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Short Report: Lesion Size Correlates with *Leishmania* Antigen-Stimulated TNF-Levels in Human Cutaneous Leishmaniasis

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Abstract. Cutaneous leishmaniasis (CL) is a worldwide disease endemic in several regions of the globe. The hallmark of CL is skin ulcers likely driven by efforts of the immune system to control *Leishmania* growth. Cytokines, such as tumor necrosis factor (TNF) and interferon-gamma can control disease progression in animal models. Nevertheless, the impact of these cytokines in CL ulcer outcome is not well established in humans. In this study, 96 CL patients from an endemic area of *Leishmania braziliensis* were enrolled for a follow-up study that consisted of clinical and immunological evaluations in a 2-year period. Statistical analysis revealed that healing time ($P = 0.029$), age ($P = 0.002$), and TNF levels ($P = 0.0002$) positively correlate with ulcer size at the time of the first clinical evaluation. Our findings suggest that ulcer size correlates with healing time and TNF levels support the use of TNF inhibitors combined with standard therapy to improve healing in CL patients with severe lesions.

INTRODUCTION

Cutaneous leishmaniasis (CL) is an endemic disease in several regions of the world. In Brazil, *Leishmania braziliensis* is the most commonly identified causal agent.[1,2] Despite the wide range of clinical manifestations, 75% of CL patients display a typical clinical picture, characterized by one or a few round ulcers with elevated borders and a necrotic center usually on the lower limbs. In addition, lymphadenopathy has been shown as an early common signal and may precede the ulcer development.[3]

Although a potent Th1 immune response may help control the *Leishmania* infection, unregulated production of proinflammatory cytokines seems to contribute to ulceration and lesion severity. Tumor necrosis factor (TNF) and interferon-gamma (IFN-γ) cytokines have been detected in tissue samples from CL patients, however their involvement in the lesion outcome is not fully understood.[4–7] The TNF seems to play a critical role in the lesion severity because TNF production is exacerbated in lesions from mucosal leishmaniasis (ML) patients[8] and in CL patients unresponsive to antimonial treatment.[9] In addition, lesion area measurements from CL patients positively correlated with the percentage of *Leishmania* antigen-specific TNF producing lymphocytes.[10]

Here, we show that a single measurement of the ulcer size at the first medical visit was an indicator of healing time and treatment duration. The size of the ulcer was positively correlated with *Leishmania* antigen-specific TNF production from circulating cells, further contributing to evidence that the immune system and distinctly TNF are linked to ulcer severity. This finding supports the hypothesis that TNF blockade may be beneficial in treating patients that present severe lesions at the first consultation.

METHODS

Patients. This study was carried out at the Piraja da Silva Reference Center (PIEJ) located in Jequie, Bahia, Brazil. This study area is endemic for CL caused by *L. braziliensis*. In a period of 2 years, 96 patients were diagnosed with CL by a positive *Leishmania* skin test and histological observation of parasites in the lesion biopsy. Patients were treated with 20 mg of Meglumine antimoniate (Sb^v)/Kg/day for 20 days and were evaluated monthly until the lesions healed. Worsening of the lesion was the criteria to indicate an additional treatment with a two or three additional course of Sb^v. All 96 patients evolved with clinical cure defined by resolution of the lesion. The clinical team had full access to patient clinical history during the study, but no access to any of the immunological data until the end of the cohort. Patients with diabetes mellitus, high blood pressure, and previous diagnosis of CL, Chagas disease, or those who had been treated previously with Sb^v were excluded from the study. The study was approved by the ethics committee of the Hospital Universitario Prof. Edgard Santos (Salvador, Bahia) following the guidelines for human experimentation and the Brazilian Ministry of Health regulations for research involving humans. All patients or parents from minors participating in the study signed informed consent forms.

Laboratory diagnosis. The skin test was performed by intradermal injection of 25 μg of *Leishmania* antigen as described previously.[11] The test was considered positive if the individual presented an induration of more than 5 mm in diameter 48 hours after the injection. A biopsy was performed under local anesthesia with a 3.5 mm punch (Baker Cummins Pharmaceuticals, Inc., Miami, FL). Paraffin-embedded sections were stained with hematoxylin-eosin and anti-*Leishmania* antibodies were used to identify *Leishmania* spp. parasites by immunohistochemistry.

Immunologic assays. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation (Histopaque; Sigma Diagnostics, St. Louis, MO). Three million cells/mL were cultured in 24-well microtiter plates with RPMI 1640 (Gibco, Grand Island, NY) and stimulated with 10 μg/mL of soluble *Leishmania* antigen (SLA), or Concanavalin A (Sigma), for 24, 48, or 96 hours. Enzyme-linked immunosorbent assay analysis was performed to measure the levels of the cytokines in the culture supernatants at 24 hours for TNF, at 48 hours for IL-10, or at 96 hours for IFN-γ, following the manufacturer's instructions (PharMingen, San Diego, CA).

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Statistical analysis. All statistics were performed using GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA). We applied the Spearman correlation test because the samples did not present a normal distribution after the Kolmogorov-Smirnov test. The Spearman coefficient (r_s) was used to measure the association between two variables. The Mann-Whitney test was made in the samples to evaluate mean differences to the amount of Sb^v and lesion size. Significance was considered when $P < 0.05$.

RESULTS

In this cohort, 64% (61) of the patients were male (mean age was 31.3 ± 18.9 years) and 58% (50) presented lymphadenopathy at the first clinical evaluation. Seventy-three percent (70) of the patients had one lesion at the physical examination. Sixty-five percent (62) of the patients had a lesion in the lower limbs and 27% (26) had multiple lesions. The mean lesion size was 3.0 ± 1.7 cm, measured as the largest diameter. Only patients that had one lesion were taken into account for the statistical tests and correlations to avoid confounding factors related to the measurement of multiple lesions. Healing time was considered from the beginning of the treatment until the lesion was completely healed with re-epithelization of the ulcer. The mean healing time was 85 ± 53 days. Forty-four percent (42) of the patients achieved clinical cure with one course of treatment, 37% (36) required a second course, and 19% (18) required a third course in the same fashion. No treatment failure was observed after the third therapeutic round. No mucosal disease or relapse was observed in any patient after clinical cure for up to 6 months post-treatment. Immunologic evaluation was conducted by measuring the levels of cytokines in the supernatant of PBMC after stimulation with SLA. Mean levels in the supernatant were 285 ± 414.9 pg/mL of TNF, 1770 ± 1046 pg/mL of IFN-γ, and 6.1 ± 18.1 pg/mL of IL-10. Data is summarized in Table 1.

We found that lesion size and healing time (P = 0.029) (Figure 1A), and lesion size and age of the patients (P = 0.002) (Figure 1B) are positively correlated. Of importance,

TABLE 1
Patient characteristics*

Characteristic		Value†
Male sex (%)		64
Age (yr)		31.3 ± 18.9
Adenopathy (%)		58
Lesion number		
	01 lesion (%)	73
	02–04 lesions (%)	27
Lesion location		
	Lower extremities (%)	65
	Upper extremities (%)	20
	Face (%)	5
	Chest (%)	4
Lesion size (cm)		3.1 ± 1.7
Healing time (d)		85 ± 53
Positive skin test (%)		74.5
01 course of Sb^v (%)		44
02 courses of Sb^v (%)		37
03 courses of Sb^v (%)		19
TNF-α (pg/mL)		285.9 ± 414.9
IFN-γ (pg/mL)		1770 ± 1046
IL-10 (pg/mL)		6.1 ± 18.1

*TNF-α = tumor necrosis factor-alpha; IFN-γ = interferon-gamma; IL-10 = interleukin-10.
† Plus-minus values are means ± SD.

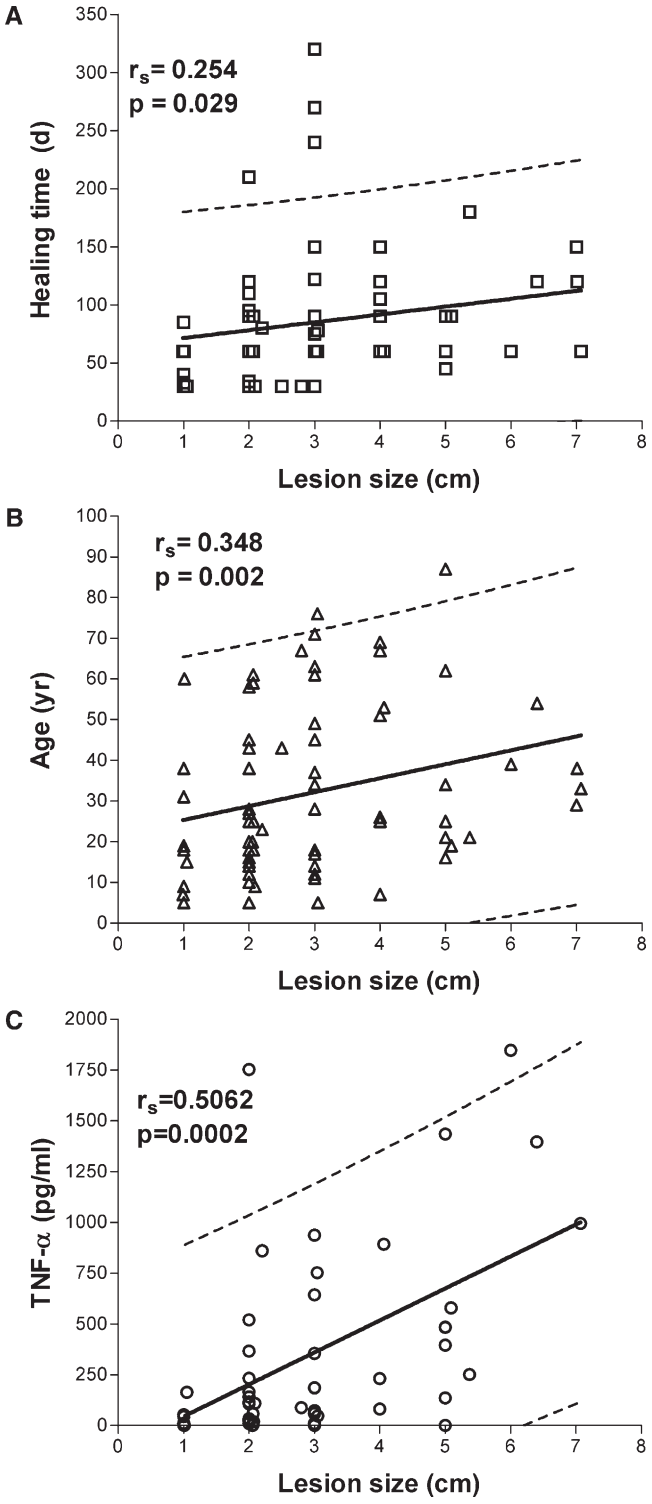

FIGURE 1. Positive correlation between lesion size and healing time, age, or tumor necrosis factor (TNF) levels on cutaneous leishmaniasis (CL) patients. (**A**) Correlation between healing time (days) and lesion size (cm) (N = 70). (**B**) Correlation between patient age (years) and lesion size (cm) (N = 70). (**C**) Correlation between TNF levels in the supernatant measured by enzyme-linked immunosorbent assay (ELISA) (pg/mL) and lesion size (cm) (N = 52). The solid line represents the linear regression and the dotted line the 95% predicted coefficient interval. The Spearman correlation coefficient is designated in each figure by "r_s."

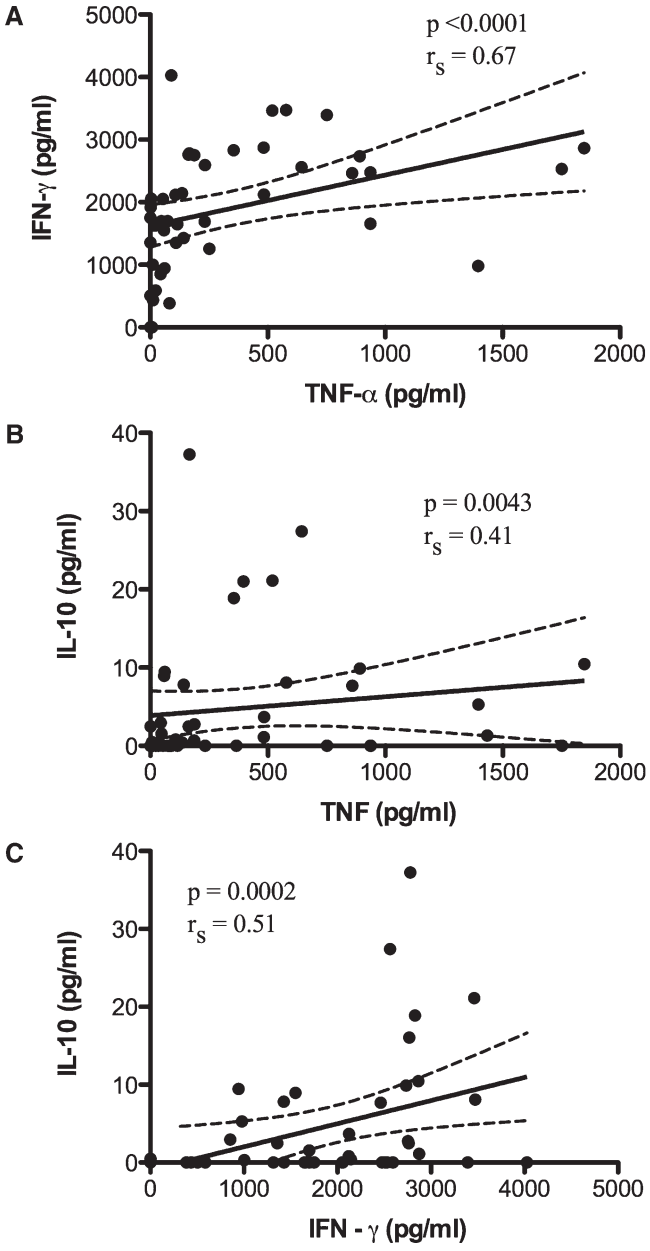

FIGURE 2. Positive correlation between cytokine levels on cutaneous leishmaniasis (CL) patients. (**A**) Correlation between tumor necrosis factor (TNF) and interferon-gamma (IFN- γ) levels (pg/mL) (N = 52). (**B**) Correlation between TNF and interleukin-10 (IL-10) levels (pg/mL) (N = 52). (**C**) Correlation between IFN- γ and IL-10 levels (pg/mL) (N = 52). The solid line represents the linear regression and the dotted line the 95% predicted coefficient interval. The Spearman correlation coefficient is designated in each figure by "r_s."

lesion size showed also a positive correlation with TNF levels (P = 0.0002) (Figure 1C), which suggests that patients with larger lesions were more likely to be older and display higher levels of SLA-specific TNF production. No correlation was observed between lesion size and IFN- γ or IL-10 levels. We also have found a positive correlation between TNF and IFN-γ (P < 0.0001, r_s = 0.67) (Figure 2A), TNF and IL-10 (P = 0.0043, r_s = 0.41) (Figure 2B), and IFN-γ and IL-10 (P = 0.0002, r_s = 0.51) (Figure 2C). As shown in Figure 3, patients presenting a larger lesion at the first medical evaluation also required more courses of Sb^v to achieve cure (P < 0.05).

FIGURE 3. Comparison between lesion size and number of courses of Sb^V on cutaneous leishmaniasis patients. Data represent mean ± standard deviation of the measurements of lesion size in the group of patients that received either a single or several (two or three) courses of Sb^V during the study. The Mann-Whitney test was performed. (*) denotes statistical significance with P < 0.05.

DISCUSSION

In this study, we have investigated clinical and immunological parameters influencing CL ulcer outcome in a cohort of 96 patients. We showed that the lesion size at the first medical evaluation positively correlated with the healing time and the number of Sb^v courses. The CL ulceration is a complex interplay among the parasite burden, the activated state of the immune system, and the repair system. Treatment of individuals with lymphadenopathy only, a stage that precedes ulceration, prevents lesion development in only 50% of the patients.[12] The CL patients followed in this report displayed similar clinical characteristics at the time of diagnosis as those described by others.[13] Age positively correlated with the size of the lesion and this association was not caused by a longer time before reaching medical assistance. Endocrine, hematological, and nutritional aspects related to aging and, more importantly, local vascular deficiencies may participate in determining the size of lesions in these individuals.[14]

Interestingly, lesion size was correlated with TNF production detected after SLA *in vitro* stimulation. Our results concur with the work of Antonelli and others that found a positive correlation between lesion size and the frequency of TNF or IFN-γ positive-lymphocytes in CL patients.[6,10] We did not find correlations between IFN-γ and IL-10 with lesion size. Severity has been associated with the expression of TNF in patients with ML,[15,16] and persistently elevated TNF serum levels have been proposed as an indicator of unresponsiveness to treatment in visceral leishmaniasis patients.[17] The TNF may be related to pathogenesis increasing the expression of epidermal growth factor and platelet-derived growth factor,[18] inhibiting anti-clotting mechanisms and promoting thrombosis leading to a prolonged healing process. Thus, carefully inhibiting TNF-associated pathways may be beneficial to the healing process and enhancement of ulcer cicatrization. The TNF inhibitors have been successfully introduced as a therapeutic option in the treatment of patients with ML,[19] and CL.[9] Nevertheless, these inhibitors have been used in a small number of patients and clinical trials are in need.

The positive correlation observed between TNF, IFN-γ, and IL-10 may be the result of a regulated response allowing efficient activation and killing of the parasite, regarding the levels of TNF and IFN-γ but reducing tissue injury by increasing levels of IL-10. Correlation of these cytokines has been consistently shown in different CL cohorts of patients.[5,6]

In this study, we showed that the lesion size on the first clinical evaluation correlates with healing time and the number of courses of treatment needed. We observed that patient age and TNF levels are also related to the ulcer size. This work may be useful for physicians, indicating the lesion size as a parameter that could be evaluated during the first clinical appointment and, additionally, it supports the rationale for the use of TNF inhibitors together with Sb^{v} in the treatment of severe ulcers caused by CL.

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