Leishmania (Viannia) guyanensis Induces Low Immunologic Responsiveness in Leishmaniasis Patients from an Endemic Area of the Brazilian Amazon Highland

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Abstract. Cutaneous leishmaniasis caused by Leishmania (Viannia) guyanensis (CL-Lguy) is endemic in the Brazilian Amazon, differing from L. braziliensis infection in clinical, diagnostic, and therapeutic aspects. T-cell reactivity to leishmanial antigens possibly involved in the pathogenesis of CL-Lguy was studied herein. Variable lymphoproliferative responses (LPRs) to Leishmania antigens were found among the 23 studied patients, and 50% of them showed low or no response to these antigens. Active disease was associated with an enrichment of leishmanial-reactive T lymphocytes, mainly TCD4+. High and low interferon (IFN)-γ producers were observed. TNF-α, interleukin (IL)-10, and IL-5 were consistently detected. CL-Lguy displayed low antibody response in comparison to L. braziliensis patients. CL caused by L. braziliensis presented positive LPRs and higher IFN-γ production but undetectable IL-5. L. guyanensis seems to induce a down-regulation of the immune system compared with L. braziliensis. This finding could explain some aspects of clinical presentation of CL-Lguy, such as high tissue parasite burden and frequent resistance to therapy.

INTRODUCTION

Cutaneous leishmaniasis (CL) constitutes a serious public health problem in Brazil, especially in the Amazon region that is responsible for 37% of notified cases.1 Sympatric circulation of seven Leishmania species have been identified in humans. L. (Viannia) guyanensis and L. V. braziliensis are the most prevalent species in the region, the former being highly endemic north of the Amazon River.2–7 In contrast to the geographic clustering of CL caused by L. guyanensis (CL-Lguy), L. braziliensis is distributed all over the country,1 and most knowledge on leishmaniasis in Brazil comes from studies with this species. Despite the epidemiologic importance of L. guyanensis, very few studies have been carried out to understand the pathogenesis of CL-Lguy.

The most common clinical features of CL-Lguy are small and numerous cutaneous ulcers (one to six lesions) usually located above the waist; minor lymphatic involvement compared with L. braziliensis infection; and rare cases of mucosal involvement.4–5 In addition, CL-Lguy differs from L. braziliensis infection because of their higher parasite burden in the lesion and lower antibody titers.4,5,8–10 The low rates of cure after meglumine antimonial therapy and frequent relapses are also peculiar of CL-Lguy.5,9

Considering the crucial role of cellular immunity for controlling parasite burden,11 it is reasonable to argue that a weak induction of immune responses by L. guyanensis could be responsible for the high therapeutic failure, which in turn should influence the frequent relapses.

A major T cell–driven component underlies the establishment of acquired immunity and protection against re-infections in L. braziliensis patients.12–15 However, few reports have addressed anti-leishmanial T-cell responses in CL-Lguy patients.16–19 The epidemiologic impact and clinical features of CL-Lguy stimulated the study of the main immunologic characteristics underlying the pathogenesis of this infection, which are notably different from L. braziliensis disease.

MATERIAL AND METHODS

Patients. Patients were from areas north of the Amazon River (Manaus, Rio Preto da Eva, Itacoatiara and Presidente Figueiredo, North Brazil) or from Rio de Janeiro State, southeast Brazil, where human cases of leishmaniasis are caused mostly by L. guyanensis2,7 or L. braziliensis,10 respectively. Twenty-three CL-Lguy patients (18 men and 5 women; mean age ± SD = 27.6 ± 14.1 years) with active skin ulcers were studied. The control groups were made up of healthy blood donors from Amazonas (C-AM, 10 donors) or from Rio de Janeiro State (C-RJ, 5 donors) and by CL patients (8 cases) from an endemic area of L. braziliensis (CL-Lbra) in Rio de Janeiro.

The diagnostic criteria were based on clinical, parasitologic, and immunologic parameters as described elsewhere.20 The parasites isolated from nine patients from the Amazon region were characterized as L. guyanensis by multilocus enzyme electrophoresis.31 L. braziliensis was the species identified in all eight patients from Rio de Janeiro. The committee of ethics of the Fundação Oswaldo Cruz approved the study. Informed consent was obtained from all individuals.

Lymphocyte proliferative response. Peripheral blood mononuclear cells (PBMCs) were used in lymphocyte proliferative response (LPR) assays as described.13 Briefly, PBMCs were purified over a Ficoll-Hypaque gradient (Sigma Chemical, St Louis, MO) and seeded in 96-well round-bottom plates (3 × 10^6/well; Nunc, Roskilde, Denmark) in a final volume of 200 µL/well. Cultures were incubated for 5 days at 37°C in a humidified atmosphere of 5% CO₂, in the presence of Leishmania antigens, mitogen (Concanavalin A [ConA], 4 µg per well; Sigma), or medium alone. Disrupted promastigotes of L. (V) guyanensis (MHOM/BR/75/M4147, Lg-Ag) or L. (V) braziliensis (MHOM/BR/75/M2903, Lb-Ag) were added at the concentration of 10 µg/well

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(an equivalent of 10^6 parasites). Sixteen hours before harvesting, 1 µCi of ^3H thymidine (Amersham International, Amersham, UK) was added to the wells. Radioactivity uptake was measured in a scintillation beta counter (1600CA; Packard Instrumental Company, Downers Grove, IL). Results were expressed as the stimulation index (SI) defined as the mean counts per minute (cpm) in wells containing antigen or mitogen divided by the background (mean counts in non-stimulated wells). Indexes ≥ 2.5 were considered positive. The background ranged from 59 to 566 cpm throughout the study.

**Leishmania reactive blast cells.** PBMCs (3 × 10^6 cells/well) were cultured in 24-well plates (Nunc) in a final volume of 2 mL/well in the presence of 50 µg of Lg-Ag or Lb-Ag under the conditions described elsewhere.11 After 5 days in culture, cells were harvested and centrifuged over a discontinuous Percoll (Sigma) gradient. Blast cells were separated, washed twice, and adjusted to 10^6 cells/200 µL in cold phosphate-buffered saline (PBS) containing 0.01% sodium azide and 10% fetal calf serum. The supernatants of cultures were collected at different times and stored at −20°C until the measurement of cytokine levels.

**Phenotypic analysis.** PBMCs and blast cells were incubated for 30 minutes at 4°C in the presence of 5 µL of monoclonal antibodies for CD3+ (T3-RD1; Coulter Diagnosis, Hialeah, FL), CD4+ (T4-FITC; Coulter Diagnosis), CD8+ (T8-RD1; Coulter Diagnosis), and CD56+ (R-Phycoerythrin; Caltag Laboratories, Burlingame, CA). After incubation, the cells were washed three times and re-suspended in a fixing solution containing 0.1% formalin in PBS before analysis. The cells were defined by forward- and side-scatter gating. Ten thousand events were acquired in each sample run, and data were analyzed with EXPO32 software in an EPICS ALTRA flow cytometer (Beckman-Coulter, Miami, FL).

**Cytokine measurement.** Cytokines were measured by ELISA in supernatant PBMCs stimulated in vitro for 1 (tumor necrosis factor [TNF]-α), 3 (interleukin [IL]-10 and IL-5), and 5 days (interferon [IFN]-γ) with Leishmania antigens. The monoclonal antibodies were purchased from Pharmingen (San Diego, CA). The procedures were performed according to the manufacturer’s instructions. Samples were tested in duplicate, and the concentration was analyzed using the SOFTmax PRO 4.0 program (Life Sciences Edition; Molecular Devices, Downingtown, PA). Results were expressed in picograms per milliliter. The minimum cytokine levels detected were 62.5 pg/mL for IFN-γ, 31.2 pg/mL for TNF-α or IL-10, and 15.6 pg/mL for IL-5.

**Immunoblot and indirect immunofluorescence assays.** After separation of the L. guyanensis and L. braziliensis antigens by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide) and electrophoretic transfer of proteins to nitrocellulose membrane,21 lanes containing molecular mass standards were cut and stored. The nitrocellulose sheets were incubated with the appropriate sera diluted 1:100 overnight, washed six times with PBS-T 0.05%, and incubated for 1 hour with rabbit anti-human immunoglobulin G conjugated to peroxidase (Sigma). After rinsing with PBS-T 0.05%, the antigen-antibody reactions were detected by peroxidase activity on H2O2 and diaminobenzidine.23 Peptide bands were expressed as kilodaltons.

Anti-Leishmania antibodies titers were detected using an indirect immunofluorescence test (IF-IgG) as described previously.24

**Statistical analysis.** Results were analyzed by Mann-Whitney test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego CA) and expressed as mean ± SD and/or median.

**RESULTS**

**LPRs to leishmanial antigens.** PBMCs from leishmaniasis patients and healthy volunteers were stimulated in vitro with Lg-Ag or Lb-Ag. In CL-Lguy patients, the intensity of the LPR after stimulation by L. guyanensis was quite variable, being negligible in 5 of 23 patients (21.8%) Figure 1. The majority of the CL-Lguy patients showed an SI for both leishmanial antigens < 10 (Lg-Ag SI = 10.4 ± 9.2; median = 9.8; N = 23; Lb-Ag SI = 5.7 ± 7.2; median = 5.1; N = 21). Lymphocytes from CL-Lbra patients proliferated in response to both leishmanial antigens (Lg-Ag SI = 16.4 ± 6.0; median = 16.2; N = 8; Lb-Ag SI = 10.0 ± 4.4; median = 7.6; N = 7). Both groups of patients had a comparable LPR to Con-A. The duration of illness or number of lesions was not associated with the magnitude of SI values.

Control individuals from the Amazon region had low SIs of LPRs in response to Lg-Ag (SI = 5.1 ± 2; N = 7/10) or Lb-Ag (SI = 3.7 ± 1.0; N = 5/10). C-RJ individuals showed negligible LPRs to both leishmanial antigens. The positive LPRs observed in C-AM healthy donors might be caused by a subclinical infection, because this area’s ecologic conditions determine high probabilities of contact with the parasite and vectors. This situation differs from the Rio de Janeiro state, where the parasite transmission is low and restricted to some areas. Moreover, it was shown that Leishmania could induce a T-cell response in unexposed individuals.

**Cell subset analysis.** The percentages of T lymphocytes (CD3+, CD4+, and CD8+) and NK cells (CD56+) were determined in PBMC and in L. guyanensis–stimulated blast T lymphocytes. The mean ± SD proportions of blood lymphocytes

![Figure 1. Lymphocyte proliferative responses of patients with active cutaneous leishmaniasis from endemic areas for L. guyanensis at north of the Amazonas river (CL-Lguy) and endemic areas for L. braziliensis at Rio de Janeiro (CL-Lbra). Peripheral blood mononuclear cells were stimulated in vitro with leishmanial antigens as described in the Materials and Methods section. Results are expressed as stimulation indexes (SIs). Each point represents one patient. Horizontal bars represent the median value for each group.](image)
from CL-Lguy (CD3$^+$ = 45.8 ± 15%, TCD4$^+$ = 32.7 ± 12%, and TCD8$^+$ = 12.3 ± 5.5%, NK = 7.3 ± 7.7%) were similar to those observed in the healthy population. An enrichment of T lymphocytes (CD3$^+$ = 74.7 ± 12%) was observed after stimulation with Lg-Ag. CD4$^+$ blast T lymphocytes (59.7 ± 19.3%) preferentially proliferated under leishmanial stimulus, whereas a slight increase was observed among CD8$^+$ T cells (18 ± 5.7%). The percentage of NK cells (7.0 ± 6.8%) did not change after parasite stimuli. In control individuals, the percentages of lymphocytes were similar before and after stimulation with Lg-Ag. In accordance with our own and other previous results, a higher CD4$^+$/CD8$^+$ ratio was also observed in CL-bra.

**Cytokine levels.** PBMCs from CL-Lguy patients produced IFN-γ after stimulation with Lg-Ag (1,908 ± 3,080 pg/mL, median = 562 pg/mL; N = 11/13) as well as Lb-Ag (1,143 ± 1,404 pg/mL, median = 698 pg/mL, N = 9/11; Figure 2). The levels of this cytokine were quite variable among CL-Lguy patients, in which lower and higher IFN-γ responders could be identified. In control groups, the IFN-γ levels were very low or not detectable, and the cytokine production was comparable to the non-stimulated cultures (143.8 ± 26.6 pg/mL, N = 12/29). Only two control individuals produced IFN-γ (670 and 169 pg/mL) in response to leishmanial antigens. In contrast, all CL-Lbra patients produced IFN-γ under leishmanial stimuli (2,956 ± 1,160 pg/mL, N = 8/8), and the mean levels were much higher in comparison to CL-Lguy (P < 0.05).

In CL-Lguy patients and C-AM controls, the production of TNF-α, IL-10, and IL-5 was determined in supernatants from cultures stimulated only with Lg-Ag, whereas in CL-Lbra patients and C-RJ controls, these cytokines were measured in cultures stimulated only with Lb-Ag. TNF-α and IL-10 were detected in all patients studied. The mean levels of TNF-α in CL-Lguy patients (1,402 ± 664 pg/mL, median = 1,624 pg/mL, N = 18/18) was higher than those detected in C-AM controls (591 ± 485 pg/mL, median = 562 pg/mL, N = 8/8; Figure 3). No significant differences were observed in the production of TNF-α in CL-Lguy and CL-Lbra patients. On the other hand, the mean production of IL-10 was similar in the CL-Lguy group (189 ± 55.2 pg/mL, median = 182.8, N = 11) and C-AM controls (188 ± 75 pg/mL, median = 152.9 pg/mL, N = 5), although it was higher than those detected in the CL-Lbra group (73 ± 32 pg/mL, median = 80 pg/mL, N = 4). IL-5 was detectable in 6 of 13 CL-Lguy patients (142.8 ± 165 pg/mL, median = 52 pg/mL; Figure 3). In contrast, in the CL-Lbra and in the control groups, IL-5 production was below the detection limit (i.e., 15.6 pg/mL).

**Humoral responses of CL-Lguy versus CL-Lbra patients.** In immunoblot assays, the mean number of recognized antigenic fractions tended to be lower among CL-Lguy patients than in the CL-Lbra group. The frequency of recognition of bands ranged from 22% to 52% in CL-Lguy, whereas in CL-Lbra, this frequency ranged from 14% to 86%. Additionally, there were qualitative differences in antigen-antibody recognition, because sera from 15 of 23 CL-Lguy patients (65%) showed a weak immunostaining, whereas sera from CL-Lbra reacted intensely to the same antigens (Figure 4). According to immunofluorescence assays, sera from 23.5% of L. guyanensis–infected patients (4 of 17 cases) had anti-Leishmania IgG antibodies (titers varying from 1:45 to 1:90), whereas 50% of CL-Lbra patients (4 of 8 cases; titers varying from 1:45 to 1:90) showed positive results. Despite the differences on protein profile exhibited by L. guyanensis or L. braziliensis, similar parasite antigens can be recognized by sera from patients infected with these two species. However, CL-Lguy patients have low rates of specific antibodies in comparison to CL-Lbra as suggested by the low intensity of immunostained bands. No association was seen between antibody titer by immunofluorescence and the number of bands recognized by Western blotting. These data suggest the humoral response is less effective in CL-Lguy than in CL-Lbra patients.

**DISCUSSION**

*Leishmania guyanensis* has been recognized as an important causative agent of ATL in the Amazon region. However, the disease caused by this parasite has particularities that differ from other *Leishmania* species. American tegumentary leishmaniasis numerous skin lesions rich in parasites, low
antigens was evident in at least 50% of the CL-Lguy patients. These facts, in association with low IFN-γ production observed in some of the patients, suggest an inability to mount an appropriate specific immune response. This defective IFN-γ production was probably caused by a parasite-specific unresponsiveness to IL-12 caused by IL-12Rβ2 chain inhibition by IL-13. 16–26

*Leishmania guyanensis* antigens were able to induce TNF-α and IL-10 production not only in patients studied here but also in healthy controls, whereas only CL-Lguy patients produced IL-5. IL-10 was probably not produced as a consequence of the immunopathogenesis of the disease. Rather, it seems that parasite antigens *per se* are able to induce cytokines involved in the macrophage innate response. However, those that develop the disease have a T-cell modulation toward the production of other Type 2 cytokines such as IL-5 and possibly IL-4 or IL-13. 16 It was shown that a transient T-cell Type 2 response predominant during early phases of the disease allows parasite growth and establishment of the infection, whereas a subsequent induction of a Type 1 profile could provide a parasite-specific effective immune response. 19 However, the precise duration of infection can be difficult to ascertain in some affected populations. These observations could have profound practical consequences in the management of *L. guyanensis* patients because a precocious therapeutic intervention could impede a subsequent development of Type 1 effector mechanisms able to help parasite control, as well as a long-lasting T-cell response. This could explain the high rates of therapeutic failure and recurrent infections in a contingent of *L. guyanensis* patients from Manaus. 5,9

The nature of the infecting parasite is another factor that can underlie differences in T-cell responsiveness profiles. However, there is a lack of awareness on possible differences in clinical parameters or therapeutic responses of patients infected with two antigenically distinct populations of *L. guyanensis* that have been identified in patients from Manaus. 7 Moreover, because the clinical outcome may also vary in certain host–parasite combinations, 27–30 it can be argued that the variation in parasite antigens can influence the modulation of an adaptive immune response toward a harmful or a protective profile.

The functional characteristics of T-cell subpopulations induced on parasite stimuli are crucial in determining immune functions. A preferential expansion of CD4+ T cells was observed after Lg-Ag stimuli, indicating that these cells were involved in the pathogenesis of active disease. 14,20 In fact, CD4+ T cells were shown to play a major role in IFN-γ or IL-13 production during active disease. 10,18 Previous results on *L. braziliensis* have already suggested the role of *Leishmania* reactive CD4+ in the development of lesions, because a decrease in these cells is observed soon after the end of therapy and in the long term after clinical cure. 14,20 A slight increase of TCD8+ cells observed in CL-guy patients studied here indicates that clones of these cells were also expanded, whereas no changes in the NK cell compartment was observed. The potential of inducing cytotoxic effects cannot be ruled out in the protective mechanism of leishmaniasis caused by *L. guyanensis*. Although we could not study CL-Lguy patients during the process of cure, it is known that increased levels of *Leishmania*-reactive TCD8+ cells are associated with healing of *L. braziliensis* infection. 13,14,20,30
A subtle balance between pro-inflammatory and effector cytokines produced by expanded T-cell populations is thought to be one of the critical components that dictate the behavior of the infection. A positive correlation between a Type 2 response and duration of the disease or the parasite load in lesions, is seen, contrasting with a Type 1 cytokine milieu that characterizes older lesions with low parasite burden.19 Our data showing a mixed Type 1 (IFN-γ, TNF-α) and Type 2 (IL-10 and IL-5) cytokine profile displayed by L. guyanensis patients is in accordance with this thought. Subtle differences in the balance of IFN-γ or TNF-α have been observed in CL-Lguy and CL-LbRa patients when results from these patients were analyzed,14,15,20,28 but Type 2 cytokines were significantly higher in L. guyanensis patients. Both IL-5 and IL-4 have been associated with active disease or severe clinical forms of leishmaniasis13,15,30 or detrimental progression of L. donovani infection.21 Thus, because the induction of IL-5 was observed only in CL-Lguy patients, it could predict an unfavorable prognosis of these patients by allowing parasite replication. Although the IL-5 levels were consistently low, it is possible that even small amounts of this cytokine can be exert significant biological functions. On the other hand, the apparently higher capacity of Lg-Ag to induce IL-10, as observed in patients and controls, could also explain the frequent episodes of re-activation in CL-Lguy patients, favoring parasite persistence and unresponsiveness to chemotherapy.17,31,32 These data reinforce the idea that not only in L. braziliensis24,35 but also in L. guyanensis26 infection, Type 2 cytokines are responsible for maintaining Leishmania replication, whereas healing of lesions requires IFN-γ and TNF-α to activate macrophages, leading to destruction of parasites.

A low percentage of patients with anti-Leishmania antibodies and a minor number of antigenic bands and reduced immunostaining characterized the humoral response of the CL-Lguy group. This feature seems to mirror a partially impaired T-cell function in helping the B-cell affinity maturation mechanisms, favoring the generation of antibodies with low affinity. Antibodies are not proven to protect patients from the disease, and their role in leishmaniasis pathogenesis is neglected. However, high antibodies titers are usually detected during the active disease or in patients suffering from severe clinical forms of leishmaniasis.5,27 During active disease, L. guyanensis infection induces a lower humoral response than L. braziliensis.5,8 These data indicate that the L. guyanensis parasite is a weaker in vivo inducer of host immune responses than L. braziliensis.

This study showed that L. guyanensis infection was able to partially impair the expansion of specific T-cell clones as suggested by the low lymphocyte proliferation and IFN-γ production under parasite stimulus. Additionally, the induction of high levels of Type 2 cytokines could also play an important role in this scenario, by impairing Type 1 responses and also allowing continuous parasite replication, which maintains the pathogenic process. The low cellular and humoral responses elicited by L. guyanensis could explain the higher parasite burden in tissues and the frequent recurrent disease observed in CL-guy in comparison with leishmaniasis caused by L. braziliensis infection.4,35,8,30,10 These results point out that therapeutic and immunoprophylactic strategies should be revised, taking into consideration the particularities of each Leishmania species.
cutaneous leishmaniasis infected by *Leishmania (Viannia) braziliensis* or *Leishmania (Viannia) guyanensis* in Brazil. *Acta Trop* 93: 49–56.


