Epidemiologic and Immunologic Findings for the Subclinical Form of *Leishmania braziliensis* Infection

Ivonise Follador, Cibele Araújo, Olívia Bacellar, Clarissa B. Araújo, Lucas P. Carvalho, Roque P. Almeida, and Edgar M. Carvalho

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

The epidemiologic and immunologic findings for 104 subjects with subclinical *Leishmania braziliensis* infection were compared with those for 29 patients with cutaneous leishmaniasis (CL) from the same area of endemicity. Subjects had a positive leishmania skin test result and remained asymptomatic during the next 4 years of follow-up were considered to have subclinical infection. Patients with CL were younger, had larger-diameter indurations after skin testing, and were more likely to have positive serologic markers than were those with subclinical infection ($P < .05$). In subjects with subclinical infection, levels of interferon-γ and tumor necrosis factor-α in lymphocyte supernatants were lower than they were in patients with CL ($P < .05$); however, mean interleukin-5 levels were slightly higher in patients with subclinical infection than in patients with CL. These data indicate that, unlike patients with CL, individuals who do not develop disease when infected with *L. braziliensis* may have the ability to modulate their immune response.

The typical clinical manifestation of American cutaneous leishmaniasis (CL) is a single ulcerated lesion with elevated borders, frequently located on the inferior limbs [1–2]. Parasite and host factors are related to the clinical forms of leishmaniasis [3–6]. The immunological response in patients with CL is characterized by a strong cellular immune response with evidence of a delayed type hypersensitivity reaction to leishmania antigen; lymphocyte proliferation; and high production of IFN-γ, the main cytokine that activates macrophages to kill *Leishmania* organisms [5, 7–9].

Subclinical leishmania infection has been reported in areas where *Leishmania chagasi,* the causative agent of visceral leishmaniasis, is endemic [9]. Compared with patients with visceral leishmaniasis, who do not produce IFN-γ when stimulated by leishmania antigen, subjects with subclinical *L. chagasi* infection demonstrate a T cell response to parasite antigen [10]. Although no previous study has been designed to evaluate the prevalence of subclinical *Leishmania braziliensis* infection, it has been reported that ~10% of healthy subjects have a positive result of a skin test for leishmania antigen in areas where *L. braziliensis* is endemic [11–12].

The present study was performed in Canoa, a rural village located in the state of Bahia, Brazil, where an outbreak of CL occurred in 1993–1997. During this period, the entire population of the village was evaluated for *Leishmania* infection. A clinical and epidemiologic survey was performed annually to determine the frequency of the disease and the frequency of subjects who had positive intradermal skin test results without disease. In this study, we evaluated the epidemio-

logic aspects of this outbreak and compared the immunologic response of patients with CL to the response observed in subjects with subclinical *L. braziliensis* infection. The clinical findings for the 29 cases of CL detected in the area during the study period have been described elsewhere [13].

PATIENTS AND METHODS

Area of endemicity and selection of subjects. This study was performed in the village of Canoa, municipality of Santo Amaro, Bahia, Brazil. Santo Amaro is located 116 km north of Salvador, the capital of the state of Bahia, at 12°33′ south latitude, 38°42′ west longitude; the village is 50 m above sea level. The mean annual temperature is 25.4°C. The village of Canoa has 163 houses distributed along a road for 2.6 km; it is located between the Subaé and Traripe rivers. The predominant vegetation is banana trees and flowering trees that grow around the houses. There is electricity but no piped water. The village is divided into 2 areas: the area from house 1 to 100 is called "Canoa de Cima" ("uptown Canoa"), and the area from house 101 to 163 is called "Canoa de Baixo" ("downtown Canoa"). Around the village is a residual area of Atlantic forest with extensive areas of old and new forest devastation, which predominate in the second ("downtown") area of the village.

In March 1993, an outbreak of CL occurred in the village. There was no information about previous cases of CL, and there have been no reported cases of Chagas' disease or schistosomiasis in the area, although malaria was reported 30 years before the outbreak of leishmaniasis. In the first year of the study, an epidemiologic survey was performed, as well as clinical and laboratory evaluations (skin testing and serologic testing for leishmania). Informed consent was obtained from the patients or their guardians, and the guidelines for human experimentation of the Federal University of Bahia were followed in conducting this clinical research. During that year, 555 of a total of 604 inhabitants were evaluated. Monthly follow-up was performed from 1993 through 1996 to detect new cases of the disease. Each year, we performed a clinical evaluation and a skin test with leishmania antigen for all healthy individuals, in an attempt to determine the proportion of patients with intradermal skin test conversion and their clinical outcomes. Subjects who had Montenegro skin test results that converted and who then remained asymptomatic during the next 4 years of follow-up were considered to have subclinical *L. braziliensis* infection.

Intradermal skin test and serologic reactions. A delayed type hypersensitivity test was performed with a soluble leishmania antigen prepared as described elsewhere [14]. Soluble *Leishmania amazonensis* antigen was also used to perform an ELISA test to detect antibodies against *Leishmania* species [15].

Immunologic studies. Lymphocyte proliferative response and cytokine levels were measured in samples of peripheral blood mononuclear cells (PBMC) from subjects with subclinical infection and from patients with CL before therapy was initiated. Individuals with subclinical infection were recruited from the group of patients who had skin test conversion. PBMC were separated from venous blood by density gradient centrifugation with use of the Ficoll-Hypaque method (Pharmacia). For proliferation assays, PBMC were adjusted to a concentration of 10^6 cells/mL in RPMI 1640 (Gibco) containing penicillin and streptomycin and supplemented with 10% AB normal human serum [7]. Aliquots of 2×10^5 cells in 0.2 mL of RPMI 1640 were cultured in triplicate in flat-bottomed microtiter plates (Linbro Chemical) and stimulated with soluble *L. amazonensis* lysate (2 μg/mL) or "pokeweed" mitogen (PWM) in a final dilution of 1:10. Cell proliferation was measured by the [³H]thymidine uptake rate after 5 days of incubation. The results were expressed as a stimulation index, which was determined by dividing the counts per minute (cpm) for stimulated cells in culture by the cpm for unstimulated cells. Levels of IFN-γ, TNF-α, and IL-5 were determined in PBMC supernatant cultures by means of the ELISA sandwich technique [16].

Statistical analysis. Data were stored in the Epi-Info program, version 6.0 (Centers for Disease Control and Prevention). The Student's *t* test, the χ^2 test, and the Mann-Whitney U test were used to compare the results for the 2 groups (i.e., subjects with subclinical infection vs. subjects with CL). Spearman's test was used to correlate the results of cytokine production tests and skin tests. Differences were considered to be statistically significant if the probability of a type I error was <.05.

RESULTS

The mean age (±SD) of the population of Canoa was 24 ± 20 years; 306 (55%) of the 555 subjects were <20 years old. Two hundred eighty-three subjects (51%) were male, and 272 (49%) were female. The number of residents in downtown Canoa was 202 (36% of the study population). The annual survey data are shown in table 1. In the first year of the study, 555 individuals were surveyed; 18 had active CL, and 11 had previous history of skin lesions with a typical scar of cutaneous disease. These 11 individuals had positive skin test results and denied having received any kind of therapy; these findings were taken to indicate a self-healing infection. Of the 526 asymptomatic subjects evaluated, 461 (88%) had a negative skin test result and 65 (12%) had a positive result.

In the second year of the study, 11 additional cases of CL were diagnosed, and 368 previously asymptomatic subjects were evaluated. In this second evaluation, 18 subjects (5%) whose first skin test result was negative converted to a positive result. In the third year of the study, 214 healthy subjects who had 2

Table 1. Characteristics of patients in a prospective study of *Leishmania braziliensis* infection and cutaneous leishmaniasis in Canoa, Santo Amaro, Bahia, Brazil, 1993–1996.

Time period	Patients evaluated	Patients with active disease[a]	Patients with prior disease with self-healing[b]	Healthy subjects With positive skin test result	With negative skin test result
Mar 1993–Mar 1994	555	18	11	65	461
Apr 1994–Mar 1995	397	11	0	18	368
Apr 1995–Mar 1996	235	0	0	21	214

NOTE. Data are no. of patients or subjects.

[a] Patients who had cutaneous leishmaniasis during the time of the study.

[b] Subjects with the scar typical of cutaneous leishmaniasis who had a past history of disease in which lesions healed without therapy.

previous negative skin test results were reevaluated, and 21 (10%) converted to a positive test result. There were no cases of CL in the third year of the study. This area continues to be clinically monitored; of the healthy individuals who had positive skin test results throughout the study, none has developed CL. All 29 patients with active disease were treated with standard antimony therapy, and, after 4 years of follow-up, none have had relapse.

A comparison of the clinical and laboratory data for subjects with subclinical *L. braziliensis* infection with those for patients with CL from the same area is shown in table 2. The patients with cutaneous disease were younger, had larger-diameter indurations after skin testing, and had a higher proportion of positive serologic test results than did those with subclinical infection. There was also a significant difference between individuals who resided in uptown Canoa and those who resided in downtown Canoa: 84.6% of patients with CL were from downtown Canoa, but fewer than one-half of the subjects with subclinical *L. braziliensis* infection (43.3%) lived in this part of the village ($P < .001$). Several socio-economic variables, such as occupation, type of house, and conditions of hygiene, were evaluated, and no significant difference was documented with respect to these variables between the 2 parts of the village.

The lymphocyte proliferative response to leishmania antigen was evaluated in 20 individuals with subclinical *L. braziliensis* infection, 20 patients with CL, and 10 healthy subjects who were not exposed to *Leishmania* organisms. In the group of individuals with subclinical *L. braziliensis* infection, the stimulation index for cultures stimulated with leishmania antigen ranged from 1 to 63. Lymphocytes from all patients proliferated when stimulated with PWM. The mean ($\pm$ SD) stimulation index for individuals with subclinical infection was higher than that for healthy subjects who were not exposed to *Leishmania* organisms (12 ± 19 vs. 2 ± 0.6; $P < .001$) and lower than that for patients with CL (84 ± 72; $P < .001$).

Figure 1 shows levels of IFN-γ, TNF-α, and IL-5 in the supernatant of lymphocyte cultures from subjects with subclinical *L. braziliensis* infection and patients with CL. The mean ($\pm$ SD) levels of IFN-γ and TNF-α among subjects with subclinical *L. braziliensis* infection were 296 ± 308 pg/mL (range, 0–2075 pg/mL) and 55 ± 57 pg/mL (range, 0–364 pg/mL), respectively. These values were lower than those for patients with CL. The levels of IFN-γ and TNF-α in patients with CL were 1546 ± 1136 pg/mL (range, 0–3321 pg/mL) and 259 ± 260 pg/mL (range, 0–904 pg/mL), respectively ($P < .001$). There was a direct correlation between IFN-γ levels and the diameters of indurations after skin testing among subjects with subclinical *L. braziliensis* infection ($r_s = 0.4123$; $P < .05$).

Both groups had low levels of production of IL-5 in supernatants of lymphocyte cultures. The mean level of IL-5 in subjects with subclinical *L. braziliensis* infection (110 ± 113 pg/mL [range, 0–679 pg/mL]) was slightly higher than that observed

Table 2. Epidemiologic characteristics and laboratory findings for individuals with *Leishmania braziliensis* infection and cutaneous leishmaniasis in Canoa, Santo Amaro, Bahia, Brazil, 1993–1996.

Characteristic or finding	*L. braziliensis* infection	*L. braziliensis* disease	P
No. of patients	104	29	—
Male sex, % of patients	40.4	58.6	.08
Age, mean years	31.9	19.4	.06
Diameter of skin test induration, mean mm	9.4	17.6	.02
Positive ELISA result, % of patients	46.2	84.6	<.005
Residence in downtown Canoa, % of patients	43.3	84.6	<.005
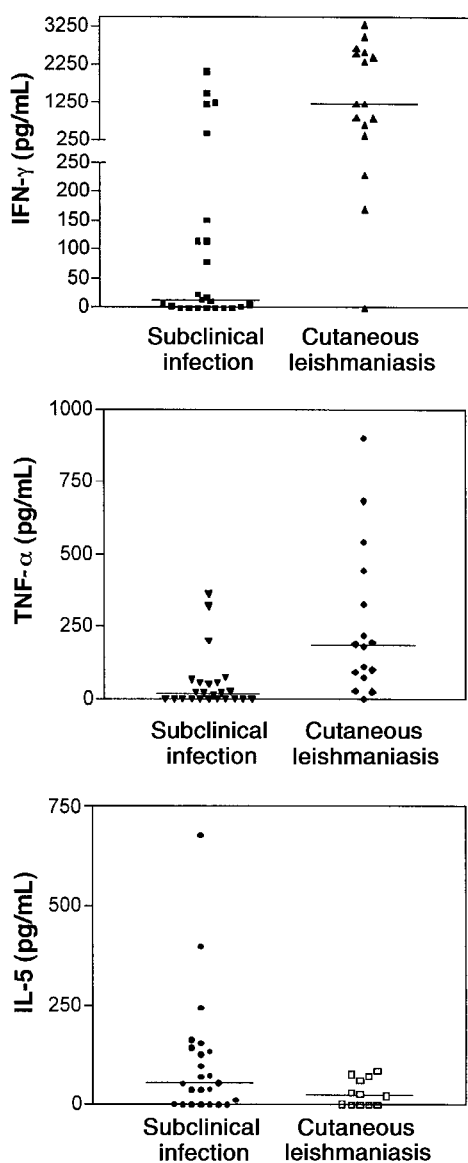
Figure 1. Profile of cytokine levels in supernatants of lymphocyte cultures from individuals with subclinical *Leishmania braziliensis* infection and in patients with cutaneous leishmaniasis. *Horizontal lines,* mean values.

in patients with CL (32 ± 34 pg/mL [range, 0–85 pg/mL]). This difference was not statistically significant ($P > .05$).

DISCUSSION

The leishmania intradermal skin test has been used to diagnose CL for >50 years. This test has high sensitivity and specificity and a good positive predictive value, making it very useful for epidemiologic and clinical studies [12, 17, 18]. The use of different species of the parasite and different forms of antigen preparation and the ability of the test to induce a delayed type hypersensitivity reaction when it is performed a second time

with only a short interval between the 2 tests [19] indicate the need for standardization of the leishmania skin test. In the present study, we used a soluble *L. amazonensis* antigen [14], and the test was repeated after 1 year. A previous study showed that no patients had leishmania skin test result conversion when the test was repeated after an interval of 1 year [20]. In the Canoa area of endemicity, of 461 individuals with negative skin test to leishmania antigen, only 8% had a positive intradermal reaction after a second skin test during 3 years of follow-up. Therefore, we assume that the conversion of the test result for asymptomatic patients was related to exposure to leishmania infection without development of disease. The possibility can not be ruled out that some of these individuals developed one or more small lesions that were not recognized as CL. However, none of the subjects had the classic ulcer typical of CL.

The frequency of subclinical *L. braziliensis* infection in the present study is similar to the percentage of individuals with a positive skin test result reported in cross-sectional studies [11, 12, 21, 22]. Environmental, parasite, and host factors may explain the appearance of the disease in only a small percentage of infected subjects. In Canoa, the *Leishmania* species isolated was *L. braziliensis,* and the phlebotomous vector was *Lutzomyia intermedia* [13]. It has been suggested that different sibling species of *Leishmania longipalpis* differ in their propensity to modulate the pathology of the disease transmitted [23]. It is also well known that a single species of *Leishmania,* such as *L. amazonensis,* can cause the whole spectrum of tegumentary leishmaniasis, including mucosal disease, CL, and diffuse CL [4].

Host factors are also involved in determining the clinical spectrum of leishmaniasis. Although patients with an evident T cell response against parasite antigen develop simple cutaneous disease [9], patients with an impaired immune response develop diffuse CL [6]. One of the important findings regarding the role of the environment in the development of CL was that the majority of cases of CL occurred in residents of downtown Canoa, an area where a heavy devastation of forest was observed. Because cases of subclinical infection were distributed equally in both parts of the village, it is possible that factors such as grade of exposure and parasite load could explain the occurrence of these different clinical forms of infection. Considering that *L. braziliensis* was the only causative agent isolated from patients and that the vector *(L. intermedia)* is associated with *L. braziliensis* infection, it is unlikely that other *Leishmania* species would be responsible for the sensitization of the subjects we studied and the different clinical forms of the infection.

The immune response in patients with CL is characterized by strong IFN-γ and TNF-α production with nitric oxide expression, which has been demonstrated in biopsies for patients with CL [24, 25]. Although these cytokines are important in the control of leishmania infection, patients develop skin ulcers characterized by an intense inflammatory reaction with few or

no parasites at the site of ulceration [25]. Therefore, it is possible that the unregulated synthesis of these cytokines may be dangerous for the host. Recent studies have shown that IL-10 modulates the immune response in patients with CL [16] and that inhibition of TNF-α production may improve the therapeutic response in patients with mucosal leishmaniasis [26]. Levels of IL-10 were not measured in the present study. We found that subjects with subclinical *L. braziliensis* infection had lower IFN-γ and TNF-α levels and higher IL-5 levels, compared with patients with CL, which indicates that, unlike CL patients, subjects who did not develop disease may have the ability to modulate their immune response. The IFN-γ and TNF-α production observed in individuals with subclinical infection are enough to control parasite growth and do not mediate tissue damage.

Acknowledgments

References

1. Llanos-Cuentas EA, Cuba CC, Barreto AC, Marsden PD. Clinical characteristics of human *Leishmania braziliensis* infections. Trans R Soc Trop Med Hyg 1984; 78:845–6.
2. Jones TC, Johnson WD, Barreto AC, et al. Epidemiology of American cutaneous leishmaniasis due to *Leishmania braziliensis braziliensis*. J Infect Dis 1987; 156:73–83.
3. Marzochi MCA, Marzochi KBF. Tegumentary and visceral leishmaniasis in Brazil: emerging anthropozoonosis and possibilities for their control. Caderno de Saúde Pública, Rio de Janeiro 1994; 10:359–75.
4. Barral A, Pedral-Sampaio D, Grimaldi G Jr., et al. Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. Am J Trop Med Hyg 1991; 44:536–46.
5. Cabello PH, Lima AMVMD, Azevedo ES, et al. Familial aggregation of *Leishmania chagasi* infection in northeastern Brazil. Am J Trop Med Hyg 1995; 52:364–5.
6. Barral A, Costa JML, Bittencourt A, et al. Polar and subpolar diffuse cutaneous leishmaniasis in Brazil: clinical and immunopathologic aspects. Int J Dermatol 1995; 34:474–9.
7. Carvalho EM, Johnson WD, Barreto E, et al. Cell mediated immunity in American cutaneous and mucosal leishmaniasis. J Immunol 1985; 135:4144–8.
8. Mendonça SC, Coutinho SG, Amendoeira RR, et al. Human American cutaneous leishmaniasis *(Leishmania b. braziliensis)* in Brazil: lymphoproliferative responses and influence of therapy. Clin Exp Immunol 1986; 64:269–76.
9. Carvalho EM, Barral A, Costa JM, et al. Clinical and immunopathological aspects of disseminated cutaneous leishmaniasis. Acta Tropica 1994; 56:315–25.
10. Barral-Netto M, Machado P, Barral A. Human cutaneous leishmaniasis: recent advances in physiopathology and treatment. Eur J Dermatol 1995; 5:104–13.
11. Badaró R, Jones T, Carvalho EM, et al. New perspectives on a subclinical form of visceral leishmaniasis. J Infect Dis 1986; 154:1003–11.
12. Davies CR, Llanos-Cuentas EA, Pyke SDM, et al. Cutaneous leishmaniasis in the Peruvian Andes: an epidemiological study of infection and immunity. Epidemiol Infect 1995; 114:297–318.
13. Follador I, Araujo C, Pinto MA, et al. Surto de leishmaniose tegumentar americana em Canoa, Santo Amaro, Bahia, Brasil [in Portugese]. Rev Soc Bras Med Trop 1999; 32:497–503.
14. Reed, SG, Badaró R, Masur H, et al. Selection of a specific skin test antigen for American visceral leishmaniasis. Am J Trop Med Hyg 1986; 35:79–85.
15. Badaró R, Reed SG, Barral A, et al. Evaluation of the micro–enzyme-linked immunosorbent assay (ELISA) for antibodies in American visceral leishmaniasis: antigen selection for detection of infection-specific responses. Am J Trop Med Hyg 1986; 35:72–7.
16. Rocha PN, Almeida RP, Bacellar O, et al. Down-regulation of Th1 type of response in early human American cutaneous leishmaniasis. J Infect Dis 1999; 180:1731–4.
17. Cuba CAC, Llanos-Cuentas EA, Barreto AC, et al. Human mucocutaneous leishmaniasis in Três Braços, Bahia-Brazil: an area of *Leishmania braziliensis braziliensis* transmission. I. Laboratory diagnosis. Rev Soc Bras Med Trop 1984; 17:161–7.
18. Gomes AC, Yamamoto YI, Capinzaiki AN, et al. Aspectos ecológicos da leishmaniose tegumentar americana. 9. Prevalência/incidência da infecção humana nos municípios de Pedro de Toledo e Miracatu, São Paulo, Brasil [in Portugese]. Rev Inst Med Trop São Paulo 1992; 34: 149–58.
19. Nascimento MDSB, Alcântara-Neves NM, Muniz MEB, et al. Induction and modulation of the immune response to leishmania by Montenegro's skin test. Trans R Soc Trop Med Hyg 1993; 87:91–3.
20. Weigle KA, Valderrama L, Arias AL, et al. Leishmanin skin test standardization and evaluation of safety, dose, storage, longevity of reaction and sensitization. Am J Trop Med Hyg 1991; 44:260–71.
21. Oliveira-Neto MP, Pirmez C, Rangel E, et al. An outbreak of American cutaneous leishmaniasis *(Leishmania braziliensis braziliensis)* in a periurban area of Rio de Janeiro city, Brazil: clinical and epidemiological studies. Mem Inst Oswaldo Cruz 1988; 83:427–35.
22. Souza WJS, Sabroza P, Santos CS, et al. Montenegro skin tests for American cutaneous leishmaniasis carried out on school children in Rio de Janeiro, Brazil: an indicator of transmission risk. Acta Tropica 1992; 52:111–9.
23. Warburg A, Saraiva E, Lanzaro GC, et al. Saliva of *Lutzomyia longipalpis* sibling species differs in its composition and capacity to enhance leishmaniasis. Philos Trans R Soc Lond B Biol Sci 1994; 345:223–30.
24. Pirmez C, Yamamura M, Uyemura K, et al. Cytokine patterns in the pathogenesis of human leishmaniasis. J Clin Invest 1993; 91:1390–5.
25. Bogdan C, Rollinghoff M, Diefenbach A. The role of nitric oxide in innate immunity. Immunol Rev 2000; 173:17–26.
26. Lessa HA, Machado P, Lima, F, et al. Successful treatment of refractory mucosal leishmaniasis with pentoxifylline plus antimony. Am J Trop Med Hyg 2001; 65:87–9.