Leishmania- Reactive CD4+ and CD8+ T Cells Associated with Cure of Human Cutaneous Leishmaniasis

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Received 10 November 1993/Returned for modification 11 January 1994/Accepted 4 March 1994

Fourteen patients suffering from American cutaneous leishmaniasis were studied. Assays of the lymphocyte proliferative response induced in vitro by Leishmania braziliensis antigens were performed. After 5 days in culture, L. braziliensis-stimulated blast T cells were harvested for CD4+ and CD8+ phenotype analysis. When results before and at the end of therapy were compared, leishmaniasis patients showed an increase in the percentage of CD8+ blast T cells and a decline in the proportion of CD4+ blast T cells in cultures. The levels of gamma interferon in T-cell culture supernatants showed a tendency to increase when the patients were cured. These results show a pattern of higher proportions of Leishmania-reactive CD8+ T cells and lower proportions of Leishmania-reactive CD4+ T cells after cure.

Human American cutaneous leishmaniasis (CL) is caused in Rio de Janeiro, Brazil, by the protozoan parasite Leishmania braziliensis, the only species of Leishmania that has been found parasitizing humans and dogs in the region (30). Transmission occurs by inoculation of the parasite at the site where the infected Phlebotominae bites the human host, producing in general a single skin ulcer. The majority of human infections are characterized by a mild form of the disease, without metastatic lesions, with healing often occurring spontaneously or after therapy. A minority of patients fail to recover and instead develop a severe, chronic form of the disease, with secondary metastatic lesions on the mucous membranes of the mouth and nose. These lesions, which are resistant to therapy, result in severe destruction of the face.

Studies of mice (10, 16, 22, 24) and humans (4–6, 8, 11, 12, 27, 33) strongly suggest that T-cell-mediated immune responses play a central role in the outcome of the disease. Various research groups (14, 20, 22, 28, 36, 38) have demonstrated that CD4+ T cells appear to induce opposing effects associated with cure or aggravation of the disease, depending on the lymphokine production profile. Recent data (31, 37, 39) point to the possibility that Leishmania-reactive CD8+ T cells may also play a role in the processes of cure of experimental leishmaniasis, by modulating CD4+ T-cell activity and/or a possible direct cytolytic effect on parasitized macrophages (15, 23, 37, 39).

In the present study, we examined the proportions of CD4+ and CD8+ Leishmania-reactive T cells after 5 days in culture in the presence of parasite antigens. Blood cells were obtained from patients with active ulcers and then again from the same patients at the end of therapy. Our aim was to investigate possible associations between the responsiveness of these T-cell populations and the healing of lesions.

Fourteen patients with CL were studied. The following criteria were utilized for diagnosis: (i) type of lesion and epidemiological data compatible with CL; (ii) positive Montenegro skin test (MST)—delayed-type hypersensitivity to leishmanial antigens; (iii) presence of immunoglobulin G and/or immunoglobulin M Leishmania-specific serum antibodies, as detected by indirect immunofluorescence; and (iv) detection of Leishmania parasites in lesions by microscopic examination of histological sections from biopsy samples or by culture in NNN modified medium (32).

All the patients came from an area in which leishmaniasis is endemic in Rio de Janeiro, Brazil. They were treated with an antimonial agent (Glucantine; Rhodia) (three 10-day courses of N-methyl-glucamime at a dose of 15 to 20 mg of Sb/kg of body weight given intramuscularly daily). The patients were considered cured when their lesions had completely healed.

All 14 patients (7 male and 7 female) had active leishmanial skin ulcers at the beginning of the study. The mean age of the patients was 35.6 ± 11.5 years, and the mean period of illness was 3.8 ± 2.9 months. Eight patients had a single lesion and six patients had two to six lesions. The MST was positive for all patients, with areas of induration ranging from 8 to 73 mm in diameter. Following cultivation in NNN culture medium, parasites were isolated from lesions of 8 of 11 CL patients examined (72.7%). All isolated parasites were characterized as L. braziliensis by G. Grimaldi, Jr., and H. Momen, using, respectively, species-specific monoclonal antibodies and isoenzyme patterns (13).

Twelve CL patients exhibited healed lesions at the end of therapy (Table 1, patients 01 to 12). Patients 13 and 14 were not cured and still had clear signs of an inflammatory reaction at the site of the lesion at the end of therapy. Patient 14 was the only one who exhibited a reappeared lesion in a follow-up examination conducted after 3 months.

The percentage of T lymphocytes (CD4+ and CD8+) as a proportion of total peripheral blood mononuclear cells (PBMC) was determined for six patients, using flow cytometry before stimulation in vitro with L. braziliensis antigens. The results were 40.6% ± 9% CD4+ and 27.1% ± 7% CD8+. Similar levels have been found in healthy populations (18). No significant differences were observed in CL patients before and at the end of therapy.

For the lymphocyte proliferative response (LPR) assays, PBMC were separated by centrifugation over a gradient of Ficoll-Hypaque (Histopaque 1077; Sigma, St. Louis, Mo.). Mononuclear cells were resuspended in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated human AB Rh+.
TABLE 1. LPRs induced by Leishmania antigens in PBMC and phenotypes of Leishmania-stimulated blast T cells after 5 days of culture*

<table>
<thead>
<tr>
<th>Patient</th>
<th>% CD4+</th>
<th>% CD8+</th>
<th>Ratio</th>
<th>% CD4+</th>
<th>% CD8+</th>
<th>Ratio</th>
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* BT, before therapy (active lesions); AT, at the end of therapy.

** SI, stimulation index.

* Patient not cured at the end of therapy.

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serum, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 1.5 mM l-glutamine, 0.04 mM 2-mercaptoethanol, and antibiotics (200 IU of penicillin per ml and 200 μg of streptomycin per ml) and were adjusted to 3 x 10⁶/ml. The cells (3 x 10⁶ per well) were distributed in triplicate in 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark). The cultures were incubated for 5 days at 37°C in a humidified atmosphere of 5% CO₂ in air in the presence of the equivalent of 10⁶ sonicated L. braziliensis promastigotes (MHOM/BR75/M2903) as antigens or 4 μg of concanavalin A (Pharmacia, Uppsala, Sweden) per well in a final volume of 200 μl per well. Sixteen hours before harvesting, 1 μCi of [³H]thymidine (Amersham International, Amersham, United Kingdom) with a specific activity of 5 Ci/mmol was added to all wells, including control wells without antigen or concanavalin A. Cells were harvested on fiber filters (Titertek; Flow Laboratories) by using a cell harvester (Titertek; Flow Laboratories). Radioactivity uptake was measured in a scintillation beta counter (Packard 1600). Results were expressed as stimulation indices defined as the mean counts in wells containing antigen or mitogen divided by background (mean counts in nonstimulated wells). Indices equal to or higher than 2.5 were considered positive. The background ranged from 260 to 410 cpm throughout the study.

The results are shown in Table 1. All patients had stimulation indices of ≥2.5 during the active phase of the disease. The differences between the L. braziliensis LPR stimulation indices before and at the end of therapy were not quite significant (P = 0.07), although a tendency to decline was observed. No significant correlation between the L. braziliensis LPR indices and the MST responses or between the L. braziliensis LPR indices and the period of illness was found. The proliferative responses to concanavalin A before and at the end of therapy were similar (data not shown). The L. braziliensis LPR indices of five healthy donors were all very low (<2.5).

In order to investigate the phenotypes of leishmanial-antigen-reactive T cells stimulated in vitro, PBMC (3 x 10⁶ per well) were cultured in 24-well plates (Nunc) in a final volume of 2 ml per well under the conditions described above. Stimulation with sonicated Toxoplasma gondii organisms (5 x 10⁶/ml) was performed as a control. After 5 days in culture, the cells were harvested and washed and then centrifuged over a discontinuous Percoll (Sigma) gradient. Blast cells were separated, washed twice, and resuspended in a fixative solution containing 0.1% formalin and 0.05% sodium azide in phosphate-buffered saline. The fixed cells were adjusted to 10⁶ cells per 200 μl in fixative solution and incubated for 30 min at 4°C in the presence of 5 μl of monoclonal antibodies for CD3⁺ (T3-RD1; Coulter Diagnosis), CD4⁺ (T4-FITC; Coulter Diagnosis), and CD8⁺ (T8-RD1; Coulter Diagnosis). After incubation, the blast cells were washed three times prior to analysis by flow cytometry (EPICS 751; Coulter). The supernatant of each culture was collected and stored at −70°C until use for determination of gamma interferon (IFN-γ) concentration.

The phenotype profile of leishmanial-antigen-reactive blast T cells following stimulation in vitro is shown in Table 1 and Fig. 1. Comparing the proportions of CD4⁺ and CD8⁺ L. braziliensis-reactive blast T cells before and at the end of therapy, we observed an increase in the percentage of CD8⁺ blast T cells (Fig. 1B) (P < 0.01), a decline in the proportion of CD4⁺ blast T cells (Fig. 1A) (P < 0.01), and a consequent reduction in the CD4⁺/CD8⁺ ratio (P < 0.01).

Three patients failed to show any increase in the percentage of CD8⁺ blast T cells at the end of therapy (Table 1; Fig. 1). Two of these patients (13 and 14) were not cured and still had signs of an inflammatory reaction at the site of the lesion. Furthermore, a re开放ed ulcer was observed in patient 14 after 3 months of follow-up. The third patient (no. 5) who failed to show increased levels of CD8⁺ cells was cured at the end of therapy. This patient, however, had already exhibited a relatively high percentage of CD8⁺ blast T cells before therapy, and this level was maintained at the end of therapy. In these three patients, as in the majority of CL patients, the levels of CD4⁺ blast T cells showed a tendency to decline at the end of therapy, although two of the patients (13 and 14) were not cured, as already mentioned (Table 1; Fig. 1A). The percentage of double-positive CD4⁺/CD8⁺ cells was observed to be much higher among L. braziliensis-reactive blast T cells (12.4% ± 3%) than among nonstimulated PBMC (5.9% ± 7%). No correlation was found between the percentage of CD4⁺ or CD8⁺ L. braziliensis-reactive blast T cells and the period of illness or the magnitude of the MST response.

These results suggest that CD8⁺ T cells could be implicated in the process of cure of CL. However, it is not clear whether the process of cure is associated only with the increased percentage of CD8⁺ Leishmania-reactive blast T cells or whether it depends on the balance of CD4⁺ and CD8⁺ cells, since a decrease in the proportion of CD4⁺ cells was also observed.

In order to verify whether the change in the profile of the CD4⁺ and CD8⁺ L. braziliensis-reactive T cells after cure was a direct consequence of the antimonial therapy, patients were also tested before therapy (n = 12) and at the end of therapy (n = 13) to determine the T-cell response to T. gondii. The phenotype profile of T. gondii antigen-reactive blast T cells following stimulation in vitro showed no significant differences before and at the end of therapy, either in the proportion of CD4⁺ T. gondii-reactive blast T cells (before-therapy mean = 44.3% ± 2% and end-of-therapy mean = 50.6% ± 2%; P = 0.35) or in the proportion of CD8⁺ T. gondii-reactive blast T cells (before-therapy mean = 31.4% ± 2% and end-of-therapy mean = 44.6% ± 2%; P = 0.12). Since the antimony did not significantly alter the CD4⁺ and CD8⁺ response to T. gondii, the changes observed in the CD4⁺ and CD8⁺ response to Leishmania infection after therapy should not be interpreted as being associated with the antimony itself.

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In murine leishmaniasis, CD4+ T cells can be either host protective or disease promoting, with the outcome of the infection depending on the profiles of cytokines released by Th1 or Th2 cells, respectively (19, 21, 29, 36). The possibility that CD4+ T cells may be able to down-regulate immune responses to leishmanial antigens has been indicated by experiments in which the activity of these cells was abolished, thus enabling observation of the host-protective effect of CD8+ T-cell clones (15). Although there exists substantial evidence suggesting that CD4+ T-cell clones may be able to induce resistance to experimental leishmaniasis, other data indicate that CD8+ T cells may also be involved in the healing process (7, 31).

In this study, samples taken from the same patients when the disease was active and then again following cure exhibited different proportions of CD4+ and CD8+ L. braziliensis-reactive T cells in culture. It is difficult to explain the mechanism for the CD4+-CD8+ switch. One possible factor could be a decrease in the parasite load or an increase of killed parasites during the healing process. However, in vitro experiments do not support this hypothesis, since no significant difference was observed when T cells were stimulated with live or killed parasites (data not shown). Recently (3), it was demonstrated that low-dose infection of mice with Leishmania major leads to protection while high doses induce severe disease.

The level of IFN-γ production by Leishmania-reactive T cells was determined by testing the supernatants from 5-day cultures of stimulated PBMC by a solid-phase enzyme-linked immunosorbsorbent assay (ELISA test kit for quantification of human IFN-γ; Holland Biotechnology, Leiden, The Netherlands). The test was carried out on culture supernatants from seven patients and five healthy donors, the latter serving as controls. The samples were analyzed in duplicate, and the results were expressed as units per milliliter. The mean levels of IFN-γ in supernatants from leishmanial-antigen-stimulated cell cultures were 123.7 ± 58.6 and 193.4 ± 70 U/ml during the acute phase of the disease and after therapy, respectively. In six of seven patients (all cured at the end of therapy), the levels of IFN-γ produced by L. braziliensis-stimulated T cells showed a tendency, albeit not significant, to be higher at the end of therapy than before therapy (active lesions). No correlation between IFN-γ levels and the relative proportions of CD4+ and CD8+ blast T cells in culture or the results of L. braziliensis LPR was seen. The controls (five healthy donors) exhibited low levels of IFN-γ (0.4 ± 0.2 U/ml) in the supernatants of PBMC cultures containing L. braziliensis antigens.

The two-tailed Mann-Whitney test and the Spearman correlation test were utilized where applicable for the statistical analysis.

IFN-γ is considered to be a lymphokine that mediates the killing of parasites by macrophages (2, 35). We demonstrated that in vitro-stimulated L. braziliensis-reactive T lymphocytes from the blood of CL patients were able to produce much higher levels of IFN-γ in culture than cells from noninfected control individuals. The IFN-γ levels were higher at the end of therapy (when the lesions were healed) than before therapy, suggesting that the capacity to induce IFN-γ production increases during the healing process.

Unfortunately, the cell types and cell functions involved in the processes of cure or aggravation of human leishmaniasis have not yet been adequately established. The present results suggest that increased levels of Leishmania-reactive CD8+ T cells and IFN-γ production may be important for the healing of lesions (2, 7) and probably also for immunological protection. These data, combined with data from further studies on cell functions, should contribute to a better understanding of the
immune mechanisms involved and aid in the selection of appropriate antigens capable of eliciting a protective immune response for use in a future vaccine.

Notwithstanding the importance of studying T-cell responses to well-characterized peptides and/or epitopes derived from leishmanial antigens (17, 26, 40), it is equally important not to neglect the study of human T-cell responsiveness to crude leishmanial antigens, for the following reasons. (i) Whole-parasite antigens, whether processed by antigen-presenting cells or not, are present in lesions. Thus crude antigen may be essential to defining the critical types of T-cell response elicited in cases of self-cure or in mild forms of the disease, as opposed to cases that exhibit severe lesions or resistance to therapy. (ii) All trials for human leishmaniasis vaccine performed so far have utilized whole parasite antigens (1), giving about 50% protection. (iii) There is evidence that even soluble antigens induce a CD8+ T-cell response (25, 34). (iv) It has been demonstrated previously (6, 9) that in immunotherapy or combined immunotherapy and antimonial therapy, using whole parasite antigens in both instances, antigens can be extremely useful in the treatment of patients who are resistant to antimonial therapy or for whom antimonial therapy is not indicated (e.g., pregnant women).

The present results show that cure in CL patients is associated with higher percentages of *L. braziliensis*-reactive CD8+ T cells in antigen-stimulated cultures. This suggests that any antigen or epitope candidate for a vaccine against leishmaniasis should elicit a specific CD8+ T-cell response in association with *L. braziliensis*-reactive CD4+ T cells.

We are grateful to D. McMahon-Pratt, C. Pirmez, and S. C. F. Mendonça for valuable discussions and for critical readings of the manuscript, to R. Nogueira for laboratory assistance, and to M. Santiago for flow cytometry analysis, and R. Pelegrino for typing the manuscript. This work was supported by grants from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, CAPES, and CNPq.

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