INFECTION AND IMMUNITY, Oct. 1989, p. 3091–3096
0019-9567/89/103091-06$02.00/0

Vol. 57, No. 10

Comparison of T-Cell Responses in Self-Limiting versus Progressive Visceral *Leishmania donovani* Infections in Golden Hamsters

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Received 18 May 1989/Accepted 17 July 1989

***Leishmania donovani* infection in golden hamsters was studied as a model for human kala-azar. After intradermal inoculation of *L. donovani* amastigotes, hamsters developed positive skin reactions (delayed-type hypersensitivity [DTH]) to parasite antigens and lymphoid cells from these hamsters proliferated to parasite antigens in vitro and transferred DTH reactivity to normal recipients. In contrast, hamsters infected by the intracardial route developed progressive visceral infections and failed to respond to skin test antigens. Spleen cells, lymph node cells, and peripheral blood lymphocytes (PBLs) from these hamsters were unresponsive to parasite antigens in vitro, and spleen cells failed to transfer DTH to normal recipients. Spleen cells, but not PBLs, displayed depressed responses to T-cell mitogens and also suppressed the proliferative response of cells from hamsters inoculated intradermally. Removal of adherent cells restored the capacity of spleen cells, but not PBLs, to respond to parasite antigens. The nonadherent population of these spleen cells also transferred DTH to normal recipients. The adherent suppressor cells, which have the characteristics of macrophages, appear to be localized to the spleen and are apparently not responsible for the failure of peripheral lymphoid cells to respond to antigen. These studies suggest that hamsters with visceral infections develop a population of antigen-reactive cells and that in the absence of suppression these cells may express functional activities, including the capacity to elicit DTH responses.**

Human visceral leishmaniasis (VL) caused by infection with *Leishmania donovani*, an obligate intracellular protozoan parasite of macrophages, is characterized by marked immunological dysfunction. Despite the production of high levels of immunoglobulin, much of which is not specific to the parasite, the overall manifestation of the immunological response is consistent with T-lymphocyte unresponsiveness. In contrast to self-healing cutaneous infections, skin test responses (delayed-type hypersensitivity [DTH]) are conspicuously absent in patients with visceral disease. In addition, lymphocytes from these patients fail to proliferate or produce lymphokines after in vitro stimulation with antigen or, in some cases, T-cell mitogens (6, 7, 10, 11, 13–15, 24). There is no clear demonstration that suppressor T cells play a role in this apparent host anergy (24), and patients usually exhibit responsiveness to parasite antigens after successful chemotherapy (7, 13).

While experimental murine *L. donovani* infections have provided insight into the nature of the immune response to this parasite, they are generally self-limiting and are difficult to compare with active human infections, which are progressive and often fatal in the absence of treatment. In contrast, golden hamsters, like humans, are highly susceptible to *L. donovani* and develop anemia, hepatosplenomegaly, hypergammaglobulinemia, and cachexia associated with a progressive accumulation of parasites in visceral organs after intracardial (i.c.) parasite inoculation (1, 4, 5, 9, 29). However, when infected by the intradermal (i.d.) route, some hamsters may also develop self-limiting infections and express a degree of resistance to a challenge infection (9). These self-limiting patterns of infection may be similar to those seen in humans who develop mild or inapparent disease after infection with *L. donovani* (2). Thus, *L. donovani* infections in hamsters may be more appropriate models than those in mice for the study of human kala-azar.

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In this paper we contrast the immunological responses which develop in hamsters having progressive visceral infections (i.c. inoculated) with those which develop in hamsters having self-limiting infections (i.d. inoculated) and compare both responses with those observed in humans.

MATERIALS AND METHODS

Animals. Inbred male LHC and outbred LVG hamsters (*Mesocricetus auratus*) were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

Parasites and animal inoculation. The 2S strain of *L. donovani*, originally isolated from a kala-azar patient in the Sudan in 1961 (30), was maintained by serial passage in LVG hamsters. Amastigotes were obtained from homogenized spleens of chronically infected animals. Inbred LHC hamsters were inoculated i.c. with 10 million amastigotes in 0.1 ml of phosphate-buffered saline or i.d. with 50 million amastigotes in a hind footpad. Parasites were counted from Giemsa-stained impression smears of the spleen and liver as described previously (29).

Antigen. Antigen for lymphocyte proliferation assays was prepared by sonicating promastigotes (3×10^8/ml) in saline. The optimum concentration of antigen was titrated using responding spleen cells from hamsters inoculated i.d. with *L. donovani*, and samples of the stock preparation were frozen at −70°C. Fresh dilutions of antigen were made for each experiment and used at a final concentration of 6×10^5 sonicated promastigotes per ml. For skin test antigen (leishmanin), 2×10^8 promastigotes were suspended per ml of 0.5% phenol–saline and stored at 4°C.

Lymphocyte proliferation assay. Suspensions of single cells from the spleen and lymph nodes were made by grinding these organs in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) in a loose tissue grinder and passing the cells through a 64-μm nylon mesh. After washing, cells were suspended in RPMI 1640 supplemented with sodium bicarbonate, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-

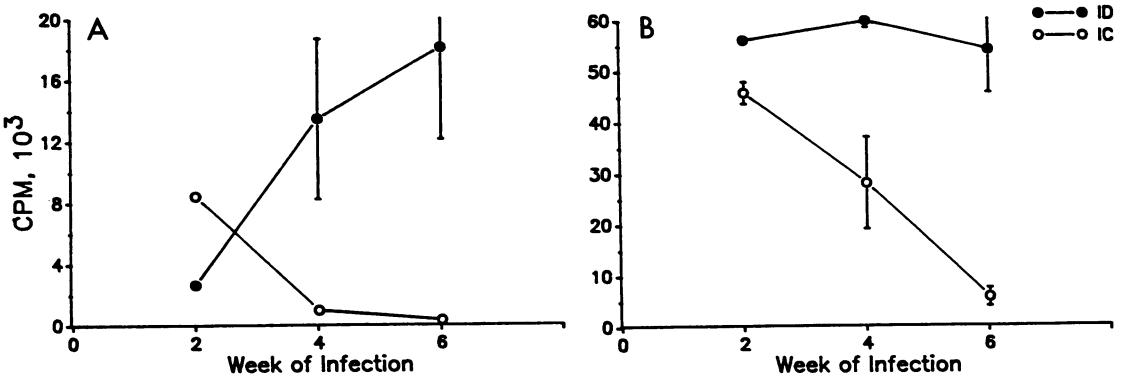

FIG. 1. Proliferative response to antigen (A) or ConA (B) by spleen cells from hamsters infected either i.c. (IC) or i.d. (ID) with *L. donovani* amastigotes. Each value represents the mean counts per minute ± standard error of triplicate cultures from four hamsters.

2-ethanesulfonic acid), 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 200 mM L-glutamine, and 5×10^{-5} M β-mercaptoethanol and plated in triplicate at 2.5×10^5 per well in 96-well flat-bottomed plates (Linbro, Flow Laboratories, Inc., McLean, Va.). The appropriate dilutions of antigen or concanavalin A (ConA) (2 μg/ml) were added, and the plates were then incubated at 37°C in 5% CO_2–95% air for a total of 3 days for mitogen and 4 days for antigen stimulation. At 18 h before they were harvested, plates were pulsed with 1 μCi of [^{3}H]thymidine per ml.

In some experiments, spleen cells from i.c. infected or normal hamsters were treated with mitomycin C (40 μg/ml for 45 min at 37°C), washed, and cultured with an equal number of spleen cells from i.d. infected hamsters.

Plastic adherence and recovery of adherent cells. Plastic tissue culture dishes were preincubated with serum-containing medium for 30 min at 37°C to improve adherence. Thereafter, 10×10^6 spleen cells per ml of medium were incubated for 2 to 3 h at 37°C. The nonadherent cells were decanted and the adherent cells were washed and removed by vigorous pipetting after incubation with 0.02% EDTA (17).

Depletion of hamster B or T cells. Hamster B cells were removed from nonadherent fractions of spleen cells by panning on anti-immunoglobulin-coated plates as described previously (32, 34). T cells were depleted using monoclonal antibodies (MAbs) 38 and 20 (kindly provided by Pamela Witte, University of Texas, Austin) and complement. MAb 38 binds a cytotoxic subpopulation of hamster T cells, while MAb 20 reacts with most hamster T cells (31). Treatment of normal hamster spleen cells with MAb 20 and complement reduced proliferative responses to ConA by 99%.

RESULTS

Responses to ConA and leishmanial antigen. The capacity of spleen cells from infected hamsters to respond to either ConA or parasite antigen was assessed at various times after i.c. or i.d. inoculation of *L. donovani* amastigotes. The level of in vitro spleen cell proliferative responses to antigen in the i.d. inoculated group increased progressively over a 6-week period, while responses to ConA remained constant (Fig. 1). In contrast, spleen cells from the i.c. inoculated group responded to antigen at 2 weeks after infection, but similar cells from hamsters infected for 4 to 6 weeks failed to proliferate. Additionally, a significant depression in the ability of spleen cells to respond to ConA was noted in the i.c. inoculated group at 4 or 6 weeks after infection. At 6 weeks after infection, the average splenic parasite burden in the i.c. inoculated group was 6×10^8 amastigotes, whereas the average parasite number in the i.d. inoculated group was $<2 \times 10^5$, the level of parasite patency detected by microscopic examination of impression smears.

In i.c. inoculated hamsters, maximal tissue parasitization occurs in the spleen, liver, and bone marrow. To determine whether levels of tissue parasitization influenced the capacity of cells to respond in vitro, lymph node cells and peripheral blood lymphocytes (PBLs), both of which contained negligible numbers of parasites, were assayed for their response to antigen. PBLs, lymph node cells, and spleen cells from the i.c. inoculated group all failed to respond to antigen when tested at 6 weeks (Fig. 2). In contrast, all cell populations from the i.d. inoculated group gave strong responses to antigen under similar conditions. Thus, the lack of response to antigen in the i.c. inoculated group is not limited to sites of parasite infection.

Suppression of responses. To determine whether the failures of lymphoid cells to respond to antigen noted in i.c. inoculated animals were due to active suppression, antigen-reactive cells from i.d. infected hamsters were cocultured with equal numbers of mitomycin C-treated spleen cells, lymph node cells, or PBLs from i.c. inoculated hamsters and stimulated with *L. donovani* antigen or ConA. Spleen cells, but not PBLs or lymph node cells, from i.c. inoculated hamsters significantly suppressed the capacity of cells from i.d. inoculated hamsters to respond to antigen and mitogen

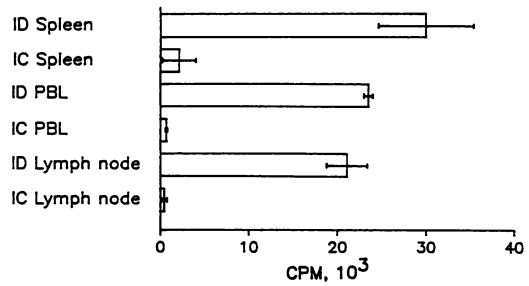

FIG. 2. Proliferative response to leishmanial antigen by spleen cells, lymph node cells, and PBLs 6 weeks after either i.d. (ID) or i.c. (IC) inoculation of *L. donovani*-infected hamsters. Each value represents the mean counts per minute ± standard error from four or more hamsters.

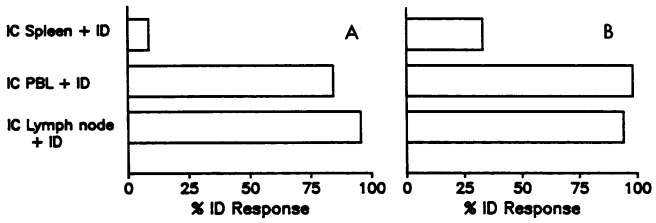

FIG. 3. Suppression of proliferative response to antigen (A) and ConA (B). A total of 2×10^5 spleen cells, lymph node cells, or PBLs from i.c. (IC) infected hamsters were mitomycin C treated and cocultured with an equal number of cells from i.d. (ID) infected animals. Values are expressed as the percentage of the control (response to cells from i.d. infected animals plus mitomycin C-treated normal cells).

(Fig. 3). The levels of spleen cell-mediated suppression were approximately 60% for ConA responses and 90% for antigen-specific responses.

To determine the nature of the suppressor cells, spleen cells from i.c. infected hamsters were separated into plastic-adherent and -nonadherent fractions, treated with mitomycin C, and cocultured with equal numbers of responsive spleen cells from i.d. infected hamsters before stimulation with ConA or parasite antigen. The results (Table 1) show that spleen cells from i.c. infected animals suppressed responses to both ConA and antigen and that suppressor activity is limited to the adherent cell fraction. The adherent cell population contained more than 90% esterase-positive cells (data not shown). The capacity of these cells to suppress cells from i.d. inoculated hamsters was not altered after treatment with MAbs cytolytic for either total T cells (MAb 20) or cytotoxic T cells (MAb 38) (Table 2), suggesting that suppression was probably mediated by macrophages.

Effects of adherent cells on proliferative responses. To determine whether antigen-reactive cells are present in i.c. infected hamsters, but fail to respond to antigen because of the activity of adherent suppressor cells, plastic-nonadherent fractions of spleen cells and PBLs from hamsters infected for 6 weeks were cocultured with mitomycin C-treated normal spleen cells (as a source of antigen-presenting cells) and stimulated with either ConA or parasite antigen. Whereas unfractionated spleen cells failed to proliferate, the nonadherent fraction responded strongly to antigen stimulation (Table 3). Removal of adherent cells also increased the proliferative response of spleen cells from i.c. infected hamsters to ConA by almost fourfold. Removal of B cells by panning on anti-immunoglobulin-coated plates (data not shown) did not reduce the antigen-specific response of the nonadherent fraction. In contrast, removal of adherent cells had no effect on the capacity of PBLs to respond to parasite antigen, suggesting that the lack of response by these cells may not be due to the presence of adherent suppressor cells similar to those found in the spleen.

TABLE 1. Suppression of lymphocyte proliferation by adherent cells from i.c. infected hamsters[a]

Cell population	Thymidine incorporation (cpm ± SE, 10^3)	
	Antigen	ConA
Normal nonadherent + i.d.	11.3 ± 2.6	ND
Nonadherent i.c. infected + i.d.	11.9 ± 2.2	23.2 ± 2.2
Normal adherent + i.d.	20.5 ± 2.2	32.8 ± 0.8
Adherent i.c. infected + i.d.	1.9 ± 1.0	2.3 ± 1.0
Unfractionated i.c. infected + i.d.	5.6 ± 1.1	8.3 ± 0.7

[a] Cells were fractionated by adherence to plastic. Cells being tested for suppressive activity were treated with mitomycin C, washed, and added to 2.5×10^5 spleen cells from hamsters infected by the i.d. route. ND, Not determined.

TABLE 2. Effect of anti-hamster T-cell antibody on the suppressive activity of spleen cells from i.c. infected hamsters[a]

Treatment	Thymidine incorporation (cpm ± SE, 10^3)	
	Antigen response	ConA response
i.d. spleen alone	12.8 ± 0.6	25.8 ± 2.9
i.c. spleen alone	0.5 ± 0.5	1.3 ± 1.2
i.c. spleen (MAb 38 complement)	0.5 ± 0.5	0.9 ± 0.9
i.d. spleen + i.c. (MAb 38 complement)	3.4 ± 0.6	7.6 ± 0.9
i.d. spleen + i.c. (MAb 20 complement)	2.6 ± 2.1	3.5 ± 0.6
i.d. spleen + i.c. spleen (complement)	2.6 ± 0.3	2.4 ± 0.1

[a] MAb 20 binds to most hamster T cells, while MAb 38 binds to a cytotoxic subpopulation (32). i.c. and i.d., Spleen cells from hamsters infected i.c. and i.d., respectively. (MAb + complement) and (complement) indicate that spleen cells from i.c. infected hamsters have been treated with monoclonal anti-hamster T-cell antibody and complement or complement only, respectively.

Skin test reactivity and transfer of DTH. Hamsters were skin tested by inoculating 50 μl of leishmanin in one footpad and phenol-saline in the other. The increase in footpad thickness was measured at 24 and 48 h. Intradermally inoculated hamsters showed an increase in footpad thickness in response to antigen starting at 2 weeks of infection. No positive skin test responses were observed in i.c. inoculated hamsters at any time during infection. Figure 4 shows the differential skin test response to leishmanial antigen at 15 days after infection.

To determine whether the lack of skin test reactivity in i.c. inoculated hamsters was due to a failure of recruitment of antigen-reactive cells to the test site, antigen-responsive cells from the spleen and lymph nodes of i.d. inoculated hamsters were inoculated locally with antigen into the footpads of normal hamsters or i.c. inoculated hamsters at 4 weeks of infection. The colateral footpad received antigen alone. Intracardially infected hamsters receiving 3×10^6 to 4×10^6 cells from i.d. infected hamsters plus antigen failed to develop a local cutaneous response, while normal hamsters responded with positive DTH reactions (Fig. 5).

The ability of cells from i.c. infected hamsters to transfer DTH to normal hamsters was also tested. Total spleen cells

TABLE 3. Effect of depletion of adherent cells on proliferative response of lymphoid cells from i.c. infected hamsters[a]

Cell type	Thymidine uptake (cpm ± SE)	
	Antigen response	ConA response
Spleen		
Unfractionated i.c. infected	1,222 ± 577	8,802 ± 331
Nonadherent i.c. infected	11,155 ± 786	35,707 ± 1,516
PBLs		
Unfractionated i.c. infected	1,635 ± 394	ND
Nonadherent i.c. infected	1,782 ± 608	ND
Unfractionated i.d. infected	23,108 ± 2,313	ND
Nonadherent i.d. infected	31,958 ± 2,559	ND

[a] The various cell poulations (2.5×10^5) were cultured with half the number of either mitomycin C-treated normal spleen cells or PBLs. ND, Not determined.

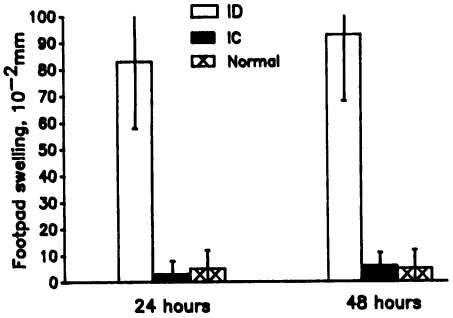

FIG. 4. Skin test responses to leishmanial antigen of hamsters infected i.d. (ID) or i.c. (IC) with *L. donovani* 2 weeks previously. Hamsters were inoculated with 50 μl of leishmanin in one footpad and phenol-saline in the colateral pad. Values represent the mean ± standard deviation of the difference in footpad swelling at 24 and 48 h in four hamsters.

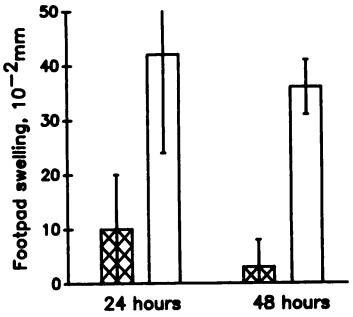

FIG. 6. Ability of spleen cells from i.c. infected hamsters to transfer DTH locally. Unfractionated (▩) or plastic-nonadherent (□) spleen cells (3×10^6) from i.c. infected hamsters were inoculated with antigen into one of the hind footpads of normal hamsters. The colateral pad received antigen alone. Values represent the mean ± standard deviation of the difference in footpad swelling at 24 and 48 h in four hamsters.

or the plastic-nonadherent fraction of these cells from i.c. infected hamsters (DTH negative) was injected, with antigen, into the footpads of normal hamsters. The nonadherent fraction of infected spleen cells, but not the total unfractionated cell population, elicited a positive skin reaction in normal recipients, suggesting that adherent cells, similar to those which suppress in vitro proliferative responses, may also inhibit the functional capacity of spleen cells to confer DTH reactivity to recipient hamsters (Fig. 6).

DISCUSSION

In this study we have compared the immunological responses which develop in hamsters with self-limiting, nonvisceralizing *L. donovani* infections with those which occur in hamsters which develop progressive visceral disease. Not surprisingly, i.d. inoculated hamsters develop positive correlates of cell-mediated responses, including DTH reactivity, to skin test antigens. In addition, both spleen cells and PBLs from these hamsters proliferate in response to in vitro stimulation with parasite antigens. This is in marked contrast to the responses observed in i.c. inoculated hamsters, which fail to exhibit positive DTH reactions during infection and whose cells fail to proliferate to antigen during most stages of infection. The depressed responses to both antigen and mitogen noted in the spleen are due to the development of an adherent suppressor cell population. In the absence of suppressor cells, nonadherent cells from viscerally infected hamsters were capable of transferring DTH reactivity to normal hamsters, suggesting that these cells may also have the functional capacity to participate in cell-mediated responses.

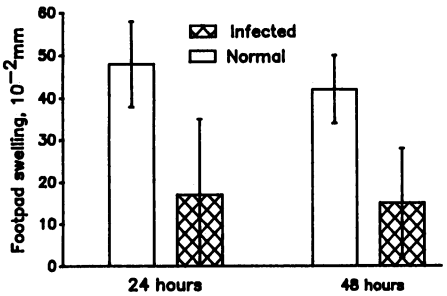

FIG. 5. Local transfer of DTH. A total of 3×10^6 antigen-responsive cells from i.d. infected hamsters were inoculated with antigen into one of the hind footpads of normal hamsters or hamsters infected i.c. with *L. donovani* 4 weeks previously. The colateral pad received antigen alone, and the difference in footpad thickness is presented. Values are the mean footpad swelling at 24 and 48 h ± standard deviation in five or more hamsters.

Adherent suppressor cells have been shown to depress lymphoproliferative response in several intracellular infections, including human diffuse cutaneous leishmaniasis (23), lepromatous leprosy (25, 26), and experimental infections of mice with *Leishmania major* (27) and *L. donovani* (18–20). In murine VL, such adherent suppressor cells have been found to develop and resolve in parallel with levels of splenic parasitization (18). In contrast to studies in murine leishmaniasis, there are only a limited number of studies addressing immunosuppression during VL of hamsters. Nickol and Bonventre (21), in contrast to the results reported here, found that nylon wool-nonadherent T cells suppress antigen-specific proliferative responses. The reasons for the differences between our results and those of the aforementioned study are not clear. In the previous study, hamsters infected for 3 weeks after i.c. parasite inoculation were used as a source of antigen-reactive cells, and it is possible that these cells were more susceptible to suppression than were cells from i.d. inoculated animals. Also, the previous study did not investigate suppression by adherent cell populations, so it is possible that adherent cell populations, if they had been tested, might also have exhibited suppressor activity. However, our results demonstrate that suppression is limited to adherent cells. The fact that the cells which exhibit suppressor activity are greater than 90% esterase positive and that suppressor cell activity is maintained after treatment with anti-T-cell antibodies suggests that suppressor cells are more likely to be macrophages than T cells.

Although antigen-reactive cells are uniformly undetectable in human VL, PBL responses to mitogens are variable. In a recent study of Sicilian leishmaniasis patients, cryopreserved cells collected during active disease were able to suppress phytohemagglutinin responses by autologous cells collected after drug cure (8). In contrast, a number of studies have shown that patient cells respond normally to mitogens (7, 13), and antigen-specific proliferative responses could not be restored by either depletion of monocytes (7) or T cells of the suppressor phenotype (24). In hamsters, we could not demonstrate suppressor cells in the peripheral blood. It is therefore difficult to attribute the failure of PBLs to respond to antigen to active suppression, although it is possible that

PBLs are suppressed by a mechanism different from the one operating in the spleen. Alternatively, the lack of response in the periphery may possibly be due to the selective recruitment of antigen-reactive cells to areas of high antigen load, such as the spleen. Intravenous inoculation of antigen is known to withdraw antigen-reactive cells from the lymphatic circulation to the spleen (28), thus making them undetectable in the periphery. Hahn et al. (12), inoculating mice with a high dose of sheep erythrocytes and transferring peritoneal cells to *nu/nu* mice, showed that antigen-sensitized T cells capable of mediating DTH, as well as those helping antibody production, were absent from peritoneal exudates but were present in the spleen. In a similar study, Milon et al. (16), transferring DTH-mediating cells into irradiated mice that had been exposed to a high dose of antigen or activated B cells or both, showed that retention of antigen-sensitized T cells in the spleen was dependent on the presence of both a high dose of antigen and activated B cells. The situation in VL may be similar, since there is a constant antigenic stimulation due to parasite proliferation in the viscera and B cells are actively producing large amounts of nonspecific, as well as *L. donovani*-specific, antibody. It is therefore possible that this high antigen load in the spleen could trap antigen-reactive cells for extended periods. However, trapping of antigen-reactive cells in parasitized tissues does not explain our failure to observe DTH responses in i.c. infected hamsters after the local transfer of sensitized cells plus antigen. Thus, it is possible that circulating suppressor factors or antibody or both may influence cell function in peripheral sites. In this light, a humoral factor(s) which suppresses mitogen- or antigen-induced activation of T cells has been described from sera of *Leishmania braziliensis*-infected hamsters (22), as well as from kala-azar patients (3, 33). It is also possible that parasitization of the bone marrow and spleen may alter the capacity of hematopoietic cells to respond to inflammatory mediators.

In summary, the patterns of clinical disease, as well as the immunological responses which develop during *L. donovani* infections, are similar in both humans and golden hamsters. Our observation that antigen-reactive cells may be detected in the spleen, but not in peripheral lymphoid tissues, of infected hamsters suggests that similar cells may occur during human visceral infections. The functions of these splenic antigen-reactive cells, as well as the mechanisms which lead to either their absence or suppression in peripheral tissues, may help increase our understanding of the apparent anergy which accompanies human kala-azar.

ACKNOWLEDGMENTS

We thank Gerhard Schad and Thomas Nolan for their critical review of this manuscript.

This work was supported by Public Health Service grant AI-22837 from the National Institutes of Health and the United Nations Development Program/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, TDR 820332.

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