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Alterations of the Immune Response Associated with Chronic Experimental Leishmaniasis

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BALB/c mice infected with *Leishmania mexicana* developed a chronic infection usually accompanied by the appearance of metastatic lesions. Throughout the 20 weeks of observation, infected mice showed an impairment in both in vivo delayed hypersensitivity response and in vitro lymphocyte reactivity to leishmanial antigen. Four to 8 weeks after inoculation infected mice displayed a transitory enhancement of spleen cell responses to phytohemagglutinin P, concanavalin A, and lipopolysaccharide. At the same time, immunization with sheep erythrocytes resulted in a greater number of immunoglobulin M plaque-forming cells. Thereafter, responses to phytohemagglutinin P, lipopolysaccharide, and sheep erythrocytes diminished progressively, whereas reactivity to concanavalin A was markedly augmented. When cocultivated with spleen cells from mice infected for 12 to 20 weeks, normal lymphocyte responses to phytohemagglutinin P, concanavalin A, and lipopolysaccharide were drastically reduced. These results suggest a role for suppressor cells in chronic experimental cutaneous leishmaniasis.

American cutaneous leishmaniasis has been attributed to *Leishmania mexicana* and *L. braziliensis* (16). The disease generally consists of a single lesion which may regress spontaneously or after treatment, and healing is accompanied by the development of cellular responses against the parasite (19, 26). Some patients exhibit a bizarre form of the disease called diffuse cutaneous leishmaniasis (8). In these cases, a chronic nonulcerating primary lesion is followed by the appearance of disseminated nodes rich in parasites, and cellular responses against parasite antigens are usually depressed (3, 4).

Previous work in our laboratory has established that, after inoculation with 10^4 amastigotes of *L. mexicana*, different mouse strains displayed dissimilar patterns of infection (23). C57BL/6 mice developed self-resolving lesions whereas BALB/c mice showed chronic large, ulcerated lesions usually accompanied by metastases. Furthermore, recovered C57BL/6 mice exhibited significant delayed hypersensitivity response (DHR) and agglutinating antibodies against the parasite. In contrast, chronically infected BALB/c mice manifested depressed cellular and humoral responses against *Leishmania* antigens (22, 23). The present work further explored the immune responsiveness in chronic experimental leishmaniasis. Both specific and nonspecific responses were evaluated throughout the infection.

MATERIALS AND METHODS

Hosts. Inbred BALB/c female mice weighing 20 to 25 g and outbred 80-g male hamsters were obtained from the breeding unit of the Instituto Venezolano de Investigaciones Científicas.

Parasites. The human strain AZV of *Leishmania mexicana*, isolated from a typical case of American cutaneous leishmaniasis, was used (23). It was maintained in vivo by serial passages in hamsters and in vitro in Earle solution containing 0.5% lactalbumin hydrolysate (GIBCO, Grand Island, N.Y.) and supplemented with 5% fetal calf serum (GIBCO) and antibiotics (22, 23).

Infection. Leishmanial nodules, obtained from hamsters infected as previously described (22, 23), were aseptically dissected out, teased and ground in a glass homogenizer containing cold Earle solution with antibiotics. Large debris was removed, and the parasite concentration was adjusted. Mice were subcutaneously inoculated with 10^4 amastigotes into the dorsal surface of the right foot. The course of infection was assessed by periodical examination of lesions and the presence of metastases. Footpads were measured at weekly intervals with Schnelltaster calipers (H. C. Kröplin, Germany), and the size of lesions was calculated from the difference in width between the infected and the uninfected foot.

DHR. DHR was estimated by the footpad swelling after subcutaneous injection of 50 μg of soluble leishmanial antigen (SLA) (23) into the uninfected hind footpad. From 4 to 20 weeks of infection, skin reactions of three control and three infected mice were measured at 24 h and compared to zero hour readings (25).

In vitro cultures. Microcultures of spleen and

thymus cells, from three control and three infected mice, were prepared as previously described (22). Briefly, they consisted of a total of 5×10^5 lymphoid cells in 0.2 ml of RPMI 1640 (Microbiological Associates, Inc., Bethesda, Md.) supplemented with 5% fetal calf serum and contained either 5 μg of Concanavalin A (ConA; ICN Pharmaceuticals, Inc., Cleveland, Ohio) per ml, 1 μl of phytohemagglutinin P (PHA; Difco Laboratories, Detroit, Mich.) per ml, 1.5 μg of lipopolysaccharide B (LPS; Difco) from *Escherichia coli* O17:B8, 4 μg of SLA per ml or medium alone. Deoxyribonucleic acid synthesis was assayed by adding 1.0 μCi of tritiated thymidine (specific activity, 2.0 Ci/mM; New England Nuclear Corp., Boston, Mass.) for the final 18 h of a 72-h incubation period. Lymphoid cell responses of infected animals were compared to those of control cells obtained from uninfected members of the same lot of mice. Stimulation indexes (E/C) were calculated by dividing counts per minute of control cultures by counts per minute of stimulated cultures. Percent suppression or enhancement after *Leishmania* infection was calculated by the following formula. (E/C of cultures from infected animals)/(E/C of cultures from control animals) × 100 = 1% control response.

PFC responses. At intervals after infection, four control and four infected mice were sensitized by an intraperitoneal injection of 0.1 ml of 10% sheep erythrocyte (SRBC) suspensions, and 4 days later the direct immunoglobulin M plaque-forming cell (IgM PFC) assay was performed by using a modification of the method of Jerne and collaborators (9). The results are expressed as the arithmetic mean ± standard error of the mean (SEM), and groups were compared by Student's *t* test.

RESULTS

Course of infection. Figure 1 shows the course of infection in BALB/c mice inoculated with 10^4 amastigotes of *L. mexicana*. Lesions were apparent at 4 to 6 weeks, increased steadily in size, and ulcerated at approximately 8 to 10 weeks. Lesions progressively increased in size, and after 18 to 20 weeks they appeared as large ulcers. Infected mice usually developed metastases in the tail and occasionally lost the infected foot. Toward the end of the experimental period, most animals lost weight, became hunched, and showed ruffled fur.

DHR to soluble leishmanial antigen. When skin was tested 4 weeks after inoculation, mice infected with *L. mexicana* were found to develop significantly increased reactivity to SLA ($P < 0.05$). At 8 weeks, skin responses of infected and uninfected mice were not statistically different. Responses of infected animals increased slightly at 12 weeks but after 16 weeks they were progressively decreased. Minimal responses occurred 20 weeks after inoculation (Table 1).

In vitro spleen cell responses to leishmanial antigen. To assess whether the impairment in in vitro lymphoid cell reactivity to SLA observed in BALB/c mice infected for 20 to 23 weeks (22) represented a late event in chronic experimental infection, spleen cell responses to SLA were determined at 4-week intervals during the 20-week observation period. When compared to normal cells, which were moderately stimulated by SLA, spleen cells from infected animals showed, at all times, a decreased response to SLA (Fig. 2).

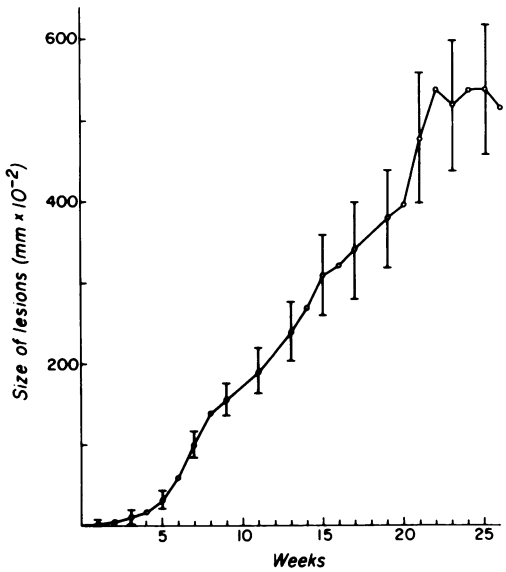

FIG. 1. *Course of lesions in BALB/c mice infected with 10^4 amastigotes of L. mexicana. (Values indicate mean ± SEM for ten mice.)*

TABLE 1. *DHR of BALB/c mice infected with Leishmania mexicana to leishmanial antigen*

Weeks after inoculation	Response[a]		
	Infected mice	Uninfected mice	P^b
4	0.48 ± 0.10	0.15 ± 0.09	0.05
8	0.16 ± 0.08	0.10 ± 0.10	NS
12	0.58 ± 0.25	0.12 ± 0.06	NS
16	0.41 ± 0.12	0.27 ± 0.08	NS
20	0.27 ± 0.03	0.18 ± 0.10	NS

[a] Responses to 50 μg of leishmanial antigen were measured at 24 h and compared to zero hour readings. Values indicate millimeters ± standard deviation.

[b] Values computed from a Student's *t* test. NS, Differences between control and infected groups not significant at the $P < 0.05$ level.

PFC responses to SRBC. Day 4 IgM PFC responses of mice immunized at different times between 4 and 16 weeks of infection with *L. mexicana* are shown in Fig. 3. Immunization at 4 and 8 weeks of infection resulted in signifi-

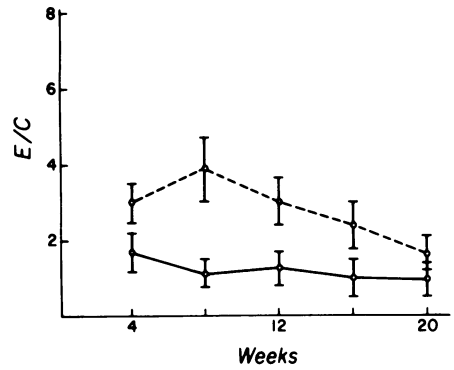

FIG. 2. *In vitro spleen cell responses of control (-----) and mice infected with 10^4 amastigotes of L. mexicana (——). Values indicate mean ± SEM for three experiments.*

cantly increased mean PFC responses ($P < 0.05$ and $P < 0.01$). However, a definite depression ($P < 0.001$ and $P < 0.001$) of PFC responses was seen in all mice immunized with SRBC 12 and 16 weeks after infection, representing 29.7 and 16% of the control responses, respectively.

In vitro lymphoid cell responses to T and B cell mitogens. Experiments were performed to determine whether the inability of BALB/c mice to control *Leishmania* infection was reflected in alterations of lymphocyte responses to mitogens. Proliferative responses of spleen cells to PHA, ConA, and LPS and of thymocytes to PHA and ConA were assessed at 4, 8, 12, 16, and 20 weeks of infection. Figure 4 shows that infection with *L. mexicana* modified spleen cell responses of BALB/c mice to mitogens. Four weeks after inoculation, there was a significant enhancement of spleen cell responses to PHA and a slight increase in the responses to ConA and LPS. At 8 weeks, spleen cell responses to mitogens were either comparable or moderately smaller than those of control cells. Thereafter, the nature of the alterations differed among the mitogen responses studied. Responses to PHA and LPS were significantly suppressed, whereas reactivity to ConA was markedly augmented. These modifications were maximal 16 weeks after inoculation. Thymocyte responses to ConA at all intervals studied were significantly greater in infected animals than in controls (Fig. 5). However, thymocyte responses to PHA were not modified after *Leishmania* infection.

Effects of spleen cells from chronically infected mice on normal lymphoid cell responses to mitogens. To ascertain whether suppressor cell activity was associated with chronic leishmanial infection, the responses of a mixture of 1.25×10^5 spleen cells from mice infected for 16 weeks and 3.75×10^5 normal spleen cells to PHA, ConA, and LPS were initially compared to those of 5×10^5 normal spleen cells. Spleen cells from infected BALB/c mice markedly suppressed normal lymphoid cell responses to these mitogens (Fig. 6). This suppressor activity was not manifest at all times of infection and spleen cells obtained 8 weeks after inoculation did not exert suppression (Fig. 7). The effect was evident at 12 weeks and maximal when cells added to normal splenocytes were obtained from mice which have been infected for 16 weeks.

DISCUSSION

These data show that experimental infection of BALB/c mice with *L. mexicana* is associated with several modifications of both specific and nonspecific immune responses. Two different effects were observed along the course of infection with 10^4 amastigotes, and a transitory enhancement of several responses was followed by a state of immunodepression. Four weeks after inoculation, mice showed a significant increase in the number of direct PFC against SRBC (Fig. 3). This may indicate the induction, by the parasite or parasite-derived products, of nonspecific helper cells as suggested for other parasitic infections (15). At the same time, spleen cell proliferative responses to PHA, ConA, and LPS were greater in infected mice than in controls (Fig. 4). This observation is consistent with a stimulation of both T and B cell compartments.

Thereafter, these early alterations were substituted by a progressive depression of several of the responses studied. Although spleens of control and infected mice were of comparable size, lymphoid cell reactivities to PHA and LPS as well as PFC responses to SRBC were markedly diminished after 12 weeks of infection (Fig. 2

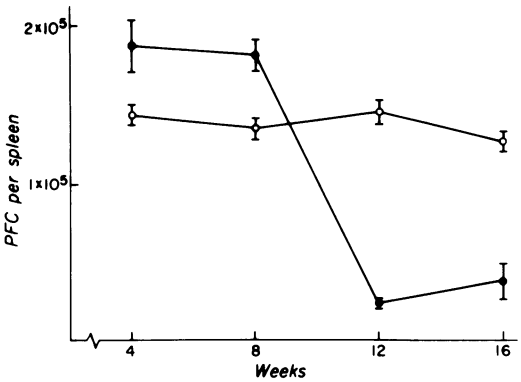

FIG. 3. *Direct PFC responses to SRBC of control (○) and mice infected with 10^4 amastigotes of L. mexicana (●). Values indicate mean ± SEM for four mice.*

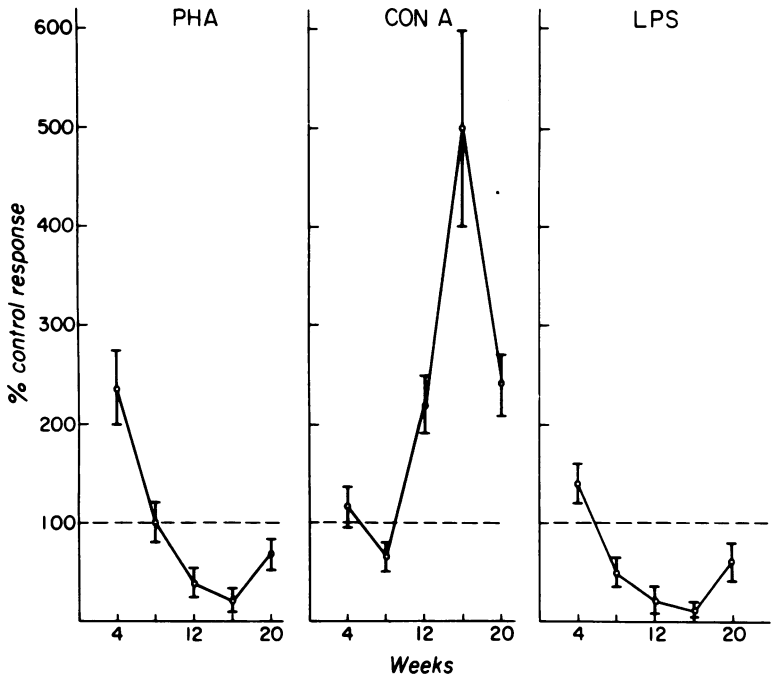

FIG. 4. *Spleen cell responses of mice infected with 10^4 amastigotes of L. mexicana to PHA, ConA, and LPS. Results are expressed as percentage of responses of control spleen cells and indicate mean ± SEM for three experiments.*

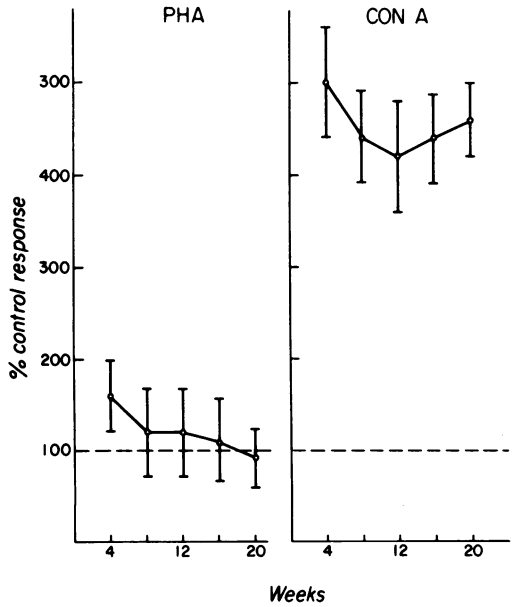

FIG. 5. *Thymus cell responses of mice infected with 10^4 amastigotes of L. mexicana to PHA and ConA. Results are expressed as percentage of responses of control thymocytes and indicate mean ± SEM for three experiments.*

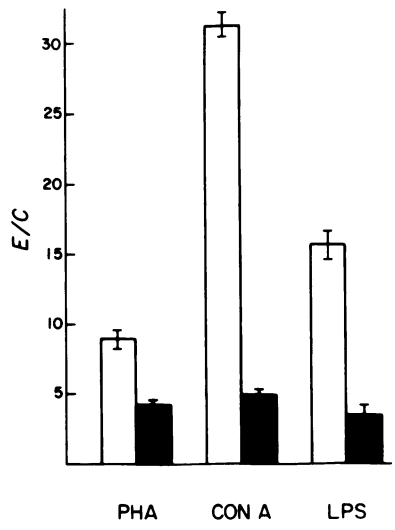

FIG. 6. *Effects of spleen cells from mice infected with 10^4 amastigotes of L. mexicana on normal spleen cell responses to mitogens. Open bars correspond to 5×10^5 control spleen cells, and closed bars correspond to a mixture of 3.75×10^5 control spleen cells and 1.25×10^5 spleen cells from mice infected for 16 weeks. Values indicate mean ± SEM for triplicate cultures.*

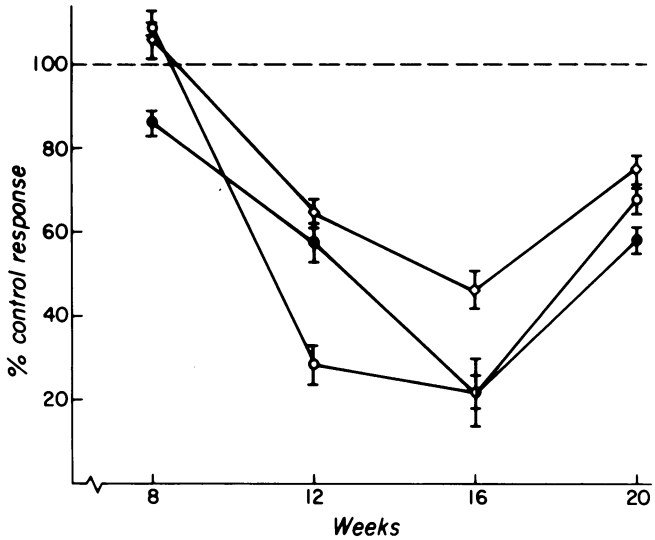

FIG. 7. *Effect of spleen cells from mice infected with 10^4 amastigotes of L. mexicana on normal spleen cell responses to PHA (◇), ConA (●), and LPS (○). Results are expressed, for a mixture of 3.75 × 10^5 control spleen cells and 1.25 × 10^5 spleen cells from infected mice, as the percentage of responses of 5 × 10^5 control spleen cells. Values indicate mean ± SEM for three experiments.*

and 3). In contrast, there was a simultaneous increase in ConA responses of spleen cells obtained from infected mice (Fig. 3). The depression of responses to PHA, LPS, and SRBC was maximal when the size of lesions had reached its peak, suggesting that a heavy parasitic load could be involved in the induction of this phenomenon (26, 27, 34). The alterations in PHA and LPS responses suggest a marked impairment of lymphoid cells since both mitogens are known to stimulate a significant number of T and B cells, respectively (1, 30). The increase in spleen cell responses to ConA observed in infected animals might reflect a selective expansion in the T cell subset which preferentially responds to this mitogen (30, 31). Alternatively, it could result from a redistribution of T cells among different lymphoid organs, with a consequent increase in the spleen content of T cells belonging to this subset. The former possibility is supported by the marked increase in thymocyte responses to ConA observed in infected animals (Fig. 5).

In relation to the responses specific to the parasite, DHR to leishmanial antigens was absent or weakly expressed in BALB/c mice along the course of infection and, although statistically significant at 4 and 12 weeks (Table 1), it never reached the values observed in infected C57BL/6 mice, which are able to control *Leishmania* infection (22). The low DHR of infected BALB/c mice to SLA was correlated with the absence of in vitro lymphocyte reactivity to this preparation (Fig. 2). The lack of reactivity to parasite antigens both in vivo and in vitro supports the notion that in disseminated forms of cutaneous leishmaniasis there is a central failure in the development of a specific cellular response to *Leishmania* rather than a defect in the peripheric expression of this response (4, 38). Although the role of cell-mediated immunity in the control of *Leishmania* infection is not well understood, its importance has been suggested by several studies of rodents infected with *L. mexicana* and *L. enriettii*. Induction of partial tolerance to leishmanial antigens, treatment with antilymphocyte serum and regional antigenic competition induced by multiple injection of bacterial adjuvants resulted in a more generalized form of leishmaniasis (5, 25). Our model differs from the aforementioned in that chronic infection arises in normal BALB/c mice which are known to develop good humoral and cellular responses to several antigens including some from parasites (17, 18, 33). However, inability of BALB/c mice to control infection with other *Leishmania* species has been reported (2, 37) and previous studies in our laboratory have also indicated that the response of inbred mouse strains to *L. mexicana* is probably under genetic control (23). Thus, failure of BALB/c mice to control infection with *L. mexicana* could reflect a basic defect in their response to the parasite.

During chronic experimental infection of BALB/c mice with *L. mexicana* there was an induction of nonspecific suppressor cells which

were able to regulate normal spleen cell responses to mitogens (Fig. 6 and 7). These cells could be responsible for both the depression of PHA and LPS responses and the decrease in PFC responses observed after 8 weeks of infection. This idea is supported by the observation that both the suppression (Fig. 7) and the impairment in responses to PHA, LPS, and SRBC (Fig. 3 and 4) reached a maximum simultaneously at 16 weeks of infection. It was interesting to observe that, although leishmanial infection was associated with increased lymphoid cell responses to ConA (Fig. 4), spleen cells from infected animals suppressed normal lymphocyte responses to this mitogen (Fig. 6). Although we do not have an explanation for this observation, it has been suggested that multiple factors might determine whether suppression or synergy will result when immune and normal cells are mixed together (11, 12, 24). Elucidation of the nature of suppressor cells described in other parasitic diseases has not been simple (10, 14, 35, 36). For example, suppressor cell activity in both T and adherent cell compartments has been described in mice infected with *Trypanosoma brucei* (10). Experiments currently in progress in our laboratory are investigating the nature of the suppressor cells that appear in chronically infected BALB/c mice. On the other hand, it has been recently reported that some antigen-stimulated Ly 1^+ T cells can induce a nonimmune set of T cells to participate in suppressor activity (6, 7). An early stimulation of Ly 1^+ T cells, which also include helper cells, followed by induction of suppression could explain the phenomena observed in BALB/c mice infected with *L. mexicana*. The possibility also exists that chronically infected mice lack the T cells which synthesize soluble factors necessary for macrophage to destroy the parasite (21, 28, 29).

Low-resistance forms of cutaneous leishmaniasis and other infectious diseases, also characterized by a spectrum of clinical manifestations, have been associated with depressed cellular immunity (4, 13, 20, 32, 38). For example, some of the studies of patients with lepromatous leprosy have revealed a depression in T lymphocyte function manifested by low responses to specific antigens and mitogens and a decrease in early rosette-forming T cells (13, 20).

Induction of suppressor cells might play a role in the pathogenesis of diffuse cutaneous leishmaniasis. Further studies of disseminated forms of cutaneous leishmaniasis, in both patients and experimental models, should give an insight into the mechanisms leading to this form of the disease.

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