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Visceral *Leishmania tropica* Infection of BALB/c Mice: Cellular Analysis of In Vitro Unresponsiveness to Sheep Erythrocytes

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In mice, infection with *Leishmania tropica* initially produced a nonspecific enhancement of the immune response to sheep erythrocytes as measured both in vitro and in vivo. Subsequently, the spleen cell responses of susceptible mice (BALB/c) to sheep erythrocytes and T- and B-cell mitogens in vitro decreased dramatically, whereas those of the resistant strain (C57BL/6) returned to normal. Analysis of the spleen cells of infected animals revealed that macrophages (the target cells of *Leishmania*) were not defective. However, both T- and B-cell-depleted splenocyte populations of infected animals lacked the ability to respond in the presence of their corresponding B- and T-cell-depleted populations of normal spleen cells. It was also observed that the addition of various numbers of *Leishmania* organisms did not alter the response of normal spleen cells in vitro. The results of cocultures of various ratios of cells from the spleen of infected and normal animals ruled out the possibility of a strong active immunosuppression. The decrease of in vitro response is attributed to the depletion of immunocompetent cells in the spleen of infected mice, which is heavily populated by null cells.

Different mouse strains, when injected with *Leishmania tropica*, produce various types of diseases covering the entire spectrum of the human cutaneous leishmaniasis (3, 4, 11, 19, 23, 24). Resistant mice (C57BL/6 and C3H) produce a small localized self-healing cutaneous lesion, accompanied by a moderate antibody response and a long-lasting delayed-type hypersensitivity reaction. The intermediate susceptible strains either develop a nonhealing lesion (DBA/2) (3) or show a progressive susceptibility with higher doses of *L. tropica* (CBA) (23).

Of particular interest is the highly susceptible strain (BALB/c), which suffers a visceral and lethal disease (15, 20) even when injected with very low doses of the parasite (13). In these mice, the infection produces high levels of antibody but a minimal or no delayed-type hypersensitivity reaction when injected with *Leishmania mexicana* (21) or *L. tropica* (20). The lack of delayed-type hypersensitivity has been attributed to the generation of specific T suppressor cells (12).

In previous studies, we observed that *L. tropica* produced a visceral infection in all strains of mice tested, regardless of their susceptibility (16). Although the resistant mice control the disease, viable organisms can be isolated from the spleen long after the healing of the skin lesion. However, in BALB/c mice, the parasite grows abundantly in the visceral organs and produces a clinicopathological picture (lymphadenopathy, splenohepatomegaly, hyperglobulinemia, and hypoalbuminemia) which closely resembles that of human kala azar (9). Based on these observations, it was suggested that BALB/c mice infected with *L. tropica* may serve as an animal model with which to study human visceral leishmaniasis caused by *Leishmania donovani* (9).

In the present paper, we report studies designed to evaluate the immunological status of infected BALB/c mice with respect to production of antibody to an unrelated antigen and to sheep erythrocytes (SRBC) and their spleen cell responses to mitogens, lipopolysaccharide (LPS), and concanavalin A (ConA). Attempts were also made to elucidate the cellular basis of their in vitro unresponsiveness to SRBC.

MATERIALS AND METHODS

Animals. Male or female BALB/c, DBA/2, and C57BL/6 mice, 8 to 12 weeks old, purchased from Iffa-Credo, L'Arbresle, France, were used.

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***Leishmania* strain.** A strain of *L. tropica* (major) kindly provided by A. Nadim, School of Public Health, Tehran, Iran, was used. This strain was isolated by Javadian et al. (14) from *Rhombomys opimus*, and biochemical typing by W. Peters revealed it to be a typical *L. tropica* major. This is the same strain used in previous studies (9, 16, 18, 20).

For injection, the organisms were isolated from the spleens of infected BALB/c mice in RPMI medium (Flow Laboratories, Irvine, Scotland) containing 10% fetal calf serum and passaged either in the same medium or in NNN medium three to six times. Promastigotes (1.2×10^6 per 0.1 ml) were injected intracutaneously at the base of the tail.

Detection of organism in the spleen. At various times after infection, the animals were exsanguinated, the spleens were teased in RPMI 1640 medium, and 5-ml cell suspensions containing 10^7 nucleated cells per ml were incubated at room temperature. The number of live promastigotes was determined every other day from 3 to 13 days of culture by using a hemacytometer and a procedure similar to that of Poulter (22).

In vivo response against SRBC. The numbers of direct antibody plaque-forming cells (PFC) were estimated 5 days after an intraperitoneal injection of 2×10^8 SRBC. Results are expressed as PFC per 10^6 nucleated splenocytes (plaques per million).

In vitro response against SRBC. Mouse spleen cells were suspended in RPMI 1640 medium (Flow Laboratories) containing 2 mM glutamine, antibiotics, and 10% heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). Immunizations were performed by the method of Mishell and Dutton (17). Spleen cells (1.5×10^7) were cultured with 6×10^6 SRBC in 1 ml of medium in plastic petri dishes (Nunclon, Poly-Labo-Block, Paris). Cultures were fed daily with 0.1 ml of nutritional cocktail containing 30% fetal calf serum. They were incubated at 37°C with 7% O_2–10% CO_2–83% N_2 and were rocked throughout the culture period. On day 5, duplicate cultures of cells were harvested by scraping the dishes with a rubber policeman, and the number of viable cells was determined in a Malassez hemacytometer. The number of antibody PFC was estimated by the method of Cunningham and Szenberg (7). Results are expressed as the number of PFC per 10^6 recovered viable cells (plaques per million).

Measurement of thymidine incorporation. A 1-ml amount of cell suspension containing 5×10^5 viable cells was incubated in a humidified atmosphere (5% CO_2–95% air) in round-bottom culture tubes (no. 2054; Falcon Plastics, Oxnard, Calif.). LPS extracted from *Salmonella enteritidis* (Difco Laboratories, Detroit, Mich.) and ConA (Miles-Yeda, Ltd. Rehovot, Israel) were used at 10 and 3 μg/ml of culture, respectively. After 48 h, 1 μCi of tritiated thymidine (1 Ci/mmol; Saclay, France) was added to each tube. After 6 h, cells were harvested on glass fiber filters with a multiple sample device (Skatron; Flow Laboratories). Results are expressed as counts per minute.

Cell separation. (i) Separation of macrophages and of nonadherent cell populations. Macrophages were separated by incubating spleen cells in 35-mm petri dishes at a concentration of 1.5×10^7 cells per ml for 5 h at 37°C.

Nonadherent cells were removed, washed, and suspended in 1 ml of medium. Petri dishes were washed with fresh medium and used immediately.

(ii) Preparation of T-cell-depleted population. T-cells were depleted from the spleen cell population by treatment with a monoclonal anti-Thy 1.2 serum (kindly provided by M. Khorshidi, University College, London) and complement. Briefly, 5×10^6 spleen cells per ml were incubated with a predetermined dilution (1/500) of anti-Thy 1.2 for 60 min at 4°C, washed once, reincubated with guinea pig serum (1/5) for an additional 45 min, and washed. The cells had lost their ability to respond to ConA but responded normally to LPS.

(iii) Preparation of B-cell-depleted population. A T-cell-rich fraction was obtained by elimination of B-cells by treatment with a rabbit anti-mouse immunoglobulin serum (Nordics Immunological Laboratory, Tilburg, The Netherlands) and complement. Spleen cell suspensions were treated as described for T-cell cytotoxicity except that anti-immunoglobulin was used at a dilution of 1/30 instead of anti-Thy 1.2. These cells had completely lost their ability to respond to LPS but responded normally to ConA.

Enumeration of B- and T-cells and macrophages. B-cells were determined by cytotoxicity as described above and by immunofluorescence. For enumeration of immunoglobulin-bearing cells, the following procedure was used. To 10^7 cells suspended in medium 199 (Institut Pasteur Production, France), 20 μl of fluorescein-conjugated rabbit globulin fraction of anti-mouse serum (Institut Pasteur Production) was added, and the cells were incubated at 37°C for 1 h. After being washed three times, the fluorescent cells were counted in a total of at least 200 cells with a Zeiss fluorescence microscope equipped with an epichromatic system. The results of two procedures for enumeration of B-cells were similar, hence only the results with cytotoxicity are presented. T-cells were enumerated by cytotoxicity as described above. Macrophages were determined by the nonspecific esterase test described by Tucker et al. (27). Stained cells were scored in a total of at least 500 cells.

Except where stated otherwise, all experiments were repeated at least three times and gave similar results. For in vitro experiments, the spleens of two to five mice were pooled in each experiment.

RESULTS

In vivo response to SRBC. The in vivo responses to SRBC of infected mice at different times after injection of 2×10^6 *L. tropica* organisms are shown in Fig. 1. Forty days after infection, the response of BALB/c mice was significantly increased. In contrast, 75 days after inoculation, mice of the two susceptible strains, DBA/2 and BALB/c, showed a significantly depressed response, expressed as PFC per 10^6 nucleated splenocytes. The difference in the responses of infected and uninfected C57BL/6 mice was not significant. In another experiment, when BALB/c mice infected 86 to 93 days previously were tested, the response was also significantly lower than that of the uninfected sex- and age-matched controls ($P < 0.001$). As previously described (9), a considerable splenomegaly was observed after infection.

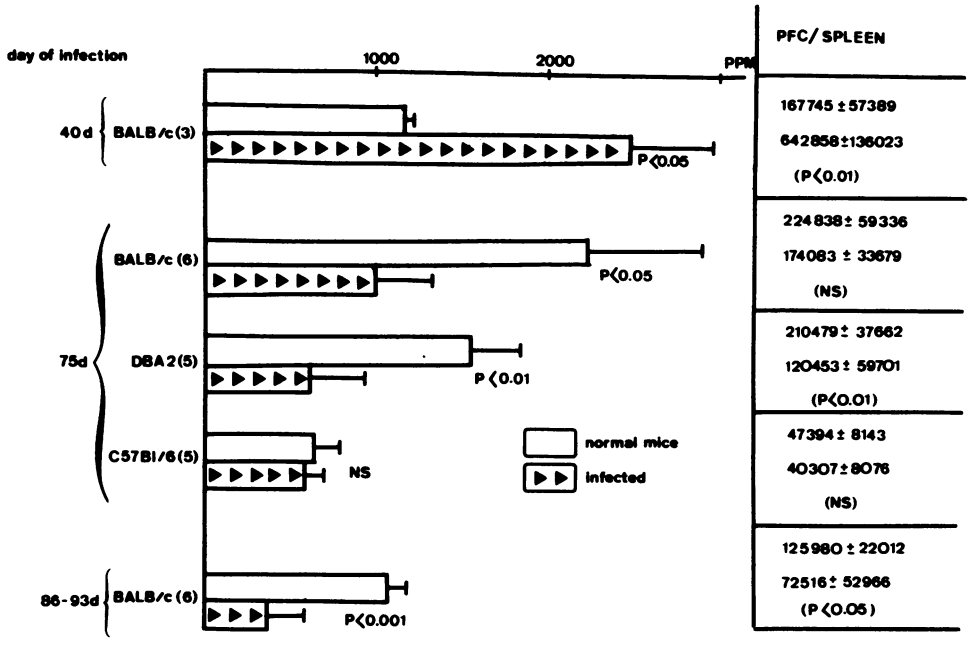

FIG. 1. In vitro response to SRBC. DBA/2, BALB/c, and C57BL/6 mice were infected on day 0 with 2×10^6 promastigotes of *L. tropica*. At different times thereafter, infected and control mice were injected intraperitoneally with 2×10^8 SRBC, and the PFC per 10^6 viable cells (PPM) of their spleen cells were determined 5 days later. The numbers in parentheses denote the number of mice tested. Differences between control mice and infected animals were analyzed by Student's *t* test. (a) Ratio of numbers of viable cells in the spleens of infected animals over those of normal animals. NS, Not significant.

When PFC were calculated on the basis of total viable cells per spleen, the depressions seen in vivo were 23 and 42% for 75 and 86 to 93 days postinfection, respectively (Fig. 1). Note that the cell suspensions prepared from the spleens of infected animals at later stages of the disease contained a large number of dead cells which were not considered in our calculation.

In vitro response to SRBC. To analyze the cellular basis of early augmentation followed by depression of the immune response in infected mice, the responsiveness of their spleen cells was tested in vitro. As shown in Table 1, 47 days after infection there was an increased response in infected animals similar to that seen in the in vivo experiments. This hyperreactivity was more apparent in the resistant C57BL/6 mice than in susceptible BALB/c mice. Later in the course of infection (97 days after inoculation), BALB/c spleen cells were nonreactive, whereas

TABLE 1. In vitro response to SRBC

Mouse strain	Expt	Days after infection	Ratio, infected/normal live spleen cells	Day of appearance of promastigotes	No. of promastigotes per ml ($\times 10^3$)	PPM response to SRBC[a]
BALB/c	1	47	1.16	5	12.0	165
	2		1.56	5	0.5	119
	1	97	3	3	172	0
	2		2.78	3	57	1
C57BL/6	1	47	1.12	U[b]	<1	207
	2		0.98	U	<1	218
	1	97	1	U	<1	87
	2		1.11	U	<1	92

[a] Expressed as (infected cells/normal cells) × 100. PPM, PFC per 10^6 viable cells.
[b] U, Undetectable up to day 13.

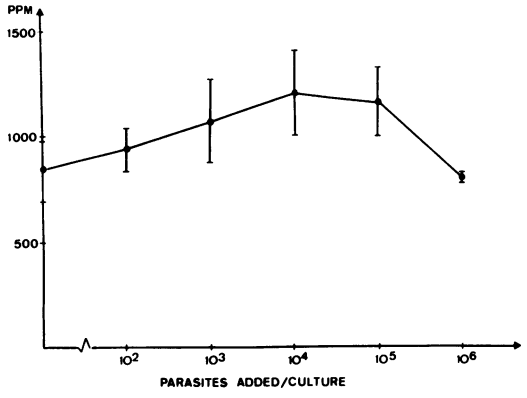

FIG. 2. In vitro PFC response of normal BALB/c spleen cells to SRBC in the presence of *Leishmania*. Various numbers of live promastigotes were added to normal BALB/c spleen cells cultured at 1.5×10^7 cells per ml. SRBC (6×10^6) were added on day 0, and the number of PFC per 10^6 viable cells (PPM) was determined on day 5.

the response of C57BL/6 mice (which had recovered from the infection at that time) returned to normal. This unresponsiveness of BALB/c mice was associated with a dramatic increase in the number of promastigotes detectable in the culture of infected spleen cells. It seemed possible that parasites could interfere directly with the production of PFC. This hypothesis was tested directly as described below.

In vitro PFC response of normal BALB/c spleen cells to SRBC in the presence of *Leishmania*. Viable promastigotes were added to normal BALB/c spleen cell cultures at concentrations varying from 10^2 to 10^6 parasites per 1.5×10^7 spleen cells. SRBC were added on day 0, and PFC responses were assayed on day 5. Under these experimental conditions, even at high concentrations of parasites, the in vitro response to SRBC was not modified (Fig. 2). Thus, it could be assumed that the suppression observed was not due to a direct effect of *L. tropica*, but rather to a defect at the cellular level of infected mouse spleen cells. Therefore, we examined the immunological activity of adherent and nonadherent cells from the spleens of infected BALB/c mice.

Immunological activity of macrophages and macrophage-depleted spleen cells from infected mice. Adherent cells were prepared from infected and normal BALB/c spleen cells. To these macrophages, nonadherent spleen cells from normal or infected mice were added in separate cultures. Each cell population was tested alone as a control. As can be seen in Table 2, nonadherent cells alone, from either normal or infected mice, could not respond to SRBC. The addition of 2-mercaptoethanol to the normal nonadherent cell cultures could partially restore the response. This was not observed in nonadherent cells of infected mice. Moreover, splenic macrophages from normal and infected mice are able to collaborate with nonadherent cells of normal spleens for the production of an anti-SRBC response. In contrast, nonadherent spleen cells from infected mice could not develop any PFC in the presence of splenic macrophages from either normal or infected mice. These results clearly indicate that, surprisingly, the unresponsiveness resides in the nonadherent lymphoid cell populations of infected animals. We therefore examined the ability of T- and B-cell-enriched populations from infected mice to respond to SRBC when cultured with complementary cell populations from normal animals.

Immunological activity of T- and B-cells from infected mice. None of the T- and B-cell-enriched populations from normal or infected BALB/c mice was able to respond to SRBC when cultured alone (Table 3). In contrast, cocultures of normal B- and T-cells gave a normal

TABLE 2. Failure of nonadherent infected spleen cells to collaborate with adherent normal spleen cells

Source of spleen cells		2-Mercaptoethanol (5×10^{-5} M)	PPM[a] on day 5		
Adherent	Nonadherent		Exp 1[b]	Exp 2[c]	Exp 3[d]
Normal	Normal	−	294 ± 21	959 ± 21	1,413 ± 390
Infected	Infected	−	0	14 ± 20	0
Normal	Infected	−	6 ± 8	11 ± 13	0
Infected	Normal	−	462 ± 96	2,361 ± 215	1,068 ± 321
	Normal	−	0	NT[e]	NT
	Normal	+	182 ± 21	NT	NT
	Infected	−	0	NT	NT
	Infected	+	19 ± 4	NT	NT

[a] PPM, PFC per 10^6 nucleated splenocytes.
[b] Day 100 of infection.
[c] Day 98 of infection.
[d] Day 75 of infection.
[e] NT, Not tested.

TABLE 3. Failure of infected T- and B-cells to collaborate with normal B- and T-cells

Source of spleen cells		PPM[a] on day 5	
B-cells (anti-Thy 1.2 + complement)	T-cells (anti-immunoglobulin + complement)	Exp 1[b]	Exp 2[c]
Normal[d]	Normal[d]	745 ± 78	1,464 ± 66
Normal[d]	Infected[d]	0	0
Infected[d]	Normal[d]	0	0
Infected[d]	Infected[b]	0	0
Normal[e]		15 ± 18	NT[f]
	Normal[e]	0	NT
Infected[e]		7 ± 10	NT
	Infected[e]	0	NT
Normal untreated cells		803 ± 14	1,704 ± 253
Infected untreated cells		3 ± 5	3 ± 5

[a] PPM, PFC per 10^6 nucleated splenocytes.
[b] Day 87 of infection.
[c] Day 78 of infection.
[d] 0.75×10^7 cells per culture.
[e] 1.5×10^7 cells per culture.
[f] NT, Not tested.

PFC response. Cocultures of normal B-cells and infected T-cells or of normal T-cells and infected B-cells were unable to respond to SRBC. These results suggest that both T- and B-cell-enriched populations from infected mice are adversely affected.

Similar defects were observed in mitogenic responses to LPS and ConA (Fig. 3). LPS response was completely abolished 78 days after infection, and ConA response was markedly decreased. The removal of T-cells did not restore the LPS response, and similarly, elimination of B-cells did not modify the ConA response of infected spleen cells.

These deficient responses could occur if necessary cell populations were reduced in number or deficient in activity, or if nonspecific suppressor cell populations were induced by infection that prevented expression of normal cell activity. Therefore, preparations of splenic cells from infected mice were examined for their ability to suppress PFC responses in normal spleen cells.

In vitro response to SRBC of cocultures of infected and normal spleen cells. Graded numbers of infected BALB/c spleen cells were added to normal spleen cells, keeping the final cell concentration constant at 1.5×10^7 cells per ml. SRBC were added on day 0, and the responses

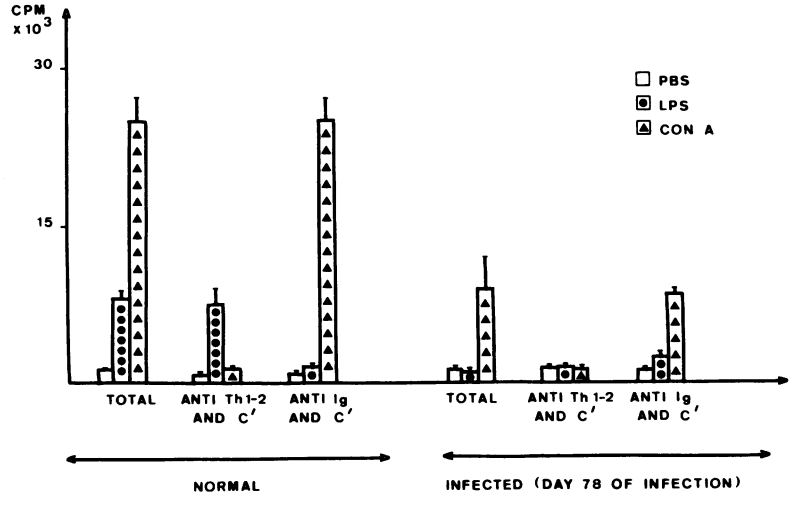

FIG. 3. In vitro responses of BALB/c spleen cells to phosphate-buffered saline (PBS), LPS, and ConA of normal or and *Leishmania*-infected BALB/c mice were determined by tritiated thymidine incorporation. C', complement.

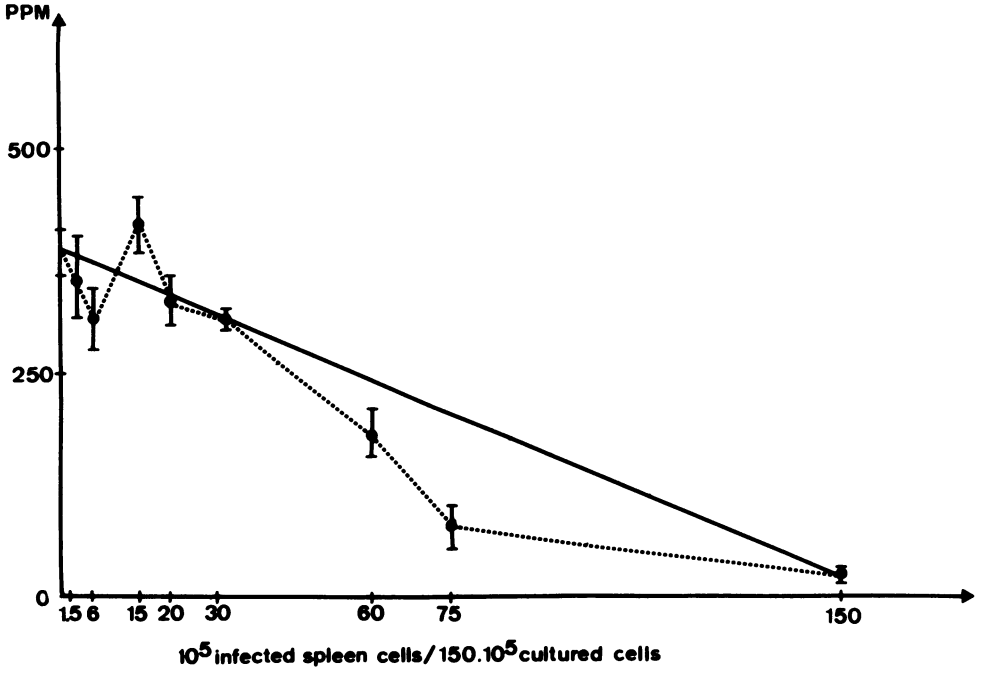

FIG. 4. In vitro response to SRBC of cocultures of infected and normal BALB/c spleen cells. Spleen cells were cultured from infected (day 107 of infection) or normal BALB/c mice separately or in various ratios of normal to infected spleen cells. The cell density was kept constant at 1.5×10^7 cells per ml. The solid line represents the response expected if no interaction occurred between the two cell populations, assuming that, for normal spleen cells, the rate of PFC per total number of cells remained constant for various cell concentrations. The expected response was calculated on the basis of 396 ± 36 PFC per 1.5×10^7 normal cells and 18 ± 18 PFC for the same number of spleen cells from infected animals. The dotted line represents the observed response.

of cocultures were assayed 5 days later (Fig. 4). No difference could be observed between expected responses (if no interaction occurred between the two cell populations) and observed responses up to a ratio of 1/5 infected to normal cells. A marginal suppression was noticed only if the ratio of infected to normal spleen cells was equal to 1. This could very likely be due to dilution of immunocompetent cells of normal spleens by unreactive cells of infected mice. Moreover, this marginal suppression could not be reversed by elimination of T- or B-cells (data not shown). These results strongly argue against the hypothesis of an immunosuppression mediated by suppressor cells.

Cellular analysis of infected and normal BALB/c spleen cells. As can be seen in Table 4, the percentage of T-cells was greatly reduced in infected animals (31 and 8% for normal and infected, respectively). A similar but weaker reduction in the percentage of B-cells was also observed. In contrast, macrophages increased from 9 to 18%, and the spleen became populated with a large percentage (45%) of null cells. The spleens of infected animals were enlarged significantly. The total number of viable cells in the spleen increased five times (81×10^6 and 409×10^6 for normal and infected, respectively). The total number of B-cells increased threefold. The T-cell increase, however, was not as dramatic (1.35-fold). A striking difference was observed between the number of macrophages and null cells (10- and 14-fold increases in infected animals for macrophages and null cells, respectively). This could explain the lower responses to SRBC and mitogens seen in vitro.

DISCUSSION

Specific and nonspecific immunosuppression appears to be a common characteristic of parasitic infections: increased susceptibility to tumors, allografts, or infectious processes, as well as impaired responses to heterologous antigens, have been described in many experimental systems (reviewed in reference 26). For instance severely depressed responses to SRBC have been observed after *Plasmodium yoelli* (2) or *Trypanosoma brucei* (10) infections. Different types of mechanisms which could explain these

TABLE 4. Cellular analysis of the spleens of BALB/c mice

Cell type	Normal cells		Infected cells (3.5 months)	
	No. ($\times 10^6$)	%	No. ($\times 10^6$)	%
Total viable cells	81	100	409	100
T-cells[a]	26	31	35	8
B-cells[a]	36	44	115	28
Macrophages[b]	7	9	73	18
Null cells[c]	13	16	185	45

[a] Determined by cytotoxicity.
[b] Determined by esterase.
[c] Calculated.

immune response defects that follow parasitic infections have been proposed. Thus, antigenic competition, nonspecific T-cell suppression, and immunoregulation by parasitic extracts have been evidenced in different experimental models (reviewed in references 5 and 6). In the present study, we demonstrated that nonspecific immunodepression also occurred during *L. tropica* infection.

However, analysis of the mechanisms of this suppression argues in favor of an abnormal differentiation of the bone marrow precursor stem cells rather than the previously described mechanisms.

Infected BALB/c mice exhibit an initial hyperreactivity (about 1 month after infection) followed by depressed responsiveness. Resistant mice also show an increased immunological reactivity but then a return to normal status. This unresponsiveness in susceptible mice cannot be due to immunosuppressive substances produced by *L. tropica*, in contrast to some other parasitic infections in which such substances have been described (i.e., *Trypanosoma cruzi* [8] and *T. brucei* [25]). In fact, there was no depression in the magnitude of the in vitro response of normal spleen cells to SRBC in the presence of a large number of *L. tropica* parasites (Fig. 2), and the titer of anti-SRBC antibody in heavily infected mice seemed to be normal (data not shown). Furthermore, when the results of in vivo immunization are expressed in terms of PFC per spleen, the depression is less impressive, although these animals harbor a very large number of parasites.

Cellular analysis of the depressed nonspecific immune responsiveness observed in vitro at late stages of infection with *L. tropica* in BALB/c mice seems to indicate that macrophages, although the target cells of the parasite, are not immunologically defective in the in vitro response against SRBC. Handman et al. (11) showed that BALB/c macrophages, unlike those of resistant strains, exhibited a defect in expression of H-2 antigen when infected with *L. tropica* in vitro. This may be important in the control of the infection but does not seem to play a major role in the response to SRBC. The defect in the in vitro response to SRBC, therefore, must reside in the nonadherent population, since these cells were incapable of responding when cocultured with normal macrophages. Both T- and B-cell populations seemed to be affected by the infection, as judged by the results of experiments performed with enriched T- and B-cell populations. In contrast to the observation of Arredondo and Pérez (1), who, in the in vitro response to mitogens, indicated the presence of suppressive cells in *L. mexicana*-infected BALB/c mice (an infection which very closely resembles that of *L. tropica*), our results indicate that the lack of in vitro response to SRBC was not mediated by suppressor cells. This is apparent from experiments with cocultures with various ratios of spleen cells from infected animals to those of normal ones. The response of normal spleen cells could not be suppressed by the addition of up to 25% of cells from infected mice. When the ratio of two cell populations reached 1:1, a slight decrease in observed PFC was seen, as compared to theoretically expected numbers (Fig. 4). This was most likely due to the dilution of immunocompetent cells of normal spleens with immunologically unreactive ones present in the spleens of infected mice. The large immunoglobulin, Thy 1.2-, and esterase-deficient, nonadherent putative null cells, which can comprise up to 45% of the spleen cell population at terminal stages of the disease, are one of the likely candidates for nonreactive cells. A similar increase of null cells in the spleen and bone marrow has been reported for other parasitic infections (28). The origins and functions of these cells remain unknown.

As mentioned earlier, *L. tropica* infection in BALB/c mice is accompanied by lymphadenopathy, hyperglobulinemia, and an initial immunological hyperreactivity. The subsequent immunodepression may represent exhaustive stimulation of T- and B-cells by the parasite. In addition, the presence of a large number of parasites in the bone marrow may alter the

normal differentiation of precursor stem cells, leading to abnormal expansion of a certain cell lineage. Studies are under way to establish the truth of these theories in the mouse model.

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