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Cell-Mediated Immunity in American Visceral Leishmaniasis: Reversible Immunosuppression During Acute Infection

EDGAR M. CARVALHO,[1] RODOLFO S. TEIXEIRA,[1] AND WARREN D. JOHNSON, JR.[2]*

University of Bahia, Salvador, Brazil[1] and the Division of International Medicine, Department of Medicine, Cornell University Medical College, New York, New York 10021[2]

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Cell-mediated immunity was assessed in 14 Brazilian patients with acute untreated American visceral leishmaniasis (AVL) and in 11 healthy patients successfully treated 1 to 14 years previously. The diagnosis of AVL was established by demonstration of leishmania in bone marrow aspirates. The responsiveness of peripheral blood mononuclear cells to *Leishmania chagasi* antigens and phytohemagglutinin was studied in vitro. Soluble preparations of *L. chagasi* antigens were obtained from frozen-thawed promastigote cultures. *L. chagasi* antigen-stimulated lymphocytes from untreated AVL patients were unresponsive and incorporated a mean of $1.2 \pm 0.5 \times 10^{-3}$ cpm after a [^{3}H]thymidine pulse. The cured AVL patients had 19.1 ± 7.2 cpm, and 15 normal control subjects had 0.8 ± 0.1 cpm. There was no difference in the response of controls and either untreated or cured AVL patients to phytohemagglutinin stimulation. Three of four untreated AVL patients responded to *L. chagasi* antigens when restudied 2 to 4 weeks after therapy. The impaired response of lymphocytes from untreated AVL patients could not be attributed to either reduced numbers of circulating T cells or the inhibitory effect of monocytes or serum factors.

Leishmania are obligate intracellular protozoa which cause a spectrum of disease in man ranging from self-healing skin ulcers to fatal visceral infections (13). American visceral leishmaniasis (AVL) is caused by *Leishmania chagasi* and clinically is characterized by fever, hepatosplenomegaly, anemia, leukopenia, and marked hyperglobulinemia (13). AVL occurs primarily in children and is usually fatal if untreated. A similar disease caused by *L. donovani* is called kala-azar in Africa and Asia.

Experimental studies in rodents have suggested that host control of leishmania infection is dependent on intact cellular immune mechanisms (1, 3, 17, 20). Immunosuppression of *L. enriettii*-infected guinea pigs with antilymphocyte serum (4) or disruption of lymphatics draining the site of skin infection (12) results in disseminated cutaneous lesions. T cell depletion of mice naturally resistant to *L. tropica* infection delays resolution of their disease (19). Studies of cell-mediated immunity in acute human visceral leishmaniasis have been limited to the demonstration of impaired delayed-type skin hypersensitivity reactions to leishmania antigens (16). This study demonstrates that peripheral blood mononuclear cells from untreated AVL patients are unresponsive to *L. chagasi* antigen in blastogenesis assays. The impaired response is specific, rapidly reversible with effective chemotherapy, and not attributable to either depletion of T cells or the inhibitory effect of serum or monocytes.

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MATERIALS AND METHODS

Subjects. The 40 subjects studied include 14 patients with untreated AVL, 11 patients with cured AVL, and 15 healthy control subjects. Patients with untreated AVL were hospitalized in Salvador, Brazil. The diagnosis of AVL was confirmed by demonstration of leishmania in Giemsa-stained bone marrow aspirates. All untreated patients had fever, hepatosplenomegaly, and hyperglobulinemia. Cured AVL patients met similar diagnostic criteria, but were successfully treated with one or more courses of pentavalent antimony 1 to 14 years (mean, 4.1 years) previously. They were asymptomatic and without hepatosplenomegaly at the time of the study. The control subjects included laboratory personnel and healthy relatives of study patients. The range and median age of subjects in the three groups were as follows: untreated AVL patients, 1 to 20 years (median, 8 years); cured AVL patients, 4 to 17 years (median, 9 years); and control subjects, 6 to 40 years (median, 19 years).

Collection, separation, and culture of mononuclear cells. Human peripheral mononuclear cells were separated from heparinized venous blood by centrifugation over a Ficoll-Hypaque gradient as previ-

ously described (9). The mononuclear cell band was washed twice in RPMI with penicillin and streptomycin (GIBCO Laboratories, Grand Island, N.Y.) and incubated (37°C) for 1 h. The cells were washed again in RPMI and resuspended in RPMI supplemented with either 15% heated (56°C for 30 min) autologous or pooled human AB serum at a final concentration of 10^6 cells per ml. Aliquots (0.2 ml each) of this cell suspension were cultured in flat-bottom microtiter plates (Linbro Chemical Co., New Haven, Conn.) for blastogenesis assays. In some experiments, cell preparations were depleted of monocytes by incubating (37°C for 45 min; 5% CO_2–95% air) 10×10^6 to 15×10^6 mononuclear cells in 6 ml of RPMI–20% fetal calf serum in plastic dishes (100 by 15 mm) (Falcon Plastics, Oxnard, Calif.). Nonadherent cells were collected, and the procedure was repeated. This resulted in a reduction of monocytes from 29 ± 4% of the original cell preparation to 3 ± 2% of the final preparation. Monocyte-depleted cell preparations were used to determine the percentages of T, B, and L lymphocytes. T cells were characterized by their ability to bind sheep erythrocytes, and B cells were characterized by detecting surface immunoglobulin with fluoresceinated goat anti-human globulin (Hyland Laboratories, Deerfield, Ill.) (14). L cells are non-T, and non-B, Fc receptor-positive cells.

Lymphocyte blastogenesis assay and mixed lymphocyte reaction. Mononuclear cell preparations contained both monocytes and lymphocytes unless otherwise indicated. Triplicate cultures were incubated (37°C; 5% CO_2–95% air) for 3 days with phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, Mo.) in concentrations which yielded final PHA dilutions of 1:10, 1:100, and 1:200. Cell cultures were incubated for 5 days with *L. chagasi* antigens in at least three concentrations ranging from 0.5 to 25 μg of protein per ml of culture. The data presented are the mean responses of triplicate cultures to the antigen or mitogen concentration giving the greatest amount of [^{3}H]thymidine incorporation. In 6-day mixed lymphocyte reactions, 10^5 cells from untreated AVL patients were used as responders or treated with mitomycin C (Sigma Chemical Co.) and used as stimulators (9). [^{3}H]thymidine incorporation was quantitated by liquid scintillation spectrometry after a 4-h pulse of cell cultures with 1 μCi of [^{3}H]thymidine (6.7 Ci/mM) (New England Nuclear Corp., Boston, Mass.) Statistical analysis utilized the Student *t* test. All populations were of equal variance, as determined by the F test.

***L. chagasi* antigen preparation.** A strain of *L. chagasi* from a Brazilian AVL patient was isolated from a bone marrow aspirate onto 5% blood agar overlaid with saline. The strain was subcultured every 2 weeks in liquid liver infusion tryptose medium (2). Promastigote forms from these cultures were washed three times in phosphate-buffered saline, resuspended in phosphate-buffered saline with 10^8 promastigotes per ml, and rapidly frozen (−70°C) and thawed (37°C) six times. The lysate was centrifuged (16,000 × *g*) for 10 min, and the supernatant was collected. *L. chagasi* antigens were quantitated by the protein content in the supernatant as determined by the method of Lowry et al. (15). The antigen preparation was stored at −20°C. Dose response curves were performed by using lymphocytes from 5 healthy patients with cured AVL and different concentrations of *L. chagasi* antigens (0.5 to 150 μg of protein per ml) in blastogenesis assays. Responses were maximal after 5 days of cell cultivation. [^{3}H]thymidine incorporation (mean counts per minute ± standard error of the mean [SEM] × 10^{-3}) after cell cultivation with 0.5, 5, 25, 75, and 150 μg of protein per ml of *L. chagasi* antigens was 2.4 ± 1.2, 11.5 ± 4.4, 13.0 ± 7.2, 14.4 ± 6.6, and 12.8 ± 1.6, respectively. Control subjects' mononuclear cells cultivated with identical concentrations of *L. chagasi* antigens yielded (in counts per minute ± SEM × 10^{-3}) 0.2 ± 0.1, 0.6 ± 0.2, 0.6 ± 0.1, 2.0 ± 0.3, and 2.0 ± 0.7.

RESULTS

A profile of 14 untreated AVL patients and their lymphocyte responses to PHA and *L. chagasi* antigens is shown in Table 1. The patients had a 6.5-month median duration of illness and were unresponsive to the intradermal injection of leishmania antigens. The mean values of several laboratory parameters (not shown) were as follows: albumin, 2.9 g%; globulin, 6.4 g%; hematocrit, 26.2%; white blood cell count, 3,400/mm^3. Patient 10 did not respond to pentavalent antimony therapy and died of septicemia complicating severe leukopenia 2 months after these studies were performed. There were no other deaths in this group, and all other patients responded well to PHA. In contrast, lymphocytes from all patients except number 12 were unresponsive to *L. chagasi* antigens. Lymphocytes from patients 9, 12, and 13 were active in the mixed lymphocyte reactions, both as responders and stimulators.

The mean of the lymphocyte response to PHA and *L. chagasi* antigens of the 14 untreated AVL patients, the 11 patients with cured AVL, and the 15 healthy controls is shown in Table 2. Lymphocytes from cured AVL patients responded to *L. chagasi* antigens, whereas the response of the untreated AVL group was almost identical to that of the normal controls. There were no significant differences in the response of the three groups to PHA.

Four patients with untreated AVL were restudied after successful pentavalent antimony therapy (Table 3). Bone marrow aspirates were negative for leishmania at this time. Three of the initially unresponsive patients had enhanced responses to *L. chagasi* antigens 2 to 4 weeks posttherapy. The fourth subject was restudied 1 week after treatment and exhibited no change. There were no differences in the pre- and posttherapy responses to PHA in these patients.

The absolute number of peripheral blood lymphocytes was below the lower limits of normal in patients 1, 3, 4, 7, and 13. Their quantitative

TABLE 1. *Profile of untreated AVL patients and lymphocyte response to PHA and L. chagasi antigen*

Patient	Age (years)	Sex	Duration of illness (mo)	[^{3}H]thymidine incorporation	
				PHA[a]	*L. chagasi*[b]
1	3	M	4	118.4 ± 3.5	0.2 ± 0.1
2	7	M	8	125.4 ± 4.7	0.3 ± 0.1
3	5	F	1	105.1 ± 2.3	0.9 ± 0.4
4	7	M	2	40.1 ± 1.0	0.8 ± 0.1
5	5	M	8	44.8 ± 5.8	0.4 ± 0.1
6	19	M	4	28.7 ± 1.4	0.2 ± 0.1
7	3	M	24	28.4 ± 3.6	1.3 ± 0.6
8	20	M	6	22.2 ± 3.1	0.2 ± 0.1
9	14	M	12	11.0 ± 0.1	0.8 ± 0.2
10	10	M	7	1.0 ± 0.5	0.2 ± 0.1
11	18	M	6	28.8 ± 3.2	0.6 ± 0.2
12	20	M	9	35.8 ± 8.4	8.0 ± 1.4
13	14	M	7	17.9 ± 0.4	2.0 ± 0.4
14	7	M	6	8.5 ± 0.8	0.8 ± 0.2

[a] The mean counts per minute ± SEM ($\times 10^{-3}$) of control unstimulated 3-day mononuclear cell cultures was 0.5 ± 0.1. Data are given as mean counts per minute (± SEM) $\times 10^{-3}$.

[b] The mean counts per minute ± SEM ($\times 10^{-3}$) of control unstimulated 5-day mononuclear cell cultures was 0.3 ± 0.1. Data are given as mean counts per minute (± SEM) $\times 10^{-3}$.

TABLE 2. *Lymphocyte response to PHA and L. chagasi antigen in AVL patients*

Type of subject	[^{3}H]thymidine incorporation[a]	
	PHA	*L. chagasi*
Untreated AVL (14)[b]	44.0 ± 11.0[c]	1.2 ± 0.5[d]
Cured AVL (11)	62.7 ± 13.9	19.1 ± 7.3
Control (15)	62.6 ± 7.0	0.8 ± 0.1

[a] The mean counts per minute ± SEM ($\times 10^{-3}$) of control unstimulated cultures was 0.4 ± 0.1 and 0.3 ± 0.1 after 3 and 5 days of cultivation, respectively. Data are given as mean counts per minute (± SEM) $\times 10^{-3}$.

[b] The number of patients studied in each group is indicated within parentheses.

[c] Untreated AVL versus cured AVL, $P > 0.3$; untreated AVL versus controls, $P > 0.1$; cured AVL versus controls, $P > 0.99$.

[d] Untreated AVL versus cured AVL, $P < 0.025$; untreated AVL versus controls, $P > 0.4$; cured AVL versus controls, $P < 0.02$.

counts were 1,248, 1,125, 975, 1,665, and 1,475 lymphocytes per mm^3, respectively. Lymphocyte subpopulations were enumerated in 11 untreated AVL patients. Cell percentages for the group were similar to those of controls. The mean percent ± 1 standard deviation of T, B, and L cells was 64 ± 14, 7 ± 4, and 10 ± 7, respectively, in untreated AVL patients and 74 ± 9, 8 ± 3, and 13 ± 7 in 11 control subjects. The numbers of T cells for patients 6, 10, and 13 were two standard deviations below that of the control group. Studies using autologous and AB serum did not demonstrate a serum inhibitory effect on the ability of lymphocytes from patient 13 to bind sheep erythrocytes. Patients 6 and 13 had normal numbers of T cells when restudied 2 weeks after antimony therapy. No correlation could be made between either the absolute lymphocyte count or the numbers of T, B, and L lymphocytes and the in vitro response of peripheral blood lymphocytes to *L. chagasi* antigens. Patients were unresponsive to this antigen, whether T cell numbers were normal or depressed.

The responsiveness of mononuclear cells from untreated AVL patients to *L. chagasi* antigens was not restored by cultivation of cells for 5 days with pooled AB serum. Cultivation of cells from five patients with *L. chagasi* antigens and autologous serum resulted in a mean of 1.0 ± 0.2 $\times 10^{-3}$ cpm, and the mean was 1.2 ± 0.5 $\times 10^{-3}$ cpm when pooled AB serum was included in the media. Control unstimulated cultures had a mean of 0.5 ± 0.2 $\times 10^{-3}$ cpm.

Depletion of monocytes from the mononuclear cell preparation also did not enhance the lymphocyte response in blastogenesis assays. Monocyte-depleted cultures incubated with *L. chagasi* antigens and autologous serum for 5 days incorporated [^{3}H]thymidine in an amount (0.5 ± 0.1 $\times 10^{-3}$ cpm) which was no greater than either control unstimulated cultures (0.3 ± 0.1 $\times 10^{-3}$ cpm) or cultures containing up to 29% monocytes (0.8 ± 0.2 $\times 10^{-3}$ cpm).

DISCUSSION

In this study, lymphocytes from untreated AVL patients did not respond to leishmania antigens in blastogenesis assays. This finding supports the hypothesis that visceral leishmaniasis is associated with impaired cell-mediated immunity. It is consistent with the observation that these patients do not respond to the intradermal injection of leishmania antigens with a

TABLE 3. *Lymphocyte response to PHA and L. chagasi antigen in AVL patients before and after therapy*

Patient	[^{3}H]thymidine incorporation			
	Before therapy[a]		After therapy[b]	
	PHA	*L. chagasi*	PHA	*L. chagasi*
6	28.7 ± 1.4	0.2 ± 0.1	28.9 ± 1.0	6.6 ± 0.5
8	22.2 ± 3.1	0.2 ± 0.1	ND	3.0 ± 0.5
9	11.0 ± 0.1	0.8 ± 0.2	12.1 ± 1.9	6.5 ± 0.8
11	28.8 ± 3.2	0.6 ± 0.2	36.7 ± 5.5	0.6 ± 0.1

[a] The mean counts per minute ± SEM ($\times 10^{-3}$) of control unstimulated cultures before therapy was 0.3 ± 0.1 and 0.7 ± 0.2 after therapy. Data are given as mean counts per minute (± SEM) $\times 10^{-3}$.

[b] Patients 6, 8, and 9 were restudied 2, 4, and 3 weeks after therapy, respectively. Patient 11 was restudied 1 week after therapy. ND, Not done.

delayed-type hypersensitivity reaction (16). The response to specific antigens was restored to normal within 4 weeks of successful chemotherapy in three of four patients. Similarly, Manson-Bahr reported conversion of skin tests from negative to positive within 6 weeks of therapy and positive skin tests in 80% of cured AVL patients within 2 years of treatment (16).

The lymphocyte response to PHA and mixed lymphocyte cultivation was not suppressed in untreated AVL patients. This specificity in immunosuppression is similar to that observed in untreated lepromatous leprosy. These patients have a normal blastogenic response to PHA and other bacterial antigens despite a lack of response to specific *Mycobacterium leprae* antigens (7). Ghose et al. have reported an impaired PHA response of lymphocytes from 8 of 15 Indian kala-azar patients (8). One can only speculate as to whether their patients may have differed in severity of disease or factors, such as nutritional status, which could result in a generalized suppression of lymphocyte reactivity (5, 18). It is noteworthy that the only untreated AVL patient in this study who did not manifest a normal PHA blastogenic response died despite appropriate chemotherapy.

Depletion of small lymphocytes in thymus-dependent regions of the spleen and lymph nodes has been documented in untreated fatal visceral leishmaniasis (22). Rezai et al. have also reported reduced numbers of circulating T cells in Iranian kala-azar patients (21). In the present study, five untreated AVL patients had low absolute numbers of circulating lymphocytes, and three patients had reduced numbers of T cells. The mechanism and significance of these observations are not clear. However, since patients with normal total lymphocyte counts and normal ratios of T, B, and L cells were equally unresponsive to *L. chagasi* antigens, these findings alone do not explain the immunosuppression observed.

Monocytes and serum factors are recognized inhibitors of lymphocyte responses to antigen. Ellner demonstrated that patients with active tuberculosis who had low responses to purified protein derivative in vitro had restoration of their responses after depletion of adherent cells from Ficoll-Hypaque-derived mononuclear cell preparations (6). In the present study, depletion of adherent cells did not enhance lymphocyte responsiveness. Cultivation of cells in pooled AB serum rather than autologous serum also failed to increase the lymphocyte proliferative response to *L. chagasi* antigens. A specific impairment of the response of lymphocytes from untreated lepromatous leprosy patients to *M. leprae* antigen similarly could not be attributed to serum factors (7).

Enhanced T suppressor cell activity represents a plausible explanation for the immunosuppression observed in acute AVL. Howard et al. have suggested that the susceptibility of BALB/c mice to *L. tropica* infection is a consequence of suppressor T cell generation and impaired cell-mediated immunity (10). Diminished delayed-type hypersensitivity reactions induced by suppressor cells were leishmania specific and could be transferred to normal BALB/c mice with spleen cells. Studies utilizing mouse radiation chimaeras indicate that susceptibility to *L. tropica* infection is determined by descendents of hematopoietic cells and not by host environmental factors (11). The systemic study of functional lymphocyte populations and other factors that modulate lymphocyte responsiveness may provide an explanation for the reversible immunosuppression observed in visceral leishmaniasis.

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