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CYTOKINE PROFILE IN MONTENEGRO SKIN TEST OF PATIENTS WITH LOCALIZED CUTANEOUS AND MUCOCUTANEOUS LEISHMANIASIS

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SUMMARY

American tegumentary leishmaniasis presents as two major clinical forms: localized cutaneous leishmaniasis (LCL) and mucocutaneous leishmaniasis (MCL). The immune response in leishmaniasis is efficiently evaluated by the response to *Leishmania* antigen through the Montenegro skin test (MST). Both LCL and MCL present positive response to MST, indicating that the patients present cell-mediated immunity against the parasite - *Leishmania*. In spite of the presence of immunity in MCL, this is not sufficient to stop disease progression and prevent resistance to treatment. In this study we demonstrated interleukin (IL) 2, 4, 5 and interferon (IFN) gamma expression in biopsies of MST of ten patients with American tegumentary leishmaniasis. The obtained results were compared between LCL (n = 5) and MCL (n = 5) patients. The MST of MCL patients displayed a higher expression of IL-2, IL-4 and IL-5, in comparison to LCL. There was no significant difference in IFN-gamma expression between groups. The obtained results suggest the role of IL-4 and IL-5 in the maintenance of the immunopathogenic mechanism of the destructive lesions that characterize MCL.

KEYWORDS: Human cutaneous leishmaniasis; Immunopathology; Cytokines; Delayed-type hypersensitivity; Skin.

INTRODUCTION

Leishmaniasis is a disease caused by protozoa of the genus *Leishmania*, which can occur as the tegumentary or visceral form. It is an endemic anthropozoonosis found in 88 countries around the world, with an estimated 12 million cases worldwide and 1.5 to 2 million new cases occurring annually, 1 to 1.5 million cases are of tegumentary leishmaniasis and 500,000 cases of visceral leishmaniasis[33]. In Brazil, around 30,000 tegumentary leishmaniasis cases are reported annually[3], the majority of them caused by *Leishmania (Viannia) braziliensis*[31].

Clinical manifestations of tegumentary leishmaniasis are diverse, but the most frequent forms in Brazil are localized cutaneous leishmaniasis (LCL) and mucocutaneous leishmaniasis (MCL). LCL is characterized by the presence of a single or few ulcers, usually present in exposed areas of the body, usually healing after therapy. MCL is characterized by the presence of infiltrated lesions in the oral, nasal, pharyngeal and laryngeal mucosa membranes and skin of the surrounding areas. It usually manifests months or years after healing of the localized cutaneous lesion. These lesions are usually resistant to therapy, often disfiguring the face[17].

The evolution of the disease depends on the species of the protozoa and the host's immune response[10,28]. In human and experimental leishmaniasis, immunity is predominantly mediated by lymphocytes. In experimental cutaneous leishmaniasis, using isogenic mouse strains infected with *Leishmania (Leishmania) major*, a paradigm was established associating a subpopulation of CD4+ T cells producing mainly IFN-gamma (T helper 1 cells = Th1 cells) to the resistance, and CD4+ T cells producing mainly IL-4 and IL-10 (T helper 2 cells = Th2 cells) to susceptibility[18,27]. However, in the patients with LCL and MCL, the pathogenesis does not seem to be related only to the difference in the host's susceptibility, as not many parasites are seen in the lesions, even in the most severe mucocutaneous form of the disease. This condition differs from what is observed in the lesions of the susceptible mouse, with diffuse cutaneous leishmaniasis, where there is a high expression of IL-4 and lots of parasites. Furthermore, the delayed-type hypersensitivity reaction to MST, counterpart of the T-cell mediated immunity, is positive in both forms of the disease. Indeed, in MCL this immune response does not prevent the aggressive evolution of the disease[28]. Differently, in this context, cytokines may play different roles in the pathogenesis, being more related to the maintenance of the inflammatory reaction in the tissue. Studies in this context have identified both Th1 and Th2-type cytokines in *Leishmania* antigen-stimulated peripheral blood mononuclear cell supernatant in LCL[11,13,15] and also in MCL[13]. PCR studies of lesion samples have shown Th1 profile LCL and both Th1 and Th2 in MCL[26]. On the other hand, immunohistochemistry analysis demonstrated both Th1 and Th2 -cytokine expression in LCL lesions[14]. These apparently controversial observations might be due to the different assays used and to the comparison of peripheral blood mononuclear cells and *in situ* inflammatory cell results.

A positive MST is an indication of the presence of cell-mediated immune response of the host against *Leishmania*. In the present study, we

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demonstrated the expression of IFN-gamma, interleukin (IL) 2, 4 and 5, in MST biopsies of LCL and MCL in order to address the immunopathogenic mechanism in these different forms of leishmaniasis.

MATERIAL AND METHOD

The sample study consisted of ten patients (eight males and two females) aged 19 to 82 years (mean age of 49.5 years), with confirmed diagnosis of American tegumentary leishmaniasis. The five LCL and five MCL patients had just started the specific treatment for the disease. The study was approved by the Ethics Research Board of the Institutions (University of São Paulo and Santo Amaro Medical School). Informed consent was obtained from all patients. Nine patients were from different regions of Brazil and one patient was from Bolivia (patient 3). The mean ± standard deviation (SD) time of disease was 8 ± 6.2 months in LCL and 20.1 ± 19.9 years in MCL.

The diagnosis of leishmaniasis was confirmed by: the direct parasitological test, histopathological assessment including the demonstration of Leishmania antigen by immunohistochemistry in the lesions[32], positive MST, and detection of anti-Leishmania antibody in the serum (by indirect immunofluorescence technique and ELISA).

Montenegro skin test: A sample of 100 mL of dead Leishmania promastigote suspension, in phenolic and sodium fluoride solution (two to three millions microorganisms per mL, called Montenegro antigen, produced at Adolfo Lutz Institute, in São Paulo, Brazil) was injected intradermally in the arm of each patient[21].

MST biopsies were obtained before specific treatment in eight patients, and 15 and 18 months after treatment in two patients with LCL.

The MST evaluation was carried out 72 hours after antigen inoculation, by measuring two diameters of the resulting papule. The mean of the two diameters was calculated for statistical analysis. MST with papule = 5 mm, was considered positive[4].

The biopsy samples were taken from the center of the erythematous papule using a 4-mm punch, under local anesthesia with 2% xylocaine, after the MST clinical evaluation. The control skin sample of the assessed cases consisted of normal skin biopsies from the contralateral arm.

Detection of cytokines in the skin by immunohistochemical technique: Skin fragments were placed in sterile saline solution, later pre-frozen in isopentane and finally frozen in liquid nitrogen at -180 ºC. Fragments were kept at -80 ºC and later sectioned in cryostat.

Cytokines were detected by the immunohistochemical method, using anti-human IL-2, anti-human IL-4, anti-human IL-5 and anti-human IFN-gamma purified monoclonal goat IgG, purchased from R&D Systems (MN, USA). Four-µm cryostat sections were immersed in acetone for 10 minutes, dried at room temperature, followed by immersion in water for five minutes. After serial incubation to prevent nonspecific staining with 0.03% hydrogen peroxide in Tris solution, pH 7.4, and afterward with bovine serum albumin for one hour at 37 ºC, the sections were incubated with the primary antibody for 12 hours at 8 ºC. That was followed by incubation with goat biotinylated antibody IgG (R&D Systems, MN, USA) and with peroxidase-conjugated avidin for 30 minutes at 37 ºC. The slides were washed with 0.01 M phosphate-buffered saline, pH 7.2 (PBS) after each incubation step. The reaction was developed using 0.06% hydrogen peroxide and 0.3 mg/mL 3,3'-diaminobenzidine (Sigma Chemical, USA) in PBS. Counterstaining was performed with Harry's hematoxylin (Sigma Chemical, USA). The cytokines were identified in a light brown color.

Semi quantification of the cytokine staining: Immunostaining of the cytokines in the dermis was analyzed and semi-quantified by two independent investigators, by attributing scores as follows: zero for negative immunolabeling; 1+ for up to one third of positive stained mononuclear cells in the dermal cell infiltrate; 2+ for more than one third up to two thirds of positive stained mononuclear cells in the dermal infiltrate; 3+ for more than two thirds of positive stained mononuclear cells in the dermal infiltrate. In case of discordance between the two independent investigators, an independent third investigator provided consensus about the final score.

Statistical analysis: We analyzed the arithmetic mean and median of the results from the two groups of patients and analyzed them by Friedman's and Fisher's tests. The level of significance was set at 0.05.

RESULTS

Intensity of the Montenegro skin test: The results of the MST in LCL and MCL are shown in Table 1.

Table 1
Intensity of the Montenegro skin test, obtained from the two papule diameters (mm), in both studied groups, localized cutaneous leishmaniasis (LCL) and mucocutaneous leishmaniasis (MCL) at the time of diagnoses and at the time of the present study

Groups n = number of cases		At diagnoses	Present study
LCL (n = 5)	Mean ± Sd*	9.9 ± 3.7	10.2 ± 4.7
	Range	7 - 15	6 - 18
	Median	8	10
MCL (n = 5)	Mean ± Sd*	21.7 ± 14.7	13.3 ± 3.0
	Range	10 - 45	10 - 17.5
	Median	14	14
		†$p < 0.01$	No significance

*Sd: Standard deviation; † Fisher test

The mean value of the MST intensity, evaluated at the time of the patients' diagnoses, was significantly higher in the MCL when compared to the LCL group ($p < 0.01$). On the other hand, there was no significant difference regarding the MST evaluation when the groups were tested again before the biopsy procedure. In addition, we did not find any statistical difference between the intensity of the MST, at the diagnostic procedures as well as at the time of the study, in both LCL and MCL patients.

Detection of cytokines in Montenegro skin test: The median values of the cytokines in LCL and MCL are shown in Table 2.

Table 2
Detection of cytokines by immunoperoxidase technique, in the Montenegro skin test of localized cutaneous leishmaniasis (LCL) and mucocutaneous leishmaniasis (MCL)

Groups		IFN-γ	IL-2	IL-4	IL-5	Within subjects effects *
LCL	Mean ± Sd*	2.2 ± 0.8	1 ± 1	0.6 ± 0.9	0.6 ± 0.5	*$p < 0.05$
	Range	1 - 3	0 - 2	0 - 2	0 - 1	
	Median	2*	1	0	1	
MCL	Mean ± Sd*	2.8 ± 0.4	2.4 ± 0.9	3 ± 0	3 ± 0	No significance
	Range	2 - 3	1 - 3	0	0	
	Median	3	3 *	3 **	3 **	
Between groups effects**		No significance	*$p < 0.05$	**$p < 0.01$	**$p < 0.01$	

*,** Friedman test; Sd: Standard deviation.

There was a predominance of Th1 cytokine profile in the Montenegro skin tests in LCL. The statistical analyses showed a predominance of IFN-γ in comparison to the other three cytokines ($p < 0.05$).

Both types of cytokine profiles (Th1 and Th2) were observed in MCL patients.

No cytokine expression was observed in the normal control skin of all patients studied.

The statistical analyses showed a higher expression of IL-2, IL-4 and IL-5 in the dermal infiltrate of MCL when compared with LCL patients ($p < 0.01$).

DISCUSSION

The immune response in cutaneous leishmaniasis is characterized by a cell-mediated immune response, with a wide participation of humoral immunity[6]. The cellular immune response in leishmaniasis is clinically evidenced by Montenegro skin test[23,28].

In this study, the results of the intensity of the MST were different at the diagnosis and at the time of the present study, showing that the MST can change clinically with a second inoculation, as it has been demonstrated by other investigators[1,24]. In spite of that, the MST remains positive before and after treatment, and therefore, the MST response is independent from the treatment[8], although we cannot be certain about the changes of the MST intensity after treatment, as this has not been measured by the authors. In comparison with *in vitro* studies, the treatment does not change the immunological response of the patients with LCL, as demonstrated by some authors[1,13,30]. DA-CRUZ *et al*.[13] demonstrated in peripheral blood of patients with localized cutaneous leishmaniasis caused by *L. braziliensis*, that the type of cytokine remains the same, i.e., the Th1 type, before and after the treatment. This fact supports our findings in the MST that was performed after treatment in two patients of LCL. However, in MCL patients, a change in cytokine profile after treatment has been observed *in vitro*, as the observed Th1 and Th2 types, changed to Th1 type[11,13].

The population of T lymphocytes present in the granulomatous infiltrate of the lesion of tegumentary leishmaniasis was mainly composed by CD4+ cells, and a lower proportion of CD8+ cells[6,9,22,25]. The subtypes of CD4+ T cells (Th1 and Th2 profile) have been characterized by many investigators in all forms of tegumentary leishmaniasis.

The immune response of the New World LCL caused by *L. braziliensis*, is characterized by a Th1 response, with a predominance of IFN-gamma expression *in vitro* and *in situ* and a very low or sometimes absent expression of IL-4, IL-5 and IL-10[1,2,5,11-15,20]. In relation to other cytokines, such as IL-2, they have been demonstrated *in vitro* and *in situ*, with a low expression. Additionally, the immune response of the Old World LCL caused by *L. major, L. tropica* and *L. aethiopica*, is characterized by this same cytokine profile (Th1), also observed by many investigators[19,29]. However, in acute and chronic lesions of LCL, studied *in situ* and with stimulation of peripheral blood mononuclear cells by *Leishmania* antigen, the cytokines in acute lesions show a tendency to the Th1 type, whereas, in chronic lesions, there is a tendency to a mixed cytokine type[19,29].

MCL is characterized by a mixture of Th1 and Th2 responses, which has been well established by two investigators using different techniques, both *in situ* and *in vitro*[26]. The cytokines observed in MCL are IL-4, IL-5, IL-10 at a higher proportion in comparison to LCL[11,13,14], but lower than in diffuse cutaneous leishmaniasis[4,22].

The Th1 response in MCL is more intense than in LCL, as demonstrated by the IFN-gamma and IL-2 that are observed at high proportions *in vitro* and *in situ*, and an exacerbated lymphoproliferative response to *Leishmania* antigen *in vitro*[4,6,31].

In the present study, we demonstrated a difference in the cytokine profile identified in the MST of patients with LCL and MCL. There was a predominance of Th1 response in the MST of LCL patients and both types, Th1 and Th2, in the MST of MCL patients. We observed a significantly higher expression of IL-2 ($p < 0.05$), IL-4 and IL-5 in MCL in comparison to LCL ($p < 0.01$). Initially, we thought that a low expression of IL-2 in the LCL group was due to a technical problem,

but the literature shows how weakly this cytokine is expressed in this group; its expression is a little higher in MCL, although at a very low frequency. Perhaps this cytokine is important for the initiation of the immune response, but not for its maintenance. Finally, the results showed no difference in IFN-gamma expression in both groups studied.

The predominance of IL-4 and IL-5 in Montenegro skin test of MCL may suggest that these cytokines participate in the modulation of the inflammatory reaction caused by the high amount of IFN-gamma. The destructive lesions may occur when the high quantities of IFN-gamma are not controlled by the Th2 cytokines[7].

In this study, the identification of the immunological aspects demonstrated in the MST is similar to those seen in ATL lesions. We suggest that the MST can be used as a mirror of the disease itself. These aspects have also been demonstrated by other authors in studies about the inflammatory response in the MST[16,22,26]. On the other hand, these studies did not compare the *in situ* MST immunopathological characteristics in the two forms of the disease. Further studies are needed to compare the expression of cytokines in leishmaniotic lesions and MST biopsies simultaneously. Perhaps, we will be able to study the immune response of these two clinical forms during a phase of the disease when there are no lesions, but only the positive response of the MST. This reaction has 83.7 to 100% of sensitivity, it is very specific for the *Leishmania* genus and it stays positive for a long time after the cure or even throughout life[17,23,28]. We must remember that the blood lymphocytes represent 5% of all lymphocytes and that these cells stay in circulation for only one hour, so the study using these techniques sometimes do not actually show the reality of the disease.

The Montenegro skin test may show earlier changes, before the symptoms appear in the mucosa of the patients, so it is the only test able to assess the patient's immunological reaction to the *Leishmania* antigen, before the mucocutaneous form appears. The MST might be able to answer a question that is so often formulated: When does the localized form change to the mucocutaneous form?

RESUMO

Perfil de citocinas na intradermorreação de Montenegro em doentes de leishmaniose cutânea localizada e cutâneo-mucosa

A leishmaniose tegumentar americana apresenta duas formas clínicas mais comuns: a leishmaniose cutânea localizada e a leishmaniose cutâneo-mucosa. A imunidade da leishmaniose é avaliada pela resposta ao antígeno *Leishmania* através da Intradermorreação de Montenegro. Estas duas formas apresentam resposta positiva, indicando que o paciente apresenta imunidade celular contra o parasita *Leishmania*. Apesar da presença da imunidade celular na leishmaniose cutâneo-mucosa, esta não é suficiente para barrar a progressão da doença e a resistência ao tratamento. Neste estudo, detectamos quatro citocinas por imunohistoquímica, IL-2, IL-4, IL-5 e IFN-gama nas biópsias da intradermorreação de Montenegro de pacientes com leishmaniose tegumentar americana (n = 10), cinco com leishmaniose cutânea e cinco com cutâneo-mucosa. Os resultados mostraram uma alta expressão significativa de IL-2, IL-4, IL-5 na leishmaniose cutâneo-mucosa comparada com a leishmaniose cutânea localizada, mas sem diferença significante na expressão do IFN-γ entre os grupos. Estes resultados sugerem a importância da participação da citocina IL-4 e IL-5 na manutenção do mecanismo imunopatogênico das lesões destrutivas da forma cutâneo-mucosa.

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