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Visceral Leishmaniasis in Congenic Mice of Susceptible and Resistant Phenotypes: Immunosuppression by Adherent Spleen Cells

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Visceral leishmaniasis is one of several parasitic diseases of humans characterized by immune suppression. A murine model of disseminated leishmaniasis utilizing inbred strains of specific genetic constitution was used to study the mechanisms of immunosuppression elicited during the course of infection. Resistant (Lsh^r) and susceptible (Lsh^s) strains of mice were challenged with amastigotes of *Leishmania donovani* and evaluated as to immune status at intervals between 2 and 40 weeks after challenge. The proliferative responses of splenic lymphocytes to T-cell mitogens, a B-cell mitogen, and parasite antigens were measured to evaluate the relative immune status of parasitized mice and noninfected control mice. Lymphocytes from resistant C3Heb/FeJ (C3H) mice responded normally to concanavalin A and phytohemagglutinin throughout the course of infection. Parasite antigen responses appeared 2 weeks after challenge of C3H mice and remained vigorous for periods up to 6 months. In contrast, immune suppression during infection was profound in both the curing (C57B1/10) and noncuring (B10.D2) phenotypes of Lsh^s congenic mice. Both Lsh^s strains developed severe infection as evidenced by high parasite burdens in the liver and spleen 4 to 5 weeks after challenge; splenic lymphocytes taken from these mice between 2 and 8 weeks became increasingly unresponsive to the T-cell mitogens as well as to parasite antigens. The noncuring B10.D2 mice which suffered chronic infection continued to be suppressed for as long as 40 weeks. C57B1/10 (curing) mice, in contrast, cleared infection between 12 and 16 weeks. After spontaneous recovery or elimination of parasites by antimonial drug therapy, the response of spleen cells to T-cell mitogens or parasite antigens were restored to normal. The spleen cells from the Lsh^s strains of mice obtained during the height of infection suppressed the proliferative responses of spleen cells from their uninfected counterparts upon cocultivation in vitro. Removal of adherent cells from the suppressive spleen cell populations restored normal mitogen responses. On the basis of adherence characteristics, phagocytosis, and morphology, the suppressor was identified as a macrophage population which appears to be responsible for a nonspecific immunosuppression of Lsh^s mice with significant parasite burdens of *L. donovani*.

Bradley et al. (7, 8) showed originally that, by evaluating the severity and course of infections which ensue after challenge, inbred strains of mice can be classified as innately resistant or susceptible to *Leishmania donovani*. Some mouse strains restrict parasite multiplication (Lsh^r), while others permit significant multiplication in the liver and spleen (Lsh^s), so that a 100-fold increase in tissue forms may be attained during the initial month of infection. The Lsh gene determines innate resistance by governing macrophage functions required to inhibit the growth of *L. donovani* (11), and the gene locus has been mapped to mouse chromosome 1 (9). The gene locus governing resistance to *Salmonella typhimurium* (It_y) and the Lsh gene are inseparable by traditional genetic analysis (30). In contrast, acquisition of immunity to murine-disseminated leishmaniasis is under the control of one or several genes associated with the major histocompatibility complex of mice (3, 4). Inbred strains of mice demonstrating an Lsh^s phenotype may exhibit contrasting patterns of long-term infection. Both curing and noncuring strains of mice develop considerable parasite burdens after intravenous (i.v.) challenge, but curing mice are able to acquire immunity and eliminate parasite burdens after several months of infection. In contrast, noncuring strains fail to respond immunologically, and thus the parasite burden is maintained chronically.

We used a congenic pair of mice exhibiting either a cure or noncure phenotype to evaluate their respective immune status during a prolonged course of *L. donovani* infection. In this paper, a severe immune suppression is demonstrated in both the B10.D2 (noncure) and C57B1/10 (cure) strains of mice. The suppression was sustained in B10.D2 mice but was ultimately relieved in the C57B1/10 mice. We show that a population of adherent spleen cells of infected mice was responsible for nonresponsiveness of lymphocytes to mitogen and *L. donovani* antigen stimulation. Elsewhere, we show that a parasite-specific T-cell-mediated suppression also arises in B10.D2 (noncure) mice, which appears to prevent recovery from the disseminated leishmaniasis (26).

MATERIALS AND METHODS

Animals. Inbred mice of strains C3Heb/FeJ (C3H), C57B1/10SnJ (C57B1/10), and B10.D2/nSnJ (B10.D2) were purchased from Jackson Laboratory, Bar Harbor, Maine. C57B1/10 ($H\text{-}2^b$) and B10.D2 ($H\text{-}2^d$) are congenic strains, the B10.D2 possessing the $H\text{-}2^d$ region of the DBA/2 strain on the C57B1/10 background.

Leishmania amastigotes and promastigotes. *L. donovani* Sudan strain 1S amastigotes were harvested from the spleens of infected hamsters as described previously (13). *L. donovani* promastigotes were obtained by allowing splenic amastigotes to transform in minimum essential medium plus 20% heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) and subsequently maintained by serial culture at 26°C. Promastigotes fixed in 2% paraformalde-

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hyde, washed four times in phosphate-buffered saline, and resuspended in RPMI 1640 medium were used as parasite antigen in the splenic lymphocyte assays.

Amastigotes and promastigotes were enumerated by mixing parasites with specified numbers of Formalin-fixed chicken erythrocytes. The ratio of parasites to nucleated erythrocytes was then determined microscopically. Viability was estimated by acridine orange-ethidium bromide staining (24).

Determination of parasite burden in infected mice. Unless otherwise stated, infection in mice was initiated by i.v. injection of 10^7 amastigotes in 0.2 ml of Hanks balanced salt solution. Enumeration of amastigotes in liver and spleen cells was accomplished by microscopic evaluation of Diff-Quick (American Scientific Products, Obetz, Ohio)-stained tissue imprints by the method of Stauber (34). The ratio of parasites to nucleated liver or spleen cells multiplied by the organ weight in milligrams provides an estimate of the parasite burden expressed as Leishman-Donovan units (8).

Splenic lymphocyte responses to mitogen and parasite antigens. Lymphocytes were obtained by mincing splenic tissue in Hanks balanced salt solution. Single-cell suspensions were adjusted to 5×10^6 cells per ml in RPMI 1640 medium plus 20% heat-inactivated fetal calf serum, and 0.1-ml volumes were distributed into round-bottomed multiwell tissue culture plates (Costar, Cambridge, Mass.). Mitogens or parasite antigens were added in 0.1-ml volumes of RPMI 1640 medium without serum to a final concentration of 3 μg of concanavalin A (ConA; purified ConA provided by D. Behnke, Cincinnati, Ohio), 4 μg of phytohemagglutinin (PHA; Wellcome Research Laboratories, Beckenham, England), 10 μg of lipopolysaccharide (LPS) from *Escherichia coli* (Sigma Chemical Co., St. Louis, Mo.), or 10^7 paraformaldehyde-fixed promastigotes per ml. After cultures were incubated for 48 h at 37°C in a humidified, 5% CO_2 atmosphere, 25 μl of RPMI 1640 medium containing 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) was added to each well. Eighteen hours later, cells were collected on glass-fiber filters, and the incorporation of [^{3}H]thymidine by spleen cells was measured. Data are expressed as corrected counts per minute, i.e., the arithmetic mean of counts per minute from stimulated cultures minus the mean counts per minute of control cultures. Alternatively, data are expressed as percent suppression. All samples were cultured and processed in quadruplicate.

Antimonial drug therapy. Trivalent antimony methyl glucantime (glucantime) in phosphate-buffered saline was administered intraperitoneally on four consecutive days to infected mice at 400 mg of antimony per kg of body weight. Glucantime (obtained from the Walter Reed Army Institute of Research, Washington, D.C.) contained 33.7% antimony by weight, and the dosage given resulted in a greater than 99% reduction in parasite burden of the infected mice.

T-cell enrichment of splenic lymphocytes. Mouse spleen cells were enriched for T cells and depleted of macrophages and B cells by passage through nylon-wool columns (16). Cell yields were approximately 22 to 27% of spleen cells from uninfected mice and 13 to 18% of spleen cells from infected mice.

Macrophage enrichment of spleen cells. Spleen cells were enriched for macrophages by selective adherence to plastic petri dishes by the method of Hathcock et al. (15). The cells obtained were approximately 75% macrophages as determined by morphological criteria from stained cytofuge preparations and by selective binding of carbonyl iron.

Interleukin 2 assay. The production and assay of interleukin 2 (IL 2) from normal and infected spleen cells was by the method of Gillis et al. (12). Samples (20 ml) of spleen cell suspension (5.0×10^6/ml) with or without 5 μg of ConA per ml were incubated in tissue culture flasks (75 cm^2) in an upright position for 48 h at 37°C under 5% CO_2. At the completion of the incubation, ConA was added to the unstimulated control cultures, and the cells were removed by centrifugation at $400 \times g$ for 10 min. Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, N.J.) (10 mg/ml) was added to the supernatants and mixed for 10 min to adsorb the residual ConA (17). The IL 2-containing supernatants were filter sterilized and stored at −20°C.

The IL 2-dependent CTLL-2 cell line (12) was used to assay IL 2. The CTLL-2 cell line (obtained from George Deepe, University of Cincinnati, Cincinnati, Ohio) was maintained in Murphys medium (RPMI 1640 medium with 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] buffer, 2 g of sodium bicarbonate per liter, 10% heat-inactivated fetal calf serum, 200 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 5×10^{-5} M 2-mercaptoethanol) supplemented with 10% of a standard rat IL 2 preparation. CTLL-2 cells were washed and resuspended in Murphys medium, and 0.1-ml volumes containing 4×10^3 cells were distributed to wells of flat-bottomed 96-well microtiter plates. IL 2 was assayed by adding 0.1 ml of twofold serial dilutions of the ConA supernatants to the CTLL-2 cells. Cultures were incubated for 24 h at 37°C, and [^{3}H]thymidine (1 μCi per well) was added during the final 5 h of incubation. The cells were collected on glass-fiber filters, after which incorporation of [^{3}H]thymidine by the CTLL-2 cells was determined. The data are expressed as the highest dilution of supernatants yielding counts more than 30% of the maximum obtained with the standard 10% IL 2 preparation (12).

Activation of tissue macrophages in *L. donovani*-infected mice. Mice were challenged i.v. with 1.6×10^4 *Listeria monocytogenes*. After 48 h, the spleen and a portion of the liver were excised, weighed, and homogenized as a 10% (wt/vol) suspension in brain heart infusion broth (GIBCO). Dilutions were plated in brain heart infusion agar, and bacterial colonies were counted 48 h after incubation at 37°C. Resistance to *L. monocytogenes* was expressed as the difference in the $\log_{10}$ of bacteria in the liver or spleen between uninfected and *L. donovani*-infected mice 48 h after the challenge. The assay provided an accurate index of systemic macrophage activation, because the number of viable bacteria in the liver and spleen correlated directly with resistance as measured by 50% lethal dose determination (5).

RESULTS

Course of infection. C3H, C57B1/10, and B10.D2 mice were challenged i.v. with 10^7 viable *L. donovani*, and the parasite burdens in the liver, spleen, and bone marrow were determined at intervals during the course of infection. The kinetics of infection in the mouse livers (Fig. 1) demonstrate the Lsh^r phenotype of C3H mice (7) and the Lsh^s phenotype of the C57B1/10 and B10.D2 congenic mice (3). The susceptible (Lsh^s) C57B1/10 mice eventually reduced parasite numbers to insignificant levels (cure), whereas B10.D2 mice maintained a stable chronic infection (noncure), as expected (3).

Mitogen-stimulated lymphocyte proliferation. *L. donovani* infection had no significant effect on the responses of spleen

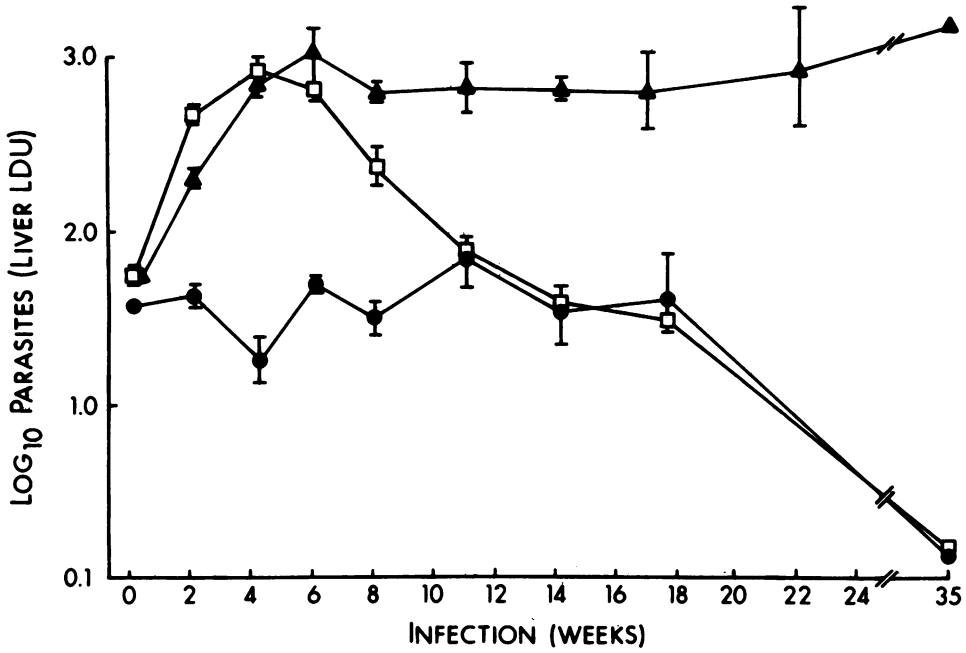

FIG. 1. Kinetics of *L. donovani* infection in inbred mouse strains. Mice were injected i.v. with 10^7 viable *L. donovani*. Parasite burdens were determined from stained imprints of liver slices (34). Leishman-Donovan units (LDU) (8) were calculated by the formula number of amastigotes/number of nucleated cells × organ weight (milligrams). Data are expressed as $\log_{10}$ Leishman-Donovan units ± standard error of the mean. Symbols: ●, C3H; □, C57B1/10; ▲, B10.D2.

cells to ConA for the first 2 weeks after challenge. After 4 weeks, however, a loss in the ability of spleen cells from infected C57B1/10 and B10.D2 mice to respond to mitogens became apparent. T-cell responses to ConA were virtually abolished between 6 to 12 weeks of infection (Fig. 2). A similar suppression was observed when splenic lymphocytes of infected C57B1/10 and B10.D2 mice were stimulated with PHA and LPS (data not shown). Spleen cells from C57B1/10 (cure) mice regained normal mitogen responsiveness after 12 to 18 weeks of infection. No restoration of mitogen re-

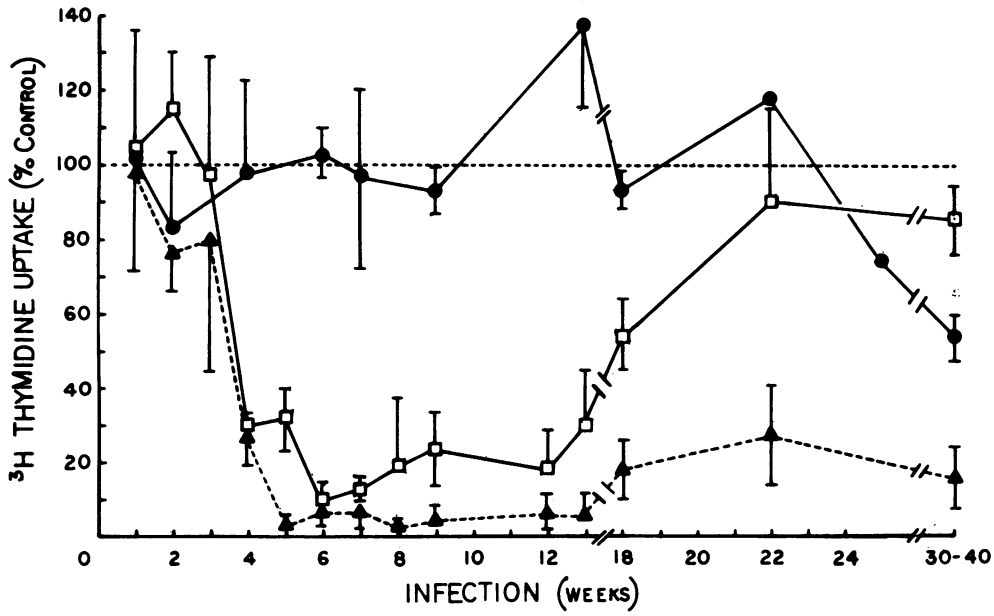

FIG. 2. Lymphocyte proliferative responses to ConA during the course of *L. donovani* infection. Mice were injected i.v. with 10^7 viable *L. donovani*. At indicated intervals, splenic lymphocytes (5.0×10^5 per well) were cultured with 3.0 μg of ConA per ml for 66 h. [^{3}H]thymidine (1.0 μCi per well) was added for the final 18 h, and the incorporation of radiolabel was determined. Results are expressed as percentage of the response of spleen cells from uninfected control animals. Data are an average of between two and five separate experiments at each time point ± standard error of the mean. Symbols: ●, C3H; □, C57B1/10; ▲, B10.D2.

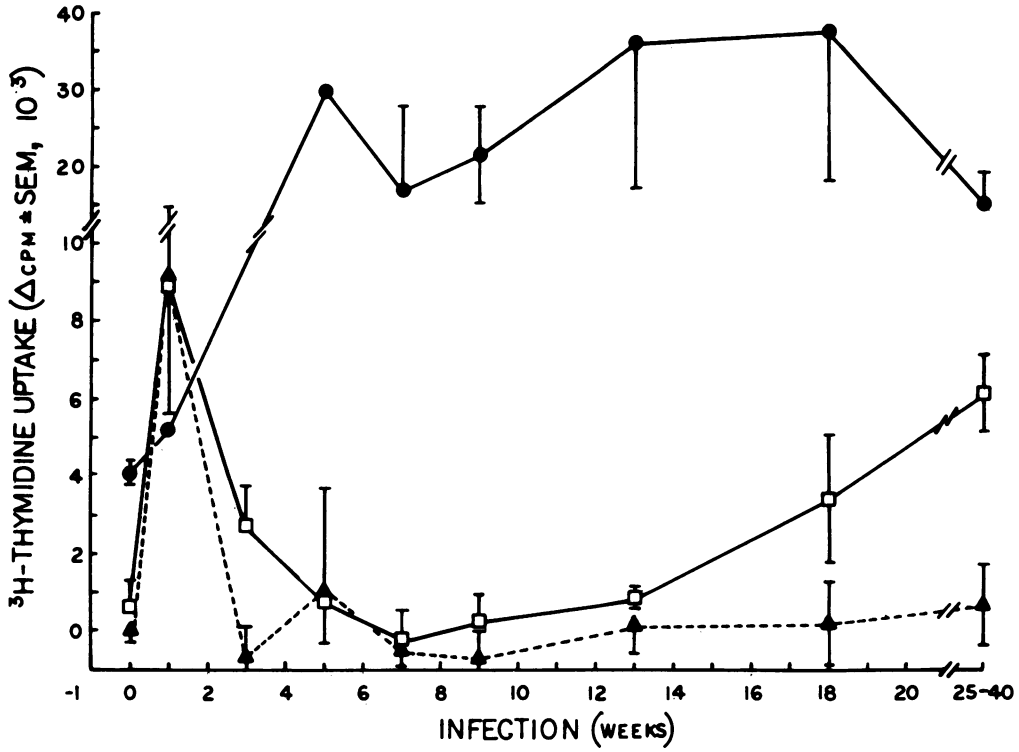

FIG. 3. Lymphocyte proliferative responses to parasite antigens during the course of *L. donovani* infection. Mice were injected i.v. with 10^7 viable *L. donovani*. At indicated intervals, splenic lymphocytes were cultured with parasite antigens (5.0×10^6 fixed promastigotes per ml) for 66 h. [^{3}H]thymidine (1.0 μCi per well) was added for the final 18 h, and the incorporation of radiolabel was determined. Values are the average of between two and five separate experiments at each time point ± standard error of the mean. Symbols: ●, C3H; □, C57B1/10; ▲, B10.D2.

sponses in infected B10.D2 (noncure) mice, in contrast, occurred at any time after the establishment of the suppressed state. *L. donovani* infection of C3H (Lsh^r) mice did not result in suppression of lymphocyte responses to ConA (Fig. 2), PHA, or LPS (data not shown).

Parasite antigen-induced lymphocyte proliferation. Paraformaldehyde-fixed promastigotes were used as antigen to stimulate sensitized splenic lymphocytes of infected mice (Fig. 3). Lymphocytes from both C57B1/10 and B10.D2 responded transiently to parasite antigens 2 weeks after infection but became markedly suppressed several weeks later. Approximately 18 weeks into the infection, spleen cells from C57B1/10 mice regained the capacity to respond to *L. donovani* antigens. B10.D2 mice, however, failed to regain parasite antigen responses. In contrast, infected C3H mice rapidly developed significant antigen-specific responses which persisted for 18 weeks, after which they began to diminish.

Recovery of proliferative responses after elimination of parasites by glucantime treatment. Because spleen cells from C57B1/10 (cure) mice recovered the capacity to respond to mitogens and parasite antigens, it was of interest to deter-

TABLE 1. Restoration of lymphocyte proliferative responses in B10.D2 mice treated with glucantime[a]

Infected	Glucantime treatment	Days after glucantime	Proliferative responses [corrected cpm ± SEM × 10^{-3}][b]			
			ConA	PHA	LPS	Promastigote antigens
No	No		132.0 ± 5.7 (100)	16.8 ± 1.1 (100)	43.4 ± 0.4 (100)	0
Yes	No		0.2 ± 0.5 (<1.0)	0.7 ± 1.0 (4)	7.4 ± 1.2 (17)	1.6 ± 0.9
Yes	Yes	11	95.9 ± 12.5 (73)	33.6 ± 5.2 (200)	24.3 ± 1.6 (56)	5.0 ± 1.1
No	No		235.3 ± 10.5 (100)	23.1 ± 1.7 (100)	94.6 ± 3.3 (100)	0
Yes	No		6.1 ± 3.2 (3)	0.1 ± 1.1 (<1)	12.5 ± 2.4 (13)	1.6 ± 0.4
Yes	Yes	15	216.2 ± 7.8 (92)	40.3 ± 4.4 (175)	68.6 ± 2.3 (73)	4.1 ± 1.5

[a] Mice were infected i.v. with 10^7 viable *L. donovani*. Five weeks later, mice were treated on four consecutive days intraperitoneally with 400 mg of antimony per mg of body weight. Lymphocyte proliferative responses were determined 11 and 15 days after the glucantime regimen.

[b] Spleen cells (5.0×10^5 per well) were incubated for 66 h in the presence of 3.0 μg of ConA, 3.0 μg of PHA, 10.0 μg of LPS, or 5.0×10^6 killed promastigotes per ml. [^{3}H]thymidine (1.0 μCi per well) was added for the last 18 h, and the incorporation of radiolabel was determined. The corrected counts per minute was calculated as the difference between counts per minute of cultures containing mitogen or parasite antigen and counts per minute of cultures without mitogen or antigen. The value in parentheses is the percentage of control.

TABLE 2. Suppression of mitogen-stimulated proliferation of normal spleen cells by spleen cells from infected mice[a]

Mouse strain	No. of spleen cells per well		Proliferation [corrected cpm ± SEM × 10^{-3}][b]		
	Uninfected mice	Infected mice	ConA	PHA	LPS
C57B1/10	5×10^5		139.4 ± 6.0	22.0 ± 1.0	75.7 ± 1.6
	1×10^6		244.3 ± 6.4	25.3 ± 2.7	87.9 ± 5.4
		5×10^5	0.0	0.0	43.5 ± 1.2
	5×10^5	5×10^5	0.6 ± 0.7	2.2 ± 0.5	43.1 ± 1.3
B10.D2	5×10^5		62.2 ± 2.0	33.7 ± 2.6	20.9 ± 3.6
	1×10^6		146.7 ± 2.7	42.4 ± 3.4	54.8 ± 1.9
		5×10^5	2.5 ± 1.5	3.8 ± 1.2	16.0 ± 0.3
	5×10^5	5×10^5	3.1 ± 0.8	6.6 ± 2.4	31.5 ± 0.4

[a] Mice were infected i.v. with 10^7 viable *L. donovani*.

[b] Spleen cells were incubated 66 h in the presence of 3.0 μg of ConA, 3.0 μg of PHA, or 10.0 μg of LPS per ml. [^{3}H]thymidine (1.0 μCi per well) was added for the last 18 h and the incorporation of radiolabel was determined. The corrected counts per minute was calculated as the difference between counts per minute of cultures containing mitogen and counts per minute of cultures without mitogen ± standard error of the mean.

mine whether spleen cells from infected B10.D2 mice would also regain lymphocyte responsiveness after the elimination of parasite burdens by antileishmanial drug therapy. The proliferative responses of splenic lymphocytes from glucantime-treated mice were determined 11 and 15 days after drug treatment (Table 1). The responses of spleen cells from the drug-treated animals to mitogens and to parasite antigens were restored to normal levels.

Suppression of lymphocytes from infected animals. Spleen cells from 6-week-infected C57B1/10 or B10.D2 mice were mixed at a ratio of 1:1 with spleen cells from uninfected animals and subsequently stimulated with mitogens. Spleen cells from 6-week-infected mice of both Lsh^s strains suppressed lymphocyte responses of cells from uninfected animals to ConA, PHA, and LPS (Table 2). Thus, the data show that *L. donovani* infection elicits actively immunosuppressive cells in the spleens of infected animals.

Removal of adherent spleen cells restores lymphocyte responses. Splenic lymphocytes of infected B10.D2 mice were depleted of adherent macrophages and B cells by passage through nylon-wool columns. Removal of the adherent cells restored the mitogen responses of splenic lymphocytes to normal levels (Table 3). Responses to LPS were absent in the nylon-wool-nonadherent spleen cell population from both uninfected and infected animals, indicating the deletion of B cells. Although unfractionated spleen cells from infected animals suppressed the responses of normal splenic lymphocytes to all mitogens in coculture (Table 2 and 3), no suppression was evident when the nonadherent cell populations from the B10.D2 (Table 3) mice were cocultured with normal splenic lymphocytes. Similar results were obtained with cells from infected C57B1/10 mice (data not shown). These results indicate, therefore, that suppressed mitogen responses of splenic lymphocytes in *L. donovani*-infected mice are due to nylon-wool-adherent cells and confirm previously reported data of Murray et al. (25).

Adherent spleen cells from infected mice suppress normal mitogen responses. Adherent spleen cells from infected C57B1/10 mice substantially suppressed the ConA-stimulated proliferation of spleen cells from uninfected mice when cocultured at ratios as low as 1:10 (Table 4). Unfractionated spleen cells from infected animals were suppressive at ratios of 1:5 or less. The addition of adherent spleen cells from infected C57B1/10 mice to cultures of nylon-wool-passed nonadherent spleen cells from infected mice were also suppressive at ratios of 1:5 and 1:10 (Table 4).

Systemic macrophage activation in *L. donovani*-infected mice. Activated macrophages may inhibit mitogen responses of lymphocytes (14, 18, 31, 33), and thus it was of interest to monitor systemic macrophage activation of *L. donovani*-infected mice. An assay with *L. monocytogenes* conveniently measured the degree of macrophage activation in

TABLE 3. Restoration of mitogen-induced proliferation of spleen cells from infected B10.D2 mice after removal of nylon-wool-adherent cells

Spleen cell population[a]	Proliferation [corrected cpm + SEM × 10^{-3}][b] (% of control)		
	ConA	PHA	LPS
Uninfected	116.0 ± 8.0 (100)	55.3 ± 2.5 (100)	56.4 ± 4.3 (100)
Infected	5,2 ± 2.5 (5)	1.8 ± 0.7 (4)	8.4 ± 0.9 (15)
NWNA uninfected[c]	118.6 ± 4.5 (100)	54.7 ± 1.7 (100)	2.2 ± 0.3 (NA)[d]
NWNA infected	84.2 ± 4.0 (71)	57.9 ± 3.4 (106)	2.0 ± 0.9 (NA)
Uninfected + uninfected	191.3 ± 13.7 9100)	92.1 ± 2.8 (100)	80.6 ± 7.2 (100)
Uninfected + infected	3.3 ± 1.3 (2)	1.5 ± 3.1 (2)	21.8 ± 1.3 (27)
Uninfected + NWNA uninfected	202.7 ± 4.3 (100)	106.0 ± 3.7 9100)	75.6 ± 3.7 (100)
Uninfected + NWNA infected	176.3 ± 3.7 (87)	120.9 ± 5.2 (114)	69.6 ± 7.4 (92)

[a] Spleen cells were isolated from uninfected animals and animals infected i.v. 7 weeks previously with 10^7 viable *L. donovani* amastigotes. Microtiter wells containing 5×10^5 cells of each cell population were incubated for 66 h in the presence or absence of mitogens. [^{3}H]thymidine (1.0 μCi per well) was added for the last 18 h, and the incorporation of radiolabel was determined.

[b] The corrected counts per minute was calculated as the difference between counts per minute of cultures containing mitogen and counts per minute of cultures without mitogen ± standard error of the mean.

[c] Nylon-wool-nonadherent (NWNA) cells were obtained by the passage of spleen cells through nylon-wool columns and elution with warm medium.

[d] NA, Not applicable.

TABLE 4. Suppression of ConA-induced proliferation of lymphocytes by plastic-adherent spleen cells from infected C57B1/10 mice[a]

Primary spleen cell population (5×10^5 per well)	Secondary spleen cell population	No. of secondary spleen cells added per well	Proliferation corrected (cpm ± SEM $\times 10^{-3}$)[b]	% of Control
Uninfected			155.9 ± 12.0	100
Infected			6.1 ± 2.6	4
Uninfected	Infected[c]	5.0×10^4	158.1 ± 4.8	101
Uninfected	Infected	1.0×10^5	120.2 ± 4.1	77
Uninfected	Uninfected adherents	5.0×10^4	190.5 ± 15.4	122
Uninfected	Uninfected adherents	1.0×10^5	230.9 ± 18.6	148
Uninfected	Infected adherents	5.0×10^4	6.4 ± 2.1	4
Uninfected	Infected adherents	1.0×10^5	1.7 ± 2.3	1
NWNA infected[d]			85.4 ± 5.9	100
NWNA infected	Infected	5.0×10^4	78.9 ± 8.9	92
NWNA infected	Infected	1.0×10^5	56.4 ± 2.9	66
NWNA infected	Infected adherents	5.0×10^4	20.8 ± 2.3	24
NWNA infected	Infected adherents	1.0×10^5	0.1 ± 1.8	<1

[a] Spleen cells were obtained from uninfected animals and animals infected i.v. 6 to 8 weeks previously with 10^7 viable *L. donovani* amastigotes. Microtiter wells containing 5.0×10^5 primary responding cells and 5.0×10^4 or 1.0×10^5 cells of a secondary spleen cell population were incubated for 66 h in the presence or absence of 3.0 μg of ConA per ml. [^{3}H]thymidine (1 μCi per well) was added for the last 18 h, and the incorporation of radiolabel was determined.

[b] The corrected counts per minute was calculated as the difference between counts per minute of cultures containing mitogen and counts per minute of nonstimulated cultures ± standard error of the mean.

[c] Samples of the total spleen cell populations of *L. donovani*-infected mice.

[d] NWNA, Nylon wool nonadherent.

mice (27). Forty-eight hours after i.v. challenge, viable listeria in the spleen and liver of *L. donovani*-infected and uninfected animals were enumerated. An activated macrophage system in *L. donovani*-infected mice is indicated by fewer bacteria recoverable from the liver and spleen as compared with control (uninfected) mice challenged with *L. monocytogenes* (5, 27). The data show that macrophages in spleens (Fig. 4) of C57B1/10 and B10.D2 mice were activated by week 4 of *L. donovani* infection. C57B1/10 mice were significantly more resistant to *L. monocytogenes* than were *L. donovani*-infected B10.D2 mice. Macrophages of Lsh^r C3H mice became slightly activated 2 weeks after *L. donovani* infection (Fig. 4) but quickly returned to a quiescent state thereafter.

IL 2 production. Reiner and Finke (32) demonstrated that spleen cells from *L. donovani*-infected BALB/c mice were deficient in PHA-stimulated IL 2 production. We wished to determine whether suppressive macrophages and deficient IL 2 production were directly correlated. Supernatants containing IL 2 were produced by ConA stimulation of spleen cells from uninfected and infected mice. Dilutions of supernatants were assayed for IL 2 activity by their ability to support proliferation of an IL 2-dependent cell line. The titers of IL 2 produced by spleen cells from C57B1/10 mice which had been infected for 6 weeks were significantly lower than those produced by spleen cells from uninfected controls (Table 5). IL 2 production by spleen cells from B10.D2 (noncure) mice was also suppressed by 6 weeks after infection and remained suppressed for 20 weeks (data not shown). In contrast, as C57B1/10 (cure) mice recovered from infection, IL 2 production returned to a normal level (data not shown).

Because the removal of nylon-wool-adherent cells from suppressed cell populations restored ConA responses (Table 3), we determined whether the removal of the adherent cell also reversed the IL 2 defect (Table 5). The data indicate that such was not the case. Whereas passage of spleen cells from 6-week-infected C57B1/10 mice through nylon wool restored lymphocyte proliferation, the deficiency in IL 2 production persisted. Additionally, nonadherent spleen cells from infected animals suppressed IL 2 production when cocultured with normal spleen cells, but these same cells did not suppress proliferative responses.

DISCUSSION

Our experiments demonstrate a pronounced suppression of immune responses during disseminated leishmaniasis of mice. The in vitro proliferation of stimulated spleen cells from Lsh^s mice (C57B1/10 and B10.D2) but not of the Lsh^r(C3H) strain was suppressed during visceralized infection with *L. donovani*. The immunosuppression of C57B1/10 and B10.D2 mice was shown to consist of an inhibition of T- and B-cell mitogen spleen cell responses (Table 2; Fig. 2) and a specific inhibition of sensitized lymphocyte responses to *L. donovani* antigens (Fig. 3). After 12 to 18 weeks of infection, C57B1/10 but not B10.D2 mice reacquired normal T-cell responses to mitogens and parasite antigens. Suppression of ConA (25) and PHA (32) responses of spleen cells from *L. donovani*-infected BALB/c mice has been observed previously by others. In addition, the data presented document suppression of B-cell proliferation as well as specific suppression of lymphocyte responses to parasite antigens. The data overall are novel because they compare immune responses and immunosuppression in congenic mice which differ only at the genetic locus responsible for acquisition of curative immunity of *L. donovani* infection.

Normal lymphocyte responses were eventually restored in the curing mice but not in the noncuring B10.D2 strain. Restoration of normal immune responses in *L. donovani*-infected mice appears to be linked with significant reduction in the numbers of amastigotes present in the liver and spleen. After 12 to 18 weeks of infection, C57B1/10 mice, which had by that time virtually eliminated the parasites, had also reacquired normal proliferative responses to parasite antigens and T-cell mitogens. In addition, chronically parasitized B10.D2 mice treated with glucantime rapidly regained normal lymphocyte responses (Table 1). The presence of actively suppressive cells in the spleens of *L. donovani*-infected susceptible mice was demonstrated by their ability to suppress the proliferation of normal spleen cell popula-

tions when cocultured in vitro. The data also show that suppressor cells were removed by passage through nylon-wool columns (Table 3 and 5) and were enriched by adherence to plastic (Table 4). Suppressive cells were also separated by repetitive treatment with carbonyl iron and a strong magnet (data not shown). On the basis of adherence to plastic and nylon wool, removal by carbonyl iron treatment, and morphological characteristics, the adherent suppressor cells present in *L. donovani*-infected C57B1/10 and B10.D2 spleens were tentatively identified as macrophages. Suppression of mitogen-induced lymphoproliferation has been reported in a variety of chronic infections (1, 10, 14, 18, 22, 29, 35) and during tumor development (20, 31). In these instances, suppressor macrophages were deemed responsible for the inhibition. Thus, macrophage-mediated suppression appears to be a general phenomenon associated with intense and prolonged antigenic stimulation. Activated spleen (2, 36) or peritoneal (2) macrophages are more suppressive than are resident cells. Macrophages within the reticuloendothelial organs of *L. donovani*-infected C57B1/10 and B10.D2 mice were activated, as evidenced by their ability to restrict the growth of *L. monocytogenes* (Fig. 4). The increased antibacterial activity of tissue macrophages was temporally coincident with suppressed lymphocyte proliferation.

It is interesting to note that the C3H mice, although classified as Lsh^r failed to clear infection for several months after challenge. The relatively small parasite burdens achieved in C3H mice would predict a rapid elimination of parasites, especially in light of the positive responses to parasite antigens exhibited by C3H splenic lymphocytes over a period of several months (Fig. 3). The rapid loss of systemic macrophage activation exhibited by the C3H mice (Fig. 4) suggests, however, that the sustained activation of macrophages required to eliminate *L. donovani* amastigotes from infected cells (13) may not be attained. It may very well be that C3H mice are noncure mice superimposed on an Lsh^r background. Evidence for this is provided by our previous observation that although *nu/nu* mice of C3H origin control intrahepatic multiplication almost as effectively as do immunocompetent C3H mice, *nu/nu* mice fail to clear infection (6) in much the same fashion as Lsh^s noncure B10.D2 mice (Fig. 1).

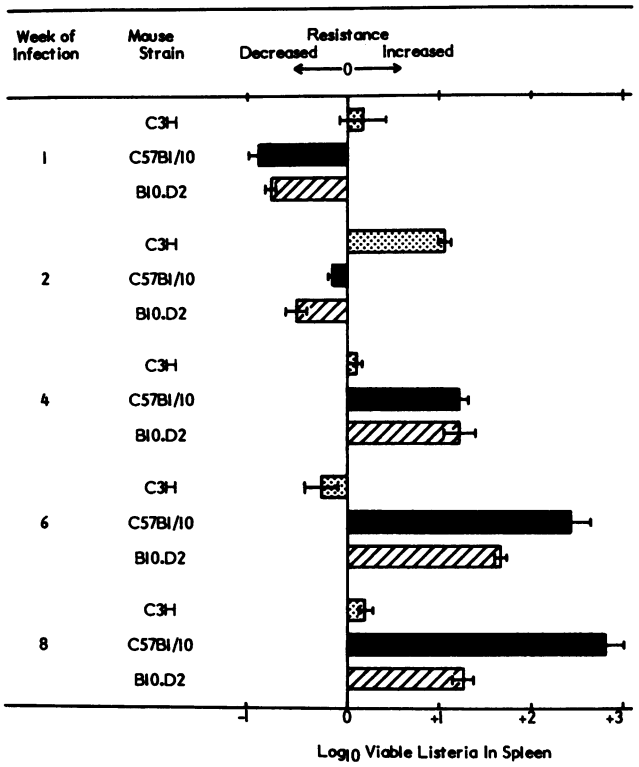

FIG. 4. Resistance of *L. donovani*-infected C3H, C57B1/10, and B10.D2 mice to *L. monocytogenes*. Mice were infected i.v. with 10^7 *L. donovani* amastigotes. Infected and uninfected mice of strain were challenged i.v. with 1.6×10^4 *L. monocytogenes*. The spleens of listeria-infected animals were excised after 48 h and assayed for viable bacteria. Resistance is expressed as the difference in viable bacteria in the spleens of the two groups of mice of each strain ($\log_{10}$ ± standard error of the mean).

TABLE 5. Failure to correlate IL 2 production with restoration of mitogen-induced proliferation of spleen cells from infected C57B1/10 mice after removal of adherent cells

Spleen cell population[a]	IL 2 titer[b]	ConA responses (% control)
Uninfected	1:16	100
Infected	1:4	9
NWNA uninfected	1:4	100
NWNA infected	1:4	80
Uninfected + uninfected	1:64	100
Uninfected + infected	1:32	18
Uninfected + NWNA infected	1:16	93

[a] Spleen cells were isolated from uninfected animals and from animals injected i.v. 6 weeks previously with 10^7 viable *L. donovani*. IL 2-containing supernatants were prepared by incubating 10^8 cells of each cell population in tissue culture flasks (75 cm^2) with 5.0 μg of ConA per ml for 48 h. Supernatants were filter sterilized and assayed for IL 2 activity. NWNA, Nylon wool nonadherent.

[b] IL 2 activity in lymphocyte culture supernatants was measured by the ability to support the multiplication of the IL 2-dependent CTLL-2 cell line. The titer of IL 2 is expressed as the highest dilution of supernatant stimulating >30% of the optimum thymidine incorporation obtained with a standard preparation of rat IL 2.

Attempts were also made to identify the mechanism by which macrophages from *L. donovani*-infected Lsh^r mice suppress lymphocyte proliferation in vitro. Release of thymidine (28) and arginase (21) by macrophages may be spurious mediators of lymphocyte nonresponsiveness. Accumulation of thymidine or arginase in lymphocyte culture fluids was excluded as a cause of T-lymphocyte suppression in our own experiments, because supernatants of suppressed spleen cells were not inhibitory (data not shown). Prostaglandin production by macrophages is also a potential factor in macrophage-mediated suppression (18, 22, 23). We have found that indomethacin only minimally restored ConA responses of spleen cells from infected animals, thus eliminating prostaglandins as a factor. The toxic effects of hydrogen peroxide also may inhibit lymphocyte proliferation (23). This possibility was also excluded, because catalase failed to restore proliferation responses. Thus, a mechanism for the macrophage-mediated suppression of lymphocyte proliferation in vitro during visceral leishmaniasis remains to be established.

Reiner and Finke (32) detected a defect in IL 2 production of splenic lymphocytes from *L. donovani*-infected BALB/c mice upon stimulation with PHA. Our data confirm these findings in that spleen cells from infected C57B1/10 and B10.D2 mice produced significantly less IL 2 in response to

ConA stimulation than did spleen cells from uninfected mice. IL 2 production by spleen cells from cured C57B1/10 mice was normal, as was spleen cell proliferative response. The IL 2 defect, however, does not appear to be a direct consequence of suppressor macrophages, because their removal by adherence restored proliferative responses but failed to augment IL 2 production (Table 5).

The immunosuppression of murine leishmaniasis demonstrated in this study was limited primarily to in vitro correlates of immunological functions. We are attempting to correlate the macrophage-mediated suppression as measured in vitro with in vivo correlates of immune status. Elsewhere (26), we show that in addition to the adherent cell-mediated suppression, B10.D2 (noncure) mice also develop parasite antigen-specific T-cell suppression which appears to contribute to the noncure pattern of disseminated leishmaniasis in that mouse strain.

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