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Comparative Study of American Cutaneous Leishmaniasis and Diffuse Cutaneous Leishmaniasis in Two Strains of Inbred Mice

HILDA PÉREZ,* BEVERLY ARREDONDO, AND MERCEDES GONZÁLEZ

Instituto Venezolano de Investigaciones Científicas, Centro de Microbiología y Biología Celular, Caracas, Venezuela

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Two *Leishmania* strains, AZV (isolated from a typical case of American cutaneous leishmaniasis) and AMP (from a case of diffuse cutaneous leishmaniasis), were studied in C57BL/6 and BALB/c mice. After infection with 10^4 amastigotes of either strain, C57BL/6 mice developed self-resolving lesions lasting 20 to 23 weeks and showed both delayed hypersensitivity response to leishmanial antigen and specific agglutinating antibodies. On the other hand, BALB/c mice infected with 10^4 AZV or AMP amastigotes developed chronic, large, ulcerated lesions and showed impaired cellular and humoral responses to the parasite. When BALB/c and C57BL/6 mice received 10^2 AMP amastigotes, patterns of infection were similar to those observed after inoculation of 10^4 amastigotes. In vitro studies revealed that spleen cells from AZV- or AMP-infected C57BL/6 mice showed an increased DNA-synthetic response to leishmanial antigen, concanavalin A, and phytohemagglutinin. Spleen cells from AZV- or AMP-infected BALB/c mice showed an increased response to concanavalin A and diminished responses to leishmanial antigen, phytohemagglutinin, and lipopolysaccharide.

American cutaneous leishmaniasis (ACL) is endemic from Yucatan to Argentina, and the disease can be acquired in almost every conceivable terrain, from an altitude of 2,500 m in the cordilleras to the swamps and forests of Amazonia (1). Human disease is due to infection with *Leishmania mexicana* or *L. braziliensis* (19). In most instances, infection consists of a single lesion, which may heal spontaneously or after treatment (22). Recovery from infection is usually associated with the development of delayed hypersensitivity response (DHR) to leishmanin and immunity to reinfection (22, 30). However, infected humans occasionally develop an abnormal condition called diffuse cutaneous leishmaniasis (DCL) (8), in which enormous masses of parasitized macrophages infiltrate extensive nonulcerated dermal areas. These patients do not show DHR to leishmanin, and treatment produces only slight and temporary improvement (8).

Immunological unresponsiveness to leishmanin in patients suffering from DCL is apparently specific (6), but its biological basis is not understood. Some authors have proposed that in Venezuela DCL is caused by a different *Leishmania* species, *L. pifanoi* (25, 26). On the other hand, there is also evidence which suggests that DCL is probably due not to a different type of parasite but to an immunological defect of the human host (9).

We have previously presented experimental evidence that in mice, the clinical course of *L. mexicana* infections is largely determined by the genetic constitution of the infected host (H. Pérez, F. Labrador, and J. Torrealba, Int. J. Parasitol., in press). The present work compares the immune responses of C57BL/6 and BALB/c mice to two human strains of *Leishmania*, one isolated from a typical case of ACL and the other isolated from a case of DCL.

MATERIALS AND METHODS

Hosts. Inbred C57BL/6 and BALB/c female mice weighing 20 to 25 g and outbred 80-g male hamsters were used as hosts. The animals were obtained from the breeding unit of the Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela.

Parasites. Human strains of *L. mexicana* (19), AMP (isolated from a case of DCL [10]) and AZV (obtained from a typical case of ACL), were used. Both strains grow luxuriantly in NNN and other media, and their inoculation in hamsters is rapidly followed by the appearance of large histiocytomas packed with amastigotes. In our laboratory, AMP and AZV are maintained in vivo by serial passages in hamsters and in vitro in Earle solution containing 0.5% lactalbumin hydrolysate (Grand Island Biological Co. [Gibco], Grand Island, N.Y.) supplemented with 5% fetal calf

serum, 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (Schwarz/Mann Laboratories, New York, N.Y.), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Gibco) (E/lac medium).

Infection. Amastigotes were obtained from hamsters 6 to 7 weeks after subcutaneous inoculation of 2×10^8 amastigotes into the dorsal surface of the right foot. Leishmanial nodules were aseptically dissected out, teased, and ground in a glass homogenizer containing cold Earle balanced saline solution with added antibiotics. Suspensions were filtered through sterile gauze to remove large debris. Parasites were counted in a hemocytometer, and the concentration was adjusted to the required dose. Groups of 10 to 20 mice were infected by subcutaneous inoculation of 10^4 or 10^2 amastigotes into the dorsal surface of the right foot.

Course of infection. The course of infection was assessed by periodical examination of lesions and the presence of metastases. Footpads were measured at weekly intervals with Schnelltester calipers (H. C. Kröplin, Frankfurt, West Germany), and the sizes of lesions were calculated from the differences in width between infected and uninfected feet.

Skin test. DHRs of 20-week AZV- and AMP-infected mice were estimated by footpad swelling after subcutaneus injection of 50 μg of leishmanial antigen into the left hind footpad. Footpad reactions were measured as above at 24 h and compared with the 0-h reading (28). Uninfected mice served as controls.

Antibodies to *Leishmania*. Sera were obtained from 20-week-infected mice. Pooled sera from five mice were inactivated at 56°C for 30 min, and their reactivity to *Leishmania* was measured by direct agglutination of Formalin- and trypsin-treated promastigotes grown in E/lac medium (23).

In vitro studies. Microcultures, established in plastic microculture plates (Linbro IS-FB-96-TC; International Scientific Instruments, Inc., Cary, Ill.), consisted of 5×10^5 spleen cells in 0.2 ml of RPMI 1640 (Microbiological Associates, Bethesda, Md.) containing either 5 μg of concanavalin A (Con A; ICN Pharmaceuticals, Inc., Cleveland, Ohio) per ml, 1 μl of phytohemagglutinin P (PHA; Difco Laboratories, Detroit, Mich.) per ml, 2.5 μg of lipopolysaccharide B (LPS) from *Escherichia coli* 017:B8 (Difco) per ml, 4 μg of leishmanial antigen from AZV or AMP per ml, or medium alone. Spleen cells were obtained from 20- to 23-week AZV- and AMP-infected mice. Media were supplemented with 5% fetal calf serum (Gibco), 2 mM glutamine (Gibco), and penicillin-streptomycin mixture (100 U/ml and 100 μg/ml, respectively) (Gibco). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. DNA synthesis was assayed by adding 1.0 μCi of tritiated thymidine (specific activity, 2.0 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for the final 18 h of a 72-h incubation period. Cultures were harvested by aspiration of cells onto glass-fiber filters, using a multiple automated sample harvester (MASH-II; Microbiological Associates). Dry filters were placed in a universal scintillation cocktail (Aquasol; New England Nuclear Corp.), and radioactivity was measured in a liquid scintillation spectrometer (Packard Tri-Carb, model 3320; Packard Instrument Co., Inc., Downers Grove, Ill.). The data are expressed as mean counts per minute of three replicate cultures ± the standard error of the mean or as stimulation indexes, calculated by subtracting the counts per minute of control cultures from those of stimulated cultures.

RESULTS

Course of lesions in C57BL/6 and BALB/c mice infected with 10^4 AMP or AZV amastigotes. In C57BL/6 mice, infections with 10^4 AZV and AMP amastigotes followed similar patterns (Fig. 1a). Nodes at site of inoculation were palpable at 2 weeks and increased rapidly in size, and between 6 and 8 weeks they had ulcerated. The sizes of these lesions were maximal between 10 and 12 weeks, when they were covered with a crust. Thereafter, the lesions started to heal, and by 23 weeks the animals had recovered from infection. In contrast, lesions in AZV- or AMP-infected BALB/c mice developed more slowly during the first 7 weeks (Fig. 1b). However, after 8 weeks, lesions in BALB/c mice started to progress rapidly, and between 10 and 12 weeks they had ulcerated. By 20 weeks, while C57BL/6 mice were healing, BALB/c mice showed large, ulcerated lesions. BALB/c infected mice usually developed metastatic lesions in the tail, of which a number still persisted at the end of the experimental period (26 weeks). Loss of the infected leg was also observed.

DHR. AZV- or AMP-infected C57BL/6 mice developed good DHRs to 50 μg of leishmanial antigen. On the other hand, infected BALB/c mice responded weakly to the antigen (Table 1).

Agglutinating antibodies. Table 2 shows the agglutination titers of sera taken from normal and 20-week AZV- or AMP-infected C57BL/6 and BALB/c mice. Sera of normal C57BL/6 or BALB/c mice did not agglutinate Formalin- and trypsin-treated promastigotes at the starting dilution of 1:2. Infected BALB/c mice showed very low agglutination titers. In contrast, C57BL/6 mice infected with either strain developed good titers of agglutinating antibodies.

In vitro responses of AZV- and AMP-infected mice to leishmanial antigens. Several observations were made when in vitro responses to leishmanial antigens from AZV and AMP were investigated (Fig. 2). First, normal spleen cells from either C57BL/6 or BALB/c mice showed increased DNA synthesis when cultured with leishmanial antigen. Second, lymphocytes from C57BL/6 mice recovering from AZV or AMP infection incorporated significantly more thymidine than did controls after stimulation with homologous leishmanial antigen. Third, lymphoid cells from AMP- or AZV-infected

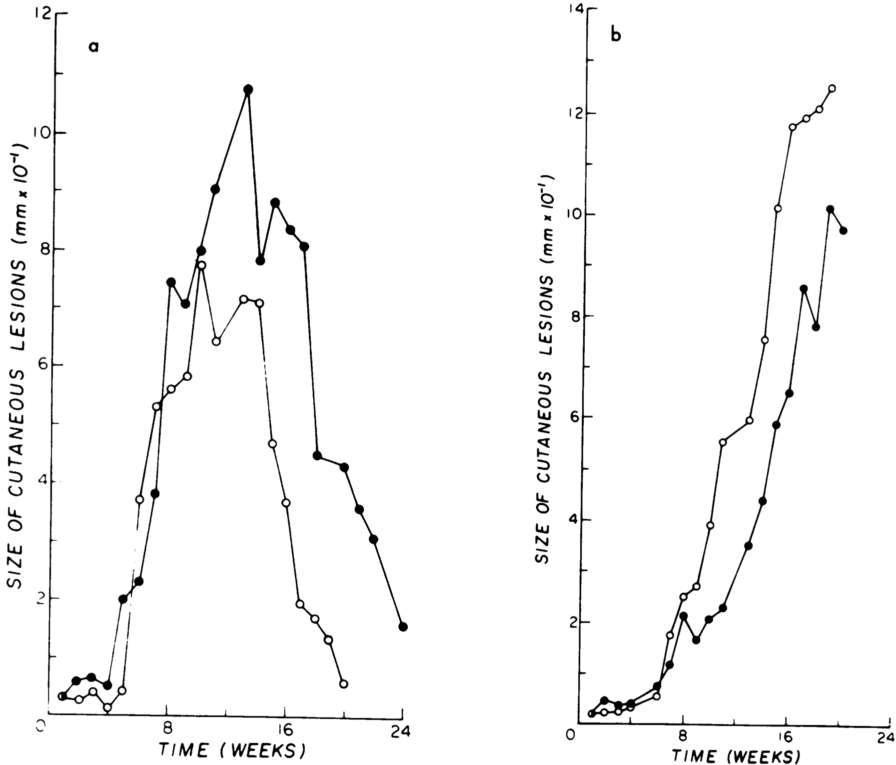

FIG. 1. *Comparisons between courses of primary infection with 10^4 AZV (●) and AMP (○) amastigotes in C57BL/6 (a) and BALB/c (b) mice.*

TABLE 1. *DHRs of 20-week AZV- and AMP-infected C57BL/6 and BALB/c mice*

Mouse strain	*Leishmania* strain[a]	DHR to[b]:	
		AZV leishmanial antigen	AMP leishmanial antigen
C57BL/6	Control	0.02	0.03
C57BL/6	AZV	1.03	
C57BL/6	AMP		0.65
BALB/c	Control	0.14	0.16
BALB/c	AZV	0.35	
BALB/c	AMP		0.30

[a] Animals received 10^4 amastigotes of the infecting *Leishmania* strain.

[b] DHR to 50 μg of leishmanial antigen. Footpad reactions were measured with Schnelltester calipers at 24 h and compared with the 0-h reading.

TABLE 2. *Antileishmanial agglutination titers of pooled sera[a] from 20-week AZV- and AMP-infected C57BL/6 and BALB/c mice*

Mouse strain	*Leishmania* strain	Agglutination titer[b]
C57BL/6	Control	0
C57BL/6	AZV	32
C57BL/6	AMP	32
BALB/c	Control	0
BALB/c	AZV	4
BALB/c	AMP	8

[a] Pooled sera from five infected and five noninfected controls of each mouse strain. All sera were tested with AZV Formalin- and trypsin-treated promastigotes.

[b] Titers are expressed as the reciprocal of the highest serum dilution giving a positive agglutination.

BALB/c mice demonstrated a markedly decreased in vitro response to homologous antigen.

In vitro responses of AZV- and AMP-infected mice to mitogens. Table 3 shows, for both mouse strains, the effects of AZV and AMP infection on lymphocyte responses to T- and B-cell mitogens. When compared with control cells, lymphocytes from C57BL/6 infected with either AZV or AMP showed increased reactivities to both PHA and Con A. LPS responses were similar to those of controls. On the other hand, cells from infected BALB/c mice showed decreased PHA and LPS responses and increased DNA synthesis when cultured with Con A.

Course of lesions in C57BL/6 and BALB/c mice infected with 10^2 AMP amas-

tigotes. Since infection with 10^4 amastigotes induced severe, chronic infection in BALB/c mice, experiments were carried out to investigate whether a lower dose of parasites might induce a different clinical course of infection. Groups of 10 BALB/c and 10 C57BL/6 mice were infected with 10^2 AMP amastigotes, and their lesions were examined at weekly intervals. Figure 3 compares the courses of infection with 10^2 AMP amastigotes in C57BL/6 and BALB/c mice. In C57BL/6, infection with AMP produced small lesions which appeared in about 7 weeks. These lesions healed in about 20 weeks. In BALB/c, infection produced lesions which progressed slowly during the first 9 weeks but thereafter increased rapidly in size and ulcerated at 14 weeks. At this time, metastatic lesions also appeared in the tail. After 16 weeks, 10^2-AMP-infected BALB/c mice showed large, ulcerated lesions.

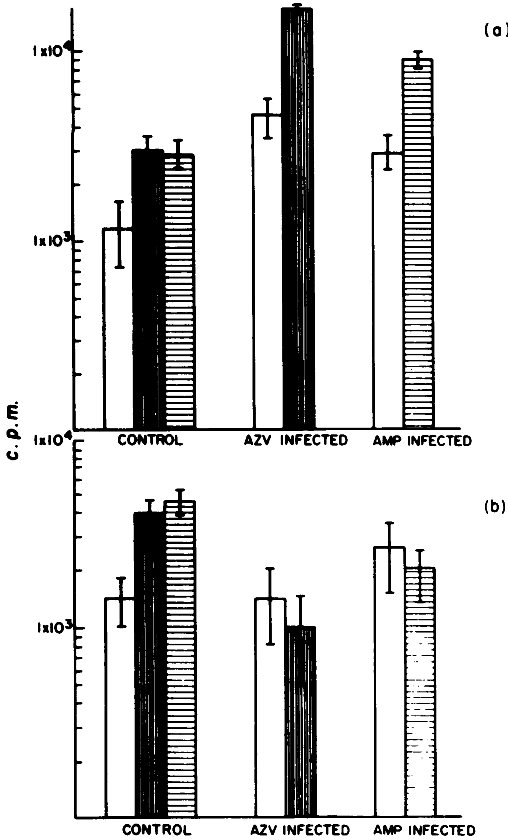

FIG. 2. *In vitro spleen cell responses of C57BL/6 (a) and BALB/c (b) mice when cultured with AZV (bars with vertical lines) or AMP (bars with horizontal lines) antigen or medium alone (open bars). Cells were obtained 20 to 23 weeks after infection with 10^4 amastigotes of either Leishmania strain.*

DISCUSSION

DCL is an uncommon manifestation of cutaneous leishmaniasis, and it is not clear whether it is due to a different type of parasite, to a peculiar host response, or to both (6). The ex-

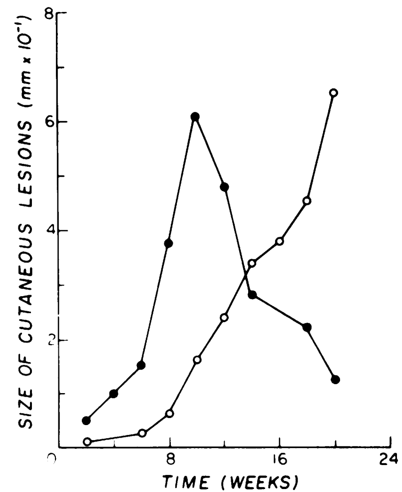

FIG. 3. *Comparison between the courses of primary infections with 10^2 AMP amastigotes in C57BL/6 (●) and BALB/c (○) mice.*

TABLE 3. *Mitogen responses for spleen cells from AZV- and AMP-infected C57BL/6 and BALB/c mice*[a]

Mouse strain	*Leishmania* strain	Stimulation index[b] with:		
		PHA	Con A	LPS
C57BL/6	None	30,109 ± 1,564	148,854 ± 10,423	44,564 ± 2,596
C57BL/6	AZV	41,763 ± 2,096*	173,447 ± 8,548	32,062 ± 2,930*
C57BL/6	AMP	37,922 ± 4,580	186,850 ± 9,755	35,834 ± 3,951
BALB/c	None	45,859 ± 4,016	53,588 ± 6,679	38,104 ± 4,910
BALB/c	AZV	32,726 ± 1,055*	132,897 ± 7,925*	409 ± 299*
BALB/c	AMP	13,402 ± 3,332*	86,856 ± 6,793*	12,438 ± 2,520*

[a] Spleen cells were obtained 20 to 23 weeks after infection with 10^4 amastigotes of either *Leishmania* strain.
[b] Stimulation indexes were calculated as described in the text. *, Significant ($P < 0.05$) by Student's t test; for each mitogen, responses from infected animals were compared with those of controls.

perimental evidence presented here strongly suggests that DCL is due to an immunological failure of the infected host. Comparison between the courses of infection with two strains of *Leishmania,* one isolated from a case of DCL and the other isolated from a case of ACL, in C57BL/6 and BALB/c mice revealed that patterns of infection were largely determined by the host. After infection with either strain (10^4 amastigotes), C57BL/6 mice developed self-healing lesions which stimulated good levels of cellular and humoral responses, as evidenced by the production of specific antibodies and in vivo DHR. Also, there was an increased in vitro lymphocyte reactivity to leishmanial antigen and moderate increases of their responses to T-cell mitogens, PHA and Con A. LPS responses were comparable to those of the controls. In contrast, BALB/c mice infected with either strain of *Leishmania* (10^4 amastigotes) developed chronic ulcerated lesions. Both DHR and production of specific antibodies were impaired, and in vitro lymphocyte responses to leishmanial antigen were decreased even below the levels of stimulation observed for control cells. PHA responses, although moderately diminished, were still significant. Con A responses were markedly increased, and there was a diminished response to LPS.

Patterns of responses of C57BL/6 and BALB/c mice to a low inoculum of parasite (10^2 AMP amastigotes) were essentially the same as those described for 10^4 amastigotes, and, whereas BALB/c developed chronic ulcerated lesions, C57BL/6 only had a mild infection. Inoculation with low doses of parasites has been postulated as a possible immunoprophylactic approach to human leishmaniasis (29). Our results, however, imply that in nonresponsive individuals, a low dose of parasite, otherwise inducing a mild infection, may lead to a chronic infection.

Recent studies have demonstrated that BALB/c mice also fail to control *L. tropica* (35) and *L. donovani* (5) infections, and there is also evidence which suggests that mouse susceptibility to *Leishmania* has a genetic basis (4, 5). Since BALB/c mice are known to develop cellular and humoral responses to other antigens (20, 21), including those of parasites (33), unresponsiveness to *Leishmania* is probably a specific event. Thus, BALB/c mice appear to mimic DCL patients in whom anergy seems to be specifically associated to *Leishmania* infection (6).

The nature of the mechanisms controlling the immune response to cutaneous leishmaniasis is not understood. Different types of cells, T and B lymphocytes and macrophages, could be involved. Leishmaniae are intracellular parasites of macrophages, and it has been suggested that these cells could be directly involved in the destruction of the parasites (30). Data so far obtained are controversial (24), but it has been demonstrated that macrophages from *L. tropica-* and *L. enrietii*-infected mice and guinea pigs (14, 36) do not support in vitro growth of the parasite as well as normal macrophages do. On the other hand, in vivo experiments have shown that, in mice, T-cell deprivation before *L. tropica* infection results in a chronic infection (28). Protective immunity can be transferred by spleen, lymph node, and peritoneal cells from *L. tropica*-infected mice, but not by immune serum alone. However, immune serum alone will enhance immunity transferred by peritoneal cells (24). Although immune serum apparently fails to confer protective immunity, in vitro studies have demonstrated that serum from immune guinea pigs could inhibit growth of *L. enrietii* promastigotes and that coating of *L. enrietii* amastigotes by immune guinea pig serum renders the parasite susceptible to intracellular killing in normal macrophages (24). Thus, it has been suggested that immunity to *Leishmania* may depend on synergism between antibodies and cells (24, 30). In our studies, BALB/c mice which failed to control *Leishmania* infection also showed suppressed cellular and humoral responses to the parasite. However, in vitro studies of mitogen responses revealed a differential effect of *Leishmania* infection on T and B lymphocytes. B cells not only were unable to mount a specific antibody response against the parasite but also showed a marked decrease in their ability to respond to LPS in vitro. LPS is considered to mimic B-cell activation by antigens (3). Mouse cells are particularly sensitive to its mitogenic action, and even spleen cells from mice tolerant to the antigenic carbohydrate moiety of LPS proliferate as well as do those of normal mice (2). Recent evidence indicates that LPS activates only a subset of B cells (13). Our results suggest that this subset could be particularly affected by the infection. On the other hand, Con A responses were significantly increased, and PHA responses, although moderately diminished, were still significant. It has been suggested that T-cell subsets can be distinguished by their relative responsivenesses to PHA and Con A (32). One subset appears to respond preferentially to Con A and is relatively sessile and radiosensitive (31), and evidence suggests that at least a portion of Con A-reactive cells may exert an inhibitory effect upon several T and B responses (27). The increased Con A response in *Leishmania*-infected BALB/c mice in which specific cellular and humoral responses were

suppressed might reflect the expansion of this T-cell subset. Experiments currently in progress in our laboratory are investigating this possibility.

There is now evidence for the occurrence of immunosuppression in several animal and human protozoal infections (11, 12, 15-18, 34). However, in *Leishmania* infections this has not been well documented. *L. donovani*-infected hamsters have a diminished capability to reject skin homografts (1) and to produce antibodies against chicken ovalbumin (7). Also, patients suffering from DCL responded normally to lepromin and tuberculin but showed an impaired response to dinitrochlorobenzene (6).

Antigenic competition, T- or B-cell tolerance, and activation of suppressor cells are some of the possible mechanisms by which immune responses to leishmanial infection may be suppressed. The importance of other factors affecting the host-parasite relationship, such as host genetic constitution and nutritional state and the magnitude of the infecting inoculum, could add further complexity to this system. Elucidation of suppressor mechanisms in experimental models could be of great value in defining immunoprophylactic approaches in human leishmaniasis.

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