Pearce E, Carvalho EM. Evidence of a T helper type 2 activation in human schistosomiasis. *Eur J Immunol* 1996;26:1399-403.
- de Waal Malefyt R, Haanen J, Spits H, Roncarola MG, te Velde A, Figdor C, et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* 1991;174:915-24.
- Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991;147:3815-22.
- 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. *Exp Parasitol* 1996;84:188-94.